METHODS IN MOLECULAR BIOLOGY™

Volume 281

Checkpoint Controls and Cancer

Volume 2
Activation and Regulation Protocols

Edited by

Axel H. Schönthal

HUMANA PRESS

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Analysis of RB Action in DNA Damage Checkpoint Response

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Summary

Cell cycle checkpoints play a key role in maintaining genome stability by monitoring the order and integrity of cell division events. Checkpoints induced by DNA damage function to limit the propagation of potentially deleterious mutations. The retinoblastoma tumor suppressor (RB) is a critical effector of DNA damage checkpoint function by eliciting G1-phase cell cycle arrest following genotoxic stress. Here, we describe methodologies for evaluation of three facets of RB action in the DNA damage checkpoint response: (1) transcriptional repression of E2F-regulated genes (cyclin A reporter assay); (2) induction of cell cycle arrest (Brd-U incorporation assay); and (3) inhibition of DNA double-strand break accumulation (phosphorylated-histone H2A.X detection). Together, this combination of techniques allows the evaluation of RB action in the coordinated checkpoint response to DNA damage.

Key Words: Retinoblastoma tumor suppressor; checkpoint; DNA damage; cisplatin; cell cycle arrest; phosphorylated H2A.X; murine embryo fibroblast; transcriptional repression; bromodeoxyuridine; DNA double-strand breaks

1. Introduction

Cell growth and division requires the coordinated regulation of numerous events. To ensure the fidelity of division, cells have evolved a number of complex checkpoint mechanisms that monitor the replication process, ensuring that events are completed correctly and in the proper order ***(1)***. Of particular importance are DNA damage checkpoints, which sense genotoxic stress and elicit a coordinated response to eliminate the propagation of potentially deleterious mutations ***(2)***. A critical component of the response to DNA damage is induction of cell cycle arrest ***(3)***. Delays in cell cycle progression are thought to allow time for damaged DNA to be repaired. Different components of checkpoint pathways are inactivated at high frequency in the genesis of human

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

tumors, highlighting the critical importance of these processes for maintaining genome stability and protecting against development of cancer ***(4)***.

The retinoblastoma tumor suppressor (RB) plays an essential role in executing the DNA-damage checkpoint signal for cell cycle arrest ***(5,6)***. RB is a potent negative regulator of cell cycle progression and its ability to control cell cycle progression is achieved by regulated phosphorylation ***(7)***. Active, hypophosphorylated RB inhibits cell cycle progression by binding to E2F transcription factors ***(8)***. This interaction inhibits the ability of E2Fs to transactivate key genes required for S-phase entry and DNA synthesis ***(8)***. Moreover, active RB recruits chromatin-modifying proteins to actively repress the transcription of E2F-regulated genes ***(9)***.

DNA damage checkpoints are thought to impinge on RB action through both p53-dependent and -independent mechanisms. However, the best characterized response is p53 dependent. Following DNA damage, the p53 protein is stabilized and activated by post-translational modifications. This results in increased levels of the cdk2 inhibitor $p21^{cip1}$, a key transcriptional target of p53. Inhibition of cdk2 by $p21^{cip1}$ results in accumulation of the active, hypophosphorylated form of RB, and cell cycle arrest ***(6)***.

Functional analysis of the role of RB in the response to DNA damage can be done by several complementary approaches. Below, we describe three methodologies that can be used to evaluate critical facets of RB action following DNA damage. Firstly, we describe techniques used to assess the role of RB in transcriptional repression of E2F-regulated genes (cyclin A-luciferase assay ***[10]***) and in the induction of cell cycle arrest following exposure to genotoxic stress (Brd-U incorporation ***[5]***). Finally, we describe an approach to directly probe the critical role that functional checkpoints play in protecting cells from the accumulation of double-strand DNA breaks following genotoxic stress (phosphorylated-histone H2A.X [γ-H2A.X] staining ***[11,12]***). In principle, these approaches are applicable to the study of any agent that damages DNA.

2. Materials

2.1. Cell Culture, Transfection of Reporter Construct, and DNA Damage

1. Rb wild type (*Rb*+/+) and null (*Rb*-/-) murine embryonic fibroblasts (MEFs). Prepare MEF cultures by standard methodologies by breeding heterozygous (*Rb*+/–) mice, available from Jackson laboratories; strain name: $129S\text{-}Rb1^{tm1Tyj}$.
2. Dulbecco's modification of Eagle's medium (DMEM, Cellgro, Cat. no. 10-017-CV) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Cat. no. S12450), 100 units/mL penicillin–streptomycin, and 2 m*M* L-glutamine (Gibco-BRL).
3. Dulbecco's phosphate-buffered saline (PBS), tissue culture grade, without calcium and magnesium (Cellgro, Cat. no. 21-031-CV).

4. 1X Trypsin-EDTA solution (Cellgro, Cat. no. 25-052-CI).
5. 60-mm tissue-culture dishes.
6. 6-well tissue-culture dishes.
7. 12-mm circular glass coverslips (Fisher), sterilized and ready to use.
8. Relevant plasmids: cyclin A-luciferase reporter construct (–608 CycA-Luc; *see* **ref. *10***), CMV-β-galactosidase expression vector, control vector (e.g., pcDNA3, Invitrogen).
9. 0.25 *M* $CaCl_2$: dissolve in ddH_2O; filter (0.2 μm) sterilize and store in 50-mL aliquots at –20°C.
10. 2X BES-buffered solution (2X BBS): 50 m*M* *N,N*-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, adjust pH with 10 *N* NaOH to 6.95 in ddH_2O, filter (0.2 μm) sterilize and store in aliquots at –20°C.
11. Inverted fluorescence microscope (Zeiss).
12. Clinical-grade cisplatin (cis-Diaminedichloroplatinum; CDDP; Bristol Oncology), sterile. Store at room temperature.
13. Microcentrifuge.
14. Probe sonicator (e.g., Branson Sonifier 450).
15. Galacto-Star β-galactosidase assay reagents (Tropix, Cat. no. BM100S).
16. 5X Reporter Lysis Buffer (Promega, Cat. no. E397A).
17. Luciferase Assay System (Promega, Cat. no. E1501).
18. Borosilicate culture tubes, 12 × 75 mm (Fisher, Cat. no. 14-961-26).
19. Luminometer (e.g., Femtomaster FB 12, Zylux Corp.).

2.2. Inhibition of Brd-U Incorporation by DNA Damage

1. CDDP-treated *Rb*+/+ and *Rb*-/- MEFs on coverslips in 6-well plates.
2. Cell proliferation–labeling reagent, Brd-U/FdU (Amersham Pharmacia, Cat. no. RPN201).
3. 3.7% (v/v) formaldehyde in PBS: dilute fresh from 37% (w/w) stock solution (Fisher).
4. 0.3% (v/v) Triton X-100 (Fisher) made fresh in PBS.
5. Jeweler's forceps (Fisher, Cat. no. 08-95F).
6. Immunofluorescence (IF) buffer: 0.5% v/v Nonidet P-40 (Fisher), 5 mg/mL (w/v) bovine serum albumin (Sigma) in PBS; store at 4°C.
7. 1 *M* $MgCl_2$.
8. DNase I, RNase-free (10 U/μL) (Roche, Cat. no. 776 785), store at –20°C.
9. Monoclonal rat anti-Brd-U antibody (Accurate Scientific, Cat. no. YSRTOBT-0030), store at –80°C.
10. Donkey anti-rat IgG, Rhodamine-red X-conjugated (Jackson Immunoresearch, Cat. no. 712-295-153).
11. 1 mg/mL (w/v) Hoechst 33258 (Sigma, Cat. no. B2883), store at 4°C.
12. Microscope slides (Fisher, Cat. no. 12-544-7).
13. Gel/Mount (Biomeda Corp., Cat. no. M01).
14. Inverted fluorescence microscope (Zeiss).

2.3. Detection of DNA Double-Strand Breaks by γ-H2A.X Staining

1. CDDP-treated *Rb*+/+ and *Rb*–/– MEFs on coverslips.
2. 0.3% (v/v) Triton X-100 in PBS.
3. IF buffer (*see* **Subheading 2.2., item 7**).
4. Mouse monoclonal anti-γ-H2A.X (ser139), clone JBW301 (Upstate, Cat. no. 05-636), store at –20°C.
5. Rhodamine-red-X-conjugated affiniPure goat anti-mouse IgG (Jackson Immunoresearch, Cat. no. 115-295-062), store at –80°C.
6. Hoechst 33258.
7. Microscope slides.
8. Gel/Mount.
9. Inverted fluorescence microscope.

3. Methods

3.1. Cell Culture and Transfection for Reporter Assay

3.1.1. Cell Culture

1. Seed approximately 3×10^5 cells per 60-mm dish in DMEM supplemented with 10% FBS and penicillin–streptomycin.
2. MEFs should attach to the tissue-culture dish within 4–6 h.

3.1.2. Cell Transfection

1. Prepare purified plasmid DNA stocks at 1-mg/mL concentration in TE buffer.
2. Add 2 µg –608 CycA-Luc reporter construct (or other luciferase reporter constructs of interest), 1 µg CMV-β-galactosidase expression vector, and 5.5 µg control vector to 1.5-mL Eppendorf tubes (8.5 µg total DNA per 60-mm dish; *see* **Note 1**). Include a dish of cells transfected only with 8.5 µg of control vector to serve as a baseline for luciferase and β-galactosidase activity.
3. Add 250 µL 0.25 *M* $CaCl_2$ to DNA and mix by pipetting.
4. Add 250 µL 2X BBS solution and mix by gentle inversion.
5. Incubate tubes at room temperature for 20 min.
6. Add DNA/$CaCl_2$/BBS solution to cells dropwise.
7. Inspect the cells for the presence of precipitate using an inverted microscope (×20 magnification is sufficient; *see* **Note 2**).
8. Return cells to tissue-culture incubator (37°C, 5% CO_2).
9. Sixteen hours after addition of precipitate, wash cells three times briefly with PBS.
10. Inspect dishes to ensure removal of precipitate.
11. Add fresh medium to cells and return to incubator.

3.1.3. Treatment With DNA-Damaging Agent

Twenty-four to thirty-six hours post-transfection (i.e., approx 8–20 h after removal of precipitate), add cisplatin to a final concentration of 32 µ*M* or vehicle control to cell culture media.

3.1.4. Preparation of Cell Lysate for Luciferase/β-Galactosidase Assay

1. Sixteen hours after the addition of cisplatin, aspirate medium and wash cells with PBS.
2. Add 0.75 mL of 1X trypsin-EDTA solution to dishes.
3. Incubate 3–5 min at 37°C, confirm that cells have detached using inverted microscope.
4. Inactivate trypsin by adding an equal volume of medium containing 10% FBS.
5. Transfer suspended cells to 1.5-mL Eppendorf tubes.
6. Pellet cells at 16,000*g* for 10 s in microcentrifuge.
7. Carefully aspirate medium from cell pellet.
8. Resuspend cell pellet in 0.5 mL PBS.
9. Repeat **step 6**.
10. Aspirate PBS.
11. Resuspend cell pellet in 100 µL of 1X reporter lysis buffer (diluted 5X with sterile water).
12. Incubate tubes on ice for 20 min.
13. Sonicate samples with three brief pulses at 30–40% output (constant duty cycle).
14. Centrifuge tubes at 16,000*g* in microcentrifuge at 4°C for 10 min.
15. Transfer cleared lysate to new 1.5-mL Eppendorf tube.
16. Place tubes on ice.

3.1.5. β-Galactosidase Assay

1. Add 150 µL of Galacto-Star reagent (substrate diluted 1:50 in diluent according to the manufacturer's instructions) to a borosilicate tube.
2. Add 1 µL of cleared lysate to Galacto-Star reagent. Swirl to mix.
3. Incubate at room temperature protected from light for 1 h (*see* **Note 3**).
4. Following incubation, analyze and record β-galactosidase activity for each sample in luminometer.

3.1.6. Luciferase Reporter Assay

1. Add 70 µL of luciferin (prewarmed to room temperature) to a borosilicate tube.
2. Add 15 µL of cleared lysate from the first sample and swirl to mix (*see* **Note 4**).
3. Immediately analyze luciferase activity in luminometer.
4. Record luciferase activity and repeat **steps 1–4** for remaining samples (*see* **Note 5**).

3.1.7. Quantitation and Documentation

1. To normalize for transfection efficiency between samples, determine the ratio of luciferase to β-galactosidase activity for each sample (divide luciferase activity by β-galactosidase activity).
2. Set the normalized luciferase activities for untreated *Rb*+/+ and *Rb*–/– MEFs to 100%.
3. Relative luciferase activity for CDDP-treated samples is then represented as a percent of the untreated control for both *Rb*–/– and *Rb*+/+ MEFs.
4. Plot the relative luciferase activity for each sample (*see* **Fig. 1**; *see* **Note 6**).

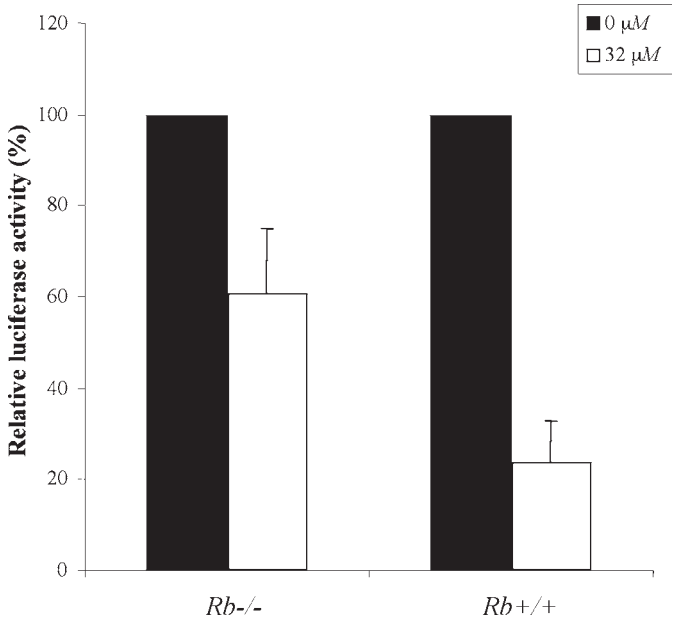

Fig. 1. *Rb*+/+ and *Rb*–/– MEFs were transfected with 608CycA-Luc reporter construct and CMV-β-galactosidase expression vector and treated with 0 or 32 μ*M* CDDP for 16 h. Cell lysis and assay of luciferase and β-galactosidase activity were performed as described. Shown is the mean from two experiments ± s.d.

3.2. Bromodeoxyuridine Incorporation Assay

3.2.1. Cell Culture and Drug Treatment

1. Plate *Rb*+/+ and *Rb*–/– MEFs (approx 1×10^5 cells/well; *see* **Note 7**) on coverslips in 6-well plates (four coverslips per well; *see* **Note 8**). Ensure enough dishes of both *Rb*+/+ and *Rb*–/– MEFs are prepared for analysis of the desired number of drug doses. Cells should be 60–70% confluent before addition of CDDP to ensure cells are in exponential growth phase.
2. Freshly prepare working solutions of CDDP (0, 8, 16, and 32 μ*M*) in prewarmed tissue culture media.
3. Remove dishes from incubator and carefully and completely aspirate the medium from each dish.
4. Replace with media containing desired CDDP doses and return cells to tissue culture incubator (37°C, 5% CO_2).
5. Incubate *Rb*+/+ and *Rb*–/– cells with CDDP for 16 h.
6. After completion of drug treatment, aspirate media and wash cells three times for 5 min with prewarmed tissue culture medium. Return dishes to tissue culture incubator for each wash step.

3.2.2. Brd-U Labeling

1. Prepare working solution of cell proliferation–labeling reagent (1:1,000 dilution) in prewarmed tissue culture medium (*see* **Note 9**).
2. Aspirate medium carefully from each dish and replace with medium containing cell proliferation-labeling reagent.
3. Return dishes to tissue culture incubator for 8 h.

3.2.3. Fixation

1. Following Brd-U labeling, aspirate medium from dishes.
2. Wash cells gently with PBS.
3. Fix cells at room temperature with 3.7% formaldehyde in PBS for 15 min, 2 mL/well.
4. Aspirate formaldehyde.
5. Add PBS to wells.
6. Coverslips in PBS may be stored in the dark at 4°C, wrapped in foil.

3.2.4. Brd-U Staining

1. Transfer one or two coverslips from each well to a fresh 6-well dish containing PBS (*see* **Note 8**).
2. Aspirate PBS.
3. Add 0.3% Triton X-100 in PBS to wells to permeabilize the cells.
4. Incubate dishes at room temperature for 15 min.
5. Aspirate 0.3% Triton X-100 and replace with PBS.
6. Prepare primary antibody solution by mixing the following in IF buffer in one solution:
   a. 1:50 1 *M* $MgCl_2$.
   b. 1:500 rat anti-Brd-U antibody.
   c. 1:500 DNase I (*see* **Note 10**).
7. Using jeweler's forceps, carefully transfer coverslips to a humidified chamber prepared as illustrated in **Fig. 2**. Make sure that cells are on the surface that is facing up.
8. Pipet 35 µL primary antibody solution directly onto each coverslip.
9. Place lid on chamber and incubate at 37°C for 45 min.
10. Carefully transfer coverslips to a clean 6-well dish containing PBS. Make sure that cells remain face up during all wash steps.
11. Wash coverslips three times for 5 min with PBS at room temperature.
12. Prepare secondary antibody solution by mixing the following in IF buffer:
    a. 1:100 donkey anti-rat, Rhodamine-red-X-conjugated.
    b. 1:100 Hoechst (10 µg/mL final concentration).
13. Carefully return coverslips to the humidified chamber containing a clean sheet of parafilm. Make sure that cells are face up.
14. Pipet 35 µL secondary antibody solution directly onto each coverslip.
15. Place lid on chamber and incubate at 37°C for 45 min.
16. Return coverslips to labeled 6-well dish containing PBS.

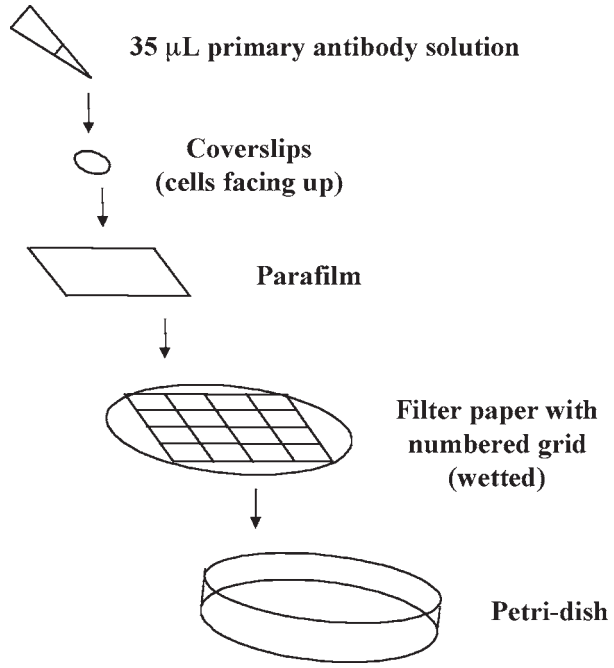

Fig. 2. Diagram of immunofluorescence staining in a humidified chamber of fixed and permeabilized cells grown on glass coverslips.

17. Wash coverslips three times for 5 min with PBS at room temperature.
18. Ensuring cell side is face down, mount coverslips on slides using Gel/Mount.
19. Store slides in the dark at room temperature until Gel/Mount is dry.

3.2.5. Quantitation and Documentation of Brd-U Incorporation

3.2.5.1. Quantitation

1. Examine coverslips using fluorescence microscope (×63 objective) to quantitate Brd-U incorporation:
   a. Count the number of Hoechst-stained nuclei in a random field (excitation/emission maxima 350/460nm).
   b. Without changing fields, count the number of cells that are also Brd-U-positive (i.e., Rhodamine-red-X-positive: excitation/emission 570/590nm).
   c. Repeat **steps a** and **b** until 150–200 nuclei have been counted.
   d. Calculate the percent Brd-U-positive cells for each coverslip ([Brd-U-positive/Hoechst positive] × 100).
   e. Compare the effect of CDDP treatments vs untreated controls for both *Rb*+/+ and *Rb*–/– MEFs. For each cell type the percentage of untreated control cells

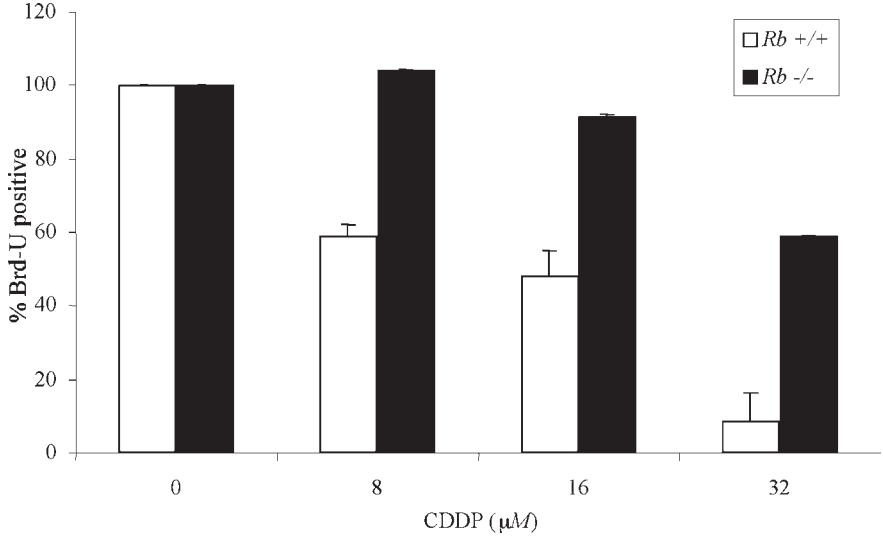

Fig. 3. *Rb*+/+ and *Rb*–/– MEFs were treated with 32, 16, 8, or 0 μ*M* CDDP for 16 h. Following extensive washing, cells were pulse labeled with Brd-U for 8 h. Fixation, permeabilization, immunostaining, and quantitation were performed as described.

staining positive for Brd-U is set arbitrarily to 100% to normalize for any differences in Brd-U incorporation between *Rb*+/+ and *Rb*–/– MEFs. The effect of drug treatment is then represented as the percent Brd-U-positive cells expressed relative to untreated control (*see* **Fig. 3** and **Note 11**).

3.2.5.2. Documentation

1. Take representative photomicrographs of selected fields.
2. Use blue (Hoechst) and red (Rhodamine-red-X) channels to obtain photomicrographs of the same field (*see* **Fig. 4**).

3.3. Detection of DNA Double-Strand Breaks by γ-H2A.X (ser139) Staining

3.3.1. Cell Culture and Drug Treatment

1. Culture *Rb*+/+ and *Rb*–/– MEFs to 60–70% confluence (approx 1×10^5 cells/well) on coverslips in 6-well plates (four coverslips per well).
2. Freshly prepare working solutions of CDDP (32 and 0 μ*M*) in prewarmed tissue culture medium.
3. Remove dishes from incubator and carefully and completely aspirate the medium from each dish.
4. Replace with medium containing 32 or 0 μ*M* CDDP and return cells to tissue culture incubator.

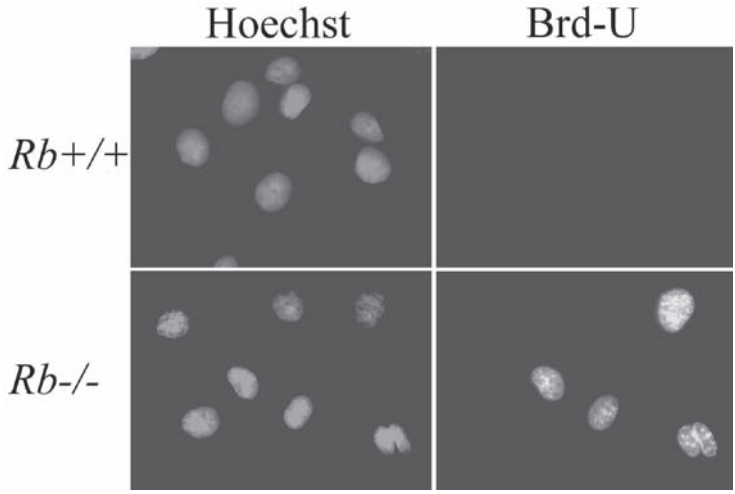

Fig. 4. *Rb*+/+ and *Rb*–/– MEFs were treated with 32 μ*M* CDDP for 16 h. Following extensive washing, cells were pulse labeled with Brd-U for 8 h. Fixation, permeabilization, and immunostaining were performed as described. Photomicrographs were acquired at the same magnification with equal exposure times (×63 objective).

5. Incubate *Rb*+/+ and *Rb*–/– cells with CDDP for 8 h.
6. After completion of drug treatment, aspirate medium and wash cells three times with PBS.

3.3.2. Cell Fixation

1. Remove one coverslip from each well and transfer to a new 6-well dish containing PBS (*see* **Note 8**).
2. Remove PBS and add 2 mL of ice-cold methanol (prechilled at –20°C). Transfer dishes to –20°C freezer for 10 min.
3. Completely remove methanol.
4. Add fresh PBS to wells and store in dark (wrapped in foil) at 4°C until staining.

3.3.3. Staining

1. Aspirate PBS.
2. Permeabilize cells by addition of 0.3% Triton X-100 in PBS for 20 min at room temperature.
3. Aspirate 0.3% Triton X-100 and replace with PBS.
4. Prepare primary antibody solution by diluting the γ-H2A.X antibody 1:20 in IF buffer.
5. Using jeweler's forceps, carefully transfer coverslips to a humidified chamber prepared as illustrated in **Fig. 2**. Make sure that cells are on the side that faces up.

6. Pipet 35 µL primary antibody solution directly onto each coverslip.
7. Place lid on chamber and incubate at 37°C for 45 min.
8. After incubation, carefully transfer coverslips to a clean 6-well dish containing PBS. Make sure that cells remain face up during all wash steps.
9. Wash coverslips three times for 5 min with PBS at room temperature.
10. Prepare secondary antibody solution by mixing the following in IF buffer:
    a. 1:100 goat anti-mouse Rhodamine-red X.
    b. 1:100 Hoechst (10 µg/mL final concentration).
11. Carefully return coverslips to the humidified chamber containing a clean sheet of parafilm. Make sure that cells are face up.
12. Pipet 35 µL secondary antibody solution directly onto each coverslip.
13. Place lid on chamber and incubate at 37°C for 45 min.
14. Return coverslips to labeled 6-well dish containing PBS.
15. Wash coverslips three times for 5 min with PBS at room temperature.
16. Making sure that cell side is face down, mount coverslips on slides using Gel/Mount.
17. Store slides in the dark at room temperature until Gel/Mount is dry.

3.3.4. Quantitation and Documentation of DNA Double Strand Breaks

3.3.4.1. Quantitation

Examine coverslips using fluorescence microscope (20-63X objective) to quantitate γ-H2A.X staining:

1. Count the number of cells in a random field stained with Hoechst (indicated by blue staining).
2. Without changing fields, count the number of phospho-H2A.X positive nuclei (entire nucleus should exhibit red focal staining; *see* **Fig. 5**).
3. Repeat **steps 1** and **2** until 150–200 total cells have been counted.
4. Calculate the percent γ-H2AX positive cells for each coverslip ([γ-H2AX positive/Hoechst positive] × 100).
5. The percentage of untreated *Rb*+/+ and *Rb*–/– cells staining positive for γ-H2A.X is set arbitrarily to 1.0. Compare the percent of CDDP-treated *Rb*+/+ and *Rb*–/– MEFs staining positive for γ-H2A.X to the pertinent untreated control and represent on a graph as *n*-fold increase in positive staining (*see* **Fig. 6**, and **Notes 12** and **13**).

3.3.4.2. Documentation

1. Take representative photomicrographs of selected fields.
2. Use blue (Hoechst) and red (Rhodamine-red-X) channels to obtain photomicrographs of the same field (*see* **Fig. 5**).

4. Notes

1. 8.5 µg total DNA is the optimum quantity for transfection of a 60-mm dish using the calcium phosphate method. For a control vector (i.e., to bring the quantity of

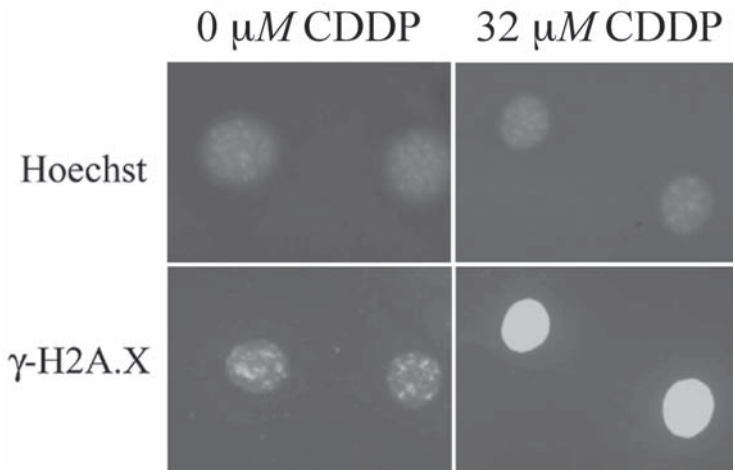

Fig. 5. *Rb*–/– MEFs were treated for 8 h with CDDP (0 or 32 μ*M*). Fixation, permeabilization, immunostaining for γ-H2A.X, and documentation of representative fields were performed as described. Images were acquired using equal exposure times and the ×63 objective.

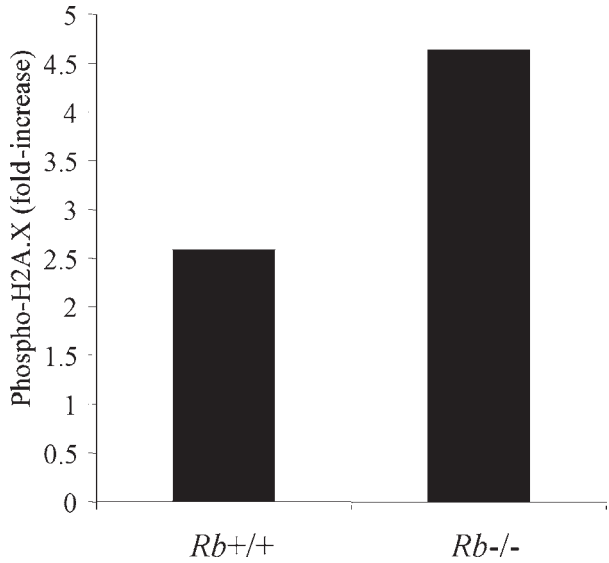

Fig. 6. *Rb*+/+ and *Rb*–/– MEFs were treated for 8 h with CDDP (0 or 32 μ*M*). Fixation, permeabilization, and immunostaining for γ-H2A.X were performed as described. Effect of CDDP treatment on γ-H2A.X levels in *Rb*+/+ and *Rb*–/– MEFs is represented as n-fold increase vs respective untreated cells (not shown).

DNA to 8.5 µg) we typically use an empty vector that is identical to a vector used in the transfection but that does not contain the insert (e.g., pcDNA3).
2. The formation/presence of black, granular precipitate ensures the quality of the transfection reagents (i.e., 0.25 *M* $CaCl_2$ and 2X BBS). Poor precipitate formation is often owing to incorrect pH of the 2X BBS solution.
3. We routinely perform β-galactosidase assays initially and carry out luciferase assays during the 1 h incubation period.
4. This is a time-sensitive assay. Process all samples on an equal time scale.
5. Low β-galactosidase readings (those comparable to untransfected cells or cells transfected without β-galactosidase plasmid) indicate poor transfection efficiency, while low luciferase readings (those comparable to lysis buffer alone or cells transfected without a reporter plasmid) indicate weak promoter activation. Make sure that plasmids were quantitated accurately and that the cells were transfected according to protocol while in exponential growth phase. Consistently high readings of luciferase or β-galactosidase activity could indicate an excess of plasmid in the transfection. In this case, less plasmid can be transfected.
6. We routinely analyze luciferase activity with at least two independent experiments, with each condition analyzed in triplicate.
7. It is imperative that cultures used for evaluation of Brd-U incorporation be in exponential growth phase.
8. We typically process only one or two of the fixed coverslips from each well in case of errors during staining. Thus, the other coverslips serve as backups in the event of mistakes during processing.
9. Brd-U is light sensitive. Add to tissue culture media in a dark tissue-culture hood, and limit light exposure (as with any fluorophore) during staining.
10. We recommend using DNase I only from Roche. DNase I purchased from other companies has produced poor results, likely owing to excess enzyme activity.
11. The percent of untreated *Rb*–/– cells staining Brd-U positive is typically slightly higher than *Rb*+/+ cells. We arbitrarily set the percent of both untreated *Rb*+/+ and *Rb*–/– MEFs staining positive for Brd-U to 100% to normalize for differences in Brd-U incorporation between these cultures. The effect of each drug dose in both *Rb*+/+ and *Rb*–/– cells is then determined relative to the pertinent untreated control.
12. When scoring γ-H2A.X staining, score only nuclei that exhibit completely focal red staining as positive. Untreated cells typically have low levels of γ-H2A.X staining, and we consider this weak staining as negative (*see* **Fig. 5** for examples).
13. Under the experimental conditions described (i.e., *Rb*+/+ and *Rb*–/– MEFs treated with 32 µ*M* CDDP for 8 h) we routinely observe approx 40–50% γ-H2A.X positive staining in *Rb*–/– cells and 20–25% in the *Rb*+/+ cells.

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2

Interaction Between the Retinoblastoma Protein and Protein Phosphatase 1 During the Cell Cycle

Norbert Berndt and John W. Ludlow

Summary

The functions of the retinoblastoma protein (pRb) are in part regulated by reversible and cell cycle-dependent phosphorylation. While the regulation of pRb by cyclin-dependent kinases (Cdks) has been studied extensively, the role(s) of protein phosphatase 1 (PP1) in controlling pRb are only partially understood. In this chapter, we will describe experimental approaches to investigate the interactions between pRb and PP1. Methods will be presented to study the cell cycle-dependent dephosphorylation of pRb by various PP1 isozymes, the specificity of PP1 isozymes for distinct pRb phosphorylation sites, the dephosphorylation of pRb associated with apoptosis, and the cell cycle- and pRb-dependent phosphorylation of PP1.

Key Words: Retinoblastoma protein (pRb); protein phosphatase 1 (PP1); cyclin-dependent kinase (Cdk); tumor suppressor; protein dephosphorylation; protein complex; cell cycle; checkpoint control; restriction point; G1/S transition; mitosis; cancer; phosphorylation site-specific antibodies.

1. Introduction

The retinoblastoma protein (pRb) is the prototype of mammalian tumor suppressors and regulates the entry into and passage through the S phase of the cell cycle. pRb plus its upstream and downstream partners have recently been referred to as pRb pathway. Its established components are pRb itself; the cyclin-dependent kinases (Cdks), which phosphorylate and inactivate this protein as cells prepare for the G1/S transition; the regulators of these kinases, such as cyclins D, E, and A, as well as Cdk inhibitors; and finally, the downstream effectors of pRb, the transcription factors of the E2F family (for recent reviews, *see* **refs. *1–7***). This pathway is now being recognized as very important insofar as in the vast majority of human cancers, at least one member of the pRb pathway is malfunctioning ***(8,9)***. Since pRb is now being considered an integral part of the checkpoint controlling the G1/S transition, the original

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

definition of a checkpoint bears repeating: The dependence of an event B upon the completion of a prior event A is due to a checkpoint, if a loss-of-function mutation exists that relieves that dependence ***(10,11)***.

Although the significance of each of the aforementioned proteins is not in dispute, we would like to suggest granting membership in this pathway to another protein—PP1, which has recently been shown to control the phosphorylation state of pRb. Support for this idea comes from several observations: PP1 dephosphorylates pRb at the end of mitosis ***(12)*** and apparently keeps pRb in a dephosphorylated state throughout G1 ***(13)***. Furthermore, PP1α was found to interact with pRb in a two-hybrid screen and to co-immunoprecipitate with pRb ***(14)***. Consistent with these observations, PP1α ***(15)*** as well as the other two PP1 isozymes γ and δ (Rui-Hong Wang and Norbert Berndt, unpublished) are found in a complex with pRb throughout interphase ***(15)***. Meanwhile, all three mammalian isoforms of PP1 dephosphorylate pRb at the end of mitosis; however, they prefer distinct subsets of phosphorylation sites ***(16)***. While these data strongly suggest that PP1 is an important regulator of pRb function, it is also evident that pRb can function as a PP1 inhibitor ***(17)***. In addition to the emerging role(s) of PP1 in the cell cycle, apoptotic stimuli induce PP1α, which then appears to dephosphorylate pRb, presumably in order to recruit it to caspase-mediated destruction ***(18)***. We hope that this short introduction has convinced the reader that the interaction between pRb and PP1 is important for the regulation of both the cell cycle and apoptosis.

In this chapter, we will describe strategies and methods to analyze interactions between pRb and PP1. We will focus our attention on (a) the cell cycle-dependent dephosphorylation of pRb by PP1, (b) the apoptosis-associated dephosphorylation of pRb by PP1, and (c) the phosphorylation of PP1 by Cdks. Obviously, we have examined the PP1-pRb interactions only in a few cell lines, but we would like to point out that these techniques may be employed in or modified to accommodate a wide variety of experimental settings. There is increasing evidence that both regulation and functions of pRb as well as PP1 is context specific (for review, *see* **refs. *6,19–22***). Thus, it is likely that both the nature and extent of the PP1–pRb interactions vary with the cell type (i.e., the cell-specific proteome), the developmental status or previous history of the cell, or environmental signals. The techniques rely in large part on the use of phosphorylation site-specific antibodies.

2. Materials

1. Phosphate-buffered saline (PBS) (available in tablet form from Sigma-Aldrich). Dissolve one tablet in 200 mL of water to yield 137 m*M* NaCl, 2.7 m*M* KCl in 10 m*M* Na_2HPO_4/KH_2PO_4 phosphate buffer, pH 7.4. Store at room temperature.

2. Cytosine arabinoside (araC) (Sigma-Aldrich). Prepare a 100 m*M* stock solution in PBS. Store in small aliquots at –20°C.
3. Aphidicolin (Sigma-Aldrich). Prepare a stock solution of 5 mg/mL in DMSO and store at –20°C.
4. Mimosine (Sigma-Aldrich). Prepare a 10 m*M* stock solution in phosphate-buffered saline and store at –20°C.
5. Nocodazole (Sigma-Aldrich). Prepare a stock solution of 5 mg/mL in DMSO and store at –20°C.
6. Okadaic acid (Calbiochem). Prepare a stock solution of 5 μ*M* in DMSO and store at –20°C.
7. EBC buffer: 50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 0.5% NP-40. Store at 4°C.
8. Kinase reaction buffer: 20 m*M* Tris-HCl (pH 7.5), 20 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol, 150 m*M* NaCl, 0.5% NP-40, supplemented with freshly added protease and phosphatase inhibitors: 2 μg/mL aprotinin, 1 m*M* benzamidine, 0.5 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.1 m*M* PMSF, 25 m*M* NaF, 1 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4 (*see* **Note 1**). Store at 4°C.
9. NET-N buffer: 20 m*M* Tris-HCl (pH 8.0), 100 m*M* NaCl, 1 m*M* EDTA, 0.5% NP-40, 4% bovine serum albumin (BSA). Store at 4°C.
10. Phosphatase reaction buffer: 20 m*M* imidazole (pH 7.0), 150 m*M* NaCl, supplemented with 1 μg/mL aprotinin, 1 m*M* PMSF, and 1 m*M* $MnCl_2$ (*see* **Note 1**).
11. RIPA buffer: 50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 1% (v/v) NP-40, 0.1% (w/v) SDS, supplemented with freshly added protease, protein kinase, and phosphatase inhibitors: 2 μg/mL aprotinin, 1 m*M* benzamidine, 0.5 μg/mL leupeptin, 1 μg/mL pepstatin A, 0.1 m*M* PMSF, 0.5 m*M* EDTA, 100 m*M* NaF, 1 m*M* Na_3VO_4, and 5 μ*M* okadaic acid (*see* **Note 1**).
12. 2X SDS sample buffer: 125 m*M* Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 10 % (v/v) 2-mercaptoethanol. Store at room temperature.
13. SDS-PAGE molecular weight standards: BioRad. The high and low molecular weight standards contain proteins with M_r = 200, 116, 97, 66, 45, 36, 31, 21.5, and 14.4 kDa, respectively.
14. Antibodies to PP1α: Ab1, raised against residues 316–330 and thus recognizing the dephosphorylated form of PP1α ***(15,23)***; Ab2, raised against residues 294–309 and thus recognizing PP1α regardless of its phosphorylation state (provided by Emma Villa-Moruzzi, University of Pisa, Italy) ***(24)***; Ab3, raised against a peptide containing residues 316–323 and a chemically phosphorylated Thr320 (provided by Angus C. Nairn, Rockefeller University, New York, NY) ***(25)***.
    Isoform-specific antibodies to PP1α, PP1γ1, and PP1δ are also available from Santa Cruz Biotechnology.
15. Antibodies to pRb: sc-102, specific for the centrally located A/B pocket region, and sc-50, specific for a peptide derived from the C terminus (both available from Santa Cruz Biotechnology). Phosphorylation site-specific antibodies for

Table 1
Phosphorylation Sites in pRb. Site Specificity of Different Cdks and Effects of Phosphorylation

Site	Cdk/cyclin *(Reference)*	Effect of phosphorylation *(Reference)*	Antibody available
S005	4/D1-2/E-2/A *(32)*		
S249	4/D1 *(32)*		yes
T252	4/D1 *(32)*		
T356	4/D1 *(32)*	Decreases G1 arrest *(33,34)*	yes
T373	4/D1-2/E *(32)*		yes
S608	4/D1-2/A *(32)*		
S612	2/E-2/A *(32)*		yes
S780	4/D1 *(35)*	Disrupts binding to E2F *(35)*	yes
S788	4/D1 *(32,36)*		
S795	4/D1 *(32,36)*; 2/E-2A *(32)*	Abolishes growth suppression *(36)*	yes
S807	4/D1 *(32)*	Decreases G1 arrest *(33,34)* Disrupts binding to Abl *(37)*	yes
S811	4/D1 *(32)*	Decreases G1 arrest *(33,34)* Disrupts binding to Abl *(37)*	yes
T821	2/E *(32)*; 2/A *(37)*	Decreases G1 arrest *(33,34)* Disrupts binding to LXCXE motifs *(32)*	yes
T826	4/D1 *(32,37)*	Disrupts binding to LXCXE motifs *(32)*	yes

pRb were originally made in the laboratory of Sibylle Mittnacht *(16)* but are now available for various sites and from different companies: pS249, pT356, pS612, pS780, pS807, pS811, pT821, pT826 (BioSource); pS249, pT356, pT373, pS780, pS795 (Santa Cruz Biotechnology). This panel of antibodies covers 10 of the 14 commonly phosphorylated sites in pRb (*see also* **Table 1**).

16. Proteins: The following colleagues kindly made available to us recombinant baculovirus preparations encoding human pRb (Barbara Driscoll, Children's Hospital Los Angeles); Cdk2, cyclin A, and cyclin E (David O. Morgan, University of California, San Francisco, CA); Cdk4 and cyclin D1 (Charles J. Sherr,

St. Jude's Children's Research Hospital, Memphis, TN). Expression vectors encoding PP1α were generated in NB's laboratory; cDNAs encoding mammalian PP1γ1 and PP1δ were kindly provided by Ernie Lee, Medical College of New York, Valhalla, NY.

3. Methods

3.1. Cell Synchronization

The protocols described here for cell synchronization are based on inducing specific cell cycle blocks and are guaranteed to work with the cell lines described here: MG63 human osteosarcoma cells and CV1-P monkey kidney cells. Although the principles of these techniques have proven effective in countless other cell types, the concentrations of the drugs and/or the length of exposure to them may have to be varied.

The success of any cell cycle experiment involving such synchronization procedures obviously depends on the degree of synchrony of a cell population. We recommend that with these or any other cell lines the cell cycle status of synchronized cells should always be verified by flow cytometric analysis of the DNA (also referred to as fluorescent-activated cell sorting, or FACS). This is typically being done by setting up sister cultures that are exposed to the same concentrations of the synchronizing agent as the experimental cultures. Detailed descriptions of methods related to cell synchronization and cell cycle analysis can be found in references ***(26)*** and ***(27)***.

3.1.1. CV1-P Cells

1. Synchronize cells in G0/G1 by incubation in methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum (FBS) for 72 h.
2. To obtain cells enriched in S phase, incubate G0/G1 cells for 16 h in DMEM containing 0.5% hydroxyurea and 10% newborn calf serum (NCS). This arrests the cells at the G1/S boundary. Then release the cells for 3 h into S phase by using medium plus NCS but without the hydroxyurea.
3. To obtain cells in M phase, incubate cells arrested at G1/S for 18 h with DMEM plus 10% NCS and 0.4 µg/mL nocodazole. Collect mitotic cells by a "shake-off."
4. Replate the cells in different dishes, incubate further for several hours, and collect cells at different time points.

3.1.2. MG63 Cells

1. Plate 1×10^6 cells in 75-cm^2 flasks (or approx 13,000 cells/cm^2) in RPMI-160 medium containing 0.5% FBS, and incubate for 48 h at 37°C. These cells should be arrested in G0/G1 (*see* **Note 2**).
2. To stimulate re-entry into the cell cycle, add fresh medium containing 10% FBS to the cells. These cells may be harvested for up to 16 h to obtain cells in various stages of the G1 phase.

3. To obtain a cell population arrested in late G1, incubate serum-deprived cells obtained in **step 1** for 24 h in the presence of fresh medium containing 10% FBS and 600 μ*M* mimosine (*see* **Note 2**).
4. To obtain a cell population arrested at G1/S, incubate serum-deprived cells obtained in **step 1** for 24 h in the presence of fresh medium containing 10% FBS and 2.5 μg/mL aphidicolin. For S-phase-enriched cells, wash aphidicolin-treated cells repeatedly with PBS to remove the drug and finally incubate for another 4 h in the presence of 10% FBS (*see* **Note 2**).
5. To obtain mitotic cells, incubate serum-deprived cells obtained in **step 1** for 30 h in the presence of fresh medium containing 10% FBS and 50 ng/mL nocodazole.

3.2. Cell Cycle-Dependent Dephosphorylation of pRb by PP1

The observation that the phosphorylation status of pRb is directly or indirectly controlled by PP1 was first reported in 1993 by several groups using different approaches ***(12,14,28)***. While these studies suggested that pRb undergoes dephosphorylation during mitosis, experiments with phosphatase inhibitors also indicated that PP1 activity might be required during G1 to maintain pRb in the dephosphorylated form ***(13)***. Apart from the dephosphorylation of pRb itself, there is also the interesting issue of complex formation between pRb and PP1. Studies in a number of cell types suggest that various forms of PP1 bind to pRb in a cell cycle-dependent manner ***(14,15,17,29–31)***, and that this binding involves the participation of a third protein ***(29)***. The question whether different PP1 isoforms dephosphorylate specific sites in pRb is important, because the phosphorylation of pRb at different sites has distinct functional consequences (examples are given in **Table 1**). Until recently, investigating the phosphorylation or dephosphorylation of pRb had to rely on techniques that require metabolic labeling of cells with rather large amounts of radioactivity. With the availability of phospho-specific antibodies to a large subset of pRb phosphorylation sites, this appears no longer necessary.

3.2.1. Dephosphorylation of pRb During Exit From Mitosis

1. Prepare mitotic CV1-P cells as described in **Subheading 3.1.1.**.
2. Release the cells from the nocodazole-induced mitotic block and collect them for up to 6 h.
3. Separate equal amounts of protein (100 μg) per time point via SDS-PAGE and transfer them to Immobilon-P membranes. Probe the membranes with the appropriate antibodies (*see* **Note 3** for our Western blotting protocol).
4. Incubate the membranes with either sc-102 (to quantitate the overall amount of pRb) or the various phospho-specific antibodies (use 1 μg of antibody per 100 μg of cellular protein) (*see* **Note 4**).

3.2.2. Specificity of PP1 Isoforms for Different pRb Phosphorylation Sites In Vitro

1. Express mammalian recombinant PP1 in *E.coli* and purify as described ***(38)***.
2. Use recombinant baculoviruses encoding pRb, Cdk2/cyclin A, Cdk2/cyclin E, or Cdk4/cyclin D1 to infect 10^7 Sf9 cells ***(18)***. A detailed protocol for generating pRb and Cdks from baculovirus infected insect cells can be found in references ***(39)*** and ***(40)***, respectively.
3. Lyse cells by incubating them for 10 min at 4°C in kinase reaction buffer and immunoprecipitate pRb from pRb-overexpressing cells as described in **Subheading 3.3.1.**. Retain a small portion of nonphosphorylated pRb as a control.
4. Mix the lysates from Cdk/cyclin-overexpressing cells with the immunoprecipitated pRb, add 1 m*M* ATP, and incubate for up to 45 min at 30°C with occasional mixing (*see* **Note 5**).
5. After the phosphorylation reaction, it is crucial to wash the protein G beads exhaustively (at least six times) with ice-cold EBC buffer to remove the protein kinases and ATP. Retain one-fifth of the sample to examine the efficiency of pRb phosphorylation.
6. Divide the remainder of sample into four equally sized aliquots. Incubate equal amounts of the sample with either approx 20 U of PP1α, PP1γ, or PP1δ individually, or approx 20 U of a mixture or all three isoforms at 30°C for 20 min.
7. Wash the dephosphorylated pRb again at least six times with ice-cold EBC buffer, and finally resuspend the sample in SDS sample buffer, separate by SDS-PAGE, and perform western blotting with the appropriate phospho-pRb antibodies (*see* **Notes 3** and **6**).

3.2.3. Specificity of PP1 Isoforms for Different pRb Phosphorylation Sites In Vivo

1. Synchronize CV1-P cells as described in **Subheading 3.1.1.**.
2. Lyse 5×10^5 cells by sonication in 1 mL EBC buffer supplemented with 1 µg/mL aprotinin and 1 µg/mL PMSF (*see* **Note 1**).
3. Preclear the lysates by adding 75 µL protein A Sepharose diluted 1:1 in NET-N.
4. Immunoprecipitate the PP1 isoforms from cells corresponding to 100 µg of protein.
5. Wash the immunoprecipitates three times with 1 mL NET-N and twice with phosphatase reaction buffer.
6. To assess the overall pRb-directed phosphatase activity of distinct PP1 isoforms, mix immunoprecipitated PP1 with ^{32}P-labeled immunocomplexed pRb (this can be prepared as described in **Subheading 3.3.1.**) and incubate at 30°C for 30 min.
7. Stop the reaction by adding 2X SDS sample buffer, boil for 10 min, spin for 1 min at full speed, separate labeled proteins by SDS-PAGE, and visualize by autoradiography (*see* **Note 7** for a recommendation regarding quantitation of band intensities).

8. To assess the activity of individual PP1 isozymes against specific pRb phosphorylation sites, mix immunoprecipitated PP1 isoforms with lysates obtained from mitotic cells, carry out the phosphatase reaction as described previously, and analyze the reaction products by western blotting with antibodies to the pRb phosphorylation sites in question. It is worth mentioning that in our study, PP1 isoforms isolated from G1 or M phases markedly differed in their ability to dephosphorylate certain sites (pS807 and pT356) in pRb ***(16)***.

3.3. Apoptosis-Associated Dephosphorylation of pRb by PP1

Several lines of evidence suggest that pRb plays a negative role in apoptosis (for reviews, *see* **refs. *5,41***). Consistent with this function, pRb is being destroyed by a caspase in response to apoptotic stimuli ***(42–44)***: the major cleavage reaction liberates a 5 kDa fragment from the C-terminus of pRb. More recent results suggest that for pRb to be proteolyzed, it has to be present in dephosphorylated form ***(18)***. The reader will recall that traditionally, countless laboratories assessed the overall phosphorylation state of pRb by simple Western blot, assuming that phosphorylated pRb produces a band corresponding to 110 kDa, whereas dephosphorylated pRb produces a band of 105 kDa. Therefore, the larger of the two pRb fragments resulting from caspase-mediated cleavage can very easily be confused with the dephosphorylated version of pRb. To distinguish between proteolysis and dephosphorylation, a combination of two antibodies may be used. One of these (sc-50) reacts with the C-terminus of pRb and will thus reveal the amount of intact pRb, regardless of the phosphorylation state.

3.3.1. Analysis of pRb Following Induction of Apoptosis in Synchronized HL-60 Cells

1. To obtain HL-60 cells synchronized at the G1/S boundary, incubate the cells for 16 h in RPMI-1640 medium supplemented with 10% FBS and antibiotics (100 U/mL of penicillin G and 100 µg/mL of streptomycin) and 5 µg/mL aphidicolin.
2. Wash the cells repeatedly to remove aphidicolin. To induce the apoptotic response, add araC to a final concentration of 10 µ*M*. Incubate the cells for 2 h.
3. Preincubate 1×10^6 cells with 2 mL of phosphate-free medium for 30 min, then replace with 2 mL of fresh phosphate-free medium containing 200 µCi ^{32}P-inorganic phosphate and incubate for 2 h in the presence or absence of araC (*see* **Note 8**).
4. Remove the drug and the remaining radioactive label by washing the cells at least four times in complete medium. Then incubate the cells further in complete medium. Collect the cells at different time points for up to 6 h, and wash at least four times with PBS (*see* **Note 9** for a comment on the time course of pRb dephosphorylation). Set aside a small aliquot for protein determination. Cells can be stored frozen at –20°C at this stage.
5. Using the small aliquot from **step 4**, lyse the washed cells in EBC buffer and determine the protein concentration. Then lyse the frozen cells as before, and if

necessary, increase the protein concentration to approx 1 mg/mL by adding BSA (*see* **Note 10**).

6. To preclear the lysate, add 1 µg of mouse IgG plus a 1:1 mixture of 40 µL of protein G-sepharose:EBC buffer in 1 mL of EBC buffer to 1 mg of protein.
7. Centrifuge the suspensions and mix the supernatants with 2 µg of the pRb antibody sc-102; rotate overnight at 4°C. Add 20 µL of protein G-Sepharose beads and incubate for another 4 h. Centrifuge the beads and wash at least six times with EBC buffer at 4°C.
9. Separate the immunocomplexed pRb via SDS-PAGE.
10. Carry out immunoblotting with the two pRb antibodies sc-102 and sc-50 (*see* **Note 3**). sc-102 reacts with all of the pRb present. sc-50 reacts only with intact pRb. A shift to a smaller apparent molecular mass as revealed by sc-102 thus indicates dephosphorylation, as long as sc-50 produces pRb bands of the same intensity.

3.4. Cell Cycle-Dependent Phosphorylation of PP1 by Cdks

Shortly after mammalian PP1α was found to be inhibited in vitro by Cdk-mediated phosphorylation at a unique residue, Thr320 ***(45)***, it was shown that the *S. pombe* homolog of PP1, dis2, is also phosphorylated at the corresponding Thr in vivo at the onset of mitosis ***(46)***. Using an antibody specific for the phosphorylated form of PP1α, Kwon et al. demonstrated a few years later that mammalian PP1α undergoes mitotic phosphorylation at Thr320 ***(25)***. Our finding that a phosphorylation-resistant, constitutively active PP1α mutant causes pRb-dependent cell cycle arrest in G1 ***(47)*** suggested that PP1α is also phosphorylated in late G1. And indeed, Thr320 in pRb-associated PP1α was shown to be phosphorylated before the G1/S transition **(15)**. The techniques used in this study form the basis for the methods described herein.

3.4.1. Immunoprecipitation of PP1 From Radioactively Labeled Cells

1. Plate and synchronize MG63 cells in 75 cm^2 flasks as described in **Subheading 3.1.2.**.
2. Once the desired point in the cell cycle has been reached, preincubate the cells with 2 mL of phosphate-free medium containing 5% FBS for 30 min, then replace with fresh phosphate-free medium containing 1.0–1.5 mCi of $^{32}P_i$ per 1×10^6 cells and incubate for 2.5 h (*see* **Note 8**).
3. To harvest the cells, remove the radioactive medium and discard it into appropriately shielded radioactive waste containers. Rinse the monolayer of cells three times with serum-free medium and discard as above. Lyse the cells in the flask with 1.2 mL ice-cold RIPA buffer. This is sufficient to just cover the cells evenly (if, for any reason, the size of the flask or well has to be changed, you should aim for at least 15 µL per cm^2). Leave on ice for 10 min, and then tilt the flask so that the liquid can be collected from one corner of the flask. Transfer lysates to Eppendorf tubes. If convenient, lysates can be stored at –20°C until further use.

4. Determine the protein concentration and adjust each sample to about 1 mg/mL of protein by adding BSA (*see* **Note 10**). Pre-clear the lysates by incubating them for 30 min on ice with 50 µL of a 1:1 suspension of protein A–Sepharose:RIPA buffer. Then remove the Sepharose A beads by a short centrifugation at 1500*g* for 2 min at 4°C and carefully transfer the supernatants to fresh tubes on ice. Discard the pellet into a radioactive waste container.
5. For immunoprecipitation, add a fresh 50 µL of Sepharose A suspension and the PP1α antibody Ab2 (8 µg) or the phospho-PP1α antibody Ab3 (4 µg). Rotate the samples end-over-end for 2 h at 4°C and collect the beads by centrifugation for 15 sec at 4°C at top speed (14,000*g*). Aspirate the supernatants with a 25-gage needle and discard into radioactive waste. Add fresh RIPA buffer, mix gently, and centrifuge. Repeat three times.
6. Finally, mix the beads containing radioactively labeled PP1 with 50 µL of 2X concentrated SDS sample buffer and boil for 5 min. Samples are now ready to be examined by SDS-PAGE.
7. With radioactively labeled samples, expose the dried gel to x-ray film and visualize phosphorylated PP1 by autoradiography. With nonlabeled samples, perform a Western blot with Ab3 (*see* **Notes 3** and **11**).

3.4.2. Co-Immunoprecipitation of PP1 and pRb From Nonlabeled Cells

1. Prepare cells as described in **Subheading 3.4.1.**, except that no metabolic labeling of cells is required.
2. For immunoprecipitation of pRb from 2×10^6 MG63 cells, use 10 µg of sc-102; otherwise follow the protocol described in **Subheading 3.3.1.**.
3. Separate the immunocomplexes via SDS-PAGE, and carry out Western blotting (*see* **Note 3**) with three antibodies—sc-102 for pRb, Ab1 for PP1α, and Ab3 for phosphorylated PP1α (*see* **Note 12**).

4. Concluding Remarks

Although a cell cycle function for pRb has been known since 1989, the study of this important protein is still attractive to many laboratories. One major reason for the continued interest in pRb is the intriguing idea to target pRb for novel cancer therapies, and another may be that the presumably many functions and interactions of pRb still pose severe puzzles. It is obvious that, therefore, pRb is being studied in a wide variety of model systems and techniques. In this chapter, we have described several approaches to study the interaction between PP1 and pRb, which has been neglected so far. Given that further conceptual and technical breakthroughs (such as the realization that pRb and PP1 are involved in apoptosis, and the availability of phosphospecific antibodies for the majority of sites phosphorylated in pRb) will occur, new and important questions will undoubtedly arise. Therefore, the methods described herein have to be considered as guidelines.

Table 2
Preparation of Concentrated Stock Solutions

Reagent	Concentration	Times final	Solvent	Store at
Aprotinin	2 mg/µL	1000	H_2O	–20°C
Benzamidine	1 *M*	1000	H_2O	RT
Leupeptin	50 µg/mL	100	H_2O	–20°C
Pepstatin	1 mg/mL	1000	H_2O	–20°C
PMSF	100 m*M*	1000	DMSO	RT
EDTA	0.5 *M*	1000	H_2O	RT
NaF	1 *M*	10–40	H_2O	RT
Na_3VO_4	100 m*M*	10	H_2O	RT

5. Notes

1. For all supplements to be added freshly it is most convenient to prepare concentrated stock solutions as follows, and according to **Table 2**:
2. Serum deprivation, mimosine, and aphidicolin typically arrest eukaryotic cells with the same DNA content: FACS analysis reveals that these cells contain a G1 amount of DNA—i.e., these cells have not yet replicated their DNA. FACS analysis of cells that have been released from these various blocks for 4–24 h reveals that these three conditions are not equivalent. Thus, cells released from serum deprivation need the most time to reach S phase, cells released from mimosine need a few hours to reach S phase, whereas aphidicolin-treated cells enter S phase almost immediately. In summary, serum-deprived cells are believed to be in G0/G1. Mimosine—chemical name: α-amino-β-(*N*-[3-hydroxy-4-pyridone])propionic acid—is a plant amino acid that arrests cells late in G1 ***(48)***, and its mechanism of action may involve induction of $p27^{Kip1}$ ***(49)***. Aphidicolin arrests cells at the G1/S boundary by inhibiting DNA polymerase ***(50)***.
3. We have used the following protocol for Western blotting experiments involving pRb and PP1 in the past: After electrophoresis, transfer proteins to Immobilon-P membranes at a constant voltage of 30 V. Using the Novex Minicell, this should be accomplished in 1–2 h. Incubate the membrane overnight in 5% non-fat dry milk in TBS (25 m*M* Tris-HCl, pH 8.0, 137 m*M* NaCl, 2.7 m*M* KCl). Incubate the membrane for 2 h with the primary antibodies diluted in TBS containing 0.2% Tween-20 (TBS-T) and 2% BSA. If convenient, this step can be performed overnight as well. Wash the membrane with TBS-T three times for 15 min, and incubate for 1 h with the secondary antibody diluted with 2% BSA in TBS-T. Wash again three times for 15 min and visualize immunoreactive bands with the ECL reagents (Amersham). All incubations are at room temperature.
4. Make sure to harvest sufficient sample so that each pRb phosphorylation site can be examined individually in a separate western blotting experiment. It is not recommended to "strip" the membranes and reprobe with another pRb antibody.

5. The aim of this experiment is to generate pRb that has been phosphorylated by a distinct agent or a "cocktail" of Cdk/cyclin complexes. Therefore, the Cdk/cyclin complexes should be in excess of pRb to shorten the reaction time, while the overall amounts of protein should be adjusted such that there is sufficient sample to visualize by western blotting. Assuming that (1) 50 ng of pRb phosphorylated in a particular site is enough for this purpose (*see also* **Note 5**), (2) this experiment involves at least 10 phospho-specific antibodies, and (3) the phosphorylation reactions proceed to only 50% completion, one would have to use at least 1 µg of pRb per PP1 isoform to be tested in this experiment.
6. Originally, the phosphorylation of pRb was performed in the presence of [γ-^{32}P]ATP ***(51)***. In that case, in order to address the question of site specificity, ^{32}P-labeled pRb had to be digested by a protease (we used chymotrypsin successfully [Barbara Driscoll and Norbert Berndt, unpublished]), and the peptides were separated by two-dimensional phosphopeptide mapping. Nowadays however, while it may still be a good idea to include radioactively labeled ATP to assess the overall level of phosphorylation, the phosphorylation site-specific antibodies are more convenient to address the issue of site specificity.
7. If necessary, the band intensities of the Western blot can be analyzed with a scanning densitometer (Hoefer Scientific Instruments) and quantitated with GS370 v.3 software. This approach should yield an accurate assessment of the time course of pRb dephosphorylation for different sites.
8. Phosphate-free medium is easily available commercially—for example, from Irvine Scientific, Santa Ana, CA. To obtain phosphate-free FBS, dialyze the required amount against an excess of phosphate-free medium overnight. To minimize the pipetting steps and, therefore, possible contamination of the workspace with radioactivity, it is best to prepare metabolic labeling experiments as follows: Determine the number n of samples/wells/flasks, the volume V of the medium that is needed per sample, and the amount of radioactivity R needed per sample. Prepare $(n + 1) \times V = a$ mL of medium, add $(n + 1) \times R = b$ µCi, and mix well. Then add the predetermined volume of medium containing the radioactivity to each sample.

   As already pointed out in **Subheading 3.1.**, in order to double-check the degree of synchrony of metabolically labeled cells, it is advisable to set up identically treated sister cultures (except that radioactive label is omitted) and examine them by FACS analysis.
9. This protocol describes the procedure for araC-induced apoptosis in HL-60 cells synchronized at the G1/S boundary. In this case, dephosphorylation of pRb was maximal 45 min after terminating the exposure to araC, whereas caspase-mediated cleavage of pRb was not detectable until 105 min after terminating the exposure to araC ***(18)***. Assuming that this protocol may be of interest for researchers studying other experimental models of apoptosis (cell types, cell cycle phase, apoptosis-inducing drugs, "death substrates" that are also substrates for PP1), these times may vary. Therefore, we recommend performing a time course of dephosphorylation of the protein in question.

10. In very dilute protein solutions, proteins often adsorb to tube walls, or aggregate, or become degraded. The addition of BSA is recommended to protect precious sample proteins from these unspecific processes. If the protein concentration is higher than 1 mg/mL, then this is not necessary; instead dilute all samples to 1 mg/mL with the appropriate buffer (e.g., the one the samples are stored in).
11. It would be of interest to also determine the phosphorylation of the other two PP1 isozymes, γ and δ. As phosphorylation site-specific antibodies for these are not yet available, an alternative approach would be to immunoprecipitate these PP1 isoforms, transfer them to membranes, and then probe them with phosphothreonine antibodies (PharMingen).
12. It is recommended to run this experiment in duplicate. After electrotransfer of the proteins, cut the membranes in two pieces along the BSA molecular weight standard (66 kDa), with pRb (M_r = 110 kDa) on the upper half and PP1 (M_r = 37 kDa) on the lower half. Done this way, you can avoid "stripping" membranes and reprobing them with a different primary antibody. Probe the upper halves with sc-102, and the lower halves with Ab1 and Ab3, respectively.

Acknowledgments

The authors thank Angus C. Nairn for providing the phosphorylation site-specific antibody to PP1α and Sibylle Mittnacht for providing phosphorylation site-specific antibodies to pRb. N. B. would like to thank Cathy Liu and Rui-Hong Wang for their contributions to this work. This work was supported in part by grants from the NIH (R01 CA54167) and the T. J. Martell Foundation (to N. B.).

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3

Generation of p53 Target Database Via Integration of Microarray and Global p53 DNA-Binding Site Analysis

Suxing Liu, Asra Mirza, and Luquan Wang

Summary

The completion of the human genome sequence and availability of cDNA microarray technology provide new approaches to explore global cellular regulatory mechanisms. Here we present a strategy to identify genes regulated by specific transcription factors in the human genome, and apply it to p53. We first collected promoters or introns of all genes available using two methods: GenBank annotation and a computationally derived transcript map. The "FindPatterns" program is then used to search sequences in regulatory regions that match the p53 DNA-binding consensus sequence, resulting in the p53 Target Database. This database collects human genes that have at least one p53 DNA-binding sequence in their regulatory region. cDNA microarray was also used to identify genes that respond to p53 at a genomic scale. Integration of the microarray data and the p53 Target Database should greatly enrich direct p53 target genes. Taqman® analysis and quantitative chromatin immunoprecipitation analysis are used to validate the *in silico* prediction and microarray data. Enrichment factor analysis is used to demonstrate that *in silico* prediction greatly enriches for genes that are transcriptionally regulated by p53 and assists us to identify other signaling pathways that are potentially connected to p53. The approaches can be extended to other transcription factors. The methods shown here illustrate a novel approach to the analysis of global gene regulatory networks through the integration of human genomic sequence information and genome-wide gene expression analysis.

Key Words: p53; transcription; DNA binding; microarray; bioinformatics; Taqman® analysis; chromatin immunoprecipitation.

1. Introduction

The publication of the nearly complete human genome sequence has marked a significant milestone in biology. The development of high-density cDNA microarray technology is also having an increasing impact as a direct result of the Human Genome Project, owing to the increasing collection of genes, or expressed sequence tags (ESTs). Integration of bioinformatic methods with

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

high-throughput gene-expression analysis provides new ways to analyze global cellular regulatory mechanisms. In this chapter, we describe a strategy to identify genes regulated by specific transcription factors in the human genome, using a combination of computational methods and genome-wide expression analysis. We have used the tumor suppressor gene product p53 as the first example. The extension of these methods to other transcription factors is straightforward.

The p53 protein is a tumor suppressor involved in regulating cell cycle progression and apoptosis ***(1,2)***. Mutations in p53 are the most frequent alterations observed in cancers. p53 acts as a sequence-specific DNA-binding transcription factor that binds to a sequence with two copies of the decamer motif 5'-RRRCWWGYYY-3', separated by 0 to 13 bp of random sequence, where R = G or A, W = T or A, Y = C or T ***(3)***. Although the number of identified p53 target genes keeps growing, it is conceivable that many p53 target genes remain to be identified. Knowledge of genes that respond to p53 and contain one or more p53 consensus binding sequences in their regulatory regions will greatly assist the identification of direct p53 target genes, some of which could be potential targets for cancer chemotherapeutic drugs.

1.1. Global p53 DNA-Binding Site Analysis

The binding of a transcription factor to a specific, often short DNA sequence in the promoter region of a target gene is necessary for transcriptional regulation. The general approach we employed is to first identify transcription factor DNA-binding site(s) in the genome. However, many potential binding sites may be found randomly throughout the genome due to their short and degenerate nature. This problem can be partially circumvented by searching a specialized human promoter database. A flow chart, shown in **Fig. 1**, illustrates an *in silico* analysis to identify the p53 target genes via detection of potential p53 DNA-binding sites in GenBank™. The first step is to collect human regulatory region sequences of all genes available from GenBank™. Two methods can be used: GenBank™ annotation and a computationally derived transcript map ***(4)***. The first method takes advantage of annotated features available in GenBank™ entries to identify the regulatory region of human genes, which have mostly been characterized experimentally (*see* **Subheading 3.1.** and **Fig. 1**). It has been shown that wild-type p53 can mediate sequence-specific transcriptional activation through an internal promoter. For instance, functional p53 DNA-binding sites in the third intron of the *GADD 45* gene and the first intron of Hdm2 are necessary for their transcriptional activation ***(5,6)***. Intronic sequences in GenBank™ should thus be included in the search for p53 DNA-binding sequences. A subset of promoters and introns should be taken and confirmed manually for the accuracy of the collection. Our study ***(4)*** indicated that there were 6541

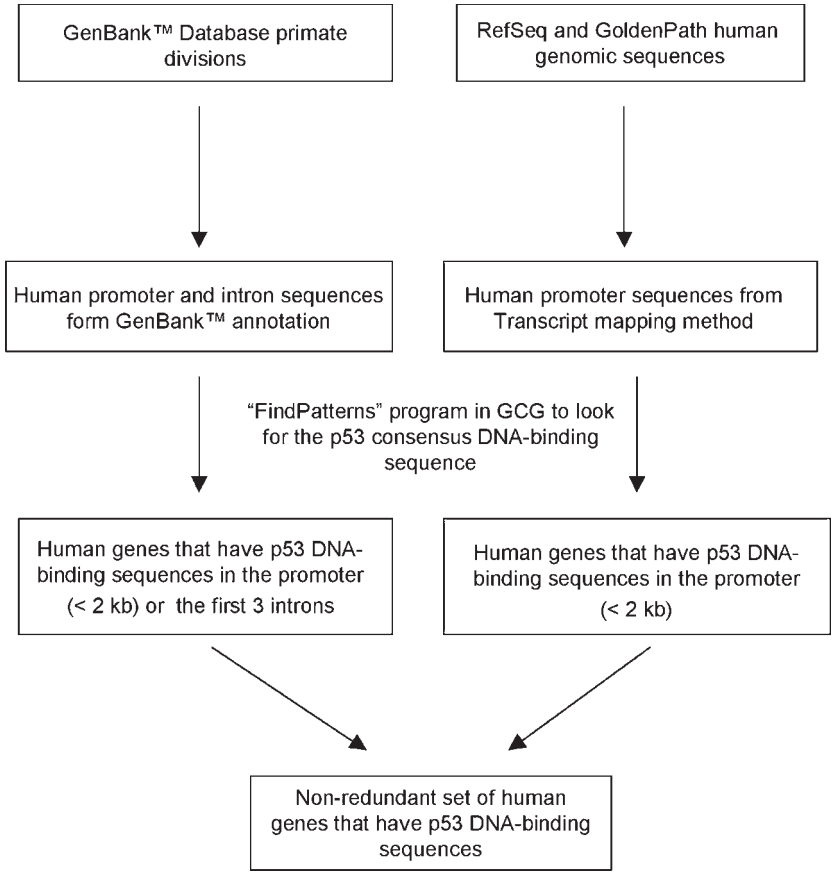

Fig. 1. A flow chart of *in silico* global p53 DNA-binding site analysis.

annotated human 5'-flanking sequences and 24,659 annotated human intronic sequences from GenBank™ Release 120 (October 2000). Alternatively, the Eukaryotic Promoter Database (EPD) could be used. The EPD has a collection of experimentally characterized eukaryotic Poly II promoters *(7)*. As of February 2003 (Release 73), there were 1871 human promoters. The EPD database is available at website: http://cmpteam4.unil.ch.

Only a small portion of the gene structures in the human genome have been experimentally characterized, hindering the genome-wide studies. As the complete human genome sequence becomes available, the second method, which maps transcripts onto a genomic template to locate the promoter sequence, will greatly extend promoter collection (*see* **Note 1**). Human mRNA sequences are first extracted from GenBank™ and mapped to the genomic sequences

(*see* **Subheading 3.2.**). Both finished and draft human genomic sequences were used as templates to identify or to extend the 5'-flanking region of many existing mRNA sequences for which there was either no annotated promoter sequence or short promoter sequence in their GenBank™ records. The *in silico* prediction should be evaluated by using a sample of promoters annotated in GenBank™ as "internal controls." In our previous study, we took 150 promoters annotated in GenBank™. 132 (88%) were perfectly predicted by the transcript mapping. The remainder (12%) differed from GenBank™ annotated promoters owing to either a different transcript start site of the annotated gene due to gene duplication, or a 5' alternative splice variant ***(4)***.

The second step is to search for matched p53 consensus DNA-binding sequence(s) in these regulatory regions. A number of programs (FindPatterns, ProtfileSearch, MatInspector, and PatScan, and so on) have been well established for searching nucleotide-sequence databases for matches to short sequences, including transcription factor binding sites ***(8)***. However, only FindPatterns allows us to include a flexible insertion in the search (*see* **Note 2**). Therefore, FindPatterns was used to search for p53 consensus sequences with an insertion of 0 to 13 random nucleotides between two p53 decamers (*see* **Subheading 3.3.**). The efficiency of computational analysis should be evaluated using known functional p53 response elements within the human genome. As a result, two sets of genes are generated which have at least one p53 consensus DNA-binding sequence in their regulatory region: one from GenBank™-annotated promoters or introns, the other from the predicted promoter sequences mapped by transcripts. These are further collapsed to yield a nonredundant set of genes (*see* **Subheading 3.3.**), designated as the p53 Target Database, available on the website of *The Journal of Biological Chemistry* at http://www.jbc.org/ ***(4)***.

To validate the *in silico* prediction, a small set of genes can be randomly selected and tested for their p53 responsiveness using Taqman® analysis (*see* **Subheading 3.4.**). Taqman® analysis is a sensitive and powerful tool for quantification of steady-state RNA levels, which permits the analysis of a large number of samples with high sample-to-sample reproducibility ***(9)***. This method is well suited for validation of the *in silico* prediction and microarray data described in **Subheading 1.2.**. In our previous study, of 13 genes randomly selected from the p53 Target Database for Taqman® analysis, 11 were responsive to p53 expression ***(4)***.

1.2. Microarray Analysis of Human p53-Responsive Genes

Comparable to the global p53 DNA-binding site analysis, high-density DNA microarray technology provides a new opportunity to conduct genome-wide analysis of p53-responsive genes. p53 specifically recognizes and binds to

regulatory sequences of target genes, whose transcription is up- or down-regulated as a consequence. A well-controlled model system is essential for microarray-based expression analysis. In our system, we used an adenoviral vector to mediate transduction of wild-type p53. The human ovarian tumor cell line 2774qw1, which carries mutant p53, was chosen based on its high infectivity with adenovirus ***(10)***. The concentration of rAd-p53 used was optimized to ensure the greatest pro-apoptotic effect, while the same concentration of rAd-empty vector showed minimal viral effect ***(11)***. To correlate expression profiles with p53 biological activity, temporal profiles of apoptotic response and cell cycle progression was monitored on infected cells at the same time points where RNA was isolated for microarray hybridization ***(11)***. A pilot experiment was carried out using Taqman® analysis (*see* **Subheading 3.4.**) with 5 apoptosis- or cell cycle-related genes ($p21^{WAF1/Cip1}$, caspase 3, bax, cyclin B1, and bcl-2) ***(11,12)***. Since we wished to identify p53-responsive genes whose RNA levels varied during the process of apoptosis and cell cycle arrest, we collected mRNA ***(11)*** at six time points (4, 8, 12, 16, 20, and 24 h post infection) for microarray hybridization (*see* **Subheading 3.4.**). To obtain an overall profile of gene expression, a total of six Incyte GeneAlbum microarrays (approx 60,000 cDNAs), representing 33,615 individual genes, were screened (*see* **Subheading 3.5.**). Using this approach, we have identified 1501 genes that respond to p53 ***(11)***.

1.3. Integration of the Microarray Data With the Global p53 DNA-Binding Site Analysis

Although microarray technology offers a relatively simple approach for directly examining thousands of genes at once, a major limitation of the technique is that the analysis yields a mixed readout of direct and indirect p53 target genes. The major obstacle to the *in silico* prediction of transcription-factor binding sites is that it contains too little diagnostic information to distinguish between functional transcription-factor DNA-binding sites and random occurrences of DNA sequences. Defining the overlap between p53 responsive genes identified by microarray with p53 target genes predicted *in silico* should greatly enrich direct p53 target genes ***(4,11,12)***. The integration is performed at the transcript level (*see* **Subheading 3.6.**). Transcripts with a defined promoter are used to match the transcripts on microarrays, and consequently a relationship table can be built between a promoter and a transcript on microarrays (*see* **Note 3**). By integration of the microarray data with the p53 Target Database, it was found that 361 p53-responsive genes were present in the p53 Target Database, suggesting that the observed p53 responsiveness of these genes may be mediated directly by sequence-specific p53 binding ***(11)***.

The interactions of transcription factors with DNA have been extensively studied in vitro ***(13)***. However, it is crucial to know whether p53 does bind in cells to DNA-binding sites predicted *in silico* and to measure the level of occupancy on these sites. The most direct way is the use of the quantitative chromatin immunoprecipitation (ChIP) technique (*see* **Subheading 3.7.**). The ChIP assay utilizes formaldehyde to covalently attach transcription factors to their natural DNA-binding sites in vivo. DNA sequences are identified by extracting the crosslinked protein–DNA complexes from cells, immunoprecipitating with antibodies that recognize p53, and analyzing the co-precipitated DNAs by Taqman® analysis. Using this technique, we showed that p53 occupies the predicted binding sites in vivo in 6 out of 6 of the p53-activated genes tested ***(4)***, and 8 out of 10 of the p53-repressed genes tested ***(11)***.

Furthermore, we developed an enrichment factor analysis ***(4)*** to estimate the enrichment of p53 target genes by integration of microarray data and the binding site analysis (*see* **Subheading 3.8.**). The enrichment factor (EF) is a parameter that estimates the significance of an observed degree of p53 target genes predicted *in silico* from pools of genes which show differential expression in microarray experiments ***(4)***. T0 is the ratio of genes directly regulated by p53 in the human genome to those that are not regulated by p53. T1 is a similar ratio for the genes in the p53 Target Database. The EF is defined as the comparison of these ratios (EF = T1/T0), such that an EF >1 indicates enrichment for genes regulated by p53 in the p53 Target Database (*see* **Note 4**). Since the actual number of genes in the human genome is not known, we use the cDNA microarray to represent a large random subset of the complete genome and use hybridization experiments to determine the p53-responsive genes within the subset. Therefore, the lower boundary for the EF can be estimated from the following equation:

$$EF > [R_{target}/(T_{target} - R_{target})]/[R_{random}/(T_{random} - R_{random})]$$

where R_{target} is the number of p53-responsive genes observed in the microarray experiments that are in the p53 Target Database, T_{target} is the total number of genes on the microarrays that are in the p53 Target Database, R_{random} is the number of p53-responsive genes observed in microarray experiments, and T_{random} is the total number of genes on the microarrays.

1.4. Identification of Signal Transduction Pathways That Are Potentially Connected to the p53 Pathway

The p53 Target Database could not only predict direct p53 target genes, but also help to identify other signaling pathways that are potentially connected to the p53 pathway. The rationale is that if p53 mediates some of the effects of certain signaling pathways, the differential expression data derived from the

stimulation of that pathway would give increased EF values (since the expression of p53 target genes would be induced or repressed). To test this hypothesis, we used a set of microarray data derived from two human normal mammary epithelial cells with the addition of TGF-β1 for 24 h; withdrawal of EGF for 2 d; addition of IFN-α for 24 h; and addition of IFN-γ for 24 h, respectively ***(14)***. The microarray data were integrated with the p53 Target Database (*see* **Subheading 3.6.**). The EF analysis (*see* **Subheading 3.8.**) gave a relatively high EF (2.3) to TGF-β1 treatment (24 h), while EFs of other treatments were close to background (EF = 1). The majority of genes affected by TGF-β1 (16 out of 22) contained p53 DNA-binding sequences in their regulatory region. This suggested that p53 might be a mediator of the growth-suppressive effects of TGF-β1 ***(4)***.

1.5. Applications to Other Transcription Factors of Interest

The methods described here can be applied to other transcription factors of interest. How well the consensus DNA-binding sequence of the transcription factor is defined is a key element leading to the success in identifying target genes. For example, forkhead transcription factor plays a critical role in cell survival signaling ***(15)***. We aligned six functional forkhead transcription factor binding sequences and derived a consensus sequence for forkhead transcription factor binding, WAARYAAAYW (W = A or T, R = A or G, Y = C or T). A similar approach was then applied to the forkhead transcription factor consensus DNA-binding sequence as described in **Subheadings 3.1., 3.2.,** and **3.3.**. The search yielded 437 human genes having at least one forkhead transcription factor binding site in the promoter region. Furthermore, TGF-β2 was selected and confirmed experimentally as a transcriptional target of forkhead transcription factor ***(15)***.

Another set of microarray experiments was performed with mRNA samples isolated from the human ovarian tumor cell line 2774qw1 at 4, 8, 12, 16, 20, and 24 h post infection with 10^{10} particles/mL rAd-p21 or rAd-empty vector. 159 genes showed differential expression in response to $p21^{WAF1/Cip1}$ expression ***(10)***. Because $p21^{WAF1/Cip1}$ is not a transcription factor, other transcription factors must be responsible for the observed differential expression of the genes. Similarly, the extensive search was performed with all the consensus DNA-binding sequences of human transcription factors from the TRANSFAC database ***(16,17)*** in the promoter regions of p21-responsive genes as described in **Subheadings 3.3.** and **3.6.**. EF analysis was then performed as described in **Subheading 3.8.** The results suggest E2F transcription factor family members (out of 759 human transcription factors analyzed) are the most likely candidates responsible for the differential expression of genes in response to $p21^{WAF1/Cip1}$ ***(10)***. This agrees with the theory that

induction of $p21^{WAF1/Cip1}$ results in the inhibition of cyclin/CDK activity, leading to the dephosphorylation of the retinoblastoma protein and inhibition of E2F-mediated transcription ***(18)***.

2. Materials

2.1. Electronic Resources

1. GenBank™ primate divisions—ftp://ftp.ncbi.nlm.nih.gov/genbank/.
2. Human mRNA RefSeq database—ftp://ftp.ncbi.nlm.nih.gov/refseq.
3. EPD database—http://cmpteam4.unil.ch.
4. p53 Target Database at *The Journal of Biological Chemistry*—http://www.jbc.org/.
5. Human working draft genomic sequences through the University of California at Santa Cruz—http://genome.ucsc.edu.

2.2. Hardware

The information system described in this chapter can be set up on most UNIX-based machines (Sun, HP, SGI, and Compaq). One setup example: Sun Microsystems® Enterprise 6500, 8 GB of memory, 100 GB of hard disk space, and SunOS 5.7 operating system.

2.3. Software

1. Non-commercial software described in this chapter is written using PERL 5.0 (http://www.perl.org) within a UNIX operating system.
2. A relational model database is used to organize all the sequence and analysis results—for example, Oracle (http://www.oracle.com), Sybase (http://www.sybase.com), or MySQL (http://www.mysql.com).
3. FindPatterns program (Wisconsin Sequence Analysis Package, Genetics Computer Group, Madison) is used for p53 DNA-binding sequence search.
4. Sequence alignment software AAT (Michigan Technological University, Houghton, Michigan) ***(19)*** is used for transcript mapping.
5. RepeatMasker (http://ftp.genome.washington.edu/RM/RepeatMasker.html) is used for screening and masking interspersed repeats.
6. PHRAP (http://www.phrap.org) is used for DNA sequence assembling.
7. The NCBI BLAST package is used for homology search and can be downloaded through FTP at ftp://ftp.ncbi.nlm.nih.gov/blast/.

2.4. Cell Line

The cell line, 2774qw1, is a single clone derived from the human ovarian cancer cell line 2774 ***(10)***, which was obtained from the American Type Culture Collection (ATCC). Culture conditions followed the instructions from ATCC.

2.5. Taqman® Analysis

1. MicroAmp® Optical 96-Well Reaction Plate (Cat. no. N801-0560; PE Applied Biosystems, CA).
2. MicroAmp® Optical Caps (Cat. no. N801-0935; PE Applied Biosystems).
3. Taqman EZ RT PCR Kit (Cat. no. N808-0236; PE Applied Biosystems).
4. Taqman® Universal PCR Master Mix (Cat. no. 4318157; PE Applied Biosystems).
5. ABI Prism® 7700 Sequence Detection System (PE Applied Biosystems).

2.6. Quantitative Chromatin Immunoprecipitation Analysis

1. Branson Sonifier 450 (Branson, CT).
2. Suspension buffer: 100 m*M* NaCl, 50 m*M* Tris-HCl (pH 8.1), 5 m*M* EDTA (pH 8.0), 0.02% NaN_3 (Cat. no. S2002; Sigma, MO). Filter and store at room temperature.
3. 0.5% SDS lysis buffer: 100 m*M* NaCl, 50 m*M* Tris-HCl (pH 8.1), 5 m*M* EDTA (pH 8.0), 0.02% NaN_3, 0.5% SDS (sodium dodecyl sulfate, Cat. no. 1666 924; Boehringer Mannheim, IN). Filter and store at room temperature.
4. 5% Triton dilution buffer: 100 m*M* Tris-HCl (pH 8.6), 100 m*M* NaCl, 5 m*M* EDTA (pH 8.0), 0.02% NaN_3, 5.0% Triton X-100 (Cat. no. BP151-500; Fisher). Filter and store at room temperature.
5. Mixed micelle wash buffer: 150 m*M* NaCl, 20 m*M* Tris HCl (pH 8.1), 5 m*M* EDTA (pH 8.0), 5.2% sucrose, 0.02% NaN_3, 1% Triton X-100, 0.2% SDS. Filter and store at room temperature.
6. LiCl/detergent wash: 0.5% deoxycholic acid (Cat. no. D6750; Sigma, MO), 1 m*M* EDTA (pH 8.0), 250 m*M* LiCl (lithium chloride, Cat. no. L7026; Sigma, MO), 0.5% NP40 (Cat. no. 1754 599; Roche, IN), 10 m*M* Tris-HCl (pH 8.0), 0.02% NaN_3. Filter and store at room temperature.
7. Buffer 500: 0.1% deoxycholic acid, 1 m*M* EDTA, 50 m*M* HEPES (pH 7.5), 500 m*M* NaCl, 1% Triton X-100, 0.02% NaN_3. Filter and store at room temperature.
8. IP buffer: 1 volume 0.5% SDS lysis buffer and 0.5 volume of 5% Triton dilution buffer. Mix before use.
9. TBS: 20 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl.
10. Proteinase K solution: 0.04 mg/mL glycogen (Cat. no. 10814-010; Gibco, NY), 0.4 mg/mL proteinase K (Cat. no. 1 373 196; Roche, IN) in TE buffer (pH 7.6) (Cat. no.1666 819; Boehringer Mannheim, IN). Prepare immediately before use.
11. Glycine (Cat. no. G8790; Sigma, MO).
12. Complete protease inhibitor cocktail tablet (Cat. no. 1836 145; Roche, IN).
13. Protein A beads (Cat. no. 16-157; Upstate Biotechnology, VA).
14. Buffered phenol:chloroform:isoamyl alcohol (25:24:1, vol:vol:vol, pH 7.9) (Cat. no. 9730, Ambion, TX).

2.7. cDNA Microarrays

The cDNA microarrays (GeneAlbum® 1 to 6) containing approx 60,000 elements were derived from human expressed sequence tag (EST) clones from Incyte Pharmaceutical, Inc. (Palo Alto, CA).

```
LOCUS       HUMIL8R    13089 bp    DNA     PRI  22-APR-1998
DEFINITION  Human interleukin-8 receptor (IL8RB) gene, complete cds.
ACCESSION   M99412
NID         g576678
KEYWORDS    .
SOURCE      human.
ORGANISM    Homo sapiens; Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
            Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 13089)
  AUTHORS   Sprenger,H., Lloyd,A.R., Lautens,L.L., Bonner,T.I. and Kelvin,D.J.
  TITLE     Structure, genomic organization, and expression of the human interleukin-8 receptor B gene
  JOURNAL   J. Biol. Chem. 269 (15), 11065-11072 (1994)
  MEDLINE   94209273
FEATURES    Location/Qualifiers
     source     1..13089
                /organism="Homo sapiens"
                /db_xref="taxon:9606"
                /tissue_lib="lambda DASH"
     TATA_signal 1096..1103
     mRNA       join(1123..1453,4402..4453,9847..12243)
                /gene="IL8RB"
     gene       1123..12319
                /gene="IL8RB"
     exon       1123..1453
                /gene="IL8RB"
                /number=1
                /evidence=experimental
     intron     1454..4401
                /gene="IL8RB"
                /number=1
                /evidence=experimental
     exon       4402..4453
                /gene="IL8RB"
                /number=2
                /evidence=experimental
     intron     4454..9846
                /gene="IL8RB"
                /number=2
```

Fig. 2. Partial sequence file of the human interleukin-8 receptor (IL8RB) gene with the locus of HUMIL8R and the accession number of M99412.

3. Methods

3.1. Identification of the Regulatory Regions of Human Genes by GenBank™ Annotation

1. Extract human sequences from primate sequences in flat file format in GenBank™ primate divisions (the downloadable file name starts with "gbpri"), which are available at ftp://ftp.ncbi.nlm.nih.gov/genbank/ (*see* **Note 5**).
2. Uncompress the downloaded files using UNIX "gunzip" tool.
3. Use a PERL script to extract the following information from GenBank™ entries (**Fig. 2**): (1) Accession; (2) Organism; (3) Origin; (4) Definition; (5) Coordinates for all the features; (6) DNA Sequence.
4. Identify an annotated transcription start site by looking for the description in the features line (*see* **Fig. 2** and **Note 5**), such as, prim_transcript, mRNA, promoter, exon 1 (*see* **Note 6**), and TATA_signal. Extract up to 2000 bp sequences

5' upstream of the transcription start site as the promoter sequence (*see* **Note 7**). For sequences with multiple transcription start site annotations, save each promoter as a different record. Assign a unique ID for each promoter record. We used the template sequence accession followed by a serial number as the unique promoter ID. For example, three promoter records can be retrieved from GenBank™ accession AB038162 for TTF1, TTF2, and TTF3 genes respectively, and we can use AB038162_1, AB038162_2, and AB038162_3 as their promoter IDs. Record the downstream transcript sequence, and associate this transcript sequence with the unique promoter ID in a database table.

5. Parse the sequences with intron annotation (coordinates) to extract individual intron sequences (*see* **Note 6**). Assign a unique ID for each intron record. We used the template sequence followed by a serial number as the unique intron ID. Record the best mRNA sequence that is associated with the unique intron ID.

3.2. Identification of the Regulatory Regions of Human Genes by Transcript Mapping

1. Download human mRNA sequences from human mRNA RefSeq database in flat file format through NCBI at ftp://ftp.ncbi.nlm.nih.gov/refseq ***(22)***. To ensure that the 5' end of mRNA is close to the transcription start site, use only mRNAs that have 5' UTR sequences for transcript mapping.
2. To retrieve human genomic sequences, download a latest version of the human working draft through the University of California at Santa Cruz at (http://genome.ucsc.edu).
3. Map the transcripts on genomic templates to locate the first exon, using sequence-alignment software AAT ***(19)*** (*see* **Note 8**).
4. To reduce the number of undesirable matches owing to interspersed repeats, screen and mask the genomic DNA sequence for interspersed repeats using the RepeatMasker program (*see* **Note 9**).
5. Extract up to 2000 bp sequences 5' upstream of the first exon as the promoter sequence (*see* **Notes 7** and **10**). Assign a unique promoter ID for each promoter record. Use the combination of genomic template and mRNA accession as the unique promoter ID. For example, NM_018400_AC063921 refers to the transcript-mapped promoter for voltage-gated sodium channel beta-3 subunit, where "NM_018400" is the mRNA accession number, and "AC063921" is the accession number of the genomic template.

3.3. Identification of Human Genes Containing One or More p53 Consensus Sequences in Their Regulatory Regions

1. Use the FindPatterns program to search for the matched p53 consensus DNA-binding sequence, 5'-RRRCWWGYYY (N = 0-13) RRRCWWGYYY-3' ***(3)***. In this motif, R= G or A, W = T or A, Y= C or T, N = any base (*see* **Note 11**).
2. Use the 2000 bp DNA sequence 5' of the transcription start site and the first three introns collected by the methods described in **Subheadings 3.1.** and **3.2.** for database search (*see* **Note 7**).

3. Sort the matched sequences with no mismatch allowed for C at position 4 or G at position 7 (*see* **Note 12**).
4. For each individual binding site match, record the following information: promoter ID (intron ID), binding site location (relative to a transcription start site or 5' end of an exon), matched sequences, and mismatches. The resulting database ***(4)*** was designated the p53 Target Database, available through The Journal of Biological Chemistry at http://www.jbc.org.
5. Validate the computational analysis with known functional p53 response elements as "internal controls" (*see* **Note 13**).
6. Update regularly the list of human genes having p53 consensus sequences, since the number of sequences in GenBank™ keeps growing, and a new version of the GenBank™ database is released bimonthly.

3.4. Taqman® Analysis

3.4.1. Adenovirus Infection and RNA Isolation

1. To introduce exogenous p53, infect 2774qw1 cells with recombinant adenovirus expressing p53 (rAd-p53) or adenovirus containing empty vector (rAd-empty vector) for 1 h.
2. Remove the recombinant adenovirus and wash cells one to two times with PBS (Gibco, BRL). Refeed cells with fresh culture medium and culture at 37°C and 5% CO_2 for 2 to 24 h as indicated.
3. Harvest cells and isolate total RNA using a CsCl purification method ***(20)***. Use total RNA directly in quantitative RT-PCR analysis.
4. Carry out PolyA selection using QIAGEN Oligotex kit (QIAGEN, CA) for microarray experiments.
5. Subject the RNA to denaturing agarose gel electrophoresis to visually verify the integrity of the RNA by the presence of 28S and 18S ribosomal RNA bands.

3.4.2. Quantitative RT-PCR Analysis

1. Prepare the reactions in a microAmp Optical 96-well reaction plate. A housekeeping gene should be included for normalizing the quantification of mRNA samples (*see* **Note 14**). The plate wells should contain no reverse transcription control (NRC) and no template control (NTC) (*see* **Note 15**). NRC is performed by replacing the $Mn(OA)_2$ in reaction buffer with $MgCl_2$, and NTC is performed by adding 5 µL of water instead of RNA.
2. Prepare a master mix of reagents in the Taqman® EZ RT PCR Kit (*see* **Table 1** and **Notes 16** and **17**). Analyze each sample in triplicate. Using a repeater pipet, aliquot 20 µL to each well. Add 5 µL RNA (10 ng/µL) to each well. The final volume in each well is 25 µL. At this time, a standard curve should be constructed and loaded onto the plate (*see* **Note 18**).
3. Cap the wells using optical strip well caps. Mix well by inverting the plate a few times and spin the plate briefly in a centrifuge to collect all volumes at the bottom of the wells.

Table 1
Master Mix Preparation for 12 Reactions in Quantitative RT-PCR Analysis

Reagent name	Volume (µL)	Final concentration
5X Taqman buffer	60	1X
25 m*M* $Mn(OA)_2$	42	3.5 m*M*
Nuclease-free water	21	—
10 m*M* dNTPs	36	1.2 m*M*
UNG (1 U/µL)	3	0.01 U/µL
rTH polymerase (2.5 U/µL)	12	0.1 U/µL
10 µ*M* Forward Primer	27	900 n*M*
10 µ*M* Reverse Primer	27	900 n*M*
5 µ*M* labeled Probe	12	200 n*M*
Total	240 [a]	

[a]Apply 20 µL Master mix per well for a final 25 µL reaction.

4. Load the plate onto the ABI Prism® 7700 Sequence Detection System. Make sure the plate is aligned properly, then run the reaction. The thermal cycling conditions (40 cycles) are set as follows: 50°C for 2 min, 60°C for 30 min, 95°C for 10 min, 95°C for 15 s, 62°C for 1 min.

3.4.3. Quantitative PCR Analysis

1. Prepare the reactions in a MicroAmp Optical 96-well reaction plate. A housekeeping gene should be included for normalizing the quantification of DNA samples (*see* **Note 14**). The plate wells should contain the NTC (no template control). NTC is performed by adding 5 µL of water instead of DNA (*see* **Note 15**).
2. Prepare a master mix of reagents for each gene (*see* **Table 2** and **Note 14**). Taqman® Universal PCR Master Mix is supplied in a 2X concentration. Each sample is analyzed in triplicates. Using a repeater pipet, aliquot 20 µL to each well.
3. Add genomic DNA template of interest up to 10 µL to each well. The final volume in each well is 30 µL. Cover the plate using Micro Amp optical caps. Mix well by inverting the plate a few times and spin the plate briefly in a centrifuge at 1000*g* to collect all volumes at the bottom of the wells.
4. Load the plate onto the ABI Prism® 7700 Sequence Detection System. Make sure the plate is aligned properly and run. The thermal cycling conditions (40 cycles) are set: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, 60°C for 1 min.

3.5. Microarray Hybridization

1. Infect 2774qw1 cells with rAd-p53 or rAd-empty vector for 1 h, and harvest at 4, 8, 12, 16, 20, and 24 h post infection. For each time point, use mRNA isolated

Table 2
Master Mix Preparation for 12 Reactions in Quantitative PCR Analysis

Reagent name	Volume (μL)	Final concentration
Taqman® Universal PCR Master Mix (2X)	180	1X
10 μ*M* Forward primer	25	700 n*M*
10 μ*M* Reverse primer	25	700 n*M*
6 μ*M* Labeled Probe	10	167 n*M*
Total	240 [a]	

[a] Total volume of 12 reactions is 360 μL. Apply 20 μL Master mix per well for a final 30 μL reaction.

from cells infected with rAd-p53 to prepared cDNA labeled with Cy5-dUTP, and use mRNA from cells infected with rAd-empty vector to prepare cDNA labeled with Cy3-dUTP.
2. Mix the two cDNA probes and simultaneously hybridize to the Incyte microarrays.
3. Compile and analyze the hybridization results from all six microarrays.
4. Examine the differential expression of several known p53 target genes to ensure the reliability of the microarray hybridization results. For a more in-depth discussion of microarray methodology, *see* **ref.** ***(21)***.

3.6. Integration of the p53 Target Database and Microarray Data

1. To build a searchable sequence database for all the transcripts represented by microarray, generate a FASTA format file for all these sequences, and use formatdb in NCBI BLAST package to format the FASTA file into BLAST format (*see* **Note 19**).
2. Use the transcript sequences associated with each promoter ID or intron ID (*see* **Subheadings 3.1.** and **3.2.**) to BLAST search against the microarray sequence database.
3. Based on the alignment score, make the association between promoter IDs (intron ID) and transcripts represented on the microarrays. Use 95% identity over 200 bp as a cutoff.
4. Save the relationship table in a relational model database for easy access.
5. Use NCBI UniGene ***(23)*** to cluster the transcripts that encode the same protein and use public accession numbers associated with all microarrray transcripts (*see* **Note 20**).

3.7. Quantitative Chromatin Immunoprecipitation Analysis

The ChIP assay is used to determine whether a given protein binds to a specific DNA sequence in vivo (*see* **Subheading 1.3.**). The DNA-protein binding complex is immunoprecipitated using p53-specific antibody, or no anti-

body added as a negative control. The enrichment of the predicted p53-binding site is analyzed by quantitative PCR on Taqman (PE 7700) with the primers either overlapping with or flanking the predicted p53-binding site. In this work, p53 is introduced either by the treatment of adriamycin or infection of adenovirus expressing p53 ***(4,11)***. As a negative control, an equal number of cells is treated either with DMSO or adenovirus containing an empty vector. The enrichment of the DNA fragments containing the predicted p53-binding site (% total input DNA) in each treatment group is equal to the amount of DNA fragments enriched by chromatin immunoprecipitation divided by the amount of total input DNA (*see* **Note 21**).

1. Start with approx 1×10^7 cells per immunoprecipitation reaction. Grow cells till they are in log phase (The cells grown in a $245 \times 245 \times 25$ mm tissue culture plate can accommodate six immunoprecipitation reactions).
2. Add formaldehyde to a final concentration of 1% directly to the cell culture medium to fix the in vivo p53 protein–DNA complexes. Fix at room temperature for 10 min (*see* **Note 22**).
3. Stop the fixation by the addition of 2 *M* glycine to a final concentration of 0.125 *M* and incubate for 5 min. Rinse the plates twice with TBS.
4. Add 6 mL of ice-cold 0.5% SDS lysis buffer containing protease inhibitors (one complete protease inhibitor cocktail tablet per 10 mL buffer). Scrape the cells off the dish using cell lifters (*see* **Note 23**).
5. Pellet the cells by spinning for 6 min at 500*g*. Aspirate the buffer and resuspend cells in 4 mL ice-cold IP buffer (*see* **Note 24**). Gently vortex the sample tubes.
6. Sonicate using a Branson Sonifier 450. Set at power setting 3 and 100% duty cycle, pulse for 20 s (*see* **Note 25**).
7. Add additional IP buffer to a final volume of 1 mL per immunoprecipitation. Aliquot 1 mL lysate into each Eppendorf tube.
8. Add 25 µL of protein A beads to each tube, mix thoroughly, and spin at full speed for 30 min at room temperature in a microcentrifuge (*see* **Note 26**). Transfer the supernatant into new Eppendorf tube and discard the beads (*see* **Note 27**). Remove 25 µL lysate to be used as total DNA input for each treatment group (*see* **Notes 21** and **23**). Store at 4°C.
9. Add a monoclonal antibody against p53 (Ab-6, Oncogene Research Products) to the chromatin extract, and mix (*see* **Note 28**). Incubate overnight on a rotating shaker at 4°C.
10. The next morning, using a wide-bore tip, add 30 µL of protein A beads to each sample. Incubate for 2 h on a rotating shaker at 4°C.
11. Microcentrifuge at 500*g* for 1 min at room temperature. Remove the supernatant and resuspend the beads in 1 mL mixed micelle buffer. Incubate for 5 min on a rotating shaker at room temperature. Precipitate the beads by spinning in a microcentrifuge at 500*g* for 1 min. Discard the supernatant.
12. Repeat **step 11** twice with mixed micelle buffer.
13. Repeat **step 11** twice with Buffer 500.

14. Repeat **step 11** twice with LiCl/detergent buffer.
15. Repeat **step 11** once with TE buffer.
16. Add 300 µL of 1% SDS, 0.1 *M* $NaHCO_3$ in TE buffer and incubate for 30 min at 65°C. Briefly microcentrifuge at full speed and transfer the eluent to a fresh Eppendorf tube. At this point, add 275 µL of 1% SDS, 0.1 *M* $NaHCO_3$ in TE buffer to the total control samples (from **step 8**) stored at 4°C. Incubate the IP samples and the total control samples at 65°C overnight. Use an air incubator or oven to prevent concentration of the samples by evaporation/condensation.
17. To reverse the protein–DNA crosslinking, add 250 µL of freshly made proteinase K solution to all the sample tubes. Incubate at 37°C for 30 min. Add 55 µL of 4 *M* LiCl and 500 µL buffered phenol:chloroform:isoamyl alcohol (25:24:1, vol:vol:vol, pH 7.9). Vortex vigorously for 1 min and separate the phases by microcentrifugation for 10 min at full speed at room temperature.
18. Transfer the upper phase to a fresh 2.0 mL Eppendorf tube and add 1.2 mL 100% ethanol. Mix thoroughly, and precipitate the DNA by storing overnight at –20°C. Microcentrifuge at full speed for 30 min at 4°C. Wash pellets with 750 µL 70% ethanol, and microcentrifuge at full speed for 5 min at 4°C. Aspirate the supernatant and allow samples to air dry for 10 min. Resuspend in 200 µL of double-distilled H_2O. Store at –20°C.
19. Use 10 µL per PCR reaction. Analyze the enrichment of specific sequences by quantitative PCR as described in **Subheading 3.4.3.** and **Note 29**.

3.8. Enrichment Factor Calculation

1. Determine the total number of genes on the microarrays (T_{random}), and the number of p53-responsive genes observed in microarray experiments (R_{random}) (*see* **Subheadings 1.3., 3.5.,** and **3.6.**).
2. Determine the total number of genes on the microarrays that are in the p53 Target Database (R_{target}), and the number of p53 responsive genes observed in the microarray experiments that are in the p53 Target Database (T_{target}) (*see* **Subheadings 1.3., 3.5.,** and **3.6.**).
3. Estimate the lower boundary of EF by substituting R_{random}, T_{random}, R_{target}, and T_{target} into the following equation: $EF > [R_{target}/(T_{target} - R_{target})]/[R_{random}/(T_{random} - R_{random})]$.

4. Notes

1. 22,034 promoters were collected using the transcript mapping method from GenBank™ Release 120 (October 2000), where approx 94% of the human genome was deposited and 63% was a working draft sequence ***(4)***.
2. For searching transcription factor DNA-binding sequences that do not require flexible insertions, matrix-based programs (e.g., ProfileSearch (http://biobase.dk/gcgmanual/profilesearch.html) or MatInspector (http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl)) may give better specificity and sensitivity.
3. The promoter database derived either via the GenBank™ annotation approach or via the transcript map approach is biased towards known genes. Many transcripts

on the microarrays are novel, and have no corresponding promoter information available. Even with the completion of human genomic sequence, the prediction of gene structure for each transcript is still challenging, unless the full-length mRNA sequence is available. Most microarrays from Incyte Pharmaceutical Inc. or Affymetrix Inc. are fabricated with partial cDNA sequences. Another issue in data integration is alternatively spliced variants. Different spliced variants may have different 5' transcription start sites. Most microarray designs do not take the splice variant into consideration owing to the complexity of the issue.

4. Compared with regular statistical methods (e.g., chi-square and Fisher exact standard test) that can be used to measure the difference between observed and expected frequency ***(24)***, EF takes biological processes into consideration. EF analysis is not suitable for experiments that have a small number of differentially expressed genes, where it is better to use Fisher exact standard test to estimate the significance of difference.
5. GenBank™ is a general nucleotide sequence database. The advantage of using GenBank™ primate divisions is that it has the best collection of nucleotide sequences derived from primates (high-throughput sequence data, like EST, is not included in primate divisions). However, there are no uniform annotations from GenBank™. For the individual submission, the author assigns keywords and feature descriptions for each sequence record, which makes it difficult to accurately retrieve the sequences based on annotation. Nevertheless, the GenBank™ database provides a good initial source of human nucleotide sequences for our purpose.
6. Exon/intron could be designated by a Roman number (e.g., exon I and exon X) or a letter (e.g., exon A and exon J).
7. The cutoff of promoter length is arbitrary and should be adjusted based on projects. With the increase of promoter length, the level of false-positive noise will increase as well. It is a good practice to keep the promoter length as close to the transcription start site as possible, as it covers the majority of documented DNA-binding sites of p53 target genes. Since most documented p53 binding sites are within 2000 bp, we chose 2000 bp as a cutoff for the promoter length. The drawback is that some functional p53 DNA-binding sites upstream of 2000 bp in the promoter may be missed.
8. AAT ***(19)*** is a local alignment software extending the BLAST algorithm by assigning fixed penalty to long gaps, and taking into account the consensus splicing signal. There is other software, i.e., SIM4 (http://pbil.univ-lyon1.fr/sim4.html) and BLAT (http://genome.ucsc.edu), which can be used for transcript mapping.
9. In addition to low-complexity DNA sequences, there are interspersed repeats in mammalian genomes. RepeatMasker is a program that screens DNA sequences for interspersed repeats. It is free to academic and nonprofit institutions at http://ftp.genome.washington.edu/RM/RepeatMasker.html. Commercial licenses are available from Geospiza at http://www.geospiza.com/products/tools/repeatmasker.htm.
10. Since human genomic sequences are still unfinished and fragmental, the promoter extraction should be done only within a continued fragment. Gaps in genomic sequences are represented as long stretches of poly (N).

11. The FindPatterns program is designed to allow searching with ambiguous nucleotide expression that matches many different sequences in the database. For example, the p53 consensus DNA-binding sequence was written as RRR CWWGYYY(N){0.13}RRRCWWGYYY, which means RRRCWWGYYY, followed by 0 to 13 of any base, followed by RRRCWWGYYY (R = G or A, W = T or A, Y = C or T). The FindPatterns program tolerates mismatches, which in turn permits searching GenBank™ for the sequences with mismatches in the p53 consensus DNA-binding sequence. Based on statistics of the known p53 target genes ***(25)***, the searches for the matched sequences are permitted to have up to three mismatches to the canonical consensus sequence.
12. Of 13 functional p53 response elements of known p53 target genes, 23 out of 26 decamers bear a cytosine at position 4, and 25 out of 26 decamers have a guanine at position 7 ***(4)***. Therefore, the matched sequences are not allowed any mismatch for C at position 4 or G at position 7.
13. The efficiency of the p53 sites search was evaluated by analyzing 10 known p53 target genes (13 p53 DNA-binding sites) ***(4)***. We found that several functional p53 response elements were not included because of parameters we set. For example, a few known p53 target genes have a nucleotide in the position 4 or 7 other than C or G in their p53 response elements, or the response element is located more than 2000 bp upstream of the transcription start site.
14. A group of endogenous control genes should be evaluated to select one that does not alter its expression upon treatments, and can be used to normalize the quantification of a mRNA target for differences in the amount of total RNA added to each reaction. The Taqman® Human Endogenous Control Plate (Cat no. 4309199, PE Applied Biosystems, CA) could be used. We have tested five human endogenous control genes encoding acidic ribosomal protein, cyclophilin, glyceraldehyde-3-phosphate dehydrogenase, β-glucuronidase, and hypoxanthine ribosyl transferase, and found that the expression of the β-glucuronidase gene showed the least alteration by p53 across the tested samples. Therefore, β-glucronidase gene was used as a housekeeping gene.
15. NRC is used to check for genomic DNA contamination in RNA samples. Mn^{2+} is crucial for reverse transcription. Doing NRC once is enough, if the same RNA samples are used in experiments with different genes. In addition, NTC must be included in each plate every time for each tested gene. This is a good control for checking for any contamination in primer/probe mix or formation of primer dimer.
16. Be sure to make enough master mix for 1.2 times the number of samples that you actually have.
17. One-step RT-PCR is ideally suited for high sample throughput and provides the additional benefit of high-temperature reverse transcription, with a single enzyme for ease of use. The enzyme is recombinant *Thermus thermophilus* (rTth) thermostable DNA polymerase, which reverse transcribes RNA to cDNA in the presence of Mn^{2+} ion and polymerizes DNA during the PCR amplification. High-temperature (60–70°C) reverse transcription with rTth DNA polymerase

permits efficient cDNA synthesis from RNA templates that contain a complex secondary structure or high G + C content.

18. For relative quantification, any stock RNA sample containing relatively abundant RNA of the target gene can be used as a template for determining a standard curve, which can be established by analyzing a serial dilution of a known concentration of an RNA sample. The standard curve also provides a validation or an insight into the PCR efficiency for a particular primer/probe set.
19. The NCBI BLAST package can be downloaded at ftp://ftp.ncbi.nlm.nih.gov/blast/, which includes all the necessary tools to run Standalone BLAST. The main advantage of Standalone BLAST is to allow users to create local customized BLAST databases using formatdb. This can be done with any file of FASTA formatted protein or nucleotide sequences.
20. Transcripts represented by microarray chip are usually redundant. There are other methods to cluster them into gene-based groups. In addition to the UniGene approach, PHRAP, available at http://www.phrap.org (Phil Green, unpublished), can be used to assemble the transcripts into gene-based groups.
21. The amount of input DNA in the reaction is a crucial parameter for the interpretation of the results. Subsequent quantitative PCR analysis of this material (referred to as "total DNA") is used to determine the equal presence of all target DNA fragments in the immunoprecipitation, and to determine the relative amounts of DNA among different samples.
22. Formaldehyde reacts with the primary amines on the DNA and RNA bases reversibly, forming a covalent bond. The use of 1% final concentration of formaldehyde for 10 min at a temperature ranging from 12°C to 37°C works well in our system. The ability to fragment and solubilize genomic DNA depends on the extent of chromatin cross-linking. Excessive crosslinking can interfere with cell breakage and effective fragmentation by sonication.
23. When harvesting multiple plates of the same treatment group, add lysis buffer to the first plate, scrape, and simply remove the lysate from one plate and add to the next. When all the plates have been harvested, pipet homogenate into a 15-mL conical tube.
24. At this point, the lysate in IP buffer can be frozen at –80°C.
25. The extent of cell breakage is very important and can alter the immunoprecipitation efficiency. Inappropriate sonication is often the cause of failure or inconsistent results. The time or setting of sonication must be determined empirically for each particular application. This can be monitored microscopically using trypan blue, by comparing intact cells in small, diluted aliquots of the sample taken before and after sonication. The shear size of DNA can be determined by running an aliquot from the sample before increasing the volume on a 1.5% to 2% agarose gel and visualizing with ethidium bromide. A smear of DNA with an average size of 500 bp and ranging in size from 100 to 1000 bp should be apparent.
26. It must be noted that certain classes of mouse immunoglobulins are not strongly bound by protein A. At this stage, protein A beads help to trap large DNA fragments.

27. When removing supernatant, be very careful to avoid carryover of beads, which will be a source of background. Leave approx 50–100 µL lysate with beads in the tube.
28. A set of antibodies specific to p53 was tested to select the best one for this application. We started with relatively high amounts of antibody (10 µg) per reaction. Because of crosslinking, the antibody tends to have low efficiency to recognize its target protein. It is strongly recommended that immunoprecipitations be performed in at least triplicates since we observed variations between various immunoprecipitations. As negative controls, chromatin is immunoprecipitated either in the absence of p53-specific antibody, or from control virus-infected cells. In addition, it is recommended to use a nonspecific antibody for the initial experiments to confirm that the background level from the control immunoprecipitation generates little to no signal.
29. Taqman® primers and probes were designed to generate a PCR amplicon that either overlapped with or was a few nucleotides from the putative p53 DNA-binding site. It is highly recommended that a positive control gene and a negative control gene be included in the ChIP assay. For example, we included reactions containing a known p53-binding site in the promoter of the proliferating cell nuclear antigen (PCNA) gene as a positive control, and a putative p53 DNA-binding site in the promoter of the alpha tubulin gene, which does not respond to p53, as a negative control. The ChIP assays were considered positive if the enrichment of DNA fragments containing the predicted p53 binding sequence(s) was threefold greater than the no antibody control in at least four out of five independent experiments.

Acknowledgments

We thank our colleagues for their contributions to the studies described here. We also thank W. Robert Bishop for the critical reading and comments on this manuscript.

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4

Functional Analysis of CDK Inhibitor $p21^{WAF1}$

Rati Fotedar, Mourad Bendjennat, and Arun Fotedar

Summary

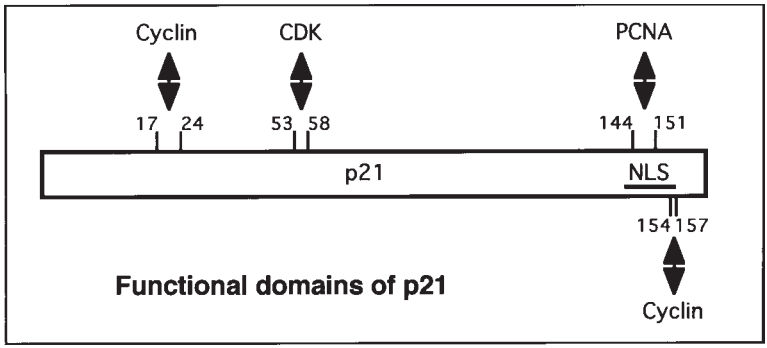
$p21^{WAF1}$ was originally identified as a protein that binds and inhibits cyclin-dependent kinases (CDKs). $p21^{WAF1}$ is recognized to have at least two separate roles—first as a CDK inhibitor, and second as an inhibitor of PCNA, an accessory protein of DNA polymerase δ. $p21^{WAF1}$ plays a critical role in the cellular response to DNA damage. Additionally, $p21^{WAF1}$ plays a role in DNA repair, apoptosis, cellular senescence, terminal differentiation, and cell cycle arrest upon extracellular signaling. $p21^{WAF1}$ protein levels are regulated both by transcriptional control by p53 and by factors other than p53, as well as by posttranscriptional regulation. Although the role of $p21^{WAF1}$ has been explained so far only by its interaction with CDKs and with PCNA, it has several other binding partners. The ability of $p21^{WAF1}$ to participate in several cellular functions has been widely studied by transfection of cells with $p21^{WAF1}$ vectors. We describe here procedures for analysis of $p21^{WAF1}$ function in mammalian cells after transfection of p21 plasmids. The procedures include inhibition of DNA synthesis, cellular localization, association with binding partners, and half-life measurements.

Key Words: $p21^{WAF1}$; CDK; cell cycle; PCNA; proteasome; ubiquitination.

1. Introduction

The CDK inhibitor $p21^{WAF1}$ plays a critical role in the cellular response to DNA damage (reviewed in ***1***). The primary targets of $p21^{WAF1}$ (hereafter referred to as p21) are the CDK–cyclins ***(2–4)***, and PCNA, an accessory protein of DNA polymerase δ ***(5)***. p21 forms complexes with a subset of CDK–cyclins to inhibit their kinase activity and with PCNA to inhibit DNA synthesis. These distinct properties map to the N-terminal and the C-terminal regions of p21, respectively **(Fig. 1)**. The N-terminal residues 1–82 of p21 bind CDK–cyclin complexes and inhibit their activity in vitro ***(6–12)***. Indeed, this region of p21 contains independent binding sites for cyclin and CDK, and both sites are required to inhibit kinase activity in vitro ***(12)***. In support of this, the crystal structure of the N-terminal region of p27 (residues 28–96) bound to active

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ




```
  1 MSEPAGDVRQ NPCGSKACRR LFGPVDSEQL SRDCDALMAG
 41 CIQEARERWN FDFVTETPLE GDFAWERVRG LGLPKLYLPT
 81 GPRRGRDELG GGRRPGTSPA LLQGTAEEDH VDLSLSCTLV
121 PRSGEQAEGS PGGPGDSQGR KRRQTSMTDF YHSKRRLIFS
161 KRKP
```

Fig. 1. Functional domains of p21. Distinct regions of p21 which bind CDK, cyclin, and PCNA are identified by amino acid residues. A putative nuclear localization signal (NLS) sequence is located within residues 140–157. The sequence of p21 is shown below and the six lysines which were mutated to arginine in this study are indicated in bold.

CDK–cyclin kinase shows that p27, like p21, has two distinct binding sites, for cyclins and for CDK ***(13)***. Interestingly, residues 154–157 in the C-terminus of p21 bind cyclin in vitro ***(11,14)***. In accord with the conclusion that both the CDK and the cyclin binding sites are required to inhibit kinase activity, a truncated p21 protein containing the C-terminal residues 114–164, and hence the cyclin binding site, does not inhibit CDK–cyclin A or E kinase activity in vitro ***(12)*** or inhibit E2F activity in vivo ***(15)***. A p21 mutant containing the N-terminal CDK-binding site and the C-terminal cyclin-binding site is able to inhibit CDK2–cyclin E activity. p21 binds PCNA and inhibits PCNA function during in vitro assays of DNA replication ***(5)***. Further studies to elucidate the basis and functional consequences of these differential interactions are awaited.

A putative nuclear localization signal (NLS) sequence is located within residues 140–157. NLS is required but not sufficient to concentrate ectopically expressed p21 in the nucleus ***(15)***. In one of the first reports on p21 localization, p21 expressed from an inducible promoter was nuclear in G1-arrested cells ***(16)***. In fact, ectopically expressed p21 and its deletion mutants are primarily nuclear ***(15)***. It has however been assumed that endogenous p21 is

always nuclear too. The subcellular localization of endogenous p21 actually varies in different cell types and probably with the cell cycle stage (M. B. and R. F., unpublished observations).

Overexpression of p21 results in cell cycle arrest in the G1 ***(2)*** and G2/M ***(17–19)*** phases of the cell cycle. The CDK–cyclin inhibitory domain of exogenous p21 most likely inhibits G1 progression by preventing the phosphorylation of retinoblastoma protein (pRb) by CDK–cyclin kinases ***(15)***. How p21 mediates G2/M arrest is not clear at present. One possibility is that p21 represses the expression of cyclin B and cdc2 ***(20)***. Up-regulation of both p21 mRNA and protein following ionizing radiation is dependent on p53 ***(21,22)***, and p21 mediates cell cycle arrest in response to the p53 checkpoint pathway ***(23–25)***. pRb acts as a downstream target of p21 in DNA damage-induced G1 arrest ***(26)***.

The role of p21 has been explained so far by its interaction with CDK–cyclin complexes and with PCNA. Besides these proteins, p21 interacts with HPV-16 E7, Gadd45, SAPK, CIZ1, XPG, calmodulin, β subunit of CK2, and CARB. p21 could play other roles, as suggested by its interaction with these proteins, although the functional significance of such interactions in vivo is not yet evident.

Although the transcriptional control of p21 by p53 and factors other than p53 plays a critical role, posttranscriptional regulation of p21 protein levels is emerging as an equally important player. We ***(15)*** and others ***(27–30)*** have shown that p21 degradation is regulated by the proteasome pathway. Indeed, p21 protein is stabilized upon treatment of various cell types with proteasome inhibitors. To directly test the role of ubiquitination in proteasome-dependent degradation of p21, we generated a p21 expression vector in which all six lysines, sites of ubiquitination, were mutated to arginine (referred to as p21K6R mutant). We present in this chapter our characterization of the p21K6R mutant as an example of how the techniques described here can be utilized to study p21 function. Exogenously expressed wild-type p21 has a half-life similar to p21K6R mutant, showing thereby that proteasome-dependent degradation of basal p21 protein is predominantly ubiquitin independent.

2. Materials

1. Incubator for cell culture.
2. Phase-contrast and confocal microscopes.
3. Microplate reader.
4. Film developer.
5. Cooled microfuge.
6. Scanner and computer for data acquisition and analysis.
7. SDS-PAGE and electroblotter equipment.

8. Sonicator.
9. Rat embryonic fibroblast (REF-52) cells are cultured in DMEM medium supplemented with 10% heat-inactivated bovine calf serum (Hyclone) and 2 m*M* L-glutamine (Gibco) in a humidified 5% CO_2 atmosphere. Other cells can be used (*see* **Note 1**).
10. The cDNAs encoding the full-length p21 protein ***(15)*** or p21K6R mutant are cloned into an SR-α based promoter. The p21K6R mutant is derived from wild-type p21 by sequential site-directed mutagenesis of the six lysine residues by PCR, and was completely sequenced to establish its identity. The proteins expressed by these vectors (p21wt and p21K6R) are epitope tagged by addition of HA (human influenza hemagglutinin nonapeptide, Tyr–Pro–Tyr–Asp–Val-Pro–Asp–Tyr–Ala) at the N-terminus.
11. *Escherichia coli* strain DH5α containing the vectors are grown in Luria-Bertani medium plus 50 µg/mL ampicilin, and the plasmids are purified using Qiagen columns (Chatsworth, CA) according to the manufacturer's instructions.
12. 2X HEPES-buffered saline (pH 7.05): 50 m*M* HEPES, 280 m*M* NaCl, 1 m*M* Na_2HPO_4.
13. 2.5 *M* $CaCl_2$.

Both the above solutions are sterilized by filtration through 0.22 micron filter and stored at 4°C for no more than 3 mo.

14. RIPA buffer: 50 m*M* Tris-HCl (pH 8.0), 150 m*M* NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 5 m*M* EDTA, 1 m*M* DTT, supplemented with 10 µ*M* aprotinin, 50 µ*M* leupeptin, 1 m*M* sodium fluoride, and 100 µ*M* orthovanadate at the time of use.
15. Protein estimation kit (Bio-Rad Protein Assay Kit).
16. TNT-low: 25 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, and 0.05% Tween-20.
17. TNT-high: 25 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, and 0.5% Tween-20.
18. Fat-free milk powder.
19. Western blot detection kit (Super Signal® West Pico Chemiluminescent Substrate, Pierce).
20. Poly-D-lysine-coated coverslips.
21. Phosphate-buffered saline (PBS): 136 m*M* NaCl, 2.7 m*M* KCl, 10.6 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, pH 7.4.
22. 8% paraformaldehyde diluted in PBS (store at 4°C up to 3 mo).
23. 10 µg/mL propidium iodide diluted in PBS from a stock of 1 mg/mL.
24. Immunofluorescence (IF) antibody diluting buffer: PBS plus 0.2% Triton X-100 and 3% BSA.
25. 1,4-diazabicyclo-[2.2.2]-octane (DABCO, Sigma) at 25 mg/mL in glycerol.
26. Nail polish.
27. Mouse monoclonal anti-HA antibody (clone 16B12, BabCO).
28. Mouse monoclonal antibody (clone SX118, BD Biosciences), which recognizes human, rat, and mouse p21 (*see* **Table 1**).
29. Horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Kirkegaard and Perry Laboratories).

Table 1
p21 Antibodies

p21 antibody source	Immunofluorescence [a]	Western blotting [b]
C-19 Santa Cruz Biotech	+	+
N-20 Santa Cruz Biotech	–	+
Clone SX118 BD Bioscience	+	+
Clone 70 BD Bioscience	+	+

[a] For detection of endogenous p21 by immunofluorescence.
[b] For detection of endogenous and transfected p21 by Western blotting.

30. Anti-rabbit IgG antibody (Biosource).
31. FITC-labeled BrdU-specific antibody (Boehringer).
32. Goat anti-mouse IgG (H+L)-Cy3 fluorochrome (Jackson).
33. FITC-labeled goat anti-mouse IgG (H+L) (Caltag).
34. PCNA-specific antibody (Oncogene, Clone 19A2).
35. Rabbit polyclonal anti-CDK2 ***(31)***.
36. Cell extraction buffer: 40 m*M* HEPES (pH 7.5), 250 m*M* NaCl, 0.1% Triton X-100 plus protease and phosphatase inhibitors.
37. Protein-A Sepharose (Pharmacia).
38. Immunoprecipitation (IP) buffer: 50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 0.5% NP-40.
39. Cycloheximide (Sigma), prepared as a stock solution (20 mg/mL) dissolved in DMSO and stored at –20°C, is used at the final concentration of 20 µg/mL to inhibit protein synthesis.
40. *N*-acetyl-Leu-Leu-norLeucinal (LLnL, Sigma), prepared as a stock solution (50 m*M*) dissolved in DMSO and stored at –20°C, is used at the final concentration of 50 µ*M* (*see* **Note 2**).

3. Methods

The methods described below outline (1) cell culture and transfection of mammalian cells with p21 expression vectors; (2) detection of proteins by Western blotting; (3) localization of transfected and endogenous p21 proteins by immunofluorescence; (4) effect of transfected p21 proteins on DNA synthesis; (5) association of p21 with PCNA and CDK2; (6) stabilization of p21 by proteasome inhibitors; and (7) evaluation of p21 protein half-life using cycloheximide.

3.1. Cell Culture, Expression Vectors, and Transfections

1. Grow monolayer cells in 100-mm dish to 80% confluence.
2. Aspirate medium by tilting dish. Wash cells with 10 mL of PBS and aspirate.

3. Add 3 mL of 1X trypsin/EDTA (just enough to cover the cells). Incubate at 37°C for 5 min. Collect cells when they begin to lift from the dish by adding 5 mL medium, to block trypsin activity, and transfer to a sterile tube.
5. Spin cells at 200*g* for 5 min.
6. Resuspend cells in 10 mL complete medium and split them into fresh 100-mm plates.
7. If the cells are split at 1/10 dilution, they will be 30% confluent after 2 d. Use cells at this confluency for transfection.
8. Aspirate medium 3 h prior to transfection. Add 9 mL fresh medium.
9. For each DNA sample, label 2 polystyrene tubes A and B (*see* **Note 3**):
   - Tube A: 50 µL of 2.5 *M* $CaCl_2$, 15–20 µg of DNA/100-mm dish, make up to 500 µL with sterile H_2O (*see* **Note 4**).
   - Tube B: 500 µL of 2X HEPES.

Add contents of tube A to tube B dropwise with fairly vigorous shaking between drops. Allow DNA preparation to sit at room temperature for exactly 20 min (*see* **Note 4**).

10. Add DNA preparation dropwise to the cells. Add to medium by tilting plate and swirling gently between additions to mix.
11. Change medium 12 h after the start of transfection.
12. Analyze cells 36 h after **step 10** (*see* **Note 5**).

3.2. Detection of p21 Protein by Western Blotting

The next step is to detect the exogenously expressed proteins. The exogenous p21 proteins are HA tagged and can be distinguished from the endogenous p21 protein by the difference in size or by using antibody that specifically detects the tag.

3.2.1. Cell Extracts

1. Wash cells obtained in **Subheading 3.1., step 12** with 10 mL of PBS and add 3 mL of trypsin. After 5 min of incubation at 37°C, cells are collected as in **Subheading 3.1., step 3** and harvested to collect cell pellet.
2. Wash cell pellet twice with PBS.
3. Lyse cells in 200 µL of RIPA lysis buffer for 30 min on ice. Lysis can be checked under the microscope.
4. Sonicate lysate briefly on ice to break chromosomal DNA.
5. Clear lysate by centrifugation in a microfuge, top speed at 4°C for 30 min.
6. Collect supernatant. Freeze in aliquots at –20°C until use.

3.2.2. Protein Estimation

1. Protein estimation is performed in duplicate using Bio-Rad solution in 96-well plate as follows:

- Blank : 160 µL H_2O + 40 µL Bio-Rad solution.
- Standards: 15 µL of BSA solution at 250, 125, 62.5, 31.25, and 15 µg/mL + 145 µL H_2O + 40 µL Bio-Rad solution.
- Sample : 2 µL of cell extract + 158 µL H_2O + 40 µL Bio-Rad solution.

2. After 10 min of incubation at room temperature, the 96-well plate is read using the microplate reader at 595 nm.

3.2.3. Immunoblotting

1. Resolve 50 µg of cell extract on 12% SDS-PAGE.
2. Transfer proteins onto the nitrocellulose membrane using a semi-dry blotter (*see* **Note 6**).
3. Block the nitrocellulose with 5% milk in TNT-low buffer for 1 h at room temperature. Shake on a platform agitator.
4. Wash the membrane two times, 5 min each time, with TNT-low buffer.
5. Incubate the blot overnight at room temperature with primary antibody diluted in TNT-low buffer containing 0.05% sodium azide. Use anti-HA antibody diluted to 1:1,000 to see only exogenously expressed HA-tagged p21, or p21-specific antibody diluted 1:1,000 to see both exogenous and endogenous p21 proteins.
6. Wash the blot three times, 5 min, with TNT-high buffer.
7. Incubate the nitrocellulose membrane with goat anti-mouse HRP antibody (1:2,000), diluted in TNT-low buffer containing 0.05% sodium azide, for 1 h at room temperature.
8. Wash the blot three times, 5 min each time, with TNT-high buffer.
9. Process the nitrocellulose membrane for ECL as instructed by the supplier. The nitrocellulose membrane can be reprobed with anti-tubulin or anti-actin antibodies (*see* **Note 7**).

Immunoblotting extracts prepared from REF-52 cells transfected with different amounts of p21 plasmid vector are shown in **Fig. 2**. For subsequent experiments it is necessary to choose the plasmid concentrations as discussed in **Note 4**.

3.3. Localization of p21 by Immunofluorescence

The localization of a protein plays a critical role for its function. The method described below can be used to detect both endogenous and exogenous p21 proteins.

1. Grow cells 24 h on poly-D-lysine-coated coverslips (*see* **Note 8**) to obtain 30% confluency, and transfect cells as in **Subheading 3.1.**. Use appropriate dish depending on how many coverslips are required (30-mm dish can hold four coverslips).
2. Wash cells with PBS in the tissue culture dish.

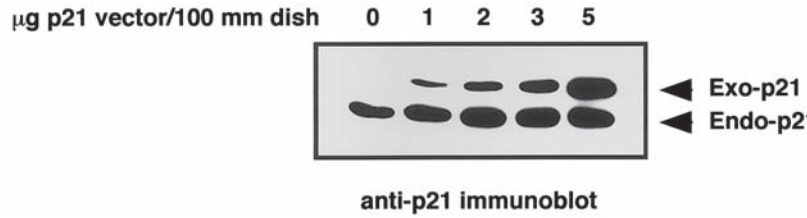


Fig. 2. Immunodetection of transfected p21. REF-52 cells were transiently transfected with different amounts of HA-tagged wild-type p21 vector as indicated. Extracts prepared at 36 h after the start of transfection were analyzed using p21-specific antibody (clone SX118, BD Biosciences). The transfected p21 migrates slower than endogenous p21.

3. Place coverslips individually in 6-well tissue culture plate. Fix cells successively with 2% paraformaldehyde for 20 min at 37°C and 70% ethanol for 10 min on ice.
4. Permeabilize cells with PBS containing 3% BSA, 0.2% Triton-X100, and 0.05% sodium azide for 10 min at room temperature.
5. Remove coverslips from the 6-well culture dish onto a sponge pad in a box.
6. Stain for exogenous HA-tagged p21 protein using 50 μL of anti-HA specific antibody diluted 1:1500 in PBS containing 3% BSA, 0.2% Triton-X100, and 0.05% sodium azide for 1 h at 37°C.
7. Transfer coverslips to 6-well tissue culture dish and wash three times, 10 min each time, with PBS.
8. Follow by incubating with 50 μL of FITC-conjugated goat anti-mouse diluted 1:500 in PBS containing 3% BSA, 0.2% Triton-X100, and 0.05% sodium azide for 1 h at 37°C. All subsequent incubations are done in the dark.
9. Wash the coverslips three times, 10 min each, with PBS.
10. Incubate the coverslips with 50 μL of 0.01 mg/mL propidium iodide (PI) in PBS for 5 min at room temperature,
11. Wash with PBS three times.
12. Add a drop of DABCO/glycerol on glass slides. Place coverslip with cell surface down on DABCO drop. Press slide with coverslip gently with paper towels and seal the coverslips with nail polish.
13. We examine cells on a Nikon Optiphot microscope equipped for epifluorescence and phase-contrast microscopy. For analysis, we collect images using a Bio-Rad Confocal Imaging System.

Localization of endogenous p21 in asynchronous REF-52 cells is primarily peri-nuclear (**Fig. 3**). Exogenously expressed protein is both nuclear and peri-nuclear, but *see* **Note 9**.

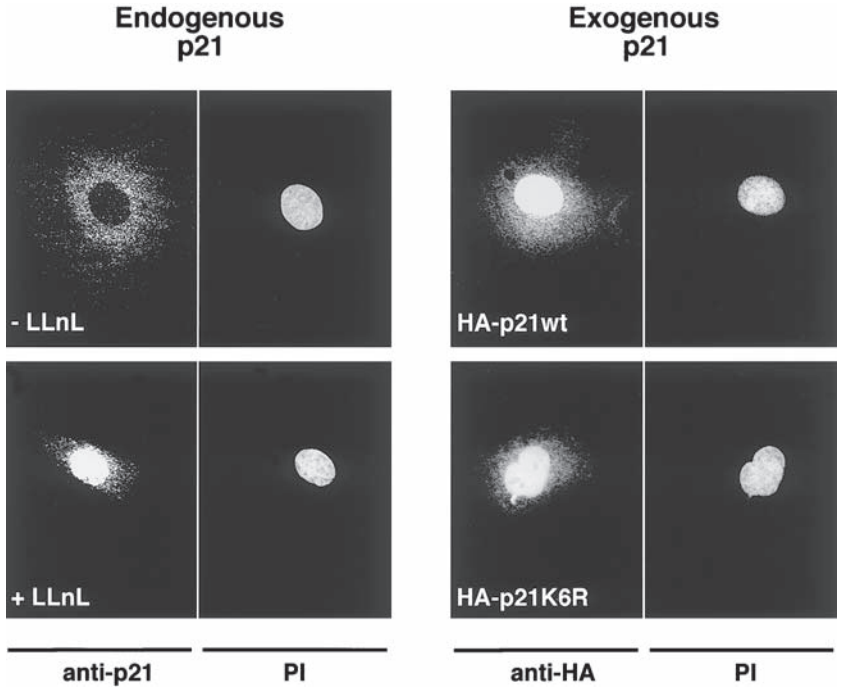


Fig. 3. Subcellular localization of HA-p21 and endogenous p21. REF-52 cells were transiently transfected with HA-tagged wild-type p21 or HA-tagged p21K6R mutant vectors and the subcellular localization was determined by double staining with anti-HA antibody and propidium iodide. Epitope-tagged wild-type p21 and p21K6R mutant are both nuclear and peri-nuclear. Endogenous p21 in REF-52 is predominantly peri-nuclear. Endogenous protein was detected using 1:1000 dilution of p21 antibody (clone SX118, BD Biosciences). Notably, in the presence of the proteasome inhibitor 50 μ*M* LLnL, p21 concentrates in the nucleus.

3.4. Effect of Transfected p21 on DNA Synthesis

Exogenous expression of p21 protein is known to arrest cell cycle progression. As the efficiency of transfection is not 100%, it is necessary to identify the transfected cells. The HA-p21-expressing cells are identified by immunofluorescence using anti-HA-specific antibody and cy3-conjugated anti-mouse antibody (red). In parallel, DNA synthesis can be monitored by BrdU incorporation using FITC-labeled BrdU-specific antibody (green). This technique is very simple and precludes the necessity to select transfected cells.

1. After 36 h of transfection, pulse cells with 10 μ*M* final concentration of BrdU for 30 min.

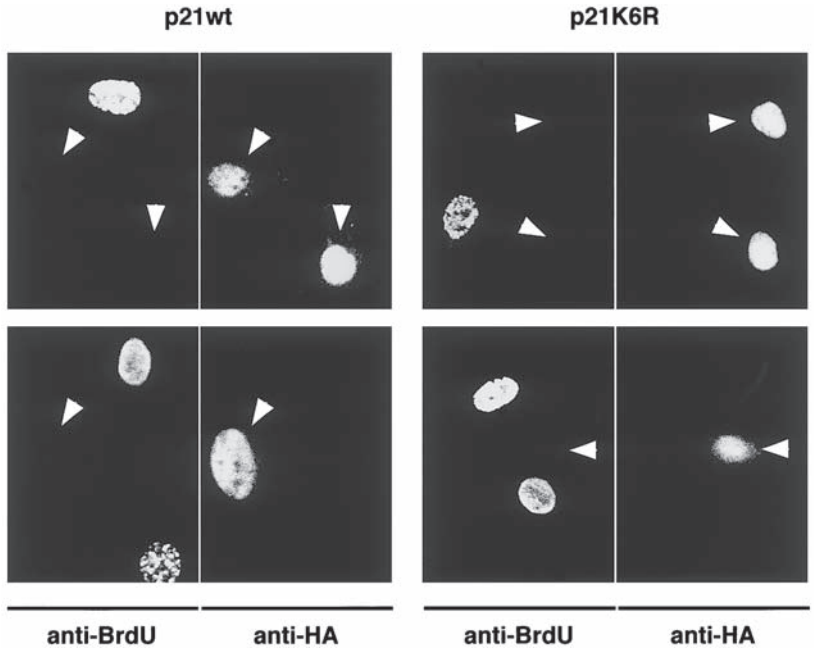


Fig. 4. Effect of p21K6R mutant on DNA synthesis. REF-52 cells transfected with HA-tagged wild-type p21 or p21K6R mutant vectors were double stained for HA and BrdU. The percentage of HA-positive cells which are also BrdU positive gives an estimate of S phase cells. Approximately 500 HA-p21-positive cells are counted in each set.

2. Follow **Subheading 3.3., steps 2–8** to identify transfected cells with anti-HA antibody. All subsequent incubations need to be done in the dark.
3. Fix cells with ice-cold 70% ethanol 10 min at 4°C.
4. Denature DNA for staining with anti-BrdU antibody with 4 *N* HCl, 0.2% Triton-X100, for 10 min at room temperature.
5. Wash the coverslips three times with PBS.
6. Add FITC-labeled BrdU-specific antibody diluted 1:50 in PBS containing 3% BSA, 0.2% Triton-X100, and 0.05% sodium azide for 30 min at 37°C.
7. Follow **Subheading 3.3., steps 11–12**.

p21K6R mutant inhibits DNA synthesis similar to wild-type p21 **(Fig. 4)**. The percentage of HA-p21 positive cells which are also BrdU positive gives an estimate of S phase cells in the transfected population, and is obtained by counting approx 500 HA-positive cells in each set.

(Number of HA-p21-positive cells that are BrdU positive × 100)/(Number of HA-p21-positive cells)

3.5. Cell Extract Preparation and Immunoprecipitation to Detect Association of p21 With CDK and PCNA

To check whether the properties of p21 protein are altered during generation of the mutant, the association of p21 with CDKs and PCNA can be tested.

1. 36 h posttransfection, collect cells as in **Subheading 3.2.1., steps 1** and **2**.
2. Lyse cells in 200 µL of cell extraction buffer for 30 min on ice. Collect cell extract by centrifugation at 10,000*g* for 10 min at 4°C.
3. Determine protein concentration of the cell extract.
4. Preclear extracts in duplicate tubes (300 µg/tube) by incubating with 20 µL of Protein A-Sepharose for 1 h at 4°C.
5. In parallel, preincubate in duplicate tubes 20 µL of Protein A-Sepharose with 2 µL of HA-specific antibody and 500 µL of IP buffer in a 1.5-mL Eppendorf tube for 1 h at 4°C. Prepare two tubes of only beads + antibody as a control to mark the position of immunoglobins and to see eventual cross-reactive bands in the absence of the extract (*see* **Fig. 5**).
6. Incubate precleared cell extracts with HA antibody–Protein A-Sepharose for 1 h at 4°C.
7. Wash beads five times with 1 mL IP buffer.
8. Elute bound proteins from beads by boiling in 20 µl of Laemmli gel loading buffer for 3 min. Do not spin to remove Protein A-Sepharose.
9. Analyze samples by 12% SDS-PAGE and Western blot using anti-PCNA (1:1000) and anti-CDK2 (1:2500) antibodies.

Both wild-type p21 and p21K6R mutant immunoprecipitate equivalent amounts of CDK2 and PCNA **(Fig. 5)**.

3.6. Determination of p21 Half-Life Using Cycloheximide

1. Transfect cells with HA-tagged wild-type p21 or p21K6R mutant vectors.
2. After 36 h, add 20 µg/mL final concentration of cycloheximide (CHX) to cells.
3. Prepare cell extracts as in **Subheading 3.2.1.** at 0 (before CHX addition) and at 1, 2, 4, 6, 8, and 10 h after CHX addition.
4. Separate equal amounts of proteins by 12% SDS-PAGE, and transfer to a nitrocellulose membrane.
5. Immunoblot with anti-p21- or anti-HA-specific antibodies as in **Subheading 3.2.3.**.
6. Scan p21 bands on film by densitometry (*see* **Note 10**).

The half-lives of wild-type p21 and p21K6R mutant are similar **(Fig. 6)**. It is important to note that measurements of the half-life using cycloheximide should be done with low amounts of transfected p21 vectors so as not to saturate the proteolytic machinery.

4. Notes

1. The transfection method described here can be used for a variety of cell types. In our hands, it works efficiently for U2OS (human osteosarcoma), HeLa (human

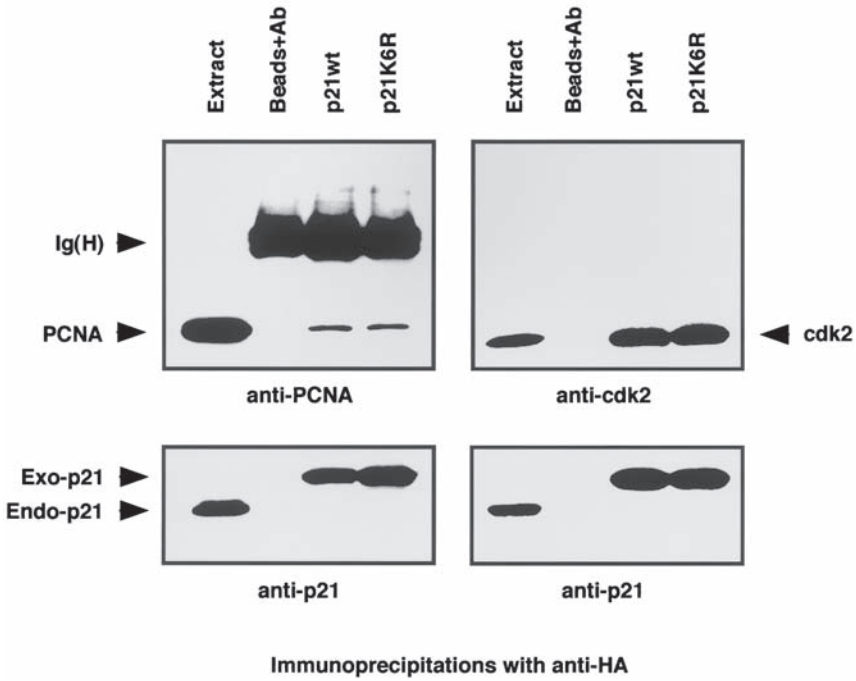


Fig. 5. p21K6R mutant associates with PCNA and cdk2. Extracts prepared from REF-52 cells transfected with HA-tagged wild-type p21 or p21K6R mutant vectors were immunoprecipitated with mouse anti-HA antibody and immunoblotted with p21-, cdk2-, and PCNA-specific antibodies. The immunoprecipitated wild-type p21 and the p21K6R mutant proteins migrate slower than the endogenous p21. A 34-kDa PCNA band is visible in immunoprecipitate of cells transfected with wild-type p21 and p21K6R mutant immunoblotted with mouse anti-PCNA antibody. The heavy (Ig-H) chain of immunoglobulin is indicated in anti-PCNA immunoblot. A 33-kDa cdk2 band is visible in immunoprecipitate of cells transfected with wild-type p21 and p21K6R mutant immunoblotted with rabbit anti-cdk2 antibody. Note that the anti-rabbit antibody does not cross react with mouse antibody. Extract (50 μg) from non-transfected cells without immunoprecipitation (Extract) is shown to indicate the position of PCNA, endogenous p21, and cdk2. As a control, Protein A-Sepharose preincubated with anti-HA antibody (without added lysate) (Beads+Ab) is used to mark the position of immunoglobulin.

cervical carcinoma), HCT116 (diploid human colon carcinoma), 293 (E1A-transformed human embryonic kidney epithelial cells), C33A (human cervical carcinoma), Saos-2 (human osteosarcoma), and NIH3T3 (contact-inhibited murine fibroblast) cell lines. All these cells are grown in DMEM supplemented with 10% fetal calf serum, 2 m*M* L-glutamine at 37°C in a humidified 5% CO_2 atmosphere.

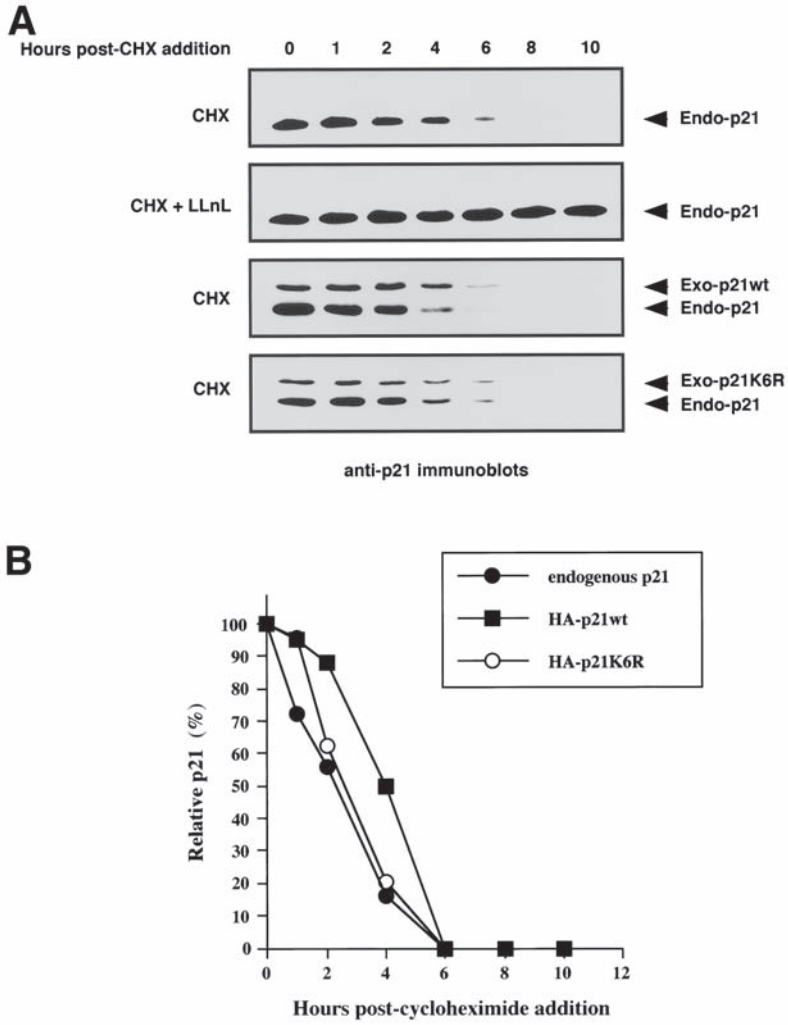


Fig. 6. Half-lives of endogenous p21, wild-type p21, and p21K6R mutant. A. The half-life of endogenous p21 was determined in extracts prepared from nontransfected cells (top panel, CHX). REF-52 cells were treated with 20 µg/mL cycloheximide and extracts prepared at the indicated times after cycloheximide addition were analyzed by Western blotting with anti-p21 antibody. Treatment of cells with 50 µ*M* LLnL for 1 h prior to the addition of cycloheximide results in the stabilization of p21 protein (CHX+LLnL). Half-life of wild-type p21 (third panel) and p21 K6R mutant (fourth panel) was determined. 36 h after the start of transfection with either wild-type p21 or p21K6R mutant vectors, REF-52 cells were treated with cycloheximide, and extracts prepared at the indicated times after cycloheximide addition were analyzed by western blotting with anti-p21 antibody. B. Wild-type p21 and p21K6R mutant exhibit similar half-life as determined by densitometric scanning of the signals.

2. LLnL has been shown to inhibit proteasome activity, but the effect of LLnL may not be specific, since it also affects lysosomal cysteine proteases (cathepsins) and calcium-activated proteases. MG132 is a more specific inhibitor of proteasome, and it modifies the terminal threonine in the 20 S proteasome involved in proteolytic degradation. It is therefore necessary to confirm that MG132 and LLnL give similar results. Further experiments can be carried out with LLnL as MG132 is very expensive.
3. Use polystyrene, not polypropylene, tubes for preparation of DNA mix for transfection. Polypropylene decrease the efficiency of transfection because it binds DNA. This leads to significant loss of the DNA estimated for transfection.
4. 36 to 48 h after the start of transfection is the best time to analyze cells. The expression of exogenous protein increases with time after transfection. The level of exogenous p21 protein expression should never be more than endogenous p21 protein. To this end, the HA-p21 vectors to be tested should never be more than 2 to 4 μg for a 60- or 100-mm dish, respectively. 1 μg/60-mm or 3 μg/100-mm dish is ideal. In case of low efficiency of transfection, the concentration of the carrier plasmid (HA vector used for cloning p21 genes) can be increased. Overexpression of p21 protein can saturate cellular interactions and give erroneous results.
5. It is critical to adjust the pH of 2X HEPES-buffered saline to 7.05 with 1 *N* NaOH. A precipitate should be seen at **Subheading 3.1., step 9**. There are two types of precipitate: a very fine precipitate (the best one) and another which is granular (less efficient than the first one in terms of transfection). If a precipitate is not observed, repeat **Subheading 3.1., step 9**. Failure to obtain a good precipitate could be owing to inaccurate pH of the HEPES-buffered saline or to the plasmid impurity. In our hands, plasmids purified using Qiagen Plasmid Kit (Chatsworth, CA) are sufficiently pure for transfection. Upon repeated failure to obtain a satisfactory precipitate, make fresh 2X HEPES-buffered saline and plasmid.
6. To test whether the transfer works, stain nitrocellulose membrane with 0.3% Ponceau red solution (Sigma) for 5 min before blocking with milk. Rinse the nitrocellulose membrane extensively before continuing with Western blotting steps.
7. Reprobe the nitrocellulose membrane, without stripping, with actin (A2066, MW 42 kDa) or tubulin (T4026, MW 55 kDa) -specific antibodies (Sigma) at 1:1000 dilution in TNT-low buffer to confirm that equal quantities of protein were loaded on gels.
8. Preparation of poly-D-lysine-coated coverslips:
   a. Make 1 mg/mL (5X) polylysine (SIGMA P7886) solution in double-distilled water (ddH_2O). Filter through 0.2-micron filter in tissue culture hood. Store the solution at 4°C in a Falcon tube. Keep sterile.
   b. Soak 1 box of coverslips (Belco Cat no. 194300012, circular 1.12 cm^2 diameter) (500/box) with 1 *N* HCl for 3–4 h in a large beaker. Stir the beaker occasionally.
   c. Wash with minimum of 1 L of ddH_2O. Aspirate ddH_2O.
   d. Add 1X polylysine in minimum volume to cover the coverslips. Incubate for 1 h. Stir the beaker occasionally.

e. Wash with 1 L of ddH_2O. Aspirate ddH_2O.
f. Transfer the beaker to the tissue culture hood.
g. Sterilize coverslips with 70% ethanol. Detach the coverslips from one another by stirring the beaker.
h. Aspirate ethanol. Let the coverslips dry in the hood. The coverslips can be stored dry in the hood. Cover beaker with aluminum foil, as polylysine is sensitive to UV.

9. Note that the inability to detect nuclear endogenous p21 may be due to lower amounts of p21 in the nucleus. The localization may be subject to change, depending on the phase of the cell cycle as well as cell-type-specific differences. For example, although p21 localization in U2OS and HCT116 cells is primarily peri-nuclear, a substantial population of cells display both nuclear and peri-nuclear staining.
10. The percentage of p21 (Relative p21 (%), **Fig. 6**) corresponds to :

Intensity of the p21 plot (arbitrary unit [a.i.]) at the corresponding time × 100/Intensity of the p21 plot (a.i.) at time 0.

Acknowledgment

The work in the authors' laboratory is supported by grants to R. F. from l'Association pour la Recherche sur le Cancer (ARC) and to A. F. from the National Institutes of Health (CA74435 and CA92321). M. B. is supported by a fellowship from ARC.

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5

Analysis of p21^{CDKN1A} Recruitment to DNA Excision Repair Foci in the UV-Induced DNA Damage Response

Lucia A. Stivala and Ennio Prosperi

Summary

The cyclin-dependent kinase (CDK) inhibitor p21^{CDKN1A} (also known as p21$^{waf1/cip1}$) is a well known player of the G1 and G2 phase cell cycle checkpoints, which are activated in response to DNA damage. In addition, p21 interacts directly with proliferating cell nuclear antigen (PCNA), thereby inhibiting DNA replication. More controversial is the role of p21 in DNA repair, since both inhibition of and requirement for nucleotide excision repair have been suggested. Since the DNA repair process occurs at discrete nuclear foci in a chromatin-bound compartment, a suitable extraction procedure is necessary to investigate the association of p21 with PCNA in these structures. This chapter focuses on biochemical and immunofluorescence methods to analyze the recruitment of p21 protein to DNA repair foci. Cellular fractionation and subsequent nuclear extraction procedures are described for Western blot analysis of p21 recruitment, as well as for protein–protein interaction studies. An *in situ* extraction protocol is also described for immunofluorescence microscopy and flow cytometric analyses of nuclear localization and cell cycle distribution of p21 recruited to DNA repair foci. The combination of these methodologies is extremely powerful to investigate in more detail the role of p21 in the UV-induced DNA damage response.

Key Words: p21^{CDKN1A}; p21$^{waf1/cip1}$; DNA repair; PCNA; DNA damage; UV-C radiation; cyclin-dependent kinase inhibitor; protein recruitment; chromatin-bound proteins; DNase I.

1. Introduction

The cyclin-dependent kinase (CDK) inhibitor p21^{CDKN1A} (also known as p21$^{WAF1/CIP1/SDI1}$) is involved in various cellular pathways, including cell cycle checkpoints, senescence, and terminal differentiation ***(1)***. In addition, p21 has been shown to interact directly or indirectly with proteins regulating gene expression, thus suggesting a role for p21 in the regulation of transcription ***(2,3)***.

Although its activity is usually associated with CDK inhibition, p21 is peculiar in that it is able to interact directly with proliferating cell nuclear antigen

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

(PCNA), thereby inhibiting DNA replication ***(4,5)***. PCNA is a co-factor of DNA polymerases δ and ε which is necessary both for DNA replication and repair ***(6)***. However, given the multiple interactions with other DNA replication and repair factors, as well as with cell cycle-related proteins (cyclin–Cdk), PCNA plays a major role in coordinating DNA transactions with cell cycle control ***(7,8)***.

More controversial is the role of p21/PCNA interaction during DNA repair, because various reports have indicated that p21 may inhibit this process in vitro ***(9–11)***, or suggested that p21 does not play a significant role in DNA repair ***(12,13)***. In other studies, the inhibitory effect was not observed ***(14–16)***, and a positive role for p21 in the nucleotide excision repair (NER) pathway was suggested, particularly for its ability to interact with PCNA ***(17–19)***. In addition, in vivo studies performed on actively repairing normal fibroblasts, showed that p21 and PCNA co-localized ***(20)***, and co-immunoprecipitated ***(21)*** as a consequence of UV-induced DNA damage. These results suggest that p21/PCNA interaction underscores a precise, though not yet elucidated, role for the successful cellular response to DNA damage.

PCNA protein adopts a trimeric ring structure ***(22)***, which is extremely stable, and must be loaded onto DNA for DNA synthesis ***(6)***. When it encircles DNA, PCNA becomes insoluble to detergents, and in this form it has been shown to be actively involved in DNA replication ***(23)***, or in DNA repair ***(24)***. Similarly, p21 protein has also been shown to become detergent insoluble, not only after UV irradiation ***(20,21,25)***, but also after oxidative DNA damage ***(26)***. Although the role of this transition during DNA repair is not yet understood, it is conceivable that it depends on tight binding with detergent-insoluble PCNA. In fact, as already mentioned, co-localization and co-immunoprecipitation of p21 with PCNA have been documented by studies on the detergent-insoluble form of these proteins associated to DNA repair foci ***(20,21)***. Since p21 recruitment has been observed only in G1 and in G2 phase cells ***(21,26)***, which are involved in DNA repair and not in DNA replication, the p21-PCNA interaction is thought to be relevant for repair events, and not merely for cell cycle arrest ***(7,8)***.

Recruitment of p21 together with PCNA to DNA repair foci is in agreement with the evidence that nucleotide excision repair factors are assembled at the site of the DNA lesion ***(27)***. Thus, when associated with PCNA, p21 may be considered a chromatin-bound protein. In order to study in more detail the involvement of p21 in DNA repair, a suitable extraction procedure is required to release the detergent-insoluble forms of p21 and PCNA. Cell fractionation procedures are thus required for extraction of both detergent-soluble and -insoluble forms, and for subsequent western blot analysis and immunoprecipitation studies.

In this chapter, we describe a procedure that enables the release of these fractions of PCNA and p21 proteins without disruption of their interaction, thus making this protocol suitable for western blot analysis and/or further immunoprecipitation studies. The procedure is based on a hypotonic lysis in the presence of a detergent, for the release of soluble proteins ***(19,21,28)***. The detergent-insoluble fraction, representing chromatin-bound proteins, is then solubilized by enzymatic digestion of DNA with DNase I ***(19,21)***. This is made possible by the ability of PCNA to slide off a linear DNA molecule ***(29)***, thereby being released into solution together with its interacting proteins, including p21 ***(21)***.

An *in situ* extraction procedure ***(30,31)***, necessary to remove the detergent-soluble proteins from cells adherent on coverslips, is also described for the immunofluorescence analysis of cellular localization of chromatin-bound p21 protein during DNA repair. A similar procedure applied to cell suspensions allows the flow cytometric analysis of the cell cycle distribution of p21 protein involved in the DNA damage response.

2. Materials

1. UV-C germicidal lamp (emission peak at approx 254 nm).
2. UV-C radiometer.
3. Fluorescence microscope.
4. Flow cytometer.
5. Standard polyacrylamide gel electrophoresis (PAGE) and protein-transfer equipment.
6. Culture medium for normal cells (or tumor cells with normal p53-p21 pathway).
7. Protease inhibitor cocktail (solution in DMSO; Sigma or Roche) without EDTA. Store in aliquots at –20°C.
8. Formaldehyde.
9. p21 monoclonal antibodies: EA10 (Oncogene Science); DCS 60.2 (NeoMarkers); polyclonal antibody C-19 (Santa Cruz Biotechnology).
10. PCNA monoclonal antibody: clone PC10 (Dako).
11. Anti-mouse secondary antibody conjugated with fluorescein isothiocyanate (FITC), Alexa 594 (Molecular Probes), or biotin.
12. Streptavidin-FITC, streptavidin-Texas Red, streptavidin-horseradish peroxidase (HRP).
13. Protein A-sepharose (Pharmacia).
14. Bovine serum albumin (BSA).
15. Peroxidase substrates for enhanced chemiluminescence (ECL) detection (Amersham Biosciences).
16. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 10.6 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, pH 7.4.
17. Physiological saline: 154 m*M* NaCl.
18. Hypotonic lysis buffer: 10 m*M* Tris-HCl (pH 7.4), 2.5 m*M* $MgCl_2$, 0.5% Nonidet P-40. Use freshly prepared solution, and immediately before use add 1 m*M* DTT

(dithiothreitol; stock is 1 *M*, store in aliquots at –20°C), 1 m*M* PMSF (phenylmethylsulfonyl fluoride; stock is 200 m*M* in isopropanol, store at 4°C), 0.2 m*M* sodium vanadate (Na_3VO_4; stock is 100 m*M;* store in aliquots at –20°C) 0.5 µM okadaic acid (stock is 150 µM in DMSO), and 100 µL/10^7 cells of protease inhibitor cocktail. Keep supplemented buffer on ice.

19. Washing buffer: 10 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 1 m*M* PMSF, and 50 µL/10^7 cells of protease inhibitor cocktail.
20. DNA digestion buffer: 2X solution is 20 m*M* Tris-HCl (pH 7.4), 20 m*M* NaCl, 10 m*M* $MgCl_2$, 20 µL/10^7 cells of protease inhibitor cocktail. Prepare fresh solution each time.
21. DNase I solution: DNase I (Sigma D-4527 or equivalent) dissolved in 20 m*M* NaCl and 0.1 m*M* PMSF just prior to use.
22. SDS-loading buffer: 65 m*M* Tris-HCl (pH 7.4), 100 m*M* DTT, 1% SDS (sodium dodecyl sulfate), 10% glycerol, 0.02% bromophenol blue. Store at –20°C.
23. PBS–Tween-20 solution: PBS containing 0.2 % Tween-20.
24. PBT solution: PBS containing 1% BSA and 0.2% Tween-20. Prepare fresh solution each time.
25. Ponceau S: 0.1% in 5% acetic acid solution. Store at room temperature.
26. Blocking solution: 5% non-fat dry milk plus 0.2% Tween-20 in PBS. Prepare fresh solution each time.
27. Propidium iodide (PI): stock solution is 500 µg/mL in double-distilled H_2O. Store at 4°C.
28. Bisbenzimide H 33258 (Hoechst 33258): stock solution is 1 m*M* in double-distilled H_2O. Store at 4°C.
29. 4',6'-diamidino-phenylindole (DAPI): stock solution is 1 m*M* in double-distilled H_2O. Store at 4°C.
30. RNase A. Use freshly prepared at 1 mg/mL in PBS.
31. Mounting medium (e.g., Mowiol) containing 0.25% anti-fading agent (1,4-diazabicycloctane).

3. Methods

The methods described below outline the procedures to investigate the recruitment of p21 to DNA repair foci in response to UV-induced DNA damage. In order to obtain reliable results, exposure of the cells to UV light under accurately determined conditions are first described (*see* **Subheading 3.1.**). The transition of p21 protein from a detergent-soluble to a detergent-insoluble state is analyzed with biochemical techniques by using cell fractionation procedures (*see* **Subheading 3.2.**). The extraction of the detergent-soluble protein is obtained with a hypotonic solution in the presence of a non-ionic detergent ***(19,21,28)***. The subsequent release of chromatin-bound proteins is obtained by an extraction procedure based on DNA digestion ***(19,21)***. The detection of p21 protein in each fraction is performed by Western blot analysis, but immunoprecipitation studies can also be performed. Recruitment of p21 to DNA repair

foci is concomitantly analyzed by means of immunocytochemical techniques and fluorescence microscopy (*see* **Subheading 3.3.**), which allow direct visualization of the protein associated with DNA repair foci. In addition, with a similar procedure, cell cycle distribution of the p21 protein is analyzed by flow cytometry ***(21,26)***.

3.1. UV Light Irradiation

UV irradiation is one of the most used methods to induce DNA damage, thereby triggering the DNA repair response in normal cells, or in tumor cells with a normal p53–p21 pathway. Since the emission spectrum of different UV lamps (often used are germicidal lamps) may vary, an accurate determination of the energy supplied to the cells, must be made.

Particular care must be taken to avoid accidental exposure to the UV-C light of the lamp. Use appropriate clothing, gloves, and protective goggles. As the optimal condition, the lamp is placed under a wooden box with an open side covered with a black sheet of paper or cloth, which can be lifted to introduce the cells .

1. Monitor the energy of the lamp (e.g., T-UV9, Philips) in the UV-C region by using a radiometer (e.g., Spectronics, USA) equipped with a detector sensitive in the 200–280 nm range.
2. Set the distance of the UV source from the working level, so that the flux of UV light will be 0.5–1 J/m^2/s. Check that the UV energy will be uniformly distributed throughout the surface to be exposed.
3. Use cells grown on petri dishes (e.g., 10-cm diameter). For immunocytochemical analysis, grow the cells attached on coverslips (24 × 24 mm) that are placed into smaller dishes (35-mm diameter). Perform all subsequent steps under a laminar-flow hood to avoid contamination during exposure. The glass window of the hood will also protect from UV light reflected from the working level.
4. Remove the culture medium and wash the cells twice with warm sterile PBS. Aspirate almost all PBS, leaving just a thin liquid layer on top of the cells.
5. Remove the dish lid and expose the cells to UV light for the period of time required to supply the chosen dose (e.g., 10 J/m^2).
6. Add back the culture medium and reincubate cells at 37°C for the desired period of time (a suitable time course ranges from 30 min to 24 h).
7. Detach cells from the petri dish with a standard trypsinization procedure.

3.2. Biochemical Analysis of p21 Recruitment to DNA Repair Foci

The analysis of p21 recruitment to DNA repair foci by biochemical techniques includes: (a) the extraction of the detergent-soluble protein; (b) the release of chromatin-bound protein by DNase I digestion; (c) the immunoprecipitation of the p21 protein complexed with PCNA.

3.2.1. Extraction of Detergent-Soluble p21 Protein

Either fresh cells or frozen pellets in 2-mL Eppendorf tubes can be used with this extraction procedure.

1. Chill fresh cells or thaw frozen samples to 0°C on ice, and resuspend in 1 mL of the hypotonic lysis buffer. The volume of lysis buffer (1 mL) is given for extraction from a cell pellet of about 10^7 cells, but it can be scaled down for smaller samples. However, for an efficient extraction of soluble nuclear proteins, add about 10 vols of lysis buffer to the cell pellet.
2. Gently resuspend the cell pellet by pipetting the sample three to four times, and allow lysis to occur for 10 min on ice (*see* **Note 1**).
3. Pellet the samples by low-speed centrifugation (300*g*, 1 min, 4°C). Collect the supernatant containing the detergent-soluble protein fraction, for protein content determination with the Bradford method. For normal human fibroblasts, about 2 mg/mL of soluble proteins are released from 10^7 cells. If this amount is lower, other soluble proteins may be released in the next washing step, meaning that extraction has not been complete (*see* **Note 2**). Determine the protein concentration also in the fraction released in the washing buffer, and if necessary add a further washing step. The detergent-soluble proteins may be analyzed by western blot (*see* **Fig. 1**) or used for subsequent immunoprecipitation studies, to be compared to the chromatin-bound protein fraction.

3.2.2. Extraction of Detergent-Insoluble p21 Protein by DNase I Digestion of DNA

After extraction of the detergent-soluble protein fraction, chromatin-bound proteins must be solubilized for Western blot analysis. The procedure described below, which is also suitable for immunoprecipitation studies ***(21)***, allows the release of DNA-bound protein complexes, and is based on DNA digestion with DNase I (*see* **Note 3**).

1. Resuspend permeabilized cells in washing buffer in order to remove as much as possible any trace of soluble proteins (*see* **Note 2**).
2. Centrifuge the samples (300*g*, 1 min), and then resuspend the pellets in a half-volume of DNase I solution (250 µL for a final digestion volume of 500 µL) containing 100–200 DNase I units/10^7 cells. After thorough resuspension, add the second half-volume of 2X digestion buffer (*see* **Note 4**). The amount of DNase I (*see* **Note 5**) to be added is dependent on the cell type (e.g., cells with a DNA content higher than the diploidy require proportionally higher amounts of DNase I, as compared with normal diploid cells).
3. Carry out the digestion for 30 min at 37°C with constant agitation in order to avoid cell sedimentation and clumping (*see* **Note 6**).
4. Pellet the samples by high-speed centrifugation (14,000*g*, 1 min) and collect the resulting supernatants, containing the DNase-released proteins, for subsequent use.

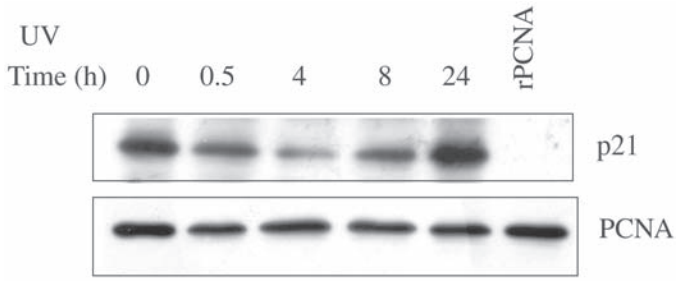

Fig. 1. Western blot analysis of the detergent-soluble p21 and PCNA proteins in cell extracts obtained from normal human fibroblasts from control (0 h) or UV-irradiated samples (10 J/m^2) collected at the indicated periods of time (0.5–24 h). Recombinant PCNA (rPCNA) was loaded as a control. The samples were probed with C-19 rabbit polyclonal antibody to p21, or with PC10 mouse monoclonal antibody to PCNA, and with secondary HRP-conjugated anti-rabbit or anti-mouse antibody.

5. Load 50 µg of detergent-soluble proteins, and a similar or higher volume (*see* **Note 7**) of the DNase-released fraction, on a 12% polyacrylamide gel for standard Western blot analysis.
6. Perform Western blot analysis with antibodies against p21 and PCNA (*see* **Fig. 2**).

3.2.3. Immunoprecipitation of Detergent-Soluble and DNase-Released Proteins

If fractionated extracts will be used for immunoprecipitation studies, perform the following steps:

1. Take a sample of soluble cell lysate containing 1.0–1.5 mg total protein, and use the whole sample of DNase-released proteins. Set apart a small volume (30–50 µL) of each fraction to be loaded on the gel as a control of the protein input.
2. Dilute both samples in washing buffer and add 10 µL of polyclonal antibody (C-19), or 0.5–5.0 µg of monoclonal antibody to p21 (EA10), for the soluble fraction. Since the amount of antibody to be added depends on the amount of target protein to be precipitated, a half-volume of antibody (e.g., 5 µL) will be sufficient for the DNase-fraction.
3. Incubate for 30 min at room temperature (RT) and then overnight at 4°C.
4. Add 100 µL of protein A–sepharose beads (10% vol/vol in 50 m*M* Tris-HCl buffer, pH 7.4) to the mixture. Incubate for 1 h at 4°C on a rocking platform (*see* **Note 8**).
5. Precipitate the beads by centrifugation at 14,000*g* for 20 min at 4°C. Remove the supernatant by gentle aspiration.
6. Wash the immune complexes on the beads three times with washing buffer. Each time, add 0.5 mL of washing buffer and re-suspend the beads by gentle vortexing. The final wash should be removed completely.

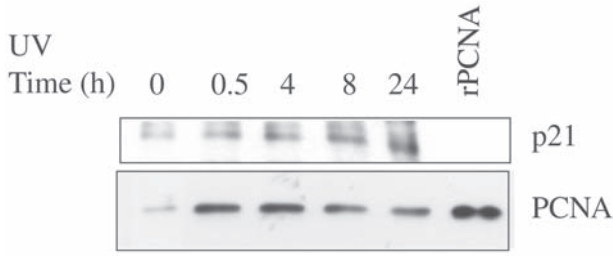

Fig. 2. Western blot analysis of chromatin-bound (detergent-insoluble) p21 and PCNA proteins released with DNase I from normal human fibroblasts after hypotonic lysis of the samples shown in **Fig. 1**. Control (0 h) and UV-irradiated cells were collected at the indicated periods of time (0.5–24 h). Recombinant PCNA (rPCNA) was loaded as a control of migration of solubilized protein. The samples were probed with C-19 polyclonal antibody, or with PC10 monoclonal antibody to PCNA, then with biotin-conjugated secondary antibodies, followed by a third step with streptavidin-HRP. Note the late recruitment of p21, as compared with the early increase in PCNA.

7. For SDS-PAGE, add 60 µL of SDS loading buffer to each sample.
8. Denature the proteins in the sample by heating to 100°C for 5 min. Centrifuge for 15 s at 10,000*g* and load the supernatant on the gel.

3.2.4. Immunoblot Analysis of Fractionated Cell Extracts or Immunoprecipitated Proteins

1. After SDS-PAGE, transfer the proteins to a nitrocellulose (or PVDF) membrane with a conventional apparatus (semy-dry or immersion).
2. Check the protein transfer to the membrane by Ponceau S staining.
3. Remove excess stain with several washes in double-distilled H_2O.
4. Block the membranes for 30 min at RT with blocking solution.
5. Incubate the membranes for 1 h at RT with monoclonal or polyclonal antibody to p21 protein (diluted 1:200 or 1:500, respectively, in PBS–Tween-20).
6. Wash the membranes four to six times (10 min each) with PBS–Tween-20, followed by a 30-min incubation in a secondary biotinylated antibody (diluted 1:2000 in PBS–Tween-20).
7. Repeat washes as in **step 6**, and then incubate for 30 min at RT with streptavidin-HRP (diluted 1:1000 in PBS–Tween-20) to amplify the immunological reaction and enhance the visualization of p21 protein in the DNase fraction. Samples obtained after immunoprecipitation do not need amplification; in this case, a two-step reaction with a secondary HRP-conjugated antibody is generally sufficient to detect the relevant signals (*see* **Fig. 3**).
8. Incubate the nitrocellulose (or PVDF) membranes with peroxidase substrates for ECL detection and expose to autoradiographic film.

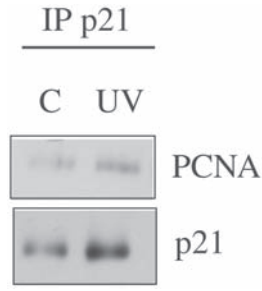

Fig. 3. Co-immunoprecipitation of chromatin-bound (detergent-insoluble) p21 and PCNA proteins released with DNase I from normal human fibroblasts. Control (C) and UV-irradiated (UV) samples were collected at 16 h after irradiation (10 J/m^2), lysed in hypotonic solution, and then processed for enzymatic digestion to release chromatin-bound proteins. Immunoprecipitation of p21 protein was performed with polyclonal antibody (C-19), and detection of p21 and PCNA in the immunoprecipitates was performed with C-19 and PC10 antibodies, respectively.

3.3. Detection of p21 Protein Bound to DNA Repair Foci Using Immunofluorescence Microscopy and Flow Cytometry

For the cytochemical determination of p21 protein by immunofluorescence microscopy and flow cytometry, only extraction of the detergent-soluble protein is necessary to remove the protein which is not associated to DNA repair nuclear structures ***(26,30,31)***. However, the two techniques require different cell-preparation procedures; thus separate descriptions are given.

3.3.1. In Situ *Extraction for Fluorescence Microscopy Analysis of Chromatin-Bound p21 Protein*

A procedure similar to that given in **Subheading *3.2.1.*** is also useful for extraction of detergent-soluble protein from cells adherent to coverslips ***(26)*** or microscope slides. However, it requires some modification, as follows:

1. Rinse the coverslips with PBS, then dip into cold double-distilled H_2O for about 4 s ***(26)***. For some cells (e.g., HeLa) dipping in physiological saline may be preferable (*see* **Note 9**).
2. Transfer the coverslips to a petri dish containing cold hypotonic lysis buffer (containing 0.1 m*M* PMSF) in which the detergent (NP-40) concentration has been reduced to 0.1%, to avoid detachment of cells (*see* **Note 10**). Keep the petri dishes at 4°C for about 10 min, with gentle agitation every 3–4 min.
3. Remove the lysis solution and wash the cells carefully with cold PBS or physiological saline, then aspirate and replace with fresh PBS (the volume is dependent on the petri dimension—e.g., for a 4-cm dish, use 2 mL).

4. Add an equal volume of 2% formaldehyde (*see* **Note 11**) solution (the final concentration is 1%) in PBS to fix the permeabilized cells (*see* **Note 12**).
5. Carry out fixation for 5 min at RT under continuous agitation. Wash the coverslips again with PBS and further post-fix in 70% ethanol. The samples can be stored in this solution at –20°C for 1–2 mo before further processing for immunocytochemical staining.

3.3.2. In Situ *Extraction for Flow Cytometric Analysis of Chromatin-Bound p21 Protein*

For flow cytometric analysis of chromatin-bound p21 protein, again follow the procedure described in **Subheading 3.2.1.** for the extraction of detergent-soluble protein, except that a lower number of cells ($1–2 \times 10^6$) is required for each sample to be examined ***(21,30)***. Keep the lysis volume to 1 mL to facilitate the complete extraction of soluble protein while reducing the number of subsequent passages and centrifugation steps. Use fresh cells in 15-mL plastic tubes.

1. After the hypotonic lysis, resuspend the cell pellet in 1 mL of cold PBS or physiological saline.
2. Fix the samples by adding 1 mL of 2% formaldehyde (the final concentration is 1%), incubate for 5 min at RT, and then remove the fixative by centrifugation (300*g*, 5 min). Resuspend the cells in 1 mL of cold physiological saline and post-fix by adding 2.3 mL of 95% ethanol (the final concentration is 70%), with gentle mixing of the two phases. The samples can be stored at –20°C up to 1–2 mo before further processing for immunocytochemical staining.

3.3.3. Immunocytochemical Staining of p21 Protein Associated to DNA Repair Foci

Described below is the method for immunostaining, which is essentially similar for lysed cells attached to coverslips or for cells in suspension. However, the procedures are described separately in order to highlight some relevant, different steps.

3.3.3.1. Cells on Coverslips

1. Remove the fixative and wash the cells with PBS.
2. Block the unspecific staining sites with PBT solution, for 15 min at RT.
3. Incubate the coverslips with anti-p21 monoclonal antibody (either EA10 or DCS 60.2, diluted 1:100 in PBT solution) for 1 h at RT; place the coverslips, with cells facing upside-down, onto a drop (50 µL) of antibody, so that a sandwich is formed on a flat parafilm strip (*see* **Note 13**).
4. Remove the antibody by placing the coverslips in petri dishes with the cells facing up, and wash three times (10 min each) with PBS–Tween-20.
5. Incubate the coverslips for 30 min at RT with anti-mouse secondary antibody (diluted 1:100 in PBT solution) by placing the cells upside-down on a drop of

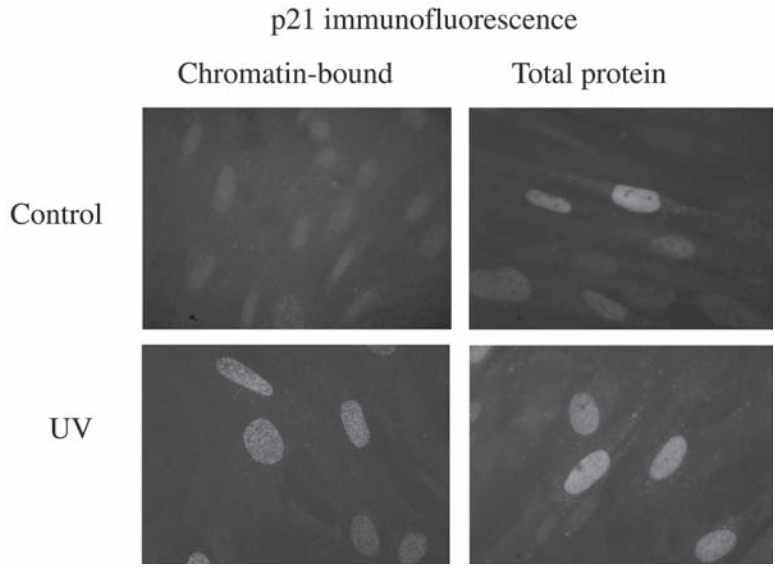

Fig. 4. Immunofluorescence staining of chromatin-bound vs total p21 protein in normal human fibroblasts before (C) and 24 h after UV irradiation (UV). Chromatin-bound protein was detected after *in situ* hypotonic lysis, to remove the detergent-soluble protein fraction. After fixation, the samples were immunostained with DCS 60.2 monoclonal antibody to p21, followed by incubation with biotin-conjugated secondary antibody, and then streptavidin-FITC. To detect total p21 protein content, whole cells were fixed and then immunostained as above. Visualization was performed with an Olympus BX51 fluorescence microscope at a ×1000 magnification.

antibody, as described above. Use antibody conjugated with FITC (green fluorescence), with Alexa 594 (red fluorescence), or better with biotin, in order to amplify the signal (*see* **Note 14**).

6. Remove the secondary antibody and wash three times (10 min each) with PBS–Tween-20.
7. If a step with a biotinylated secondary antibody was performed, then incubate with streptavidin-FITC or streptavidin–Texas Red for 30 min and then repeat washes with PBS–Tween-20, as in **step 6**.
8. Counterstain DNA with Hoechst 33258 or DAPI (0.1 μg/mL in PBS) for 2 min at RT, then remove the dye and wash the coverslips twice (5 min each) with PBS.
9. Mount the coverslips in aqueous mounting medium (e.g., Mowiol) containing anti-fading.
10. After mounting, view slides with a fluorescence microscope (*see* **Fig. 4**) equipped with filter sets for UV, blue, and green excitation of fluorescence.

11. Seal the coverslips with transparent nail polish to prevent them from drying, and to allow storage of slides at –20°C.

3.3.3.2. Cell Suspensions

1. Bring samples stored at –20°C to RT and remove the fixative by centrifugation (300*g*, 5 min). Resuspend the cells in PBT solution and incubate the samples for 15–30 min at RT to block the unspecific staining sites, as described for coverslips.
2. Remove PBT solution by centrifugation, and add 100 µL of p21 monoclonal antibody (diluted 1:100 in PBT). Either EA10 ***(21)*** or DCS 60.2 antibodies may be used with similar results.
3. Add a blank sample to be incubated with an irrelevant IgG (e.g., Sigma), to evaluate the background fluorescence ***(21)*** and to select the baseline values on the flow cytometer.
4. Incubate for 1 h at RT with frequent agitation of the tubes to avoid cell sedimentation and understaining of sedimented cells.
5. Add 1 mL of PBT solution and centrifuge (300*g*, 5 min). Remove the supernatant and wash the cells twice (10 min each) in 1 mL of PBT solution.
6. Incubate the cells in 100 µL of FITC-conjugated anti-mouse secondary antibody (diluted 1:100 in PBT solution) for 30 min at RT, with frequent agitation, as in **step 4**.
7. Repeat the washes, as in **step 5**, except that after the last wash, cells are again centrifuged and resuspended in 1 mL PBS containing 10 µg/mL propidium iodide (PI) and 1 mg RNase A to remove any RNA interfering with the PI staining of DNA (*see* **Note 15**).
8. Stain for 30 min at RT, and then overnight at 4°C (*see* **Note 16**), then measure the samples on a flow cytometer (*see* **Fig. 5**).

4. Notes

1. Resuspension of the cell pellet by pipetting may produce foam due to the presence of the detergent; however this will not compromise extraction of soluble p21 protein. Care must be taken if cells with small cytoplasm (e.g., lymphocytes) are to be used, since pipetting could result in nuclear damage and loss. In this case, pipet only two to three times.
2. When the extraction of detergent-soluble proteins is efficient, the amount of proteins released in the subsequent washing buffer should not be higher than 20% of the initial release.
3. An alternative procedure to release chromatin-bound proteins is based on the production of DNA strand breaks by sonication. This procedure is as effective as DNA digestion in releasing DNA-bound proteins in a form suitable for immunoprecipitation experiments. This would not be otherwise possible with protocols using SDS to release nuclear-bound proteins ***(25)***. However, when using sonication, care must be taken to reduce protein degradation by using a "low" setting and short pulses.

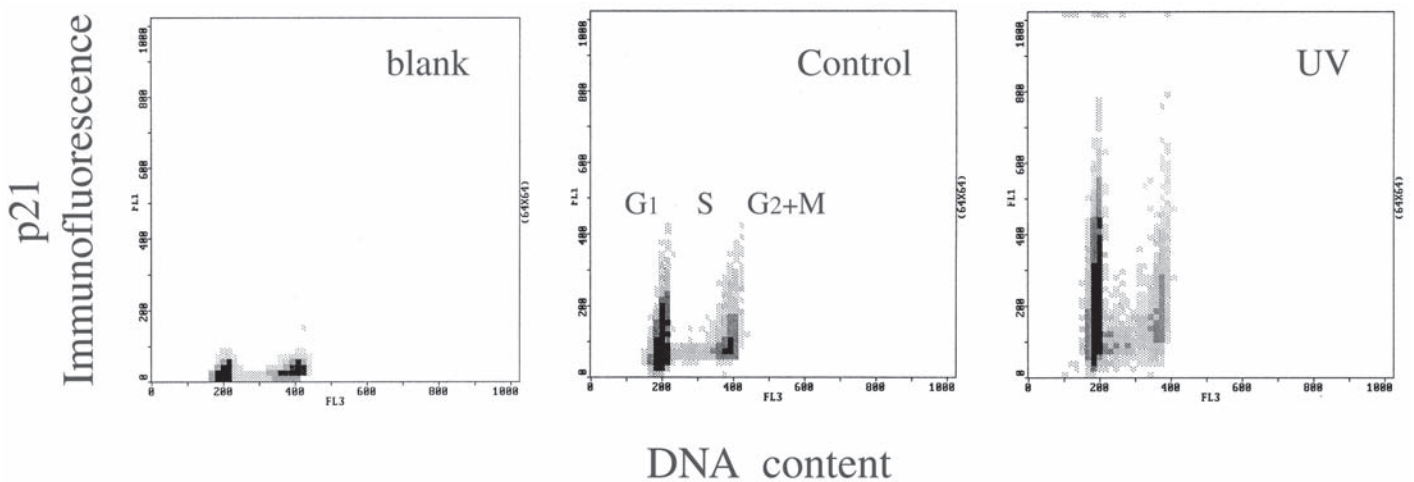

Fig. 5. Flow cytometric analysis of cell cycle distribution of chromatin-bound p21 protein in normal human fibroblasts, before (C) and after UV-irradiation (UV). Cells were collected 24 h after irradiation (10 J/m^2), and processed for *in situ* hypotonic lysis to release detergent-soluble proteins. After fixation, samples were immunostained with DCS 60.2 monoclonal antibody to p21, followed by a biotin-conjugated secondary antibody, and then by streptavidin-FITC. The sample marked as blank was incubated with an irrelevant mouse IgG antibody instead of the anti-p21 antibody, to evaluate unspecific staining. Cells were finally stained with PI for DNA content determination, and then measured with a Coulter Epics XL flow cytometer. The cell cycle compartments (G_1, S, and G_2 + M) are indicated in the control panel to show the distribution of p21 immunofluorescence. After UV exposure, the increase in p21 immunofluorecence is detectable only in the G_1 and G_2 + M compartments.

4. A good resuspension of the cell pellet in the DNase solution is a requisite for an efficient DNA digestion, and consequent release of DNA-bound proteins. The addition of 2X digestion buffer after cell resuspension is performed because of the Mg^{++} contained in the digestion buffer, which will favor cell clumping, thereby reducing the extent of DNA digestion.
5. The quality of DNase I is very important, since contaminants present in different preparations (e.g., chymotrypsin) may be responsible for proteolytic degradation. A chromatographically purified preparation (e.g., Sigma D-4527) is recommended.
6. If cell clumping occurs, a very brief sonication will help to disrupt cellular aggregates and facilitate DNA breakage.
7. Given that p21-PCNA bound to DNA constitutes only a minor fraction of the total cellular amount of these proteins, loading of proportionally higher volumes of DNase-extracts, as compared to the soluble fraction, may be required to improve the visualization of these proteins by Western blot. It is advisable to check the protein loading of different samples, by detecting another protein (e.g., actin) as an internal standard.
8. It may be useful to pre-bind the antibody to the protein A–sepharose beads, so that the complex will be incubated with fractionated cell extracts only once. This step will reduce the total time of the procedure, thereby also avoiding possible protein degradation.
9. Dipping of the cells in cold double-distilled H_2O is particularly useful with cells with large cytoplasm (e.g., with fibroblasts), since this will favor the extraction of detergent-soluble proteins. However, when using cells with a small cytoplasm (e.g., some tumor cells like HeLa), the hypotonic stress is likely to result in a substantial detachment of the cells from the coverslip.
10. Avoid to pipetting the hypotonic lysis solution directly onto the cells, since this will increase the risk of cell detachment from the coverslip.
11. Formaldehyde vapors are toxic. Solutions containing formaldehyde should be prepared in a chemical hood.
12. For some cell types (e.g., HeLa cells), the final formaldehyde concentration is increased up to 4% because it has been noticed that cells fixed with lower concentrations will loose their nuclear morphology after freezing-thawing of slides stored at –20°C.
13. The antibody sandwich formed with the coverslip on the parafilm strip will not dry under normal RT conditions, so it is not necessary to use a humid chamber. This has the advantage of reducing the incubation volume, thus saving antibody.
14. The choice of secondary antibody depends on the fluorescence emission, which has to be detected under the microscope or with the flow cytometer. In the latter case, a FITC-conjugated antibody (or streptavidin-FITC) is preferred because the fluorescence emission can be measured in the green channel (515–540 nm), thus leaving the red channel for the DNA stain (PI) emission (590–620 nm).
15. Propidium iodide (like ethidium bromide) is an intercalating agent with mutagenic properties, and therefore accidental exposure of skin or other body

parts is to be avoided. Always wear gloves, especially when handling concentrated stock solutions.

16. Formaldehyde fixation reduces the DNA stainability with intercalating dyes, such as PI. Thus, prolonged DNA staining is necessary to obtain optimal resolution of the DNA histogram, which greatly depends on the amount of fluorochrome bound to DNA.

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6

Quantitative Determination of p16 Gene Expression by RT-PCR

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Summary

In recent years, gene expression quantitation of tumor cells has become of principal importance to analyze gene patterns responsible for cancer development, progression, and resistance to treatment. Whereas semi-quantitative methods, such as Northern blotting analysis, allow only a dichotomous differentiation between positive and negative gene expressions, the real-time quantitative polymerase chain reaction (qRT-PCR) combines a large range of results with an accurate and highly reproducible quantitation of genes. In addition to forward and reverse primers, as used in a conventional PCR, the qRT-PCR system utilizes a probe that is labeled with a fluorescent dye. The probe is an oligonucleotide, homologous to a DNA sequence between the two flanking PCR primers. Degradation of the probe by the activity of the *Taq* DNA polymerase generates a fluorescent signal that will be detected by means of a laser integrated in the sequence detector. This chapter will cover the isolation of total RNA from frozen tissue, the transcription of RNA to cDNA, and the analysis of the relative gene expression by qRT-PCR. Although in principle applicable to any gene of interest, we will use as an example the qRT-PCR analysis of $p16^{INK4a}$, whose gene product is involved in cell cycle and senescence checkpoint control.

Key Words: Quantitative RT-PCR; probe; p16; gene expression; real-time PCR; RNA isolation; reverse transcription.

1. Introduction

Real-time quantitative polymerase chain reaction (qRT-PCR) is a powerful tool used for gene expression analysis, genotyping, pathogen detection, mutation screening, and DNA quantification. PCR-based techniques allow the investigator to obtain genetic information through the specific amplification of nucleic acid sequences starting with a very low number of target copies.

The principle of PCR is based on the hybridization of oligonucleotide sequences, referred to as primers, to a specific template DNA. The primers are

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

complementary to opposite strands of DNA. Upon hybridization of the template DNA with the primer, DNA synthesis starts, supported by a thermostable *Taq* DNA polymerase ***(1)***. PCR consists of three reactions: denaturation, annealing, and extension. The DNA template is denatured at high temperatures to permit annealing of the primers to the template. After annealing, the primers are extended to generate a copy of the template sequence between the two primers. A continuous cycle of denaturation, annealing, and extension follows. The result is an exponential amplification of the DNA template.

In addition to forward and reverse primers, the qRT-PCR system utilizes an oligonucleotide probe that is homologous to a DNA sequence between the two flanking PCR primers. The probe is labeled with a reporter fluorescent dye, such as 6-carboxyfluorescein (FAM), and a quencher dye, 6-carboxy-tetramethyl-rhodamine (TAMRA), attached at the 5' and 3' end, respectively. During the extension phase, the quencher can quench the reporter fluorescence only when the two dyes are close to each other, which is the case if the probe is intact ***(2)***. Degradation of the probe by the activity of the *Taq* DNA polymerase cleaves the reporter dye from the probe and generates a fluorescent signal **(Fig. 1)** ***(3,4)***. The fluorescence will be detected by means of a laser integrated in the sequence detector **(Fig. 2)** ***(1,3)***. The fluorescence signal is generated only if the target sequence for the probe is amplified during PCR. Because of these requirements, nonspecific amplification is not detected ***(5,6)***.

The PCR cycle number at which fluorescence reaches a threshold value of 10 times the standard deviation of baseline fluorescence emission is used for quantitative measurement. **Figure 3** shows a typical amplification plot, reflecting the generation of the reporter dye during amplification. The intersection between the amplification plot and the threshold is defined as the cycle threshold (CT) value. The CT value is related directly to the amount of PCR product and, therefore, related to the original amount of target present in the PCR reaction. Since the amount of genetic material added to each qRT PCR tube and its quality are not reliable parameters in order to measure the starting material, the number of copies of an endogenous control gene—referred to as a "housekeeping gene"—is also analyzed. For each experimental sample the value of both the target (e.g., p16) and the housekeeping gene (e.g., β-actin), are extrapolated from the respective standard curve equation **(Fig. 4)**. The target value is then divided by the endogenous reference value to obtain a normalized target value independent from the amount of starting material (ΔCT).

A 10-fold serial dilution of a control template is used to generate the standard curve. The resulting CT values for each input amount of template are plotted as a function of the $\log_{10}$ concentration of input amounts, and a linear trend-line is fitted to the data. The resulting slope of the line is used to determine the PCR efficiency. An ideal slope should be –3.32 for 100% PCR effi-

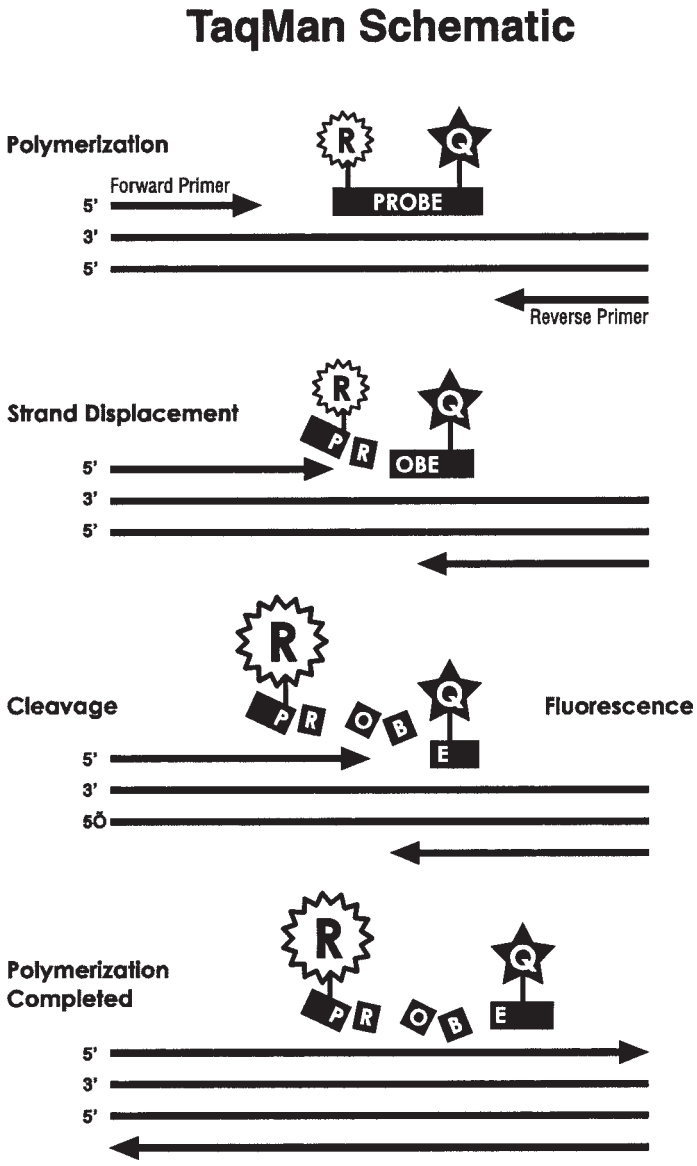

Fig. 1. Principles of quantitative real time PCR using fluorogenic probes. Forward and reverse primers are extended with Taq polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes anneals to the gene sequence between the two primers. As the polymerase extends the primer, the probe is displaced. The Taq polymerase cleaves the reporter dye from the probe. After release of the reporter dye from the quencher, a fluorescent signal is generated.

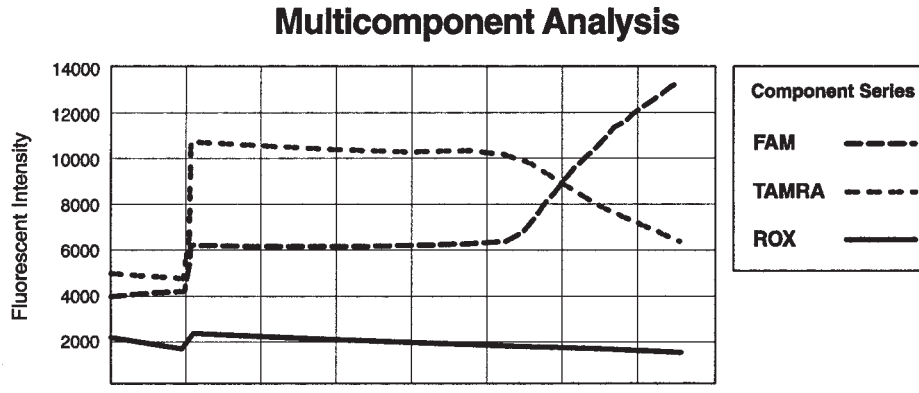

Fig. 2. Fluorescence signal plotted vs PCR cycle number. The threshold (marked with the dotted line) is arbitrarily placed well above the background noise (above 10^{-2}) and bleow the bginning of the plateau phase of the amplification curve (below 5×10^{-1}). The intersections between the amplification curves and the threshold line are entitled CT values. The distance between two CT values is cal ΔCT. 96-well optical rection plates.

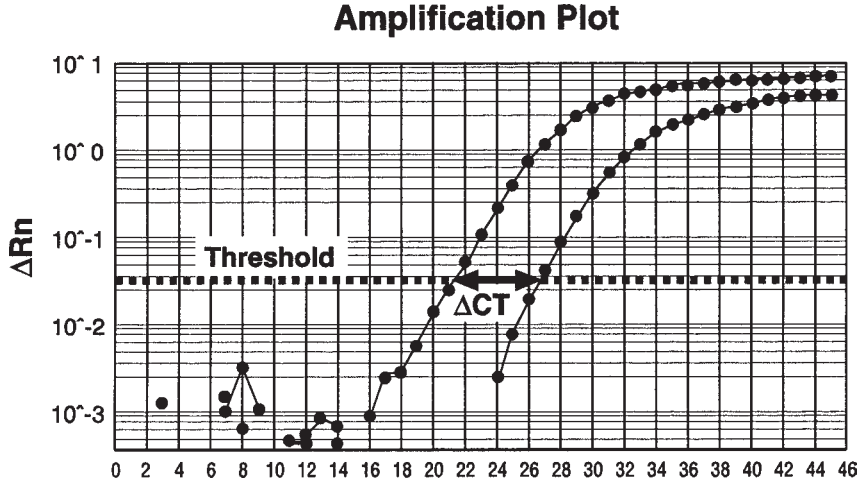

Fig. 3. Multicomponent analysis. Single fluorescent components of the reaction vs cycle numbers. A Positive TaqMan result is indicated by increasing the fluorescent intensity of the reporter dye, FAM, and by decreasing the fluorescent intensity of the second fluorescent tag, TAMRA. Another fluorescent component is ROX, which is mixed in the PCR buffer to a constant concentration and therefore may be used to normalize fluorescent signals when differences in the concentrations of the PCR reaction mix occur.

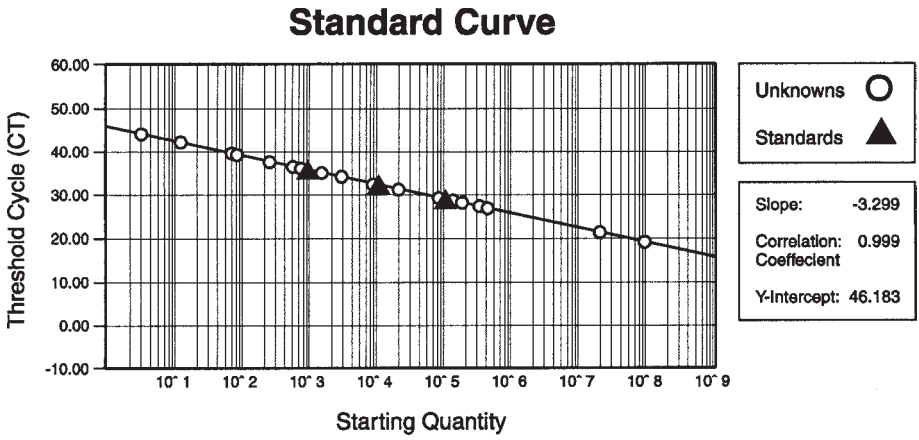

Fig. 4. Standard curve plot for calculation of PCR efficiency and quantification. A 10-fold serial dilution of a positive control template is used to generate a standard curve. The resulting CT values for each input amount of template are plotted as a function of the log10 concentration of input amounts and a linear trend-line is fit to the data.

ciency, meaning that the amount of template is doubled after each cycle. Optimal standard curves are based on PCR amplification efficiency from 95 to 100%. Most real-time PCR instruments have software that can automatically calculate the amount of template of an unknown sample from a standard curve.

To compare the results of two different TaqMan plates with each other, it is important to analyze the same standardized samples on every plate. The analyzed amount of these control samples should be multiplied by a factor in order to gain the same number in each analysis. All amounts of unknown samples analyzed in one plate should be multiplied by this factor.

$p16^{INK4a}$ belongs to a number of cell cycle-regulatory proteins and is involved in tumor suppression in the Rb and p53 pathways ***(7,8)***. $p16^{INK4a}$ blocks cell cycle progression by binding cyclin-dependent kinase (CDK 4 or 6) and inhibiting the action of cyclin D ***(9)***. By inhibiting retinoblastoma protein phosphorylation, $p16^{INK4a}$ can block the formation of an active transcriptional complex (E2F–DB) and promote the formation of a repressive transcriptional complex (Rb–E2F), which blocks cell cycle progression past the G1-S restriction point ***(10)***. $p16^{INK4a}$ alteration has been linked to tumorigenesis in different cancers, including nonsmall cell lung cancer ***(11)*** and colon cancer ***(12)***.

In this chapter, we will describe the extraction of total RNA from frozen tissue, the reverse transcription to cDNA, and the analysis of mRNA expression levels of the target gene $p16^{INK4a}$ and the internal reference gene β-actin, using quantitative real-time PCR.

2. Materials

2.1. mRNA Isolation

1. Steel mortars and pestles.
2. Guanidine thiocyanate (Fluka).
3. 1 *M* sodium citrate (pH 7.0).
4. 10% Sarcosyl.
5. 14 *M* 2-β mercaptoethanol. Store at 4°C.
6. RNAse-free H_2O.
7. Chloroform.
8. Isoamyl alcohol.
9. Water-saturated phenol (pH 5.0). Store at 4°C.
10. 2 *M* sodium acetate (pH 4.0).
11. Glycogen solution (5 mg/mL). Store at -20°C.
12. 100% isopropanol.
13. 70% ethanol.
14. 5 m*M* Tris-HCl (pH 9.0).

2.2. Reverse Transcription

1. 5X M-MLV buffer (Life Technologies): 250 m*M* Tris-HCl (pH 8.3), 375 m*M* KCl, 15 m*M* $MgCl_2$. Store at –20°C.
2. 100 m*M* dithiothreitol (DTT) (Pharmacia Biotech). Store at –20°C.
3. 10 m*M* dNTP mix (set of dATP, dGTP, dCTP, and dTTP), 1:10 dilution from 100 m*M* stock (Pharmacia Biotech). Store at –20°C.
4. 3.0 mg/mL BSA (RNA carrier, RNAse and DNAse free). Store at –20°C.
5. $pd(N)_6$ random hexamers (Pharmacia Biotech): 50 optical density, dissolved in 550 µL of 10 m*M* Tris-HCl (pH 7.5) and 1 m*M* EDTA. Store at –20°C.
6. M-MLV reverse transcriptase: 200 U/µL (Life Technologies). Store at –20°C.
7. 30,000 U/mL RNAguard RNase inhibitor (Pharmacia Biotech). Store at –20°C.

2.3. PCR

1. 5 m*M* Tris-HCl (pH 9.0).
2. Standard cDNA templates (STND), e.g., from human colon, liver, lung (Stratagene).
3. Double-distilled H_2O (RNAse and DNAse free).
4. 25 m*M* $MgCl_2$ solution. Store at RT.
5. 10X Taq buffer A (Applied Biosystems). Store at –20°C.
6. 10 m*M* dNTP mix (set of dATP, dGTP, dCTP, and dTTP) (*see* **Subheading 2.2., step 3**).
7. 5 U/µL *Taq* DNA polymerase. Store at –20°C.
8. 200 µ*M* forward primer (of both p16 and β-actin, Applied Biosystems). Store at 4°C.
9. 200 µ*M* reverse primer (p16 and β-actin, Applied Biosystems). Store at 4°C.
10. 100 µ*M* probe (p16 and β-actin, labeled with FAM and TAMRA, Applied Biosystems). Store at 4°C.

Table 1
Reaction Mixture for Solution D

Reagent	Final concentration	Amount
Guanidine thiocyanate	4.0 *M*	47.26 g
1 *M* sodium citrate (pH 7.0)	25.0 m*M*	2.50 mL
10% sarcosyl	0.5 %	5.00 mL
RNAse free H_2O		to 100.00 mL
14 *M* 2-β-mercaptoethanol	0.1 *M*	0.72 mL

11. 96-well optical reaction plates with optical caps (8 caps/strip, both Applied Biosystems).
12. ABI PRISM 7700 Sequence Detection System (Applied Biosystems) or compatible real-time cycler with sequence-detection software.

3. Methods

3.1. RNA Isolation

3.1.1. Initial Steps

1. Clean mortar and pestle sets (one set per sample) with 95% ethanol. Allow to dry and cool on dry ice for at least 4 h (*see* **Note 1**).
2. Prepare solution D without β-mercaptoethanol (*see* **Table 1**). It can be stored for 3 mo at 4°C (*see* **Note 2**).
3. Add 72 µL β-mercaptoethanol per 10 mL of solution D (only to the amount of solution D that is going to be used that day).

3.1.2. Extraction of RNA From Tissue

1. Place 5–50 mg of tissue in the cooled mortar and pestle. Add liquid nitrogen into the mortar.
2. After the liquid nitrogen has evaporated, immediately pulverize the tissue by striking the pestle with a hammer (*see* **Note 3**). Twist the pestle while it is still in the mortar. The tissue must be finely pulverized to obtain a high RNA yield.
3. Remove the pestle and transfer the pulverized tissue to a cooled 50-mL conical tube (*see* **Note 1**). Place on dry ice until you proceed with **step 4**.
4. Allow the sample to thaw for 20 s. Add 6 mL of solution D (containing 0.1 *M* β-mercaptoethanol) to the tissue and suction it up and down, using a 10-mL transfer pipet.
5. Add 0.6 mL of 2 *M* sodium acetate, pH 4; mix thoroughly by inversion.
6. Add 6 mL of phenol/chloroform/isoamylalcohol (250:49:1) solution to the sample (*see* **Note 2**).
7. Vortex the suspension for 10 s and cool it on ice for 15 min.
8. Centrifuge for 20 min at 4°C at 2000*g*. After centrifugation, RNA is present in the upper aqueous phase, whereas DNA and proteins are in the interphase and phenol phase.

Table 2
Reaction Mixture for Reverse Transcription

Reagent	Vol per 1 reaction	Vol per 10 reactions
5X M-MLV buffer	10.00 µL	100.0 µL
100 m*M* DTT	5.00 µL	50.0 µL
10 m*M* dNTP	5.00 µL	50.0 µL
BSA	1.25 µL	12.5 µL
$pd(N)_6$ random hexamers	0.25 µL	2.5 µL
RNAguard	1.25 µL	12.5 µL
M-MLV reverse transcriptase	2.50 µL	35.0 µL
Total	25.25 µL	252.5 µL

9. Transfer the upper aqueous phase to a fresh 15-mL tube on ice. Sacrifice a small amount of the aqueous phase to prevent contamination of the final sample with DNA.

3.1.3. RNA Precipitation

1. Add 10 µL of glycogen solution and 6 mL 100% isopropanol to the sample and shake vigorously. Place in a freezer at –20°C for at least 60 min.
2. Centrifuge at 2000*g* for 15 min at 4°C. A white pellet containing the RNA is visible at the bottom of the tube (*see* **Note 4**).
3. Discard the supernatant by inverting the tube and suspend the remaining RNA pellet in 7 mL ice-cold 75% ethanol. Vortex the sample and centrifuge at 2000*g* for 10 min.
4. Invert the tube to remove the supernatant and let the tube stand upside down on a clean paper towel for 1 min in order to remove additional liquid. Turn the tube upright and air-dry until no liquid droplets remain.
5. Dissolve the precipitated RNA with 40 µL of 5 m*M* Tris-HCl (pH 9.0) by pipetting carefully. The RNA is now ready for reverse transcription. It can be stored at –20°C for several weeks or used immediately for the following steps.

3.2. Reverse Transcription

1. Prepare the reaction mixture for each sample according to **Table 2**.
2. Place 25 µL of the reaction mixture into 0.2-mL PCR tubes. Add 25 µL of total RNA to each tube (*see* **Note 5**). Mix by pipetting.
3. Close the tubes tightly before placing them into the thermal cycler. Use the following conditions for the thermal cycler: 8 min at 26°C (annealing), 45 min at 42°C (elongation), 5 min at 95°C (destruction of DNase).

3.3. PCR (see *Notes 6 and 7)*

3.3.1. Initial Preparation

1. Remove all reagents except enzymes from the freezer to allow thawing at RT just before you begin the master mix preparation.
2. Make sure the 5X M-MLV buffer is at room temperature and thoroughly dissolved before pipetting. Gently rock the buffer solution to mix.

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1 1:1	STND 2 1:1	STND 3 1:1	UNKN 1	UNKN 2	UNKN 3	UNKN 4	UNKN 5	UNKN 6	UNKN 7	UNKN 8	UNKN 9
B	STND 1 1:10	STND 2 1:10	STND 3 1:10	UNKN 1	UNKN 2	UNKN 3	UNKN 4	UNKN 5	UNKN 6	UNKN 7	UNKN 8	UNKN 9
C	STND 1 1:100	STND 2 1:100	STND 3 1:100	UNKN 1	UNKN 2	UNKN 3	UNKN 4	UNKN 5	UNKN 6	UNKN 7	UNKN 8	UNKN 9
D	NTC	NTC	NTC	NRT 1	NRT 2	NRT 3	NRT 4	NRT 5	NRT 6	NRT 7	NRT 8	NRT 9
E	STND 1 1:1	STND 2 1:1	STND 3 1:1	UNKN 1	UNKN 2	UNKN 3	UNKN 4	UNKN 5	UNKN 6	UNKN 7	UNKN 8	UNKN 9
F	STND 1 1:10	STND 2 1:10	STND 3 1:10	UNKN 1	UNKN 2	UNKN 3	UNKN 4	UNKN 5	UNKN 6	UNKN 7	UNKN 8	UNKN 9
G	STND 1 1:100	STND 2 1:100	STND 3 1:100	UNKN 1	UNKN 2	UNKN 3	UNKN 4	UNKN 5	UNKN 6	UNKN 7	UNKN 8	UNKN 9
H	NTC	NTC	NTC	NRT 1	NRT 2	NRT 3	NRT 4	NRT 5	NRT 6	NRT 7	NRT 8	NRT 9

Fig. 5. TaqMan plate setup. STND, standard curve samples, run in serial dilutions (in triplicate per primer mix); NTC, no-template control samples; UNKN, unknown samples (in triplicate per primer mix); NRT, control samples without reverse transcriptase.

3. Briefly vortex, then centrifuge individual tubes of $MgCl_2$ solution, primers, probes, dNTPs, and template to ensure thorough mixing and to collect small drops off the inner walls of the tubes before pipetting.
4. Avoid vortexing or shaking enzyme solutions, which can result in denaturation.

3.3.2. Basic PCR Plate Set Up

The final 96-well PCR plate should include the following samples (*see* **Fig. 5**):

1. Three standard curve samples (STND) with known cDNA concentrations, run in serial dilutions.
2. No-template control samples (NTC), including all PCR reaction components, except the template (at least three NTC per gene).
3. Unknown samples (UNKN), each run at least in triplicate.
4. Control samples without reverse transcriptase (NRT), containing RNA instead of cDNA; at least one NRT per UNKN sample.

3.3.3. Preparation of 96-Well Plates

During this step, three 96-well plates need to be set up: (1) one plate with the master mix; (2) one plate for the dilution of the templates; and (3) one plate

Table 3
Master Mix and Primer Mix for One 96-Well Plate

a Master Mix	Amount	Final concentration
H_2O	1015.0 µL	
$MgCl_2$ solution (25 µ*M*)	525.0 µL	3.5 µ*M*
10X Taq buffer	375.0 µL	1X
dNTP mix (10 m*M* each)	300.0 µL	200 µ*M* each
Taq enzyme (5 U/µL)	25.0 µL	0.8 U/µL
Total Master Mix	2240.0 µL	
b	Primer Mix	2 Genes/Plate
Master Mix	996.0 µL	
Forward primer	10.0 µL	1.2 µ*M*
Reverse primer	10.0 µL	1.2 µ*M*
Probe	3.3 µL	0.2 µ*M*
H_2O	480.7 µL	
Total Primer Mix (each gene)	1500.0 µL	

(the 96-well optical reaction plate) that will be used for the real-time PCR reaction.

1. Prepare master mix for one plate (*see* **Table 3a** and **Note 7**). For the analysis of two genes, prepare one primer mix for the gene of interest (first primer mix for p16; **Table 3b**) plus one primer mix for the internal reference gene (second primer mix for β-actin, **Table 3b**).
2. Place 27 µL of the first primer mix (p16) in each well in rows A–D and 27 µL of the second primer mix (β-actin) in each well of rows E–H by using a multi-channel pipet.
3. Cover the plate with a plastic lid in order to avoid airborne contamination.
4. Prepare and label a second 96-well PCR reaction plate for the dilutions of the templates.
5. Dilution of STND template:
   a. Place 45 µL 5 m*M* Tris-HCl in wells 1–3 of rows A–D.
   b. Add 5 µL of the STND templates in the first three wells of row A. Mix thoroughly using a pipet.
   c. Add 5 µL from these mixes to the next row, mix, and transfer another 5 µL out of these dilutions serially to row C, in order to achieve 10-fold serial dilutions.
6. Dilution of UNKN template:
   a. Fill 20 µL 5 m*M* Tris-HCl solution in wells 4–12 of row A.
   b. Add 5 µL UNKN template per well in order to achieve a 1:5 dilution. Mix thoroughly using a multi-channel pipet.

   c. Take 8 µL out of each well in row A and add to the corresponding wells in rows B and C.
7. Dilution of NRT template:
   a. Fill 9 µL 5 m*M* Tris-HCl solution into wells 1–12 in row D.
   b. Add 1 µL of the not reverse transcribed RNA (NRT) in wells 4–12 to achieve a 1:10 dilution. Do not add any RNA or cDNA in wells 1–3.
   c. Mix thoroughly. The NRT templates must be in corresponding order to the UNKN templates.
8. After the completion of all the dilutions in the second 96-well plate, transfer 3 µL of the diluted controls and samples to the first 96-well plate with the primer mixes as follows:

   Transfer
   a. the template mix from row A to the primer mix in rows A (p16) and E (β-actin),
   b. the template mix from row B to the primer mix in rows B (p16) and F (β-actin),
   c. the template mix from row C to the primer mix in rows C (p16) and G (β-actin),
   d. the template mix from row D to the primer mix in rows D (p16) and H (β-actin).
9. Mix thoroughly with the multichannel pipet.
10. Use the multichannel pipet to transfer 25 µL from each well of the template/primer mix into the 96-well optical reaction plate. Change pipet tips after every row.
11. Seal the plate with optical caps.
12. Centrifuge the plate for 1 min at 600*g* to collect the contents at the bottom of the tubes and eliminate any air bubbles from the solution.
13. Place the plate in the real-time cycler.
14. Use the following thermal cycling conditions: 10 s at 50°C, 10 min at 95°C, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min.

3.3.4 Data Analysis

1. In the application menu of the sequence detection software, click on "Analyze," scroll to "Analyze data" to display the amplification curves.
2. Set the threshold line arbitrarily within the exponential phase of the amplification plot and well above the background noise (*see* **Fig. 2**). Use the same threshold level when comparing the CT values of the standards to one another or when comparing the CT values of the unknown samples.
3. Identify the slope and PCR efficiency of the standard curve plot. For reliable results, the efficiency needs to be 95%.
4. Generate a report file and export the file in Microsoft Excel format for further analysis.
5. Analyze the average CT value of each triplicate. Subtract the average CT value of the gene of interest from the average CT value of the internal standard (ΔCT).
6. Identify the relative level of gene expression by calculating the power of the ΔCT ($2^{-\Delta CT}$).

7. In order to adjust possible differences between two experiments, multiply the relative gene expression values of one experiment with a factor to achieve identical gene expression values for the standard samples in both experiments.

4. Notes

1. Mortars and pestles should be kept on dry ice for at least 4 h before the isolation procedure. Wrap the mortars and pestles in plastic bags to avoid frost on the surface owing to condensation. Also, precool one 50-mL disposable polypropylene conical tube and one scalpel (Number 10) for each sample in dry ice. The scalpel helps to remove the pulverized tissue from the mortars and pestles and to transfer it to the 50-mL tube. Tissue samples need to be kept frozen until the pulverized tissue is diluted in solution D because the RNA degrades rapidly at room temperature.
2. Gloves, safety glasses, and good ventilation are strongly recommended when working with the following reagents: phenol, chloroform, β-mercaptoethanol, guanidine thiocyanate, and DTT. Phenol is readily absorbed through the skin and highly toxic by inhalation. Acute poisoning by ingestion, inhalation, or skin contact with phenol may lead to death. Chloroform is listed as a probable human carcinogen. Inhalation and ingestion are harmful and may be fatal. Chloroform may cause reproductive damage after ingestion or inhalation, and dermatitis as a result of prolonged or repeated skin contact. β-mercaptoethanol, guanidine thiocyanate, and DTT are toxic by inhalation, ingestion, and through skin or eye contact.
3. If the mortar and pestle set is not made of steel, do not use a hammer to pulverize the sample. Grind the tissue thoroughly by twisting the pestle until the sample is finely pulverized.
4. A white pellet containing the RNA will be visible at the bottom of the tube after precipitation. Make sure that the white pellet remains at the bottom of the tube when discarding the supernatant and inverting the tube. The white pellet may slide easily on the inner surface of the tube and get lost during inversion of the tube. After complete drying, the pellet is not visible anymore.
5. Make sure that the precipitated mRNA is dissolved completely in 5 m*M* Tris-HCl before you continue with the reverse transcription. Use of filtered tips is strongly recommended to prevent cross-contamination.
6. Contamination is a common problem with PCRs. Contaminants easily can be amplified, due to the high sensitivity of the method. This may result in false-positive data. Change gloves frequently to avoid contamination. Be careful not to cross-contaminate reagents and samples. This can be supported by having a dedicated work area (e.g., a hood for PCR only) and a set of pipets and tips that are exclusively used for PCR.
7. The use of a master mix markedly reduces the number of reagent transfers per sample and minimizes reagent loss and sample-to-sample variations. In addition, the use of multi-channel pipets is essential to minimize pipetting errors.

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7

Measuring Cyclin-Dependent Kinase Activity

Axel H. Schönthal

Summary

The activity of cyclin-dependent kinases (CDKs) lies at the heart of cell cycle and checkpoint control. These complexes have been called the "cell cycle engine," as it is their enzymatic activity that pushes the cells through the cell-division cycle. If this activity is down-regulated, for example in response to DNA damage and the activation of checkpoint signaling, cell cycle progression is arrested until further notice. In the laboratory, the enzymatic activity of the various CDK complexes can be determined by an in vitro kinase activity assay. In this assay, individual CDK complexes are isolated with the use of specific antibodies and subsequently incubated with a protein substrate in the presence of radioactive ATP. After the termination of this reaction, the amount of radioactively labeled substrate, which reflects the extent of kinase activity, can be visualized by running it on a polyacrylamide gel and exposing it to film. Here, I describe the execution of this in vitro kinase activity assay as well as appropriate controls that need to be considered for the proper evaluation of the results.

Key Words: Cyclin; cyclin-dependent kinase; CDK; CDK activity; kinase assay.

1. Introduction

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that are present in all eukaryotic cell types, and their activity is essential for cells to progress through the cell division cycle. Each CDK is a heterodimeric complex consisting of a cyclin protein, which is the regulatory subunit, and a Cdk protein, which is the catalytic subunit. Several different cyclin proteins and several different Cdk proteins exist and are able to form a variety of heterodimeric CDK complexes that are required at distinct phases of the cell cycle ***(1–4)***.

In mammalian cells, the following CDK complexes are sequentially assembled and activated: during early and mid-G1, the D-type cyclins associate with either Cdk4 or Cdk6; this is followed by the formation of cyclinE/Cdk2 complexes later in G1. The major cellular target for these kinase complexes is the retinoblastoma (Rb) tumor suppressor protein, and hyperphos-

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

phorylation of Rb leads to the inactivation of the restriction point, which allows cells to progress towards S phase ***(5,6)***. At this time, the next CDK complex, cyclinA/Cdk2, is activated and promotes the initiation of S phase. Finally, after the completion of S phase, another CDK complex, cyclinB/Cdk1, is required to drive cells through G2 and into M phase.

In general, the enzymatic activity of the particular CDK complexes declines towards the end of the respective cell cycle phase in which they are required. For example, Cdk2-associated kinase activity fades during S phase, and the elimination of Cdk1-associated activity is essential for the exit from mitosis and the completion of cell division ***(7,8)***. This tightly controlled, cell cycle-specific regulation provides a convenient opportunity for the researcher to determine the status of the cell division cycle through the analysis of the activity of distinct CDK complexes. It therefore represents a most useful approach to investigate the consequences of checkpoint signaling, as checkpoints eventually have to impinge on CDK activity in order to pause cell cycle progression.

In our own experiments, we usually determine the activity of two different types of CDK complexes: (1) those that contain Cdk2—cyclinA/Cdk2 and cyclinE/Cdk2, because Cdk2 activity indicates G1/S progression; and (2) the CDK complex containing cyclinB—cyclinB/Cdk1, because this activity demonstrates G2/M progression. Thus, these two routines cover the most decisive parts of the cell division cycle, i.e., some of those that are affected by checkpoint signaling. It should be noted that in some instances the specific analysis of cyclinA-associated kinase activity might not reveal information pertaining to one particular cell cycle phase, the G1/S phase, as this regulatory subunit is known to not only associate with Cdk2, but also with Cdk1, which exerts its role in G2/M ***(9)***.

When the enzymatic activity of CDK complexes is analyzed in vitro, as described below in detail, the substrate of choice is histone H1 protein. This substrate works very well with all CDK complexes containing Cdk2 or Cdk1. However, in the case of Cdk4/cyclinD and Cdk6/cyclinD, histone H1 does not work as well and has to be replaced by a partial or full-length retinoblastoma protein ***(10)***, which can be purified from bacterial or insect cell expression systems ***(11,12)*** or purchased as a recombinant fusion protein from Upstate Inc., Lake Placid, NY. In either instance, whether histone H1 or retinoblastoma protein is used as a substrate, the protocol described below will work quite well to reliably determine CDK activity.

2. Materials

1. Cooled microcentrifuge (or regular microcentrifuge in cold room).
2. Acrylamide gel electrophoresis apparatus with accessories.
3. Gel dryer.

4. Beta shield for radiation protection.
5. PhosphorImager or AMBIS Radioanalytic Imaging System.
6. Scanner and computer for documentation of data.
7. Antibodies, for example anti-Cdk2 or anti-cyclinB1 from Santa Cruz Biotechnology (Santa Cruz, CA; Cat no. SC-748 and SC-752).
8. Protein A–agarose slurry, for example from Roche (Indianapolis, IN).
9. Histone H1, for example from Roche or from Calbiochem (San Diego, CA). Dissolve in 10 m*M* Tris pH 7.4 to make stock solution of 1 mg/mL. Freeze in aliquots of 50 µL.
10. ATP, for example from Roche: ATP lithium salt, 100 m*M*. Store in small aliquots at –20°C.
11. γ-^{32}P-ATP, for example Easytides from Perkin Elmer. 6000 Ci/mmol; 10 mCi/mL. Apply radiation protection procedures applicable to work with radioactive substances as per institutional regulations.
12. Protease Inhibitor Cocktail Caplets, for example, Roche Complete Mini, EDTA-free.
13. PBS (phosphate-buffered saline): 137 m*M* NaCl, 2.7 m*M* KCl, 10.6 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4.
14. RIPA lysis buffer ***(13)*** for cell lysis and immunoprecipitation: 150 m*M* NaCl, 50 m*M* Tris-HCl (pH 8.0), 1% NP-40, 0.5% DOC (deoxycholate), 0.1% SDS (sodium dodecyl sulfate). Store at 4°C. Immediately before use, add 0.2 m*M* PMSF (phenylmethylsulfonyl; stock is 200 m*M* in isopropanol; store at 4°C), 0.1 m*M* sodium vanadate (Na_3VO_4; stock is 10 m*M;* store in aliquots at –20°C), 1.0 m*M* sodium fluoride (NaF; stock is 1 *M;* store at 4°C), and protease inhibitor cocktail caplets. Keep supplemented RIPA lysis buffer on ice.
15. RIPA lysis buffer for the wash steps after the immunoprecipitation (**Subheading 3.3., steps 10** and **11**): Na_3VO_4 and protease inhibitors can be omitted in order to conserve these reagents.
16. Kinase buffer: 50 m*M* Tris-HCl (pH 7.4), 10 m*M* $MgCl_2$; immediately before use, add DTT (dithiothreitol; stock is 1 *M* in H_2O) to a final concentration of 1 m*M*.
17. Kinase reaction mix: Each reaction contains 2 µg histone H1 protein, 50 µ*M* "cold" ATP, 0.5 to 1.0 µL (= 5 to 10 µCi) γ-^{32}P-ATP, in a total of 25 µL kinase buffer. For 10 reactions (plus one extra), mix the following: 22 µL histone H1 (stock solution is 1 mg/mL), 1.4 µL of "cold" ATP (dilute a small aliquot of the 100 m*M* stock solution 1:10 with kinase buffer, then add from this 10 m*M* solution; discard the rest of the diluted ATP solution), 5 to 10 µL (50 to 100 µCi) γ-^{32}P-ATP, bring total volume to 275 µL with kinase buffer.
18. Acrylamide separating gel: To prepare 35 mL of an 11% separating gel, mix the following: 13 mL of acrylamide/bis-acrylamide mix (30% acrylamide/0.8% *N,N*-methylenebisacrylamide in H_2O), 8.75 mL 4X Tris-HCl /SDS (pH 8.8) (1.5 *M* Tris-HCl containing 0.4% SDS [sodium dodecyl sulfate]), 13.10 mL H_2O, 0.117 mL APS (ammonium persulfate, 10% stock solution in H_2O), and 0.023 mL TEMED (*N,N,N′,N′*-tetramethyl-ethylenediamine) (this recipe is according to **ref. *14***).
19. Acrylamide stacking gel: To prepare 9.9 mL of a 3.9% stacking gel, mix the following: 1.3 mL acrylamide/bis-acrylamide mix (30%:0.8%), 2.5 mL 4X Tris-

HCl /SDS (pH 6.8) (0.5 *M* Tris-HCl containing 0.4% SDS), 6.1 mL H_2O, 0.05 mL APS (10% stock solution), and 0.01 mL TEMED.

20. Protein gel running buffer: To make a 10X stock solution, add together 30.2 g TrisBase, 144.2 g glycine, and 10 g SDS (sodium dodecyl sulfate) in a final volume of 1 L H_2O. Do not adjust the pH.
21. Laemmli sample buffer: 2X stock solution is 4% SDS, 20% glycerol, 200 m*M* DTT (dithiothreitol), 120 m*M* Tris-HCl (pH 6.8), 0.02% bromophenol blue ***(15)***.
22. Fixing solution is 25% methanol, 7% glacial acetic acid in H_2O.
23. Coomassie blue staining solution is 50% methanol, 10% glacial acetic acid, and 0.12% Coomassie blue R250 in H_2O.
24. Destaining solution is 10% methanol, 10% glacial acetic acid in H_2O.

3. Methods

The methods described below outline (1) the harvest of the cell cultures; (2) the preparation of the cell lysates; (3) the purification of the cyclin-dependent kinase; (4) the performance of the kinase reaction; (5) the loading and running of the polyacrylamide gel; (6) the exposure of the gel; and (7) the further processing of the gel (*see* **Note 1**).

3.1. Harvest of the Cell Cultures

The number of cells to be prepared and cultured for the experiment has to be thoughtfully chosen and depends in large part on the details of the planned analysis. For example, it needs to be considered whether only one, or rather several, CDK complexes will be analyzed; whether Western blots or other studies will be included; and whether the treatment conditions will result in different degrees of growth inhibition or cell death (*see* **Notes 2–5** for details).

1. Culture the cells in appropriate dishes or flasks and expose them to the desired treatment.
2. At the time of harvest, discard the medium and rinse the monolayer twice with five to eight mL ice-cold PBS. Drain the PBS completely from the culture plate. Add 1 mL of ice-cold PBS. With the use of a rubber policeman or plastic cell scraper, dislodge the cells from the plate. Transfer the 1 mL PBS containing the cells into a 1.5-mL microcentrifuge tube on ice. Repeat procedure with additional plates and transfer each culture into a separate microcentrifuge tube on ice. In cases where there is more than one plate per point (i.e., when significant cell growth arrest necessitates the pooling of several plates), transfer the 1 mL PBS with the cells from the first plate onto the second plate (after the medium has been discarded and the second plate has been rinsed twice with PBS). Repeat with subsequent plate(s) if applicable. Transfer the 1 mL PBS, now containing the cells from several plates, into a microcentrifuge tube on ice.
3. Once the cells have been collected in PBS (we usually harvest up to four cultures at a time), spin the tubes in a microcentrifuge to pellet the cells. If the speed is

adjustable, use 2000*g* and spin for one minute. If the speed cannot be adjusted, spin at full speed for up to 10 s. Make sure the cells are pelleted completely (i.e., the supernatant should be completely clear).
4. Discard the supernatant (PBS) *completely*. To make sure that all of the buffer is removed, spin the tubes again for a few seconds to pellet residual drops on the wall or inside the lid of the tubes. Then remove the remainder of the buffer completely (*see* **Note 6**).
5. Transfer the tubes containing the "dry" cell pellets to a prechilled box in a –80°C freezer for storage. Under these conditions, the kinase remains active for a few weeks (*see* **Note 7**). Alternatively, process the pellets immediately for the in vitro kinase reaction (however, *see* **Fig. 1** and **Note 8**).

3.2. Prepare the Cell Lysates

The subsequent steps from here on need to be executed without delay at least to the completion of the kinase reaction (i.e., to the end of **Subheading 3.4.**), which will take a minimum of 6 to 9 h, depending on the experience of the experimenter and the number of samples to be processed. Ideally, the kinase reaction products should be loaded onto the polyacrylamide gel immediately thereafter, which will add another half hour.

1. Thaw the frozen "dry" cell pellets on ice. Alternatively, keep them on the bench for a few minutes at room temperature, but transfer them back onto ice as soon as they have thawed half way. In the case of fresh, non-frozen "dry" pellets, keep them on ice and process them promptly.
2. Add ice-cold RIPA lysis buffer to each cell pellet and vortex vigorously for a few seconds. Put back on ice. The volume of RIPA to be added depends somewhat on the size of the pellet and the amount of protein to be expected (*see* **Note 9**). In case the pellet clumps tightly and does not lyse upon vigorous vortexing, pipet up and down a few times with a 1-mL Pipetman®.
3. Incubate the lysate on ice for 10 min with intermittent, vigorous vortexing.
4. Centrifuge the tubes at maximum speed (approx 12,000*g*) for 15 min in a precooled microcentrifuge in the cold room.
5. Transfer the supernatant to a new microcentrifuge tube on ice. Be careful not to include any of the pellet. Discard the pellet and proceed with the supernatant.
6. Determine the protein concentration of each sample. Use your favorite protein determination assay kit and follow the manufacturer's instructions (*see* **Note 10**).

3.3. Purify the Cyclin-Dependent Kinase(s)

Once the protein concentration in each lysate has been established, the next step is to purify the desired CDK complex by immunoprecipitation (IP). Either anti-cyclin or anti-Cdk antibodies can be used for this procedure—as long as the antibodies are able to pull down the respective active cyclin/Cdk complex (*see* **Notes 11** and **12**).

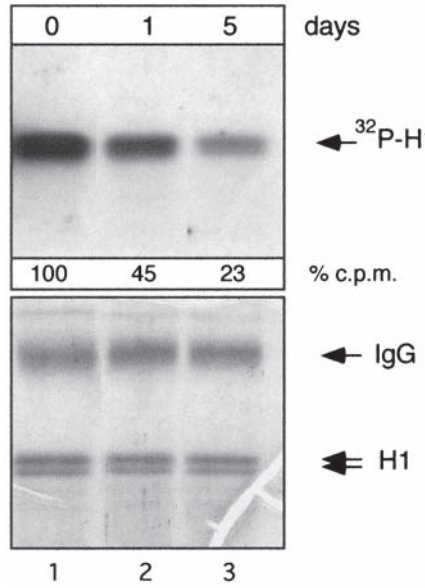

Fig. 1. Cyclin-dependent kinase assay from fresh vs stored lysates. In vitro cyclin-dependent kinase assays were performed following the procedure outlined in this chapter with lysates from the U87 glioblastoma cell line ***(16)***. Lane 1: fresh lysate was used, i.e., the immunoprecipitation and kinase assay was performed immediately following the lysis of the cells (0 d). Lane 2: an aliquot of the same cellular lysate was frozen immediately and stored at –80°C overnight, and the immunoprecipitation and kinase assay were performed the following day (1 d). Lane 3: a further aliquot of the same lysate was frozen immediately and stored at –80°C for 5 d, before the immunoprecipitation and the kinase assay were performed. For each reaction, 250 µg of total cellular lysate and 1 µg of anti-Cdk2 antibody was used. The kinase reaction products of d 0 and d 1 were stored frozen until d 5, and all three samples were separated by polyacrylamide gel electrophoresis at the end of d 5. The top panel is the autoradiograph reflecting phosphorylated histone H1 protein (^{32}P-H1; exposure for 8 h at –80°C with intensifying screen), indicating the in vitro activity of cyclin-dependent kinase. The bottom panel is the same gel stained with Coomassie blue to reveal the amount of antibody (IgG; heavy chain) and substrate histone H1 (H1) loaded in each lane. The numbers marked c.p.m. indicate the amount of radioactivity in each lane, as determined with the AMBIS Radioanalytic Imaging System. Note the loss of >50% of kinase activity during overnight storage of the lysate, and approx 75% loss after 5 d of storage.

1. Set up the IP by mixing the same amount of total cell lysate with 1 µg antibody per reaction. Use RIPA lysis buffer to adjust each reaction to the same final volume. Keep mix on ice (*see* **Notes 13** and **14**). Transfer the residual, unused portion of the total cell lysates back to the freezer for storage (*see* **Note 3**).

2. Vortex briefly to mix the components of the IP. Spin for 5 s in a microcentrifuge to collect the reaction mix at the bottom of the tube.
3. Incubate the tubes on ice for 3 to 4 h.
4. Prepare protein A–agarose slurry for addition to the IP. Calculate 10 µL of solid protein A–agarose per reaction. Transfer the total amount that is required for all samples from stock container to a microcentrifuge tube, spin full speed for 10 s, and discard supernatant. Suspend protein A–agarose pellet in RIPA buffer to make a final concentration of approx 25%. For example, for 10 IPs, make a 110 µL protein A–agarose pellet and add 330 µL RIPA to a final volume of 440 µL (this includes one extra). Do not yet add to IP (*see* **Note 15**).
5. Add ice-cold RIPA to each IP to increase the final volume to 500 µL. This will ensure better mixing during the following steps.
6. To each IP, add 40 µL of the 25% protein A–agarose slurry. Make sure the lids of the microcentrifuge tubes are securely closed (*see* **Note 16**).
7. Incubate the tubes in the cold room on a rotating (head over heels) shaker for 90 to 120 min.
8. Centrifuge the tubes for 15–20 s at full speed to pellet the protein A–agarose (which will also pellet the CDK-antibody complexes bound to it). Transfer the tubes to a rack sitting in an ice-water bath (*see* **Note 17**).
9. Remove the supernatant (i.e., the RIPA buffer), taking great care not to disturb the pellet. Discard the supernatant. *See* **Fig. 2** and **Note 18**.
10. Add 700 to 800 µL ice-cold RIPA to each pellet and vortex thoroughly for 3 or 4 s.
11. Repeat **steps 8–10** two more times.
12. After completion of the three washes with RIPA buffer, proceed with three washes using ice-cold kinase buffer. Use the same procedure as described above in **steps 8–11** (*see* **Note 19**).
13. After the final wash, remove supernatant as completely as possible (*see* **Note 20**).

3.4. Perform the Kinase Reaction

The pellet now consists of the purified CDK in a complex with antibody and protein A–agarose and is ready for the in vitro kinase reaction. It is strongly suggested to perform the following steps behind a β-shield to protect the experimenter from the radiation emitted by the ^{32}P-ATP.

1. Add 25 µL of kinase reaction mix to each microcentrifuge tube.
2. Transfer the tubes to a water bath at room temperature and incubate for 20 to 30 s to equilibrate to approximately room temperature.
3. Affix the tubes to a horizontal shaker (or onto a vortex with a fitted microcentrifuge holder) and shake the tubes vigorously for 20 to 30 min at room temperature (*see* **Notes 21–23**).
4. Take tubes off the shaker and add 40 µL of 2X Laemmli sample buffer.
5. Mix well by vortexing briefly and spin for 1 s to collect the liquid at the bottom of the tube.
6. Denature the sample by heating to 85°C for 8 to 10 min (or 95°C for 3 to 5 min) in a heating block.

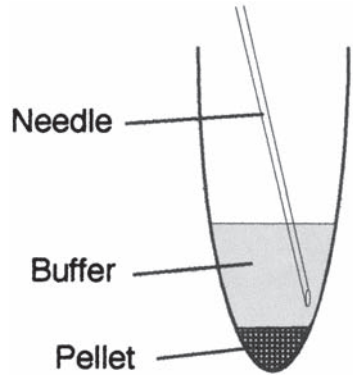

Fig. 2. Removal of supernatant from protein A–agarose pellets. During the wash steps of the immunoprecipitation (**Subheading 3.3., steps 8–13**), it is crucial to not lose any of the protein A–agarose pellet, yet at the same time wash the pellet efficiently. In order to remove the supernatant as completely as possible after each centrifugation step, we use syringe and needle to aspirate the supernatant. The opening of the needle tip is kept towards the wall of the tube. We do not insert the needle into the pellet, because this practice will occasionally clog the very fine needles, whereas with larger needles it will lead to some loss of pellet.

7. Vortex the samples for 1 or 2 s, then centrifuge at full speed for about 1 min. The samples are now ready for immediate loading onto an acrylamide gel. Alternatively, if necessary, they can be frozen and stored overnight.

3.5. Load and Run the Acrylamide Gel

Just like the steps of the previous section, the procedure described here needs to be performed behind a beta shield to protect the experimenter from the radioactivity present in the samples.

1. Place the fully assembled acrylamide gel and running apparatus behind a shield (*see* **Note 24**).
2. Load the samples. Make sure they have been centrifuged at full speed to pellet the protein A–agarose. Load only the supernatant, which contains the CDK complex, the antibodies, the histone H1 substrate, and the non-incorporated ^{32}P-ATP. Load the empty wells (if any) directly adjacent to the sample wells with 1X Laemmli sample buffer (*see* **Note 25**).
3. Run the gel at 35 to 50 V overnight.
4. The next morning, if necessary, increase the voltage to 100 V to complete the run. However, make sure that the running front does not run out of the gel into the bottom reservoir. Stop the run with the running front at least half an inch away from the end of the gel (*see* **Note 26**).

5. Disassemble the gel apparatus and keep the glass plates with the gel behind a shield.
6. Put the glass plates flat on the bench and pry them apart. Leave the gel on top of the bottom plate. With a razor blade, cut the gel as follows: (1) cut horizontally between the stacking gel and the separating gel to remove and discard the stacking gel; (2) cut the separating gel horizontally one cm above the running front to remove and discard the running front (which contains the bulk of the radioactivity (*see* **Note 26**); (3) if not all the lanes of the gel have been loaded with samples, cut vertically to remove and discard the empty lanes; (4) cut off a small piece from the bottom right corner to mark the orientation of the gel.
7. Transfer the gel into a glass tray with five gel vol of water and briefly rinse the gel. Repeat. If necessary, discard the wash into the radioactive waste.
8. Add five gel vols of fixing solution and shake gel gently on a horizontal shaker for 45 min to 1 h. Discard the solution into the radioactive waste if necessary (*see* **Note 27**).

3.6. Expose the Gel

After the components in the gel have been fixed, the gel is now ready for exposure to x-ray film. We prefer to expose the wet gel sealed into a plastic bag, rather than a dried gel, because the results will be obtained faster and we avoid potential problems such as gel cracking that could result in figures that are less than "publication quality." Depending on the results, however, we will dry the gel later on (*see* **Subheading 3.7.**).

1. Drain residual fixing solution from the gel and put the gel between two sheets of plastic (or into a small, flat plastic bag) (*see* **Note 28**).
2. Seal the plastic neatly around the gel on three sides with a heat sealer. Seal as close to the gel as possible to reduce the final size of the bag. Put the plastic bag flat on the bench and squeeze any residual liquid away from the gel. Then seal the fourth side as well. With a scissors, trim the unsealed plastic so that the remainder is a neatly sealed bag that is only slightly larger than the gel. Remove any drops of liquid from the outside of the bag.
3. With a Geiger counter, determine the approximate counts per minute (c.p.m.). Move the counter slowly across the gel and determine the spot with the highest c.p.m. The maximum number of c.p.m. is a ballpark estimate how long to expose the gel to film (*see* **Note 29**).
4. Put the sealed gel into a film cassette with intensifying screen. Assemble the film between the gel and the intensifying screen. Transfer the cassette to a –80°C freezer during exposure of the film.
5. After the appropriate time of exposure has passed, develop the film. For multiple exposures onto the same film, *see* **Note 30**. If required, expose the sealed gel again in order to obtain optimal exposures.

3.7. Process the Gel Further

If everything went well, the completion of the previous steps will have resulted in a nice autoradiograph with strong signals and exciting results. However, to establish the accuracy of the results, a few controls are needed. For example, it has to be excluded that any observed differences in kinase activity are a result of variations in the amount of antibody added to the IP, or antibody–antigen complexes lost during the wash steps of the IP, or variations in the amount of the substrate histone H1 added to or retrieved from the kinase reaction. In short, one has to verify that the same amounts of antibody and histone H1 are present in each lane of the acrylamide gel. For this purpose, the gel will be stained with Coomassie blue to visualize the relative quantity of antibody and histone H1 present.

1. Use a scissors to cut open the plastic bag containing the gel. Transfer the gel into a glass tray and add five gel vol of Coomassie blue staining solution. Incubate on a shaker for 4 to 6 h.
2. Replace the Coomassie blue staining solution with 100 mL (or more) of destaining solution. The staining solution can be collected and reused (unless it has become radioactive). Place one or two white paper towels into the tray next to the gel to soak up the blue stain. Cover the tray tightly with plastic wrap and shake for 1–2 h.
3. Replace the paper towels (which by now should have become blue) with fresh ones and continue shaking over night.
4. The next morning, two prominent bands (histone H1 [a doublet] and the IgG heavy chain) should be visible in the gel. If more destaining is required, replace the paper towels with fresh ones and heat the tray with its contents in the microwave. Do not bring to a boil, but heat until the underside of the tray is just about too hot to touch with bare hands. Transfer the hot tray back to the shaker for another hour.
5. Discard the destaining solution and add 100 mL (or more) of water for 30 to 60 min.
6. Replace the water with a solution of 5% glycerol and shake for 1 h. Treatment with glycerol reduces the chances of gel cracking during drying.
7. Drain the residual glycerol solution from the gel and place the gel onto two sheets of Whatman 3MM paper (slightly larger than the gel) in a gel dryer. Cover with plastic wrap and close the gel dryer. Run for 2 h at 80°C under vacuum with a cold trap.
8. After completion of the run, remove the dried gel (now tightly bound to the top Whatman paper) and scan it into the computer for documentation.
9. In order to quantify exactly the amount of radioactivity in each lane, expose the dried gel to an appropriate detector, such as the AMBIS Radioanalytic Imaging System (which will measure c.p.m. for each signal) or the PhosphoImager. (These measurements could also be performed earlier with the wet gel sealed into the plastic bag.)

10. Scan an appropriately exposed autoradiograph of the gel into the computer for documentation.
11. Digitally assemble the colleted data on the computer. Make a figure showing (1) the autoradiograph (visualized results); (2) the c.p.m. for each lane (quantitated results); and (3) the stained gel (control for the same amounts of antibody and histone H1 added to each reaction and subsequently loaded onto the gel). Compare to **Figs. 3** and **4**.

4. Notes

1. For the novice, it is highly recommended to start with a practice run and process no more than four samples at a time. For example, four identical cell cultures can be harvested and processed simultaneously in order to test the degree of reproducibility of the approach and, more importantly, the skill level of the experimenter. As an alternative, two identical cell cultures can be prepared and each one analyzed using two different amounts of lysate. For example, 100 µg and 300 µg from each lysate can be used. In this case, the larger lysate should yield triple the kinase activity, which would furthermore indicate that the chosen reaction conditions are within the linear range (*see* **Fig. 3**). In any case, after three to four executions of the complete assay, the experimenter should be able to reliably and reproducibly perform this experiment. As experience and confidence increase, more samples can be analyzed in parallel. An experienced professional should be able to process two sets of twelve samples in one 10-h d (the day is concluded by loading the reaction products on a polyacrylamide gel for an overnight run). For detailed time requirements and a few time-saving tips, *see* **Notes 31–33**.
2. In order to obtain sufficient material for the in vitro kinase reaction, a bare minimum of approx 3×10^5 cells is required per point. If possible, however, significantly larger numbers of cells are better (1–5 million) because it is easier to work with bigger quantities, and also allows several types of analysis in parallel. As a general rule, for many mammalian cell types, in particular rapidly growing tumor cells, the amount of cells that can be obtained from one 10-cm plate suffices. In order to set up the system, and for the purposes of obtaining high levels of enzymatic CDK activity, the cells should be kept in their logarithmic growth phase and should be allowed to reach 80–95% confluency (in this case meaning they should cover 80–95% of the available area of the culture dish). Under these conditions, most tumor cells are in their optimal growth phase and contain high CDK activity. In fact, even when these cells have just about covered nearly all of the available surface on the culture dish, they will still go through a few more rounds of rapid divisions before they slow down somewhat due to "squeezing" by the surrounding cells. Similar considerations apply to suspension cells—i.e., it is desirable to harvest them in their logarithmic growth phase in order to obtain high kinase activity. In this case, however, comparatively more material can be obtained from a 10-cm dish, which essentially represents a 10-mL suspension culture.

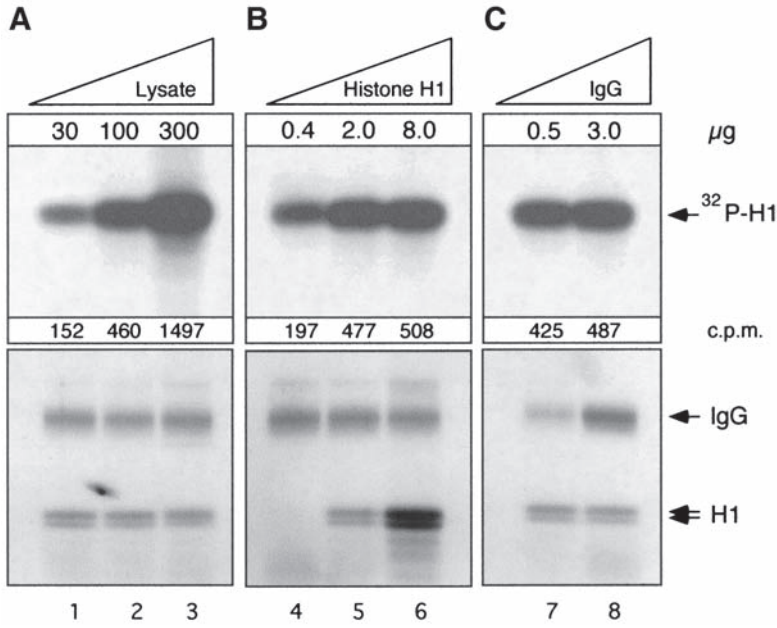

Fig. 3. Cyclin-dependent kinase assay with varying amounts of lysate, substrate, or antibody. In vitro cyclin-dependent kinase assays were performed following the procedure outlined in this chapter. Lysates from the A172 glioblastoma cell line ***(16)*** were immunoprecipitated with antibody to cyclin B1. The top panels are autoradiographs reflecting phosphorylated histone H1 protein (^{32}P-H1; exposure time 3 h at –80°C with intensifying screen), indicating the in vitro activity of cyclin-dependent kinase. The bottom panels are the same gels stained with Coomassie blue to reveal the amount of antibody (IgG; heavy chain) and substrate histone H1 (H1) loaded in each lane. The numbers marked c.p.m. indicate the amount of radioactivity in each lane, as determined with the AMBIS Radioanalytic Imaging System. In **(A)**, different amounts of cellular lysate were used for the immunoprecipitation, whereas the quantity of antibody (1 μg) and histone H1 (2 μg) was kept constant. Note that the increase in added lysate correlates with increased kinase activity in a linear fashion. In **(B)**, the quantity of the substrate histone H1 was varied, whereas the amount of cellular lysate (100 μg) and antibody (1 μg) was kept constant. Note that using more than 2 μg histone H1 does not significantly increase the overall amount of phosphorylated substrate, i.e., the difference in c.p.m. between 2 and 8 μg is <10%. In **(C)**, the amount of antibody was varied, whereas the quantity of cellular lysate (100 μg) and histone H1 (2 μg) was kept constant. Note that there is only a small increase (approx 15%) in c.p.m. when sixfold more antibody is used for the immunoprecipitation. The kinase activity obtained with the use of 3 μg antibody (lane 8) is very similar to the activity obtained with 1 μg antibody (lanes 2 and 5). Taken together, these results indicate an optimal amount of 1 μg antibody and 2 μg histone H1 in combination with 100 μg cellular lysate from A172 cells. It should be noted, however, that with the use of other antibodies and/or slower-growing cell types, significantly more lysate should be used.

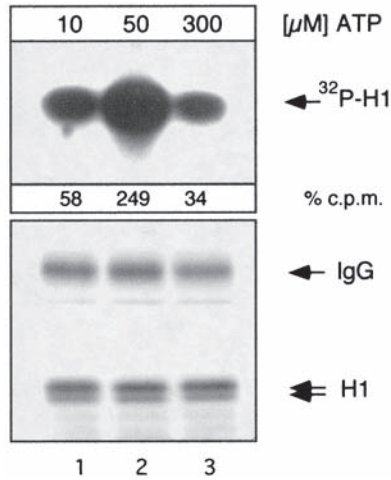

Fig. 4. Cyclin-dependent kinase assay in the presence of varying amounts ATP. In vitro cyclin-dependent kinase assays were performed following the procedure outlined in this chapter. 100 μg of total cell lysate from Raji Burkitt's lymphoma cells was immunoprecipitated with antibody to cyclin B1. The labeling of the panels is the same as the one described in the legend to **Fig. 3**. In the three kinase reactions, different amounts of "cold" ATP were included, as indicated. Everything else was kept constant between the reactions, such as the amount of radioactive ATP, histone H1 (2 μg), and reaction time. Note that there is an optimum of "cold" ATP added to the kinase reaction. Very low amounts do not optimally "fire up" the reaction, whereas very high amounts compete with the incorporation of radioactively labeled ATP into the substrate.

3. Depending on the particular cell line cultured, we routinely obtain approx 600–1000 μg of total cell lysate from a 10-cm culture dish with adherent tumor cells that were 95% confluent at the time of harvest. For each kinase reaction, 100–500 μg of total cell lysate is required, depending on which particular CDK complex is being analyzed. This amount doubles in case two different CDKs are to be analyzed in parallel. In addition, the remainder of the lysates can be separately used for other types of studies, such as Western blots to determine the levels of expression of the individual subunits of the CDK complex—such as the various regulatory cyclin proteins (cyclin A, cyclin B, and so on) or the catalytic subunits (Cdk1, Cdk2, and so on)—or the CDK inhibitors (such as $p21^{Waf1}$ and $p27^{Cip1}$), or, of course, any other protein of interest.
4. The amounts of total cellular lysate required to analyze the activity of one particular CDK can increase depending on the growth conditions of the cells. Under conditions where cells proliferate suboptimally—for example, when they are kept under low concentrations of serum growth factors, or when they are cultured beyond the point of reaching full confluency—the overall activity of CDKs will drop significantly. In this case, in order to obtain a decent signal from the in vitro

kinase reaction, double or triple the regular amount of total cellular lysate should be used for each reaction. The same may hold true for cells that proliferate very slowly even under optimal conditions, which sometimes is the case with primary cells or with cells approaching senescence.

5. Often an experiment requires exposure of cells to treatments that are cytostatic or cytotoxic. For example, treatment of cultured cells with certain chemotherapeutic drugs may result in efficient growth arrest at certain points in the cell cycle. Or, the irradiation of cells with ionizing radiation may cause a significant portion of the cell culture to be killed. In these cases, the overall yield of total cell lysate for the analysis of kinase activity can be drastically reduced. Therefore, it is recommended to set up one or two more plates of cells for this particular type of treatment; then, at the time of harvest, several plates can be pooled in order to increase the yield.
6. It is very important to *completely* remove the supernatant. Any residual drops of buffer (PBS), even the tiny ones sticking to the walls of the tube or the inside of the lid, will later on dilute the lysis buffer and cause uneven concentrations of detergent and other ingredients, such as protease inhibitors, between different samples (see **Fig. 2**).
7. It is recommended to work expediently and not let the cell pellets sit on ice for extended periods. They should be transferred to the –80°C storage as soon as possible. For this purpose, we usually harvest not more than four to six samples together, and freeze them before we attend to the next group of samples.
8. Once the cell pellets have been lysed by the addition of lysis buffer, they will have to be processed all the way to the step where the kinase reaction products are loaded onto the polyacrylamide gel. Thus, once the cell pellets have been lysed, they should not be frozen and stored, but used immediately. Freezing and storing the cells in lysis buffer even for just one day will result in appreciable loss of kinase activity. From the beginning of cell lysis to the point where the reaction products are loaded onto the gels, it will take approx 7–10 h, depending on the experience of the personnel and the number of samples to be processed. Thus, it might be more convenient to freeze the "dry" cell pellets overnight and get an early start the next day.
9. The approximate desired final concentration of protein in the total cell lysate may depend somewhat on whether or not the lysate will also be used for other purposes later on. In the case of western blots, in order to load sufficient amounts of protein into small wells of mini-gels, higher concentrations of protein (3–5 mg/mL) may be advantageous. Otherwise, for the sole purpose of achieving final volumes that can be most conveniently pipetted for the kinase assays, we usually aim for protein concentrations in the range of 1–2 mg/mL. Similarly, for purposes of accuracy during the determination of the protein concentrations of the different samples (and for more convenience during pipetting later on), we aspire to achieve comparable concentrations of proteins in the lysates from all the different samples that belong to the same experiment. Therefore, we eyeball the size of the various pellets and add correspondingly more or less lysis buffer to the cell

pellet. Depending on the treatment of the cell cultures, cytotoxic conditions can make for significantly smaller cell pellets, even when two cultures with the same treatment are pooled. In this case, adding correspondingly less lysis buffer as compared to the bigger pellets can "restore" the similarity in final protein concentration.

10. For the determination of the protein concentration in each sample, we routinely use the BCA kit from Pierce (bicinchoninic acid protein assay reagent, Pierce, Rockford, IL). We find this kit to be more accurate over a wider range of protein concentrations than other kits we have used. Also, the SDS in the lysis buffer does not seem to negatively interfere with this assay. We usually mix 10 µL of total cell lysate with 1 mL of the BCA solution (1 mL is the combined volume of solution A plus solution B) and incubate for 20–30 min in a 37°C water bath. In case we expect unusually low yield (<1 mg/mL), we may use 15 or 20 µL of lysate. The standard curve is established by using different amounts of bovine serum albumin (BSA). To each BSA sample, we also add the same volume of RIPA lysis buffer (10 µL) that is contained in the unknowns.
11. The choice of suitable antibodies is probably the most critical part of the whole experiment. Obviously, it is important to use antibodies that are able to collect the active CDK complex (i.e., cyclin-bound Cdk harboring the activating phosphorylation). However, from our experience, some of the antibodies that are commercially available and labeled in the supplier's catalog as "collecting active kinase" simply don't work for this purpose, or work at very low efficiency. Therefore, it is very important to establish in preliminary experiments with appropriate controls whether or not the respective antibodies used collect CDK complexes yielding satisfactory kinase activity. For a recommendation of good antibodies, *see* **Note 12.** A negative control is desirable as well, because other antibodies (i.e., those that are not supposed to immunoprecipitate CDK complexes) can still generate a false-positive kinase signal after extended periods of exposure of the gel to x-ray film (see **Fig. 5**).
12. We have been using two different antibodies with very good success for human cell lysates (according to the supplier, these antibodies should work similarly well with lysates from mouse cells). The first antibody is anti-Cdk2 from Santa Cruz Biotechnology (Cat. no. SC-163). This antibody collects Cdk2/cyclinA as well as Cdk2/cyclinE complexes and therefore is suitable for analyzing the kinase activity regulating the G1/S transition. The second antibody is anti-cyclin B1 from Santa Cruz Biotechnology (Cat. no. SC-752), which collects the major regulator of G2/M progression, Cdk1/cyclinB. Although it is not mentioned in the catalog that this anti-cyclin B antibody collects active kinase complexes, we found it to be the overall best-working antibody for kinase assays. It yields by far the highest kinase activity (approx 500% more than the above described anti-Cdk2 antibody) and certainly is the optimal antibody to set up the whole procedure.
13. We attempt to keep the final volume of the IP relatively small (i.e., in the range of 250 to 300 µL) in order to support the swift formation of the antigen–antibody complexes. From relatively fast, logarithmically growing cells, we use 100 µg of

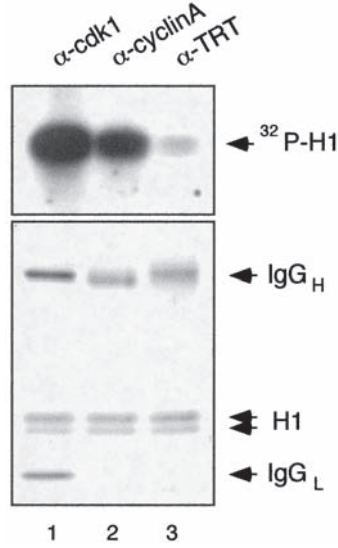

Fig. 5. Cyclin-dependent kinase assay with negative control antibody. In vitro cyclin-dependent kinase assays were performed following the procedure outlined in this chapter. 100 µg of total cell lysate was immunoprecipitated with antibodies directed either against Cdk1, cyclin A, or human telomerase reverse transcriptase (TRT) as indicated. The top panel shows the autoradiograph of a 24-h exposure. Note that the signal generated from the use of Cdk1 and cyclin A antibodies is relatively weak, as compared to the signals in **Figs. 3** and **4** (which are much shorter exposures). Furthermore, the use of the TRT antibody also generates a signal, which presumably is a false-positive signal, as a potential association of human telomerase reverse transcriptase with CDK has not been reported; i.e., it is not to be expected that the use of TRT antibodies is able to collect CDK complexes. The bottom panel, which shows the Coomassie blue staining of the top gel, demonstrates that different antibodies might stain differently. In particular, this staining reveals the light chain of the antibody (IgG_L) from some antibodies, but not from others.

total cell lysate with the anti-cyclin B1 antibody, and 250 µg with anti-Cdk2 (*see* **Note 12**). Depending on the antibody and the growth conditions of the cells (i.e., retarded or generally slow growing), significantly larger amounts of total cell lysate may be required for each IP. Into microcentrifuge tubes, we first add the RIPA lysis buffer required to equalize the final volume in all samples, then add the antibody, and finally add the lysate.

14. Because the lysed cells should not be frozen and stored for another round of kinase assays (*see* **Note 8**), all CDKs of interest have to be analyzed in parallel on the same day. That means that an additional set of microcentrifuge tubes has to be set up with the same lysates, but with a different antibody.

15. We routinely use the protein A–agarose from Roche (Cat. no. 1134515), which comes as a solution of 3 mL protein A–agarose in 5 mL buffer. Protein A–agarose from other suppliers should work as well.
16. The protein A–agarose starts to settle rapidly in solution. Therefore, during pipetting, care has to be taken that the protein A–agarose is thoroughly mixed with the RIPA buffer. We usually vortex the 25% solution after every two to three pipetting steps. This is important because otherwise unequal amounts of agarose might end up in different IPs.
17. At this point, one can conveniently compare (by visual inspection) the size of the pellets between the different tubes. They should all have the same size. If not, the cause probably was improper pipetting at the time when protein A–agarose was added to the IP (*see* **Note 16**).
18. In order to remove the supernatant, we use a 3-mL syringe instead of the regular Pipetman. We prefer a 22-gage needle (Becton Dickinson) because this size is wide enough to allow for swift flow and does not easily get clogged. With the needle opening held against the inside wall of the tube, and slowly sliding down towards the pellet as the buffer is emptied (don't touch the pellet), the buffer can be almost completely removed. In cases where the buffer is rather incompletely removed, more repeats of the washing steps are needed (*see* **Fig. 2**).
19. The three washes with RIPA buffer serve to remove the unbound proteins (ideally all the proteins that are not part of the protein A–agarose–antibody–CDK complex. The subsequent three washes with kinase buffer serve to equilibrate the mix to the kinase buffer conditions and, in particular, to remove any residual detergents from the RIPA buffer.
20. After the final wash, the size of all the pellets should be compared by visual inspection. They should all have the same size. If not, some loss occurred during the washing steps, which will lead to a reduced (inaccurate) signal from the kinase reaction. (Unequal pellet size should be indicated in the notebook). Greater care must be taken to preserve all of the agarose during the washing steps. The loss of a fraction of the pellet in some of the samples is probably the biggest danger facing the novice throughout the whole procedure, and can be a major cause for misleading results.
21. For convenience, we incubate the kinase reactions at room temperature. We have compared the efficiency of performing this reaction at room temperature vs 30 or 37°C (with the cyclinB/Cdk1 complex) and found only a small difference (an approx 25% higher signal at 37°C). Thus, unless very low overall kinase activity is expected, the reaction can be incubated at room temperature to yield a sufficiently strong signal. Alternatively, when analyzing cyclinD/Cdk4,6 complexes, investigators generally incubate the reaction at 30°C ***(11,12)***.
22. The CDK in this reaction remains bound to the protein A–agarose–antibody complex, which tends to settle to the bottom of the tube. Therefore, it is essential that the whole mix be shaken vigorously enough to keep all components well mixed during the whole incubation period.
23. With the anti–cyclin B1 antibody mentioned above and the use of fast-growing tumor cells, we routinely incubate for 20 min, which results in a strong signal

within the linear range of the reaction. With the anti-Cdk2 antibody and triple the amount of lysate, we incubate 30 min to obtain a similarly strong signal. When lysates from slowly growing cells are used, the signal intensity can be increased by incubation times somewhat beyond 30 min (and/or the use of more lysate for the IP).

24. The use of large gel sizes (16 or 20 cm width) is strongly recommended. Do not employ mini-gels. We routinely use the following gel size: 16 × 16 cm (width × height), 1.5 mm thickness, with a 14-teeth comb (the width of each well is 8 mm). This gel size very conveniently accommodates the sample volume of approx 65 µL. At 11% acrylamide and a thickness of 1.5 mm, the gel is also relatively robust for the subsequent handling (fixing and wash steps, and so on). We have successfully used higher percentage gels (up to 15%); however, here the chance of gel cracking during drying increases.
25. To load the samples onto the gel, we use 200-µL gel-loading pipet tips with extended length. The thinnest version of these pipet tips should be avoided, as those tips have a tendency to get clogged with protein A–agarose from the pellet.
26. It is important to keep the running front inside the gel because it contains all of the non-incorporated ^{32}P-ATP (which represents the majority of the radioactivity that is present in the gel). If the running front is allowed to run out of the gel and into the bottom buffer reservoir, it will require much more effort to contain and clean up the radioactive waste. In a well run experiment, the bottom reservoir will contain no measurable radioactivity. Also, it is usually not necessary to run the samples the maximum distance possible. If one expects a relatively strong signal, running the gel (i.e., the running front) only two-thirds of the distance is sufficient for good separation and a nice exposure.
27. In case the running front of the gel has not been removed completely (*see* **Note 26**), there will still be some nonincorporated ^{32}P-ATP present in the gel. In this case, the free nucleotides will diffuse out of the gel and into the fixing solution. Not only will this make the fixing solution radioactive, but a portion of the radioactivity will diffuse back into the gel and cause a significant increase in the general background of the whole gel.
28. We routinely use clear plastic bags for this purpose. The only requirement is that the bag be waterproof to prevent wetting of the film during exposure.
29. From the experience with our equipment (Ludlum Geiger Counter), we use the following lengths of exposure (with intensifying screen at –80°C): 5000 c.p.m.: 2 h 30 min (light exposure) to 4 h 30 min (stronger exposure); 30,000 c.p.m.: 40 min to 1 h 10 min; and so on. In general, with the use of anti-cyclin B1 antibodies (which give us the strongest signal), we obtain somewhere between 10,000 and 30,000 c.p.m. as the maximum signal in any given gel. In comparison, the running front (containing the nonincorporated nucleotides) yields approx 10^6 c.p.m. Extremely long exposures of the gel (24 h and above) have the potential to generate false-positive signals (*see* **Fig. 5**).
30. We routinely perform two exposures per film. From experience, we know that the phosphorylated histone H1 (the radioactively labeled substrate) is present in

the bottom half of the acrylamide gel. Therefore, we only put this part of the sealed gel onto the top half of the autoradiographic film (5 × 7 in). The bottom half of the film is not in contact with the gel. After the first exposure, we warm up the film cassette in a bacterial incubator (with vent, set at approx 30°C) for approx 15 min to adjust the cassette and its contents to room temperature. It is important to thaw everything completely to avoid (1) accidental cracking of the frozen gel; and (2) the accumulation of humidity (condensation) on the film. Then, in the darkroom, we flip the sealed gel onto the bottom (unexposed) half of the film. In this case, the gel is now upside down (with the same orientation of the samples); the bottom part of the gel (harboring the phosphorylated histone H1) is on top of the bottom part of the film, whereas the top part of the gel is below the bottom part of the film. In this case, the top part of the film (which is already exposed) is not covered by any part of the gel. After this rearrangement, the cassette is put back into the –80°C freezer for the second exposure.

31. The time requirement for the whole procedure is approx 5 d. Day 1 has the lion's share of the hands-on work. It includes **Subheadings 3.1–3.5** and requires approx 8–12 h (depending on the number of samples, but also significantly on the skill level of the researcher). Some time can be saved by harvesting the cells prior to this day and storing them as frozen "dry" cell pellets (*not* as lysates) at –80°C. Day 1 ends by loading the samples onto the gel and running the gel overnight. Day 2 has little hands-on work and requires only a few incubation steps. The gel will be fixed (1 h) and exposed to film, which may require several hours up to overnight. The following day (day 3), the gel will be stained (6 h) and subsequently de-stained, which usually is done by overnight incubation. On day 4, the gel is incubated with glycerol (1 h), dried (2 h), and then exposed in a measuring device such as the PhosphorImager or the AMBIS, which can take several hours up to overnight. Finally, on day 5, the results are scanned into the computer, assembled into a figure, and proudly presented during the lab meeting.
32. Thoughtful planning during day 1 can reduce the required time significantly. However, for the novice, it is recommended to go through the whole procedure one step at a time in order to avoid becoming confused. Once the researcher feels comfortable with the overall procedure, the following steps can be considered. (1) During the centrifugation of the cell lysates (**Subheading 3.2., step 4**), label the required microcentrifuge tubes and prepare the reagents for the determination of the protein concentration; (2) during the incubation step of the protein determination assay (**Subheading 3.2., step 6**), prepare and label the microcentrifuge tubes for the immunoprecipitation; (3) while the IP is sitting on ice (**Subheading 3.3., step 3**), do the following: equilibrate the required amount of protein A–agarose with RIPA buffer for later addition to the immunoprecipitation reaction; cast the acrylamide gel(s) and assemble the running apparatus; thaw the components of the kinase reaction buffer and mix them (store the mixture behind a beta shield until use); prepare a workstation for the wash (**Subheading 3.3., step 10**) (set up a rack in an ice water bath, have microcentrifuge and vortex nearby, and place a container to collect the supernatant to be discarded).

33. During the subsequent days, there are more options to save time if required. For example, (1) the staining and de-staining of the gel can be significantly accelerated by repeatedly heating the gel and the solution in the microwave oven (requires more hands-on work and attention); (2) one could skip the exposure of the wet gel and only expose the dried gel (danger of gel cracking, which would prevent publication-quality exposures); (3) instead of exposing the gel to autoradiographic film first, it could be directly placed into the PhosphorImager, which would combine documentation and quantitation in one step (depends on availability of PhosphorImager and resulting picture quality).

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8

Determination of the Catalytic Activities of mTOR and Other Members of the Phosphoinositide-3-Kinase-Related Kinase Family

Gary G. Chiang and Robert T. Abraham

Summary

Members of the phosphoinositide-3-kinase-related kinase (PIKK) family, which includes mTOR, ATM, ATR, and hSMG-1, play important roles in regulating the cellular response to environmental stimuli. Despite the similarity of their catalytic domain to that of phosphoinositide-3-kinase, these extremely large (>250 kDa) polypeptides function as serine/threonine protein kinases. The catalytic activities of these PIKK family members can now be measured in immune-complex kinase assays. This assay involves isolation of the kinase by immunoprecipitation and the in vitro phosphorylation of a specific substrate in the presence of radio-labeled ATP. Here we describe, in detail, the determination of PIKK catalytic activity with a standardized immune-complex kinase assay protocol.

Key Words: Phosphoinositide-3-kinase related kinase; PIKK; mTOR; ATM; ATR; hSMG-1; immune-complex kinase assay.

1. Introduction

Ataxia-telangiectasia mutated (ATM), ATM and Rad3 related (ATR), human homolog of SMG-1 (hSMG-1), a suppressor with morphogenetic effect on genitalia, and mammalian target of rapamycin (mTOR) are members of the phosphoinositide-3-kinase-related kinase (PIKK) family. Despite the similarity of their catalytic domains to that of lipid kinases, PIKK family members function as serine/threonine protein kinases in signaling pathways that control cell cycle progression and DNA damage response ***(1,2)***. In addition to the conserved catalytic domain, a hallmark feature of PIKK family members is that they are extremely large (>250 kDa) polypeptides, which makes the analysis of their function particularly challenging.

mTOR is the founding member of the PIKK family. The function of mTOR is to control cell growth in response to stimuli such as nutrients and growth

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
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factors through the regulation of protein translation. ATM and ATR both play key roles in the cellular response to genotoxic stress. The newest member of the PIKK family, hSMG-1, bridges both genome surveillance and RNA surveillance, the latter through the regulation of nonsense-mediated mRNA decay (NMD).

As mentioned previously, PIKK family members function as serine/threonine protein kinases rather than lipid kinases. ATM, ATR, and hSMG-1 preferentially phosphorylate serine or threonine followed by glutamine (S/T-Q) in their substrates. However, there is no clear phosphorylation site preference for mTOR, which has been shown to phosphorylate both serine or threonine followed by proline (S/T-P) motifs as well as serine or threonine residing in hydrophobic motifs ***(1)***.

Because PIKK family members play an important role in regulating cell function in response to its environment, an important aspect of their study is the analysis of their catalytic activity. This chapter will describe methods to analyze the catalytic activity of the PIKK family members by immune-complex kinase assay.

2. Materials

2.1. Generation of Substrates

1. pGEX expression system (Amersham Biosciences).
2. LB medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, adjust pH to 7.0 with 5 *N* NaOH. Sterilize by autoclaving for 20 min, 120°C/15 psi.
3. Ampicillin: 100 mg/mL stock in distilled H_2O, filter sterilize, store at –20°C.
4. *Escherichia coli* strains DH5α or equivalent, and BL21.
5. IPTG (isopropyl-β-D-thiogalactopyranoside): 400 m*M* stock in distilled H_2O (1000X), filter sterilize, store at –20°C.
6. Lysozyme: 50 mg/mL stock in distilled H_2O, store at –20°C.
7. GST-fusion protein lysis buffer: 20 m*M* Tris-HCl (pH 8.0), 100 m*M* NaCl, 10 m*M* EDTA, 0.5% Tween-20, 1 m*M* DTT, 10 µg/mL aprotinin, 1 µg/mL pepstatin A, 10 µg/mL leupeptin, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 20 n*M* microcystin-LR (*see* **Note 1**).
8. Glutathione (GSH) Sepharose beads.
9. GST elution buffer: 20 m*M* glutathione (reduced) in 100 m*M* Tris-HCl (pH 8.0), 100 m*M* NaCl (make fresh).
10. Prepared dialysis tubing: 12–14 kDa molecular weight cut-off.

2.2. Isolation of Kinase

1. Phosphate-buffered saline (PBS), pH 7.4: 137 m*M* NaCl, 15.2 m*M* Na_2HPO_4, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4.
2. mTOR/ATM/hSMG-1 lysis buffer: 50 m*M* Tris-HCl (pH 7.4), 100 m*M* NaCl, 50 m*M* β-glycerophosphate, 10% glycerol (w/v), 1% Tween-20 (w/v), 1 m*M*

EDTA, 10 µg/mL aprotinin, 1 µg/mL pepstatin A, 10 µg/mL leupeptin, 2 m*M* phenylmethylsulfonyl fluoride, 20 n*M* microcystin-LR, 25 m*M* NaF (*see* **Note 1**).
3. ATR lysis buffer: 25 m*M* HEPES (pH 7.4), 300 m*M* NaCl, 1.5 m*M* EGTA, 1 m*M* $MgCl_2$, 0.5% NP-40, 10 mg/mL aprotinin, 1 µg/mL pepstatin A, 10 µg/mL leupeptin, 2 m*M* phenylmethylsulfonyl fluoride, 20 n*M* microcystin-LR, 25 m*M* NaF.
4. Protein assay reagent with standards (Bio-Rad or equivalent).
5. Antibodies directed against the specific PIKK family member (e.g., anti-mTOR, anti-ATM, anti-ATR, anti-hSMG-1 antibody) (*see* **Note 2**).
6. Protein-A Sepharose or equivalent.
7. High-salt wash buffer: 100 m*M* Tris-HCl (pH 7.4), 500 m*M* LiCl.
8. Kinase wash buffer: 10 m*M* HEPES (pH 7.4), 50 m*M* NaCl, 50 m*M* β-glycerophosphate, 10% glycerol (w/v).

2.3. Immune-Complex Kinase Reaction

1. Kinase assay buffer: 10 m*M* HEPES (pH 7.4), 50 m*M* NaCl, 50 m*M* β-glycerophosphate, 10% glycerol (w/v), 10 m*M* $MnCl_2$, 1 m*M* DTT, make fresh.
3. Adenosine 5'-triphosphate (ATP), disodium salt.
4. [γ-^{32}P]-ATP, 6,000 Ci/mmol (*see* **Note 3**).
5. 4E-BP1/PHAS-I (Stratagene) or other substrate as described in **Subheading 3.1.** (*see* **Note 4**).
6. 2X SDS-PAGE sample buffer: 125 m*M* Tris-HCl (pH 6.8), 20% glycerol (w/v), 5% SDS, 10% β-mercaptoethanol (add fresh), 2 µg/ml bromphenol blue, store at 4°C.

2.4. Detection of Labeled Substrate and Kinase

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment.
2. Electrophoretic transfer equipment.
3. Immobilon-P or equivalent protein immunoblotting membrane.
4. Film for autoradiography or imaging device capable of detecting ^{32}P signals (PhosphorImager or equivalent).
5. Amido black solution: 1 mg/mL Amido Black 10B (Napthol Blue Black), 10% acetic acid, 45% methanol.
6. TBS-T wash solution: 50 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 0.2% (w/v) Tween-20.
7. 3% BSA blocking solution: 3% bovine serum albumin (fraction V, prepared by heat shock) in TBS-T, supplemented with 0.05% NaN_3. Filter through a 0.45 µm filter to remove particulates, store at 4°C.
8. 1% BSA blocking solution: 1% bovine serum albumin (fraction V, prepared by heat shock) in TBS-T. Filter through a 0.45 µm filter to remove particulates, store at 4°C.
9. Horseradish peroxidase (HRP)-conjugated protein A or appropriate HRP-conjugated secondary antibody (anti-rabbit or anti-mouse).
10. ECL substrate or equivalent chemiluminescent substrate for immunoblotting.

2.5. Stimulation and Inhibition of Kinase Activity

1. Growth medium lacking amino acids (e.g., RPMI minus amino acids) or Hanks buffered salt solution supplemented with 4.5 g/L glucose.
2. Leucine solution: 10 mg/mL leucine in distilled H_2O, filter sterilize, store at 4°C.
3. Wortmannin: 20 m*M* stock solution in DMSO. This solution is light sensitive, store at –80°C in aliquots.
4. LY294002: 20 m*M* stock solution in DMSO, store at –80°C.
5. Gammacell 40 Exactor research irradiator (MDS Nordion) or equivalent device.
6. Neocarzinostatin (Nippon Kayaku Pharmaceuticals, Tokyo, Japan), 1000X stock solution: 50 µg/mL in distilled H_2O. This solution is light sensitive, store at –80°C in aliquots.
7. Caffeine: 20 m*M* stock solution in distilled H_2O, store at –80°C

3. Methods

The methods described below outline (1) preparation of substrates; (2) isolation of kinase; (3) immune-complex kinase reaction; (4) detection of labeled substrate and kinase; and (5) stimulation and inhibition of kinase activity.

3.1. Preparation of Substrates

3.1.1. mTOR Substrates

Two established substrates for mTOR are PHAS-I/4E-BP1 and $p70^{S6}$ kinase ***(3,4)***. PHAS-I/4E-BP1 is an approx 21 kDa protein that is phosphorylated in vitro by mTOR on four sites: Thr 36, Thr 45, Ser 64, and Thr 69 ***(5)***. Although $p70^{S6K}$ is phosphorylated on eight sites in intact cells, of which five (Thr 229, Ser 371, Thr 389, Ser 404, and Ser 411) are sensitive to rapamycin ***(6)***, the major site of mTOR-dependent phosphorylation of $p70^{S6K}$ in vitro occurs at Thr 389 ***(4)***. Because PHAS-I/4E-BP1 is available commercially from Stratagene, we will only describe the preparation of $p70^{S6K}$ as a GST-fusion protein for use as a substrate here.

Using standard molecular biology techniques ***(7)***, we isolated the cDNA sequence encoding amino acids 332–414 of rat $p70^{S6K}$ by PCR, and cloned this fragment in frame into the bacterial expression vector pGEX-2T in order to produce a GST-$p70^{S6K}$ fusion protein ***(4)***. Below, we describe the isolation of the GST-$p70^{S6K}$ fusion protein with the use of GSH-Sepharose beads.

1. Grow an overnight culture of *E. coli* BL21 cells containing the pGEX-$p70^{S6K}$ plasmid in LB supplemented with 100 µg/mL ampicillin.
2. The next day, dilute the culture 1:10 in fresh LB supplemented with 100 µg/mL ampicillin. We generally prepare protein from a final culture volume of 400 mL, starting from a 40-mL overnight culture.
3. Grow the cells to an OD_{600} of 0.4–0.6 (approx 1–1.5 h).
4. Add IPTG to a final concentration of 0.4 m*M*.

5. Grow the culture for an additional 2–4 h at 37°C.
6. Harvest the cells by centrifugation at 5000*g* for 10 min.
7. Resuspend bacterial pellet in GST fusion protein lysis buffer, 1/20 of the original culture volume (e.g., 20 mL lysis buffer for a 400 mL culture).
8. Add lysozyme to 100 µg/mL.
9. Incubate on ice until solution becomes viscous, usually 10–20 min.
10. Sonicate until the viscosity of the lysate is significantly reduced (*see* **Note 5**).
11. Pellet the debris by centrifuging at 10,000*g* for 30 min, 4°C.
12. Transfer the supernatant to new 50-mL conical tube and add appropriate volume of GSH- sepharose beads (*see* **Note 6**).
13. Incubate for at least 1 h at 4°C, rotating the tube to keep the GSH-Sepharose beads from settling.
14. Centrifuge at 1000*g* to pellet the GSH sepharose, remove the supernatant, and transfer the GSH Sepharose to a fresh microcentrifuge tube.
15. Wash five times with GST fusion protein lysis buffer (*see* **Note 7**).
16. Elute the protein with GST elution buffer (*see* **Note 8**).
17. Combine the protein-containing supernatants (check by protein assay) and transfer to dialysis tubing.
18. Dialyze overnight in PBS plus 5% glycerol at 4°C.
19. Check quantity and quality of GST-p70^{S6K} by protein assay and SDS-PAGE. The yield of GST-p70^{S6K} should be approx 5–7 mg of protein per liter of culture.

3.1.2. ATM, ATR, and ATX/hSMG-1 Substrates

While many proteins, such as BRCA1, hRad17, and NBS1, have emerged as ATM/ATR substrates ***(1)***, the most common substrate utilized for ATM, ATR, and hSMG1 kinase assays is a GST-p53 fusion protein. Phosphorylation of p53 by ATM, ATR, and hSMG1 in vitro occurs primarily at Ser 15 ***(8–11)***. We will describe here the preparation of this GST-p53 fusion protein.

Using standard molecular biology techniques ***(7)***, we isolated the cDNA encoding amino acids 1–70 of p53 by PCR and cloned this fragment in frame into the vector pGEX-2T in order to produce a GST-p53 fusion protein ***(8,9)***. The purification procedure for GST-p53 is exactly the same as used for the GST-p70^{S6K} described in **Subheading 3.1.1.**. The yield of GST-p53 should be approx 5–7 mg of protein per liter of culture.

3.2. Isolation of Kinase

The first step in analyzing the catalytic activity of an individual PIKK family member is its isolation. Because antibodies are readily available for all the PIKK family members, the most straightforward method of isolation is immunoprecipitation, which we will describe here (*see* **Note 9**).

3.2.1. mTOR

1. Wash cells twice with PBS (*see* **Note 10**).

2. Add appropriate volume of mTOR/ATM/hSMG-1 lysis buffer to the cells (0.5–1.0 × 10^7 cells/mL lysis buffer) (*see* **Note 11**).
3. If adherent cells are used, keep culture plate on ice and allow cells to lyse for 20 min. If suspension cells are used, keep the tube on ice and allow cells to lyse for 20 min.
4. If adherent cells are used, use a cell scraper to collect the lysate and transfer it to a microcentrifuge tube.
5. Centrifuge lysates at 20,000*g* for 10 min to pellet insoluble material.
6. Transfer supernatant to new microcentrifuge tube.
7. Determine protein concentration of lysate using protein assay reagent.
8. To immunoprecipitate mTOR, use 2 µg of anti-mTOR antibody per 400–500 µg total protein from lysate.
9. Incubate the antibody with lysate for at least 1 h at 4°C.
10. Add 30 µL of a 50% slurry of protein A Sepharose (*see* **Note 12**).
11. Incubate the tube on a rotating shaker for at least 30 min at 4°C.
12. Centrifuge at 2500*g* for 1 min to pellet immunoprecipitated mTOR.
13. Remove the supernatant by aspiration (*see* **Note 13**).
14. Wash the immunoprecipitate three times with 500 µL of mTOR/ATM/hSMG-1 lysis buffer, centrifuging and removing the supernatant between each wash as in **steps 12** and **13** above.
15. Wash the immunoprecipitate once with 500 µL of high-salt wash buffer, centrifuge, and remove the supernatant.
16. Wash the immunoprecipitate once with 500 µL of kinase wash buffer, centrifuge, and remove the supernatant completely. Use the collected antigen-antibody complexes for the kinase reaction described in **Subheading 3.3.**.

3.2.2. ATM and hSMG-1

For isolation of ATM and hSMG-1, the procedure is similar to the mTOR protocol described above in **Subheading 3.2.1.**, but with the following modification listed in **step 8**: To immunoprecipitate ATM or hSMG-1, use 2 µg of the appropriate anti-ATM or anti-hSMG-1 antibody per 400–500 µg total protein from lysate.

3.2.3. ATR

For isolation of ATR, the procedure is similar to the mTOR protocol described above in **Subheading 3.2.1.**, but with the following modifications in the listed steps.

Step 2: Add appropriate volume of ATR lysis buffer to the cells (0.5–1.0 × 10^7 cells/mL).

Step 8: To immunoprecipitate ATR, use 2 µg of the anti-ATR antibody per 400–500 µg total protein from lysate.

3.3. Immune-Complex Kinase Reaction

This section details the steps involved in performing the kinase reaction with the immunoprecipitated PIKK family member. This protocol is identical for all PIKKs, although the substrate used in the reaction is dependent on the specific PIKK assayed.

1. Resuspend the immunoprecipitate in 30 µL kinase reaction buffer.
2. Add 1 µg substrate to each reaction.
3. To initiate the reaction, add 10 µCi [γ-^{32}P]-ATP (6000 Ci/mmol) per reaction, and ATP to a final concentration of 50 µ*M* (*see* **Note 14**).
4. Incubate the reactions for 20 min at 30°C.
5. Terminate the reactions by adding an equal volume of 2X SDS-PAGE sample buffer (*see* **Note 15**).

3.4. Detection of Labeled Substrate and Kinase

3.4.1. Detection of Labeled Substrate

As mentioned previously, a hallmark feature of the PIKK family members is their large size. The molecular weight of mTOR is 289 kDa, while the molecular weights of ATM, ATR, and hSMG-1 are 370, 305, and 390 kDa, respectively. As for substrates, 4E-BP1 and GST-p70^{S6K} have molecular weights of 21 kDa and 37 kDa, respectively. Although GST-p53 has a predicted molecular weight of 36 kDa, it migrates at a slightly higher molecular weight when resolved by SDS-PAGE.

Owing to the size disparity between the PIKK family member and the substrate, one must either resolve the samples using a 6–15% (or equivalent range) gradient gel, or use two gels—a 6% gel to resolve the PIKK member and a 10% gel to resolve GST-p53 (*see* **Fig. 1**) or GST-p70S6K (*see* **Fig. 2**). A 12.5% gel should be used to resolve 4E-BP1 (*see* **Fig. 3**). Alternatively, a common practice in our laboratory is to load the samples on a discontinuous 6%/10% (or 12.5%) SDS-PAGE gel to resolve both mTOR and the substrate on the same gel (*see* **Note 16**).

1. Resolve the samples by SDS-PAGE.
2. Electrophoretically transfer proteins to Immobilon-P or equivalent membrane (*see* **Note 17**).
3. Using prestained molecular-weight markers as a guide, cut the blotting membrane in order to separate the portion containing the PIKK family member (upper part of the membrane) from the portion containing the substrate (lower part of the membrane).
4. Expose the membrane portion containing the substrate to x-ray film for autoradiography or to a storage phosphor screen for PhosphorImager or equivalent imaging device.

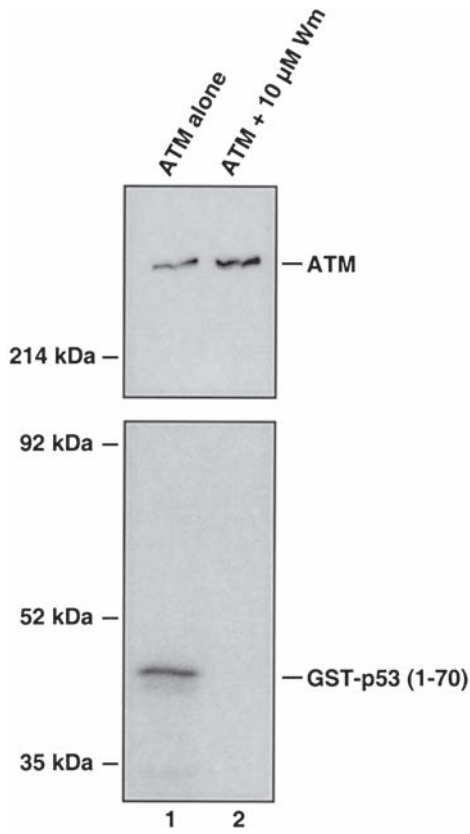

Fig. 1. Phosphorylation of GST-p53 by immunoprecipitated ATM. An in vitro kinase reaction was performed as described using immunoprecipitated ATM and GST-p53 (1–70) as substrate. Following the in vitro kinase reaction, samples were resolved on a 6%/10% discontinuous SDS-PAGE gel, transferred to a PVDF membrane, and used for subsequent analysis. Upper panel: The ATM immunoprecipitates were immunoblotted with anti-ATM antibodies. Lower panel: Autoradiogram of ^{32}P-labeled GST-p53 (1–70). Lane 1, ATM alone. Lane 2, ATM pre-incubated with 10 μ*M* wortmannin (Wm) for 30 min prior to the kinase reaction.

5. If desired, substrate amounts can be visualized by staining the membrane with Amido black or an equivalent stain.

3.4.2. Detection of Kinase

After analyzing the phosphorylation of the substrate, it is important to confirm that equivalent amounts of kinase are present in each individual reaction. This must be done to demonstrate that any difference in catalytic activity that

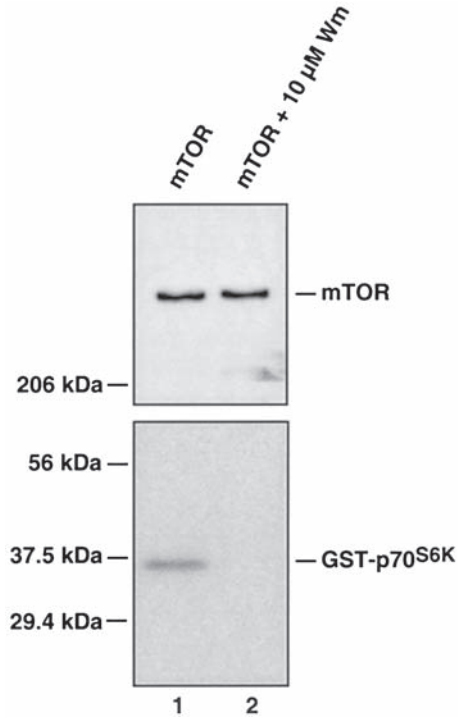

Fig. 2. Phosphorylation of GST-p70^{S6K} by immunoprecipitated mTOR. An in vitro kinase assay was performed with immunoprecipitated mTOR and GST-70^{S6K} as substrate. Following the in vitro kinase reaction, samples were resolved on a 6%/12.5% discontinuous SDS-PAGE gel, transferred to a PVDF membrane, and used for subsequent analysis. Upper panel: The mTOR immunoprecipitates were immunoblotted with anti-mTOR antibodies. Lower panel: Autoradiogram of ^{32}P-labeled GST-p70^{S6K}. Lane 1, mTOR alone. Lane 2, mTOR pre-incubated with 10 μ*M* wortmannin (Wm) for 30 min prior to the kinase reaction.

is observed is due to a change in the specific activity of the kinase and not simply due to a variation in the amount of kinase.

1. Incubate the PIKK-containing portion of the membrane with 3% BSA blocking solution with gentle agitation for at least 30 min at room temperature (RT) or overnight at 4°C.
2. Remove the blocking solution and add primary antibody solution containing antibody specific for the immunoprecipitated PIKK (*see* **Note 18**).
3. Incubate 1 h at RT with gentle agitation.
4. Wash the membrane four times with TBS-T, 8 min per wash.
5. Add secondary antibody diluted in 1% BSA blocking solution (*see* **Note 19**), and incubate for 30 min at RT with gentle agitation.

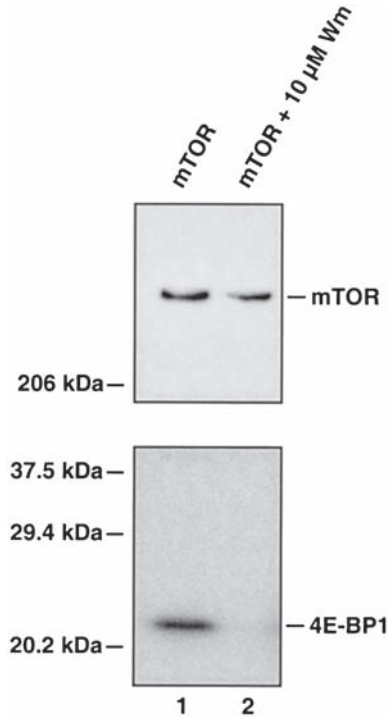

Fig. 3. Phosphorylation of 4E-BP1 by immunoprecipitated mTOR. An in vitro kinase assay was performed using immunoprecipitated mTOR and 4E-BP1 as substrate. Following the in vitro kinase reaction, samples were resolved on a 6%/12.5% discontinuous SDS-PAGE gel, transferred to a PVDF membrane, and used for subsequent analysis. Upper panel: The mTOR immunoprecipitates were immunoblotted with anti-mTOR antibodies. Lower panel: Autoradiogram of ^{32}P-labeled 4E-BP-1. Lane 1, mTOR alone. Lane 2, mTOR pre-incubated with 10 μ*M* wortmannin (Wm) for 30 min prior to the kinase reaction.

6. Wash the membrane four times with TBS-T, 8 min per wash.
7. Incubate the membrane with chemiluminescent substrate, following the manufacturer's instructions.
8. Expose the membrane to film.

3.5. Stimulation and Inhibition of Kinase Activity

This section details methods that can be used to generate samples that can serve as positive experimental controls for the stimulation of kinase activity. The pharmacological inhibition of PIKK catalytic activity, in order to serve as negative experimental controls, is also detailed in this section.

3.5.1 mTOR

Consistent with the role of mTOR as a sensor for nutrient conditions, the catalytic activity of mTOR can be stimulated by readdition of amino acids following amino acid deprivation.

1. Starve the cells of amino acids by incubating the cells for 1 h in growth medium lacking amino acids. If the appropriate growth medium lacking amino acids is not available, then cells can be starved in Hanks buffered salt solution (HBSS), supplemented with 4.5 g/L glucose.
2. Add leucine solution to a final concentration of 52 µg/mL or add complete growth medium minus the serum.
3. Incubate the cells for 15 min at 37°C.
4. Harvest the cells and process for immune-complex kinase assay as described in **Subheading 3.2.1.**.

Because of the similarity of the mTOR catalytic domain with that of PI3 kinase, the kinase activity of mTOR is sensitive to the PI3K inhibitors wortmannin and LY294002, although inhibition of mTOR occurs at much higher drug concentrations than those used for PI3K inhibition ***(12)***. Wortmannin is an irreversible inhibitor, while LY294002 acts as an ATP-competitive inhibitor ***(12)***. To inhibit mTOR activity, wortmannin can be added either to the immunoprecipitated mTOR, to intact cells, or to cell lysates at a final concentration of 10 µ*M* for 30 min prior to further sample processing. Because LY294002 is a competitive inhibitor of mTOR, it should be added to a final concentration of 10 µ*M*, 30 min prior to the kinase reaction, and must remain present during the kinase reaction.

3.5.2. ATM, ATR, and hSMG-1

When cells are exposed to DNA-damaging agents such as γ-irradiation or radiomimetic drugs, an increase in ATM-dependent phosphorylation events is observed. Furthermore, this increase in ATM kinase activity in intact cells can be measured by immune-complex kinase assay ***(8,9)***.

The procedure for the stimulation of ATM kinase activity by γ-irradiation or radiomimetic drugs is as follows:

1. Treat the cells with a 5–20 Gray (Gy) dose of γ-irradiation using a Gammacell 40 Exactor research irradiator (MDS Nordion) or equivalent device. Alternatively, incubate the cells with the radiomimetic drug neocarzinostatin at a final concentration of 50 ng/mL for 1.5 h.
2. If γ-irradiation is performed, allow the cells to recover for 1 h at 37°C. If neocarzinostatin is used, harvest the cells immediately after treatment.
3. Isolate ATM by immunoprecipitation and assay the kinase activity by immune-complex kinase assay as detailed in **Subheading 3.2.2.**.

The catalytic activities of ATM, ATR, and hSMG-1 are also sensitive to wortmannin. When wortmannin is added to immunoprecipitated ATM or ATR, the IC_{50} values are 150 n*M* and 1.8 μ*M*, respectively ***(13)***. For hSMG-1, the IC_{50} value for wortmannin is approx 100 n*M* ***(11)***. To inhibit the activity of ATM or hSMG-1, wortmannin should be added to the immunoprecipitates at a final concentration of 10 μ*M*, 30 min prior to the kinase assay. To inhibit the activity of ATR, wortmannin should be added to the immunoprecipitates at a final concentration of 30 μ*M*, 30 min prior to the kinase assay. As with mTOR, the kinase activity of immunoprecipitated ATM or hSMG-1 is inhibited by wortmannin even when added to intact cells prior to lysis. However, the same result does not hold true for ATR. Treatment of intact cells with a final wortmannin concentration of 100 μ*M* fails to achieve half-maximal inhibition of ATR by immune-complex kinase assay ***(13)***.

The kinase activities of ATM and ATR are also inhibited by caffeine. The IC_{50} for caffeine is 0.2 m*M* and 1.1 m*M* for ATM and ATR, respectively ***(14)***. In order to inhibit the activity of ATM, preincubate the immunoprecipitates with 1 m*M* caffeine for 30 min prior to the kinase reaction. For ATR, preincubate the immunoprecipitates with 10 m*M* caffeine for 30 min prior to the kinase reaction.

4. Notes

1. The kinase activity of PIKK family members, with the exception of ATR, is unusually sensitive to certain non-ionic detergents such as Triton X-100 and NP-40. This is especially apparent when using 4E-BP1 in mTOR kinase assays. Therefore, use Tween-20 in all cell lysis buffers as well as in the preparation of the GST-fusion protein substrate. As a general rule, protease inhibitors (PMSF, aprotinin, leupeptin, pepstatin A), phosphatase inhibitors (NaF, microcystin-LR), and DTT should be added to buffers immediately before use.
2. One of the anti-mTOR polyclonal antibodies that we use for in vitro kinase reactions and for western blotting is a polyclonal anti-mTOR antibody obtained from R & D Systems (Minneapolis, MN). We have also used the polyclonal anti-mTOR antibodies, mTAb1, and mTAb2, which can be obtained from Upstate Biotechnology (Waltham, MA). We have no prior experience with mTOR polyclonal antibodies obtained from Cell Signaling (Beverly, MA) or the monoclonal mTOR antibody 26E3, which can be obtained from Zymed (South San Francisco, CA). The anti-ATM antibody that we use for immunoprecipitation and Western blotting is Ab-3, obtained from Calbiochem (San Diego, CA). For ATR, we use the polyclonal anti-ATR antibody from Affinity BioReagents (Cat no. PA1-450). For ATX/hSMG-1, we use a polyclonal anti-hSMG-1 antibody that will soon be available from R&D Systems.
3. As with all procedures that involve the use of ^{32}P radioisotopes, the appropriate safeguards should be taken with respect to handling of the isotope, personal pro-

tection, and disposal of waste. Consult your institution's radiation safety office for the appropriate institutional protocols and regulatory procedures.

4. PHAS-I/4E-BP1 (Stratagene, Cat no. 206160) contains several S/T-P phosphorylation sites and is an excellent substrate for many kinases other than mTOR. PHAS-I/4E-BP1 can also be used as a substrate for ATM in vitro ***(15)***.
5. The viscosity of the solution can be easily judged by drawing the solution through a small-bore transfer pipet. If the solution can be easily drawn up into the transfer pipet, then the sonication is sufficient. Also, the color of the solution should change to a translucent "straw" color.
6. The amount of GSH sepharose used in the binding reaction depends on the binding capacity of the beads. For example, GSH sepharose from Amersham Biosciences has a very high binding capacity of approx 12 mg/mL bead volume. For a GST-fusion protein prep originating from a 500 mL bacterial culture, we generally use 400–500 µL packed bead volume.
7. The wash volume depends upon the GSH-sepharose bead volume used. For each wash, use a volume of at least 2.5× the bead volume (e.g., for a 400-µL packed bead volume, use 1 mL of GST fusion protein lysis buffer per wash).
8. For the elution of the GST-fusion protein from the GSH sepharose, a batch procedure is the most convenient. For each elution, incubate the GSH sepharose with one bed volume of GST elution buffer for 10 min at 4°C, with constant mixing to keep the glutathione sepharose from settling. After combining the protein-containing supernatants, spin the supernatant at 2500*g* to remove residual GSH sepharose that may have been carried over during removal.
9. The immune-complex kinase assay protocol is very flexible. Both endogenous and overexpressed PIKK proteins can be assayed by this protocol. One requirement when assaying overexpressed proteins is that they contain an epitope tag (such as HA or FLAG) so they can be distinguished from the endogenous proteins. One potential application of this protocol is to generate a series of PIKK mutants, overexpress these mutants in cells, and analyze for differences in kinase activity. Another potential application of this protocol is to expose the cells to different environmental conditions prior to harvest and determine whether there are differences in catalytic activity.

   Since all PIKK family members are ubiquitously expressed, the kinase activity of endogenous PIKK family members can be analyzed in many cell lines. For example, ATM and ATR are highly expressed in K562 cells. HEK 293, MCF-7, HeLa, and U87 cells are good sources of mTOR. HEK 293 cells are also a good source for hSMG-1. A sufficient amount of kinase can be immunoprecipitated from 400–500 µg of protein from cell lysates. This amount can be usually obtained from cells that are 80–90% confluent on a 6-cm plate, although this may vary depending on the cell line used (for HEK 293 cells, this is approx $3–4 \times 10^6$ cells, which yields around 600 µg of protein). To ensure that a sufficient amount of lysate is obtained for immunoprecipitation and additional manipulations such as immunoblotting of the whole cell lysate, we harvest cells from a 10-cm plate.

Once the immunoprecipitation is started, the entire procedure should be carried out to the completion of the kinase reaction, after which the samples may be stored at –80°C. Depending on the number of samples to be processed, the procedure should take 4 h to complete the kinase reactions.

10. Cells are washed twice with PBS in order to completely remove the serum-containing growth medium. Presence of excess medium will interfere with accurate protein determination following lysis. If adherent cells are used, remove the growth medium by aspiration and gently add the PBS to the plate. For a 6-cm plate, use 5 mL PBS/wash. For a 10-cm plate, use 10 mL PBS/wash. If suspension cells are used, then centrifuge the cell-containing growth medium at 1000*g* for 5 min, and remove the growth medium by aspiration, taking care not to dislodge the cell pellet. Gently resuspend the cells in 10 mL PBS. Centrifuge the cells again at 1000g for 5 min and remove the PBS by aspiration. Make sure to remove all residual PBS from the cells prior to adding lysis buffer so that the concentrations of the lysis buffer components are not diluted.
11. We try to harvest the cells at 80–90% confluency. As general practice, a 6-cm plate is lysed in 500 µL lysis buffer and a 10-cm plate is lysed in 1 mL lysis buffer. For suspension cells, add lysis buffer to the cell pellet, resuspend the pellet, and transfer the cell suspension to a microcentrifuge tube.
12. One step that can reduce the nonspecific phosphorylation of the substrate is to block the protein A Sepharose by incubation in a 1% BSA solution in PBS prior to its addition to the immunoprecipitation. This is especially helpful when using 4E-BP1 as a substrate because of the presence of S/T-P phosphorylation motifs that can be phosphorylated by the numerous proline-directed, serine/threonine kinases present in mammalian cells.
13. During wash steps, use a 27-gage needle to aspirate the supernatant in order to minimize loss of the protein A sepharose.
14. To minimize pipetting steps and to maximize consistency between samples, add a cocktail consisting of substrate, [γ-^{32}P]-ATP, and ATP to the resuspended immunoprecipitate to initiate the kinase reaction. We generally add 10 µL of the substrate/ATP cocktail to initiate each reaction, so that the kinase reaction is in a final volume of 40 µL.

    If the use of radioisotopes is not convenient, the kinase assay can also be performed in a non-radioactive format by omitting the [γ-^{32}P]-ATP, and using only unlabeled ATP in the kinase reaction. Phosphorylation of the substrate can be detected by immunoblotting with antibodies specific for phospho-p70^{S6K} (Phospho-Thr389, Cat no. 9205, Cell Signaling), phospho-4E-BP-1 (phospho-Thr 37/46. Cat no. 9459, Cell Signaling), or phospho-p53 (phospho-Ser 15, Cat no. PC461, Calbiochem).
15. At this point, samples may be stored at –80°C and processed at a later time.
16. We routinely resolve the PIKK member and the substrate on the same gel by electrophoresing the sample through a discontinuous 6%/10% or 12.5% SDS-PAGE gel. For SDS-PAGE, we use a large-format gel system (Hoefer SE600 series) that uses 16 cm (h) × 18 cm (w) gel plates. First, pour a 10% or 12.5%

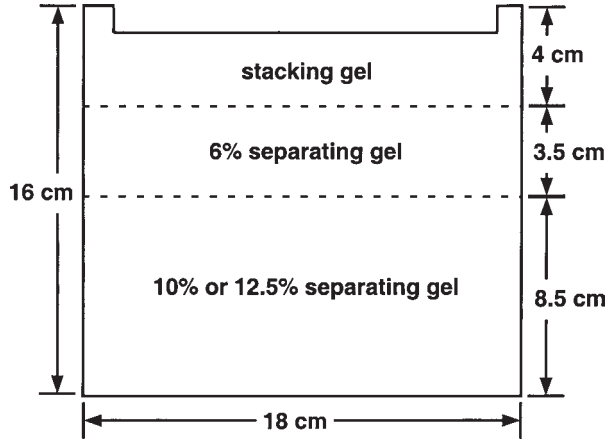

Fig. 4. Diagram of the discontinuous gel setup used to resolve the PIKK family member and the substrate on a single gel. Using a large-format gel, the lower 10% or 12.5% separating gel that will resolve the substrate is poured first and allowed to polymerize. Next, the 6% separating gel that will resolve the PIKK family member is poured and allowed to polymerize. Finally, the stacking gel is poured and the gel comb is inserted to form the sample wells.

separating gel to a height of 8.5 cm (*see* **Fig. 4**). Overlay with 0.1% SDS and allow the gel to polymerize. Remove the 0.1% SDS overlay, and pour the 6% separating gel solution to a total gel height of 12 cm (3.5 cm height for 6% gel). Overlay the 6% separating gel with 0.1% SDS and, after the 6% separating gel has polymerized, remove the 0.1% SDS overlay, pour the stacking gel, and insert the gel comb to form the sample wells.

Once the samples are loaded onto the gel, we perform the electrophoresis at constant current until the bromphenol blue dye front has exited the lower separating gel. For mTOR kinase reactions, it takes approx 150 mA × hours (i.e., mA multiplied by time, in hours) for the dye front to exit the gel using the Hoefer SE600 gel apparatus. Therefore, we often run the gel overnight for convenience. For the larger PIKK family members (ATM, ATR, and hSMG-1) it may be feasible to run the gel longer than 150 mA × h for optimal resolution of the PIKK family member on the 6% separating gel. These values are meant to serve as a guideline only; there will be variations depending on the gel size as well as the apparatus used.

17. For the electrophoretic transfer of proteins to PVDF, we utilize a Hoefer TE62 series transfer apparatus with a modified Towbin transfer buffer where the methanol and SDS are omitted (25 m*M* Tris base, 192 m*M* glycine). The transfer is performed at constant voltage, 150 V for 1 h 20 min, with a power supply rated for an output of 250 V/2.5 A. Owing to the heat generated by the high transfer

voltage and extended transfer time, it is necessary to use prechilled transfer buffer and to cool the apparatus during the transfer by chilled-water circulation. With the PIKK family members, we have had much better success with electrophoretic transfer using a tank apparatus than with a semi-dry transfer apparatus, presumably due to the large size of the PIKK family members and the extended transfer time that is required to electroblot these proteins onto the membrane. We therefore recommend the use of a tank apparatus rather than a semi-dry apparatus

18. Primary antibody solution consists of the appropriate anti-PIKK antibody diluted in 3% BSA blocking solution. The primary antibody solution can be re-used several times; retain the solution after incubation with the membrane and store at 4°C. We prefer using BSA-containing blocking solution instead of nonfat dry milk–containing buffers, because we find that the antibody solution has a longer shelf life at 4°C. For the polyclonal anti-mTOR antibody from R & D Systems, we use a concentration of 1 μg/mL for immunoblotting. For the ATM antibody, we use a concentration of 3–4 μg/mL for immunoblotting. The ATR antibody is used at a dilution of 1:2000 for immunoblotting and the hSMG-1 antibody is used at a dilution of 1:1000. These values should serve as a guideline; optimum antibody concentrations should be empirically determined for each antibody.
19. Secondary antibodies, such as HRP-conjugated protein A, HRP-anti-mouse, or HRP-anti-rabbit, are diluted in 1% BSA blocking solution. It is important to note that this solution does not contain NaN_3, which will inhibit the peroxidase reaction. We use a dilution of 1:10,000 for all the HRP conjugates. The secondary antibody solution should always be made fresh and never reused.

Acknowledgments

We thank Diane Otterness for assistance with the ATM/ATR/ATX kinase reactions. We also thank Caroline Ho for helpful suggestions and critical reading of the manuscript. This work was supported by grants CA76193 and CA97950 (to R. T. A.) from the National Cancer Institute, NIH. G. G. C. is a recipient of a Ruth L. Kirschstein NRSA Fellowship F32 CA099354 from the National Cancer Institute, NIH.

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9

CHK1 Kinase Activity Assay

Ya Wang and Hongyan Wang

Summary

Mammalian CHK1 is a Ser/Thr effector kinase that plays critical roles in the DNA damage-activated cell cycle checkpoint signaling pathway downstream of ATR (ATM and Rad3-related protein kinase). This chapter is focused on describing an assay to measure CHK1 activity in vitro. The basic mechanism of this assay is to observe the phosphorylated levels of a fragment of CDC25C containing the site that can be phosphorylated by CHK1 in vitro. This assay includes five major steps: (1) preparing extracts from the control or treated cells; (2) preparing substrate; (3) immunoprecipitating CHK1 protein from the cells; (4) assembling the kinase assay; and (5) analyzing the phosphorylated level of the substrates by CHK1. Besides CHK1, CHK2 is another important checkpoint regulator that responds to DNA damage. Because CHK1 and CHK2 share some substrates such as CDC25C in vitro, this assay could also be used for CHK2 activity assay, except that the CHK2 antibody will replace the CHK1 antibody.

Key Words: CHK1 kinase; CHK2 kinase; checkpoint; DNA damage; ionizing radiation; camptothecin.

1. Introduction

Checkpoint kinase (CHK)1 is an evolutionarily conserved protein kinase that was first identified in fission yeast ***(1)***. The identification of a homolog in mammalian cells followed a few years later ***(2,3)***. Although *chk1* is not an essential gene in fission yeast, it is essential in mice ***(1,4,5)***. CHK1 protein is an important component of the DNA damage checkpoint and of the DNA replication checkpoint ***(1,4,6–12)***. CHK1 responds to DNA damage inducers including ionizing radiation (IR), UV light (UV) ***(1,8)***, and camptothecin (CPT) ***(13)***, as well as DNA replication inhibitors including hydroxyurea and aphidicolin ***(7,9,10)***. In *Xenopus (Xe)* and in human cells, Chk1 (CHK1) is phosphorylated and activated by ATR (ATM and Rad3-related protein kinase). The ATR/CHK1 pathway responds to agents that impair DNA replication either directly (hydroxyurea, aphidicolin) or indirectly (UV, IR, and CPT) ***(4,12–16)***.

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

Mammalian CHK1 is phosphorylated on serine 317 and 345 by ATR in vitro as well as in vivo ***(4,14,16)***. Mammalian CHK1 is involved in both S phase (S) and G2 checkpoint responses after IR ***(4,16–18)***. It has been proposed that CHK1 regulates the G2 checkpoint by phosphorylating the CDC25C protein phosphatase on residue(s) that facilitate the binding of 14-3-3 proteins, thus inactivating this phosphatase. As a result, CDC25C cannot further dephosphorylate the inhibitory phosphate group on CDC2 and inactivate this kinase ***(2,3,9,19,20)***. In addition, it has been proposed that CHK1 regulates the S checkpoint by protmoting degradation of CDC25A protein phosphatase ***(13,16)***.

The recognition of CHK1 activation following DNA damage has led to increased studies of pathways regulating checkpoint response. Although CHK1 is phosphorylated and activated by ATR, the phosphorylated CHK1 in the cells treated with some types of DNA damage reagents, such as ionizing radiation, is hard to detect by the regular gel ***(4)***. However, the changes of CHK1 activity following DNA damage are easily detected ***(13,18,21)***. We describe here protocols developed to measure CHK1 activity following DNA damage induced by either IR or CPT. As CDC25C is a substrate of CHK1 ***(2)***, the basis of these protocols is to measure the phosphorylated levels of CDC25C by CHK1 in vitro. Besides CHK1, CHK2 is another important checkpoint regulator responding to DNA damage, which is phosphorylated and activated by ATM. Because CHK1 and CHK2 share some substrates such as CDC25C in vitro, this protocol could be used for CHK2 activity assay as well, except that the CHK2 antibody, not the CHK1 antibody, will be used.

The major procedures include:

1. Preparation of cellular extracts.
2. Preparation of GST-fusion fragment of CDC25C protein.
3. Immunoprecipitation (IP) of CHK1 from cellular extracts with the specific anti-CHK1 antibody.
4. Assembly of in vitro CHK1 kinase assay.
5. Analysis of the phosphorylated levels of CDC25C using gel electrophoresis.

2. Materials

2.1. Preparation of Cellular Extracts

Investigators can either prepare cytoplasmic and nuclear extracts by using the kit NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce) or prepare them in the laboratory using available reagents as follows:

1. Exponentially growing mammalian cells (human, rat, or mouse).
2. Growth medium with serum.
3. Trypsin (Gibco BRL).

4. 5 m*M* KCl buffer: 10 m*M* HEPES (pH 7.5) (stock: 0.6 *M*, pH 7.5 at room temperature), 1.5 m*M* $MgCl_2$ (stock: 1 *M*), 5 m*M* KCl (stock: 3 *M*). Immediately before use, add 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF) (stock: 100 m*M* in isopropanol), 0.5 m*M* dithiothreitol (DTT) (stock: 1 *M* in H_2O; store at –20°C) and 20 m*M* glycerophosphate.
5. 0.8 *M* KCl buffer: 10 m*M* HEPES (pH 7.5), 1.5 m*M* $MgCl_2$, 0.8 *M* KCl.
6. 1.4 *M* KCl buffer: 10 m*M* HEPES (pH 7.5), 1.5 m*M* $MgCl_2$, 1.4 *M* KCl.
7. Spectrophotometer.

2.2. Preparation of GST-Fusion Fragment of CDC25C Protein

1. BL21 bacterial cell strain (Novagen) ***(22)***.
2. Plasmid pGEX-2T (Amersham Biosciences) that was inserted with the sequences encoding human $CDC25C_{200–256}$ ***(2)***.
3. MicroSpin™ GST purification module (Amersham Biosciences).
4. Isopropylthio-β-D-galactoside (IPTG) (Amersham Biosciences; stock: 1 *M* in H_2O; store at –20°C).
5. Bacteria incubator.

2.3. IP of CHK1 from Cellular Extracts

1. Cell extracts from **Subheading 2.1.**.
2. Protein A–sepharose slurry (RepliGen).
3. CHK1 antibody (Santa Cruz, *sc-7898*) (*see* **Note 1**).
4. IP buffer: 0.5% NP-40, 1 m*M* Na_3VO_4, 5 m*M* NaF in phosphate-buffered saline (PBS) buffer (137 m*M* NaCl, 2.7 m*M* KCl, 10.6 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4). Add 0.2 m*M* PMSF immediately before use.
5. Mini-shaker.

2.4. Assembly of In Vitro CHK1 Kinase Assay

1. IP products from **Subheading 2.3.**
2. Purified GST-$CDC25C_{200–256}$ from **Subheading 2.2.**
3. Kinase buffer: 10 m*M* HEPES (pH 7.5), 10 m*M* $MgCl_2$, 10 m*M* $MnCl_2$. Add 1 m*M* DTT immediately before use.
4. ^{32}P-γ ATP.
5. Beta shield for radiation protection.
6. Heating blocks (30°C and 100°C)
7. 5X protein loading buffer: 250 m*M* Tris-Cl (pH 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol, and 500 m*M* dithiothreitol. Keep the buffer at room temperature without dithiothreitol. Add dithiothreitol just before use.

2.5. Analysis of the Phosphorylating Levels of CDC25C Using Gel Electrophoresis

1. Acrylamide gel electrophoresis equipment with the Mini-gel accessories.
2. Prestained protein marker (Cat. no. 10748, Invitrogen, Inc.).

3. Gel dryer (Bio-Rad).
4. PhosphoImager (Molecular Dynamics).

3. Methods

3.1. Preparation of Cellular Extracts

The method described here allows the preparation of cytoplasmic and nuclear extracts from mammalian cells of human, rat, or mouse. The extracts can be prepared either by using the kit NE-PER™ nuclear and Cytoplasmic Extraction Reagents (Pierce) or in a laboratory by using available reagents as follows:

1. Grow mammalian cells in 100-mm dishes (at least five dishes for each point) at 70–80% confluency (*see* **Note 2**).
2. Cell treatment: (1) IR: irradiate cells with ionizing radiation (10–20 Gy) and return the cells to 37°C (optimal CHK1 activity is increased 3–6 h after IR); (2) CPT treatment: add CPT (1–2 μ*M*) to the cell culture for 3–6 h.
3. Collect the treated and non-treated control cells by trypsinization and centrifugation (4°C, 5 min at 500*g*). After the cells are trypsinized, the samples should be kept in cold (4°C) condition.
4. Rinse once in PBS and transfer the cells to Eppendorf tubes.
5. Wash once with the 5 m*M* KCl buffer in 5 packed cell volume (PCV) and spin the cells (15 s at 12,000*g*), causing the PCV to approximately double. The following steps are based on the protocol we published previously ***(23)***.
6. Determine the new PCV. Resuspend the cell pellet in 5 PCV of hypotonic buffer.
7. Put the samples on ice for 10 min (the cells will swell). Disrupt the cell cytoplasmic membrane by three cycles of freeze-thaw: freeze the cells by putting the samples in the mixture of methanol and dry ice and thaw the cells by putting the samples in a water bath (42°C) (*see* **Note 3**). After this process, all the cytoplasmic membranes are 100% disrupted while the nuclei retain their integrity (*see* **Note 4**).
8. Add 0.11 vol of 1.4 *M* KCl buffer to the lysate and centrifuge the sample at 12,000*g*, 4°C for 20 min. Additional high-salt buffer generates an isotonic condition (approx 140 m*M* KCl final concentration). Under these conditions, the nuclei shrink back to their original size. Transfer the supernatant (cytoplasmic extracts) to a clean Eppendorf tube on ice.
9. Add an equal volume of 0.8 *M* KCl buffer to the nuclei pellets. The final concentration of KCl in the sample is approx 0.47 *M*. The high salt condition helps to extract proteins from the nuclei. Mix and shake the tube on the Mini-shaker at 4°C for 30 min.
10. Centrifuge the sample at 12,000*g*, 4°C for 20 min. Transfer the supernatant (nuclear extracts) to a clean Eppendorf tube on ice.
11. Measure the protein concentrations of the cytoplasmic and the nuclear extracts by a Bio-Rad assay (Bradford) according to the manufacturer's instructions.
12. Aliquot the sample (approx 1 mg/tube for cytoplasmic extracts and approx 500 μg/ tube for nuclear extracts) and freeze the sample at –80°C (*see* **Note 5**).

3.2. Preparation of GST-Fusion Fragment of CDC25C Protein

1. Generate the GST fusion fragment of CDC25C protein first by PCR amplifying the cDNA fragment encoding amino acids 200–256 of CDC25C (from a human cDNA library, Novagen, Inc.) and then cloning into pGEX-2T vector according to the manufacturer's instructions.
2. Transform BL21 cells with the pGEX-2T vector containing the cDNA fragment encoding amino acids 200–256 of CDC25C according to the manufacturer's instructions.
3. Grow BL21 cells from one single colony picked from the selecting plate until OD_{600} (Optical Density) value is 0.6–0.8, and then add IPTG to the cell culture (final concentration 0.5 m*M*). After 2 h of expression induction, collect the cells according to the manufacturer's instructions.
4. Purify the GST-fusion CDC25C fragment protein by using the MicroSpin™, GST purification Module (Amersham Biosciences) according to the manufacturer's instructions.
5. Measure the protein concentration, aliquot the sample (20 µg/tube), and freeze the sample at –80°C.

3.3. Immunoprecipitation (IP) of CHK1 from Cellular Extracts

1. Add 10 µL of a 50% (v/v) protein A–sepharose slurry (RepliGen) to an Eppendorf tube. Wash the protein A–sepharose slurry with lysis buffer three times by centrifuging the sample at 12,000*g* for 10 s and aspirating the supernatant.
2. Add 5 µL of CHK1 antibody (about 1 µg) to the protein A–sepharose slurry (*see* **Note 6**).
3. Add 250 µg of cytoplasmic or nuclear extracts (*see* **Note 7**) to the protein A–sepharose slurry and bring the sample to a total final volume of 250–300 µL by adding lysis buffer to the same tube (*see* **Note 8**).
4. Rotate the tube at 4°C for 4 h or overnight (*see* **Note 9**).

3.4. Assembly of In Vitro CHK1 Kinase Assay

1. Wash the sample once with cold lysis buffer and twice with cold kinase buffer without ^{32}P by centrifuging the sample (10 s at 12,000*g*) and aspirating the supernatants. Remove liquid from final wash completely (*see* Note 20 in Chapter 7). Handle this procedure carefully. The slurry contains the bound CHK1 antibody and CHK1 protein, and any loss of this sample will affect the final results.
2. Add 20 µL of kinase buffer containing 2 µg of CDC25C protein and 5 µCi ^{32}P γ-ATP. In order to have an equal volume of substrate and isotope activity in each sample, mix kinase buffer, CDC25C protein, and ^{32}P γ-ATP in one tube, and then aliquot 20 µL of the mixture to each sample.
3. Quickly spin the sample (12,000*g* for 5 s) (*see* **Note 10**) and incubate the reaction at 30°C (heating block) for 30 min.
4. Stop the reaction by adding 6 µL of 5X protein loading buffer (*see* **Note 11**). Vortex the sample, spin the sample (12,000*g* for 5 s) and boil the sample (in a

heating block at 100°C) for 5 min. After boiling, spin the sample again (12,000*g* for 5 s). Now, the sample is ready for gel electrophoresis.

3.5. Analysis of the Phosphorylated Levels of CDC25C Using Gel Electrophoresis

1. Prepare 12% SDS-PAGE using 1-mm thick spacers (in order to load the whole sample).
2. Load the prestained protein marker (5 µL) in the first well and the sample in the next well; one piece of such Mini-gel will allow a total load of nine samples (*see* **Note 12**).
3. Run the gel at 200 V for 40 min. To prevent the free isotope from running out of the gel and contaminating the buffer, keep the dye band above one-fourth from bottom of the gel.
4. Maintain the gel between 68 kD and 28 kD. The size of the CDC25C fragment is about 36 kD (*see* **Note 13**). Cut off the gel below 28 kD carefully. This piece of gel contains the dye bands and the free isotopes; put it in the isotope waste container.
5. Dry the gel by using a gel dryer (80°C for 1 h).
6. Put the dried gel on a PhosphoImager plate for exposure.
7. After finishing the exposure (*see* **Note 14**), scan the plate in the PhosphoImager.
8. Analyze the levels of phosphorylation of CDC25C by using the quantitative software in the PhosphoImager (*see* **Note 15**).

4. Notes

1. We have tested different CHK1 antibodies purchased from Santa Cruz Inc. (*sc*-7898 and *sc*-8408). Although *sc*-8408 is good for Western blot, it is not good for immunoprecipitation. We suggest using the polyclonal CHK1 antibody (*sc-7898*) for CHK1 kinase assay.
2. CHK1 protein is expressed specifically during the S and G2 phase of the cell cycle ***(24)***. In order to obtain extracts with enough CHK1 protein, it is important to keep the cells in an exponentially growing condition. Too few cells (less than 50% confluency of the culture dish) will result in less extract, and too many cells (about 100% confluency of the culture dish) will result in extracts with less CHK1 protein.
3. Cells in 5 m*M* KCl hypotonic buffer are more easily broken than those in isotonic buffer. When doing the freeze–thaw cycle, do not leave the sample in the water bath (42°C) too long. Shake the Eppendorf tube in water and when most of the sample has thawed, take the tube out and shake the tube at room temperature until the sample thaws out completely. Immediately put the sample either in the mixture of methanol and dry ice (continue the freeze–thaw cycle) or on ice (finish the freeze–thaw cycle).
4. At this step, it is suggested to confirm that 100% of the cells are lysed by observing one drop on a glass slide under the microscope.
5. Ideal concentration for the cytoplasmic extracts is >20 mg/mL, and >10 mg/mL for the nuclear extracts. The nuclear extracts are prepared in the (0.8 *M* KCl) buffer and high salt will affect IP (reducing protein binding); therefore, obtaining

the high concentration of nuclear extracts (>10 mg/mL) could reduce the high salt affection (less than 25 µL volume of extracts containing 250 µg proteins required for the IP).

6. Cut off about 1 mm of the pipet tip with scissors in order to easily pick up the protein A–sepharose slurry. In order to add an equal volume of the slurry to each sample tube, take and wash the entire volume of the slurry in one Eppendorf tube. For example, if there are ten samples, put 100 µL of the slurry into the Eppendorf tube and wash it with lysis buffer. Then add 1 mL of lysis buffer and 50 µL of CHK1 antibody to this tube. Mix well and aliquot (cut off about 1 mm of the tip with scissors) the mixture to new prelabeled Eppendorf tubes.
7. The CHK1 activity of nuclear extracts but not the CHK1 activity of whole cell lyses correlates with the S and G2 checkpoint responses ***(13,18)***. Thus, using nuclear extracts make the assay more sensitive. However, in order not to miss the changes in the CHK1 activity following DNA damage, it is better to examine the CHK1 activity separately from cytoplasmic and nuclear extracts but not from whole cell lyses.
8. The final volume of the sample depends upon the initial concentration of the extracts. The final volume of the sample should be more than 10 times the volume of the extracts (<25 µL containing 250 µg) (*see* **Note 5**).
9. This IP step can be finished after 4 h of reaction. Depending on the time schedule, the reaction could be overnight without affecting the final result.
10. Don't shake the tube after spinning it, which prevents any slurry bead splashes to the wall above the level of the buffer in the tube. Keeping all the slurry beads in the buffer is important for the normal kinase reaction.
11. The 5X protein loading buffer is very sticky, and it is easy to pick up the buffer by using a cut pipet tip. Using 5X protein loading buffer reduces the volume of the whole sample and allows loading the whole sample (approx 31 µL) into the well of the Mini-gel.
12. Load the whole sample—approx 31 µL (5 µL slurry, 20 µL reaction buffer, 6 µL 5X protein loading buffer)—into the well.
13. The red band of the prestained marker is 68 kD. Leave this band to indicate the orientation of the gel.
14. After 3 h exposure, the phosphorylation band should be visible on the PhosphoImager. It is suggested to do a reexposure overnight to obtain better signals.
15. The time requirement for the whole procedure from preparing cell extracts to getting the PhosphoImager result is approx 3 d. The work of d 1 includes preparing cell extracts and assembling the IP sample (IP overnight). The work of d 2 includes assembling kinase assays, running gels, drying gels, and placing the dried gel in a PhosphoImager cassette. On d 3, the final results could be obtained by scanning the ^{32}P signals on the cassette plate with the PhosphoImager, analyzing the data with the software in the PhosphoImager, and printing the data out.

Acknowledgment

This work was supported by NIH grants CA76203 (to Y.W.) and P30-CA56036.

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10

Assaying Cdc25 Phosphatase Activity

Ingo Hassepass and Ingrid Hoffmann

Summary

To determine the activity of all human Cdc25 phosphatases, two different methods are described. For assaying phosphatase activities of recombinant Cdc25 proteins produced in *Escherichia coli* or insect cells, a fluorimetric assay using fluorescein diphosphate (FDP) as a substrate is recommended. To analyze endogenous Cdc25 phosphatase activities of immunoprecipitates from total cellular extracts, the physiological substrate Cdk1/cyclin B1 is most sensitive.

Key Words: Cdc25; phosphatase; Cdk; cyclin; cell cycle.

1. Introduction

Progression through the cell cycle is driven by Cdk/cyclin complexes ***(1)***. The activity of these complexes is regulated by inhibitory phosphorylations of a threonine and a tyrosine residue on the Cdk subunit. These sites are dephosphorylated by a Cdc25 phosphatase, leading to activation of the kinase. In mammalian cells there are three Cdc25 proteins (Cdc25A, B, and C) ***(2)***. All three Cdc25 family members are involved in regulation of entry into mitosis ***(3)***, while Cdc25A also regulates G1/S events ***(4–6)***.

Although a colorimetric assay using p-nitrophenyl phosphate (pNPP) as a phosphatase substrate has been widely used to demonstrate phosphatase activity ***(7)***, it is only a poor substrate for all members of this phosphatase family. To determine the phosphatase activities of either bacterially expressed Cdc25 proteins, or those purified from insect cells, a fluorimetric assay using fluorescein diphosphate as a substrate is recommended. The activity of endogenous Cdc25 proteins bound to immunoprecipitates can be determined only in a two-step assay. Here, the inactive threonine/tyrosine-phosphorylated Cdk1/cyclin B1 immunoprecipitated from S-phase HeLa extracts is used as a substrate in a

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

phosphatase reaction. The resulting Cdk1/cyclin B1 kinase activity is assayed by its ability to phosphorylate histone H1.

2. Materials

1. Cooled centrifuges.
2. Fluorescence spectrophotometer. For example, from Hitachi (F-2000).
3. Acrylamid gel electrophoresis apparatus.
4. Gel dryer.
5. Radiation protection for β-radiation.
6. Adenosin 5'-[γ-^{32}P]-triphosphate, for example from Amersham (no. AA0018).
7. ATP, for example from Roche (100 m*M* [pH 7.5], stored in small aliquots at –20°C).
8. pGEX expression vectors (Amersham).
9. *Escherichia coli* host strains: XL1-blue for plasmid propagation and BL21 (DE3) for protein expression (Stratagene).
10. Ampicillin, for example from Sigma (100 mg/mL in H_2O, stored in aliquots at –70°C).
11. Isopropyl-β–D-thiogalactopyranoside (IPTG), for example from Sigma (250 m*M* in H_2O, stored in aliquots at –70°C).
12. Glutathione sepharose CL-4B, for example from Amersham (25% v/v slurry in 50 m*M* Tris-HCl [pH 7.5], 0.02% azide).
13. Reduced glutathione, for example from Sigma (dissolve the required amount in 50 m*M* Tris-HCl [pH 7.5] and use immediately).
14. Single-use columns, for example from Bio-Rad (PolyPrep columns).
15. Fluorescein diphosphate (FDP), for example from Molecular Probes (stock: 10 m*M* in 10 m*M* Tris-HCl, pH 8.5). Store in small aliquots at –20°C.
16. Hydroxyurea, for example from Sigma (prepare a 1 *M* solution with H_2O and filter-sterilize, stored at –20°C).
17. Antibodies: anti-Cdc25A, F6 Santa Cruz Biotechnology (*sc* no. 7389); anti-Cdc25B, H-85 Santa Cruz Biotechnology (*sc* no. 5619); anti-Cdc25C, H-6 Santa Cruz Biotechnology (*sc* no. 13138); anti–cyclin B1, GNS1, Santa Cruz Biotechnology (*sc* no. 245).
18. Luria Bertani (LB) medium: dissolve 10 g peptone, 5 g yeast extract, 10 g NaCl in 1 L H_2O, and sterilize by autoclaving.
19. *E. coli* lysis buffer: 50 m*M* Tris-HCl (pH 7.5), 250 m*M* NaCl, 1 m*M* EDTA, 0.1% Triton X-100 is stored at 4°C. Prior to use add: 0.1 m*M* PMSF, 10 mg/mL soybean trypsin inhibitor, 1 mg/mL aprotinin. After addition of protease inhibitors, keep on ice.
20. FDP buffer: 20 m*M* Tris-HCl (pH 8.5), 75 m*M* NaCl, 0.57 m*M* EDTA, 0.033% BSA, 1 m*M* DTT.
21. Eukaryotic cell lysis buffer: 50 m*M* Tris-HCl (pH 7.4), 0.25 *M* NaCl, 50 m*M* NaF, 0.1% Triton X-100, 5 m*M* EDTA. Before use, add 1 m*M* DTT, 1 m*M* Na_3VO_4, supplemented with protease inhibitors: 2 m*M* PMSF, 10 mg/mL soybean trypsin inhibitor, 10 mg/mL L-1-chlor-3-(4-tosylamido)-4-phenyl-2-butanon (TPCK), 5 mg/mL L-1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride (TLCK), 1 mg/mL aprotinin. Keep supplemented buffer on ice.
22. Phosphatase assay buffer (prepared freshly): 50 m*M* Tris-HCl (pH 8.0), 10 m*M* DTT.

23. Histone H1 kinase buffer (prepared freshly): 50 m*M* Tris-HCl (pH 7.5), 10 m*M* $MgCl_2$, 1 m*M* DTT, 50 µ*M* ATP, 5 µCi [^{32}P]-γ-ATP, 10 µg histone H1 (Roche) per reaction.
24. Coomassie blue stain solution: 2.5 g/L Coomassie brilliant blue R-250 (Sigma), 400 mL methanol, 100 mL acetic acid, 500 mL H_2O, stored at RT.
25. Coomassie blue destain solution: 400 mL methanol, 100 mL acetic acid, 500 mL H_2O, stored at RT.
26. 1X Laemmli buffer: % SDS (Gerbu), 10% glycerol (Roth), 5% β-mercaptoethanol, 0.002% bromphenol blue (Sigma), 0.062 *M* Tris-HCl (pH 6.8).

3. Methods

The following sections guide through the preparation and affinity purification of recombinant human Cdc25 proteins from the *E. coli* host system. After this, two methods are outlined to determine in vitro Cdc25 phosphatase activities, either from a recombinant origin using fluorescein diphosphate as a substrate or following immunoprecipitations from cell extracts with a coupled two-step assay.

3.1. Expression of Recombinant GST-Cdc25A, B, C in E. coli

1. Transform 1 µg of pGEX-Cdc25A, B, or C plasmid, respectively, in chemically or electrocompetent *E. coli* BL21 DE3.
2. Control for the correct clone using Miniprep and appropriate restriction nuclease digestion.
3. Use a single colony of the transformants to inoculate 50–100 mL of LB medium supplemented with 100 µg/mL ampicillin.
4. Grow the *E. coli* culture overnight at 37 °C and shake vigorously.
5. The following day, dilute the overnight culture 1:20 with fresh LB medium (containing 100 µg/mL ampicillin) and further incubate at 37°C for approx 3 h until OD_{600} = 0.4–0.5 (*see* **Note 1**).
6. Cool down the bacterial culture to 30°C and add β–D-thiogalactoside (IPTG, Sigma) to a final concentration of 0.5 m*M*.
7. Incubate the culture for another 3 h at 30°C under shaking and then harvest by centrifugation for 15 min at 3000*g* (Sorvall, GS3 rotor) at 4 °C.
8. The bacterial pellets can be stored at –20°C until protein isolation.

3.2. Purification of GST-Cdc25A, B , C from E. coli

Recombinant GST-Cdc25A, B, C fusion proteins can easily be purified by coupling them to glutathione *S*-transferase (GST), a 26-kDa bacterial protein that efficiently binds to glutathione sepharose ***(8)***.

1. Resuspend the bacterial pellets containing the induced GST fusion proteins in 30 mL *E. coli* lysis buffer and incubate on ice for 15 min.
2. Lyse the cells by sonification four times for 30 s, on ice.

3. Incubate the lysate further on ice for 5 min and remove cell debris by centrifugation for 30 min at 10,000*g* in the Sorvall SS-34 rotor at 4°C.
4. Wash glutathione sepharose beads (2 mL, Amersham) with 10 mL *E. coli* lysis buffer.
5. Pellet the beads by centrifugation at 3000*g* in a Megafuge R 1.0 (Heareus) for 2 min at 4°C, and remove the supernatant.
6. Add the bacterial supernatant to the beads and incubate on a wheel at 4°C for a minimum of 30 min.
7. Centrifuge the beads-bound GST-Cdc25 fusion proteins as indicated above, remove the supernatant, and wash the beads twice with 30 mL of ice-cold *E.coli* lysis buffer.
8. Resuspend the beads in 10 mL of *E. coli* lysis buffer and load on a single-use column (Bio-Rad).
9. Wash the column with 40 mL cold *E. coli* lysis buffer, followed by 10 mL of ice-cold 50 m*M* TrisHCl (pH 7.5).
10. Elute the fusion proteins at room temperature with 2 mL of 50 m*M* TrisHCl (pH 7.5) including 20 m*M* reduced glutathione (Sigma).
11. Collect the eluates in 500-µL aliquots.
12. Analyze the protein content of 10 µL of each aliquot by SDS-PAGE (10%) followed by Coomassie blue staining of the gel after gel-run (**Fig. 1**).
13. Determine the protein concentrations (*see* **Note 2**), prepare aliquots, and freeze at –70 °C.

3.3. Cdc25 Phosphatase Assay Using Fluorescein Diphosphate as Substrate

Among the exogenous substrates that can be used in Cdc25 phosphatase assays, fluorescein diphosphate (FDP) is the most sensitive. It has been shown that FDP can be dephosphorylated by tyrosine phosphatases ***(9)***. The product of this reaction is a molecule that exhibits fluorescence at 530 nm when excited at 490 nm. A phosphatase assay that uses FDP as a substrate is much simpler and is more direct than to examine the activation of phosphorylated Cdk1/cyclin B1. The FDP assay, however, is not as sensitive as using endogenous tyrosine-phosphorylated Cdk1/cyclin B1. Therefore, this assay is recommended only when recombinant GST-Cdc25 fusion protein is used, and is not suited so well for measuring small amounts of endogenous Cdc25 phosphatase activities obtained by immunoprecipitation (*see* **Subheading 3.4.**).

1. Wash the immunoprecipitate twice in FDP buffer prior to the assay.
2. Incubate recombinant or immunoprecipitated Cdc25 protein with 20 µ*M* FDP in FDP buffer for 1 h at 37°C.
3. Stop the reactions through the addition of an equal reaction volume of 1 *M* NaOH, to get a final 500 m*M* NaOH concentration.
4. Filter all reactions containing sepharose beads from immunoprecipitates through a filter Eppendorf tube (Millipore) to remove all beads from the solution.

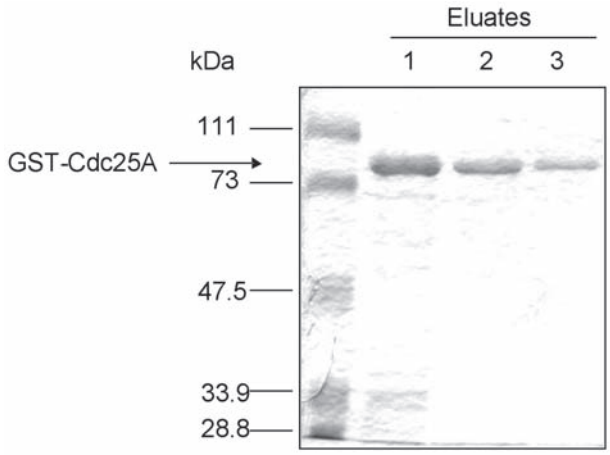

Fig. 1. Purification of GST-Cdc25A from *E. coli*. *E. coli* BL21 DE3 were transformed with pGEX-Cdc25A, and recombinant GST-Cdc25A was produced according to the protocol presented here. After cell lysis, binding to GSH-beads (Amersham), and washing, beads-bound GST-fusion proteins were eluted with 2 mL 20 m*M* reduced glutathione in 50 mM Tris-HCl (pH 7.5). Fractions of about 0.5 mL each were collected. 10 µL of each fraction was analyzed on SDS-PAGE gel and stained with Coomassie brilliant blue. Shown is the Coomassie blue-stained gel.

5. Measure the fluorescence at 530 nm with a fluorescence spectrophotometer (F-2000, Hitachi) at the excitation wavelength of 490 nm (*see* **Note 3**).

3.4. Measuring Cdc25 Phosphatase Activity In Vivo

Owing to the low abundance of Cdc25 proteins in a variety of human cell lines, it is not easy to measure Cdc25 activity. Therefore, a two-step assay has been developed ***(6)***. The assay procedures are outlined in **Fig. 2**.

For each sample that one would like to determine Cdc25 activity from, a parallel sample from S-phase-arrested cells is required. In the first step, the Cdc25 protein is isolated by immunoprecipitation using Cdc25-specific antibodies. In parallel, inactive cyclin B complex is immunoprecipitated from S-phase cell extracts. Then the two immunoprecipitates are combined and a phosphatase assay is carried out, in which immunoprecipitated Cdc25 dephosphorylates and thereby activates the cyclin B complex. In the second step, a histone H1 assay is used to determine the activity of the cyclin B1 complex from above. The amount of active Cdk1/cyclin B1 is a direct measure for the Cdc25 activity present.

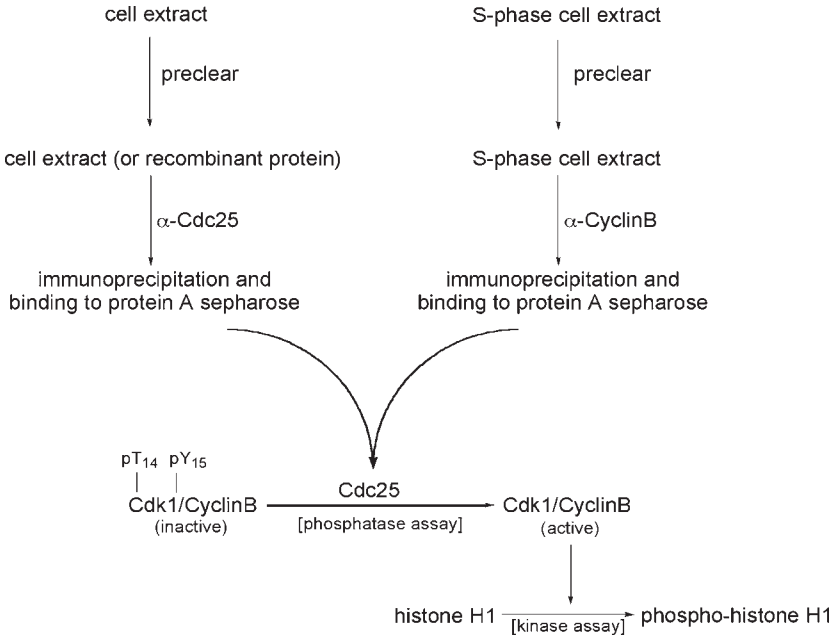

Fig. 2. Scheme for assaying endogenous Cdc25 phosphatase activity.

3.4.1. Preparing S-Phase Cell Extracts

1. Treat adherent HeLa cells (about 70% confluence) with 10 m*M* hydroxyurea (HU) for 18 h under standard cell culture conditions (*see* **Notes 4** and **5**).
2. Wash the monolayer twice with PBS.
3. Harvest the cells by trypsination followed by centrifugation.
4. Optional: freeze the cell pellet until further need.
5. Add 3 to 5 vol of eukaryotic lysis buffer (*see* **Subheading 2.**).
6. Pipet up and down to resuspend the cells.
7. Incubate on ice for 30 min with intermittent mixing.
8. Clear the lysate by centrifugation for 10 min at 10,000*g*.
9. Determine the total protein concentration by a standard assay method (for example, Bio-Rad assay system).
10. Freeze the S-phase cell extract in aliquots of 500 mg in liquid nitrogen and store at –70°C or use immediately for the anti-cyclin B immunoprecipitation.

3.4.2. Preparing Cell Extracts for Detection of Cdc25 Phosphatase Activity

1. Wash the monolayer twice with PBS (*see* **Notes 4–6**).
2. Harvest the cells by trypsination followed by centrifugation.

3. Optional: freeze the cell pellet until further need.
4. Add 3 to 5 vols of eukaryotic lysis buffer (*see* **Subheading 2.**).
5. Pipet up and down to resuspend the cells.
6. Incubate on ice for 30 min with intermittent mixing.
7. Clear the lysate by centrifugation for 10 min at 10,000*g*.
8. Determine the total protein concentration by a standard assay procedure (for example, Bio-Rad assay system).
9. Freeze the cell extract in aliquots in liquid nitrogen and store at –70°C or use immediately for the anti-Cdc25 immunoprecipitation.

3.4.3. Preclearing

1. Wash 100 µL protein A sepharose of a 25% slurry with eukaryotic lysis buffer (*see* **Note 7**).
2. Pellet the beads at 10,000*g* for 1 min.
3. Add 500 µg S-phase cell extract to the beads of one tube and fill up to 1 mL with eukaryotic lysis buffer.
4. Add 2 mg cell extract for Cdc25 activity to the beads of another tube, fill up to 1 mL with eukaryotic lysis buffer.
5. All together, two immunoprecipitations are carried out in parallel (one with anti-cyclin B and another with anti-Cdc25 antibodies, respectively), which are combined in a later step.
6. Incubate the IPs on a rotating wheel for 30 min at 4°C.
7. Pellet the beads by centrifugation for 1 min at 10,000*g*.

3.4.4. Immunoprecipitations

1. Transfer the supernatants to new reaction tubes containing 25 µL washed protein A sepharose beads.
2. Add 5 µL antibody for the IP of cyclin B1, or 10 µL antibody for the IP of Cdc25, respectively.
3. Incubate for 2 h at 4°C on a rotating wheel.
4. Spin the beads for 1 min at 10,000*g*.
5. Remove the supernatant and discard.
6. Wash the beads three times with 1 mL eukaryotic lysis buffer. After each washing step the beads are pelleted by centrifugation for 1 min at 10,000*g*. Discard the supernatants.

3.4.5. Phosphatase Assay

1. Add 500 µL phosphatase assay buffer to the beads containing the immunoprecipitated Cdc25.
2. Resuspend with the pipet, and combine with the immunoprecipitated cyclin B IP.
3. Mix well by vortexing.
4. Centrifuge at 10,000*g* and remove the supernatant completely.
5. Add 100 µL phosphatase buffer and incubate at 30°C for 15 min with constant shaking (200 rpm).

6. Centrifuge at 10,000*g* and remove the supernatant completely.
7. Without much delay, use the pellet for the histone H1 kinase assay below.

3.4.6. Histone H1 Kinase Assay

1. Add 50 µL histone H1 kinase buffer containing 10 µg histone H1 per reaction.
2. Incubate at 30°C for 15 min with constant shaking (200 rpm).
3. Stop the kinase reaction by adding 15 µL 4X Laemmli buffer.
4. Mix well and centrifuge for 2 min at 10,000*g*.
5. Load the complete supernatant on a 12.5% SDS-PAGE gel (15 × 15 cm).
6. Run at 50 V overnight.

3.4.7. Coomassie Staining and Autoradiography

1. Disassemble the glass plates with the gel after the run.
2. Cut off and discard the bromphenol blue band since it co-migrates with free [^{32}P]-γ-ATP.
3. Stain the gel in Coomassie blue stain solution for about 0.5–1 h.
4. Destain until the histone H1 bands at around 33 kDa are clearly visible and the background stain is low.
5. Dry the gel in a gel dryer for 2 h at 80°C under vacuum.
6. Expose to x-ray film (*see* **Note 8**) (**Fig. 3**).

4. Notes

1. After dilution and reincubating of the bacterial culture at 37°C, the OD is checked every 30 min. If the culture is in the linear range of growth, one can estimate the time when the culture reaches the desired OD. Therefore, if the OD is very low at the beginning, longer intervals can be adjusted.
2. The typical yield from the IPTG-induced *E. coli* culture varies over a broad range depending on many factors, like age of the transformants, kind of expressed protein, and so on. In general, Cdc25A can be expressed very well with a yield of 10 µg/µL and more in the first collected fraction. In our experience, Cdc25B and Cdc25C are expressed in similar amounts. If the yield is low, one could try to concentrate the purified fractions with Centricon tubes (Millipore) or start with 2–4 L of the IPTG-induced *E. coli* culture.
3. The signal typically depends on the spectrophotometer. With the Hitachi F-2000, we measure approx 23,000 units when 0.5 µg of recombinant Cdc25A protein is applied, and twice that when the amount of protein is doubled, whereas the background is approx 1000 units.
4. During the whole assay it is crucial to keep the extracts and the immunoprecipitated proteins on ice to prevent protein degradation and especially to inhibit dephosphorylation events. Also, all buffers, reaction tubes, and centrifuges must be cooled down prior to use.
5. Depending on the cell line, we obtain approx 800 µg of total protein per 100-mm cell-culture dish of HeLa cells at 100% confluence. In order to get "nice" signals in the appropriate time at the end of the assay, we start routinely with 500 µg

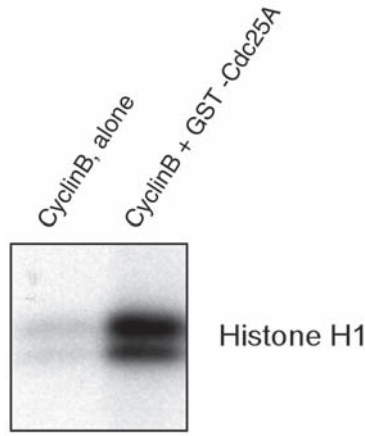

Fig. 3. Cyclin B/Cdk complex from S-phase cells is activated by GST-Cdc25A. Cyclin B1 was immunoprecipitated from S-phase cells and incubated with GST-Cdc25A purified from bacteria in a phosphatase assay (right lane) or left untreated (left lane). The cyclin B complex was then subjected to a histone H1 kinase assay and separated on 12.5% SDS-PAGE gel. Proteins were stained with Coomassie blue, and radioactively labeled histone H1 was visualized with autoradiography. Shown is the autoradiograph.

HeLa cell lysate for the cyclin B immunoprecipitation, although it is possible to use as little as 300 µg protein extract.

6. Expression levels of Cdc25 proteins are usually not high. Therefore, we routinely use 2 mg of cell extracts for the starting immunoprecipitation. HeLa cells give quite a high yield of protein (*see* **Note 5**), but for other cell lines (e.g., fibroblasts) up to 5 mg starting material may be needed.
7. For each time point, Cdc25 activity must also be measured for one sample of the S-phase-arrested HeLa cells. Therefore two immunoprecipitations per time point are performed in parallel: one immunoprecipitation with anti-cyclin B antibodies from 500 µg S-phase cells, and another IP from 2–5 mg of the cell line to be analyzed with anti-Cdc25 antibodies. Later on these two IPs are combined (*see* **Subheading 3.4.5.**).
8. If Cdc25 activity is determined from exponentially growing cells with a 2-mg starting cellular extract, generally the signal is clearly visible after 1–2 h exposure at –70°C with screen intensifier. If Cdc25 activities are followed in a time-course experiment starting with a synchronized cell population, sometimes the signals are low. In that case, exposure times up to 24 h are required.

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11

Analyzing the Regulation and Function of ATM

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Summary

We describe here the cloning of full-length ataxia-telangiectasia mutated (ATM) cDNA and characterization of its activity. Full-length ATM cDNA is cloned into an inducible EBV-based vector (pMEP4) and its expression analyzed in a stably transfected cell line. ATM protein induction is monitored by immunoblotting with antibodies against both ATM and a FLAG sequence tag in the recombinant protein. Extracts from irradiated cells are immunoprecipitated with anti-ATM antibodies, and protein kinase activity is measured using $p53_{1-44}$-specific substrate or by immunoblotting extracts with an anti-phosphoserine 15 p53-specific antibody. Missense mutations affecting ATM kinase activity are detected using in vitro mutagenesis of ATM cDNA followed by the procedures outlined above.

Key Words: ATM; Ataxia-telangiectasia; cDNA; gene cloning; inducible expression; stable cell lines; kinase activity; missense mutants; in vitro mutagenesis.

1. Introduction

Ataxia-telangiectasia mutated (ATM) is the protein that is mutated in the human genetic disorder ataxia telangiectasia ***(1,2)***. This disease is characterized by immunodeficiency, neurodegeneration, cancer predisposition, hypersensitivity to ionizing radiation, and cell cycle checkpoint defects ***(3)***. ATM is a protein kinase, belonging to a family of phosphatidylinositol-3-related kinases that respond to DNA damage. The ATM protein is rapidly activated by autophosphorylation to phosphorylate multiple proteins involved in DNA-damage recognition and cell cycle control ***(4–12)***. There is also evidence for regulation of ATM kinase at the level of transcription ***(13,14)***. The exact mechanism of activation of the protein kinase activity of ATM remains unknown, but appears to be the result of long-range changes in chromatin structure as a consequence of induced double-strand breaks in DNA ***(15)***. This stimulus causes transphosphorylation in an ATM dimer at a specific site, which apparently leads to monomerization of the dimer and activation ***(15,16)***.

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

In this report the cloning of full-length ATM cDNA and its expression in a stable cell line is described. In vitro ATM kinase activity is determined after immunoprecipitation of ATM out of cell lysates followed by phosphorylation of a GST-$p53_{1–44}$ peptide substrate in vitro. This methodology also allows for the detection of missense mutations abrogating ATM kinase activity.

2. Materials

1. pEBV/His B cloning vector (Invitrogen).
2. pMEP4 expression vector (17, Invitrogen).
3. pMAT1 construct containing full-length ATM cDNA.
4. *Escherichia coli* strain genotypes:
   a. DH5α (ϕ80*lac*ZΔM15 [*lac*ZYA-argF]. U169 *end* A1 *rec* A1 *hsd*R17 [r_k–, m_k+] *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *pho*A), BL21 ([DE3]: F- *omp*T *hsd*S_B [r_B–, MB–] *gal dcm* [DE3]).
   b. Top 10 F- *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC Δϕ80*lac*ZΔM15 Δ*lac*X74 *deo*A1 *ara*D139 Δ [*ara-leu*] 7697 *gal*U *gal*K *rps*L [Str^R] *end*A1 *nup*G).
5. Oligonucleotide primers (*see* **Tables 1–3** for details).
6. Restriction enzymes.
7. Expand™ High Fidelity PCR system (Boehringer Mannheim).
8. T4 DNA ligase, Pfu Turbo® (Stratagene).
9. Big-Dye™ Terminator V. 3.0 DNA sequencing kit (Applied Biosystems).
10. EBV-transformed lymphoblastoid cells, control (C3ABR), and A-T (L3 and ATIABR). C3ABR is part of a series (CXABR) of control lymphoblastoid cells. The ABR refers to Australia Brisbane, A-T is ataxia-telangiectasia.
11. Lipofectin™ transfection reagent (Invitrogen) and Lipofectamin 2000 (Invitrogen).
12. Hygromycin B (Boehringer Mannheim) stored at 4°C as 50 mg/mL stock solution.
13. $CdCl_2$ (Sigma). Stock solution 5 m*M* made up fresh.
14. RIPA buffer: 50 m*M* Tris-HCl (pH 7.6), 150 m*M* NaC1, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate.
15. ATM immunoprecipitation/kinase assay buffer. The buffer systems used in two different protocols are included. (a) ATM kinase immunoprecipitation buffer ***(18)***: 20 m*M* HEPES (pH 7.4), 0.15 *M* NaCl, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 0.2% Tween-20, 1 m*M* DTT, 1 m*M* Na_3VO_4, 5–10 µg/mL aprotinin, 1 m*M* NaF, 5 µg/mL pepstatin, 20 m*M* β-glycerophosphate, 5 µg/mL, leupeptin, 20 m*M*, 1 m*M* PMSF, Microcystin LR (BioMol). (b) ATM kinase immunoprecipitation buffer ***(19)***: 50 m*M* Tris-HCl (pH 7.5), 50 m*M* β-glycerophosphate, 150 m*M* NaCl, 1 m*M* NaF, 1% Tween-20, 1 m*M* Na_3VO_4, 0.3% Nonidet P-40, 1 *M* PMSF, 10% glycerol, 1X Complete™ protease inhibitors (Roche Boehringer Mannheim), 1 m*M* DTT, 2 µg/mL pepstatin, 5 µg/mL leupeptin, 10 µg/mL aprotinin.
16. Denaturing SDS loading buffer: 0.05 m*M* Tris-HCl (pH 6.8), 0.08 *M* DTT, 1% SDS, 0.1% bromophenol blue, 2% glycerol.
17. PAGE: 5% low-crosslinked polyacrylamide gel (100:1, 0.98% C), 5% (37.5:1, 2.6% C), 5% (29:1, 3.3% C) (can be used for several proteins).

18. 5% gels for ATM separation; 10% gels for detection of ^{32}P-labeled $p53_{1–44}$ (used only for ATM).
19. [γ-^{32}P] ATP: 3000 Ci/mmol (Amersham).
20. Transfer buffer: per L: 6g Tris; 3 g glycine; 20% methanol, 0.02% SDS.
21. Protein G–Sepharose™ 4 Fast Flow (Amersham Bio Sciences).
22. Protein A–Sepharose™ CL-4B (Amersham Bio Sciences).
23. GST-$p53_{1–44}$ fusion protein (1 µg/µL).
24. Two ATM antibodies were used. ATM-5BA was prepared against aGST ATM fragment in sheep (*see* **Subheading 3.2.2.**) and used for immunoprecipitations. ATM 2CI (Gentec, San Antonio) was used for immunoblotting.
25. 5X concentrated sample buffer: 0.25 *M* Tris-HCl (pH 6.8), 0.4 *M* dithiothreitol, 5% SDS, 0.5% bromophenol blue, 10% glycerol.
26. HEPES buffer: 0.15 *M* NaCl, 1.5 m*M* $MgCl_2$, and 1 m*M* EGTA (pH 7.4) containing 1 m*M* DTT, 10 µg/mL pepstatin, 5 µg/mL leupeptin, 20 n*M* microcystin, and 0.2% Tween-20 at 4°C ***(18)***.
27. High-salt buffer: 0.1 *M* Tris-HCl (pH 7.4), containing 0.6 *M* NaCl.
28. Kinase base buffer: 10 m*M* HEPES, 50 m*M* NaCl, and 10 m*M* $MgCl_2$, pH 7.4.

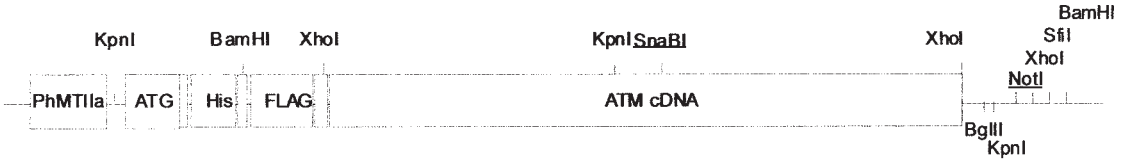
3. Methods

In the methods section an outline is provided of (1) the construction of the EBV-based vector for ATM expression; (2) production of a stable cell line expressing ATM protein; (3) ATM protein expression and determination of ATM kinase activity; and (4) in vitro mutagenesis to introduce missense mutations into ATM cDNA.

3.1. Construction of ATM Expression Vector

In order to clone full-length ATM cDNA into a mammalian expression vector λ Zap excision process (Stratagene) was used to convert ATM λ clone 7-9 ***(20)*** to a plasmid construct. This clone contains a partial 5.9-kb ATM cDNA 3' fragment.

1. Use two oligonucleotide primers to amplify a 4.7-kb ATM cDNA fragment that overlaps with the 5.9-kb fragment, both of which contain a unique SnaB1 site (*see* **Note 1**).
2. After digestion with Xhol and SnaB1 gel, purify the 4.46-kb and 5.12-kb fragments and ligate them to each other through the SnaB1 site, which results in a 9.58-kb full-length fragment.
3. Clone this fragment into the Xhol site of an EBV-based plasmid vector pEBV-His-B (Invitrogen). A hexahistidine tag sequence (His-B) is inserted upstream of ATM.
4. The hexahistidine tag–ATM cDNA is subsequently subcloned into another EBV-based vector pMEP4 that contains an inducible metallothionein II promoter called pMAT1 (*see* **Fig. 1** and **ref. *21***).
5. In addition, clone a FLAG tag sequence into pMAT1, using BamHI and XhoI, at a position upstream of the ATG and downstream of the hexahistidine tag. The



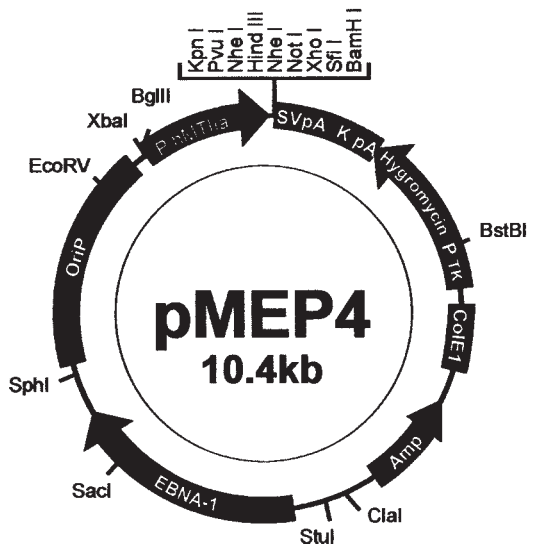


Fig. 1. The full-length ATM cDNA construct pMAT1. ATM cDNA was cloned into an EBV-based vector pMEP4 (PvuII/NotI), which contains a metallothionein II inducible promoter. This construct contains both a hexahistidine tag and FLAG tag upstream of ATM. The underlined restriction enzyme sites are unique.

hexahistidine tag is used for the purification of recombinant ATM, while the FLAG tag is used for immunoprecipitation and western blot detection of recombinant ATM. Approximately 1–2% of the clones generated in *E. coli* should contain full-length ATM cDNA.

3.1.1. Propagation of ATM Plasmids in Bacteria

1. Use the *E. coli* strain Top10 (Invitrogen) or a related strain for the manipulation of plasmid DNA to stabilize full-length ATM cDNA.
2. If intending to use another strain, make sure it does not contain a transposable element, in particular Tn10, as this has been found in ATM cDNA preparations propagated in XL1-Blue (*see* **Subheading 3.1.2.** for method of detection). Full-length intact clones tend to be the smallest colonies on the plate. Use caution when manipulating the DNA, as we have observed that the 5' region is particularly unstable.
3. Use PCR to amplify the first 1 kb of ATM to confirm its presence (PCR conditions and primers are described in **Subheading 3.1.2**) and the integrity of ATM cDNA. It is not advisable to grow large culture preparations (>200 mL) to saturation. For large-scale plasmid production, 150 to 200 mL of LB medium supplemented with 100 µg/mL ampicillin should be used to culture cells at 30°C overnight. The lower temperature appears to lower the risk of rearrangement.

3.1.2. Confirmation of the Integrity of the Full-Length cDNA

1. To confirm the presence of the 5' region of ATM, the primers NZF1 and NZR1 are used (*see* **Table 1**).
2. In order to check that no transposons are present, a series of overlapping PCRs are performed, which encompass the entire coding region of ATM (*see* **Table 2** for primers). It is our experience that the transposon inserts itself between 2311 and 3387 bp. A Bgl II digest will also pick up any differences in size for this region.
3. The Bgl II digest of intact ATM in pMAT1 should generate the following size products: 10.327 kb, 4.919 kb, 3.698 kb, and 1.066 kb. An additional band of approx 2.0–2.5 kb will be seen if a transposon is present in the ATM cDNA.

3.1.3. Further Confirmation of Integrity of Full-Length ATM cDNA

While the above methods are a useful indication of the integrity of ATM cDNA, they do not ensure that no errors have been introduced into the cDNA during amplification. Accordingly, carry out DNA sequencing of the entire ATM cDNA using a dye terminator kit as described by the manufacturer (Perkin Elmer/Applied Biosystems) to detect any substitutions.

3.2. Establishment of Stable Cell Lines Expressing Recombinant ATM

1. Typically pellet 2×10^6 exponentially growing EBV-transformed lymphoblastoid cells by centrifugation at 3000*g* and wash twice in Opti-MEM medium without fetal calf serum (FCS).

Table 1
ATM Primers for Checking the Integrity of the 5' End of ATM cDNA

Primer	Nucleotide sequence (5'-3')	Position on *ATM* ORF, bp
NZF1	CTTAATGATCTGCTTATCTGC	10–30
NZR1	CCACGAATACTTAGTTGT	901–918

PCR conditions are denaturation at 94°C for 30 s, annealing at 55°C for 80 s and extension at 72°C for 90 s. ORF = open reading frame.

Table 2
PCR Primers for Determining Integrity of Full-Length ATM cDNA

Primer	Nucleotide sequence (5'-3')	Position on *ATM* ORF, bp
ATM 1F	Atgagtctagtacttaatg	1–19
ATM 1R	Gttgacagccaaagtcttg	720–738
ATM 2 F	Gaagataccagatccttggagatttc	1075–1100
ATM 2 R	Ctcctgcacattgcattagagagttggc	2245–2272
ATM 3F	Agttgacaattggttccttgag	2311–2330
ATM 3R	Tcccttcgtgtcctggaac	3288–3307
ATM 4F	Aggacacgaagggagattc	3292–3311
ATM 4R	Ccctgaaaagtcacagag	4093–4111
ATM 5F	Gaggatttctatagatcttg	3733–3752
ATM 5R	Ctgtgcttcttataaac	4294–4310
ATM 6F	Cttaaaatatatcacctgtttg	4315–4336
ATM 6R	Ttgatgttctaaaaggc	5295–5312
ATM 7F	Ctacagccttttagaacatc	5290–5309
ATM 7R	Tctgtctcttagagattg	6397–6415
ATM 8F	Agtctacattttatgaaagtc	6421–6439
ATM 8R	Cctaaggagacctacttcc	7266–7285
ATM 9F	Cttagggaacataaaattc	7279–7297
ATM 9R	Caagaatttttccatgc	8504–8521
ATM 10F	Caagctatttggtttgagaag	8524–8544
ATM 10R	Acacccaagctttccatcc	9151–9168

PCR conditions as for Table 1 primers.

2. Resuspend in 1 mL of Opti-MEM medium containing 6 µg of pMAT1 plasmid DNA and 20 µg of lipofectin or lipofectamin.
3. Incubate at 37°C for 6–7 h in 6-well plates under an atmosphere of 5% CO_2, followed by addition of 4 mL of RPMI1640 medium + 10% FCS and further incubation for 48 h. The pMEP4 vector contains a gene that provides resistance to the antibiotic hygromycin B.

4. Accordingly, select transfectants employing 200 µg/mL of hygromycin B in the cell-culture medium. After approx 1 wk a large proportion of the cell culture dies, but by 2 wk it is evident that some cells survive.
5. It is usually possible to isolate successfully transfected cells by 3–4 wk after transfection. It is not necessary to derive single-cell clones from the survivors since all contain the full-length ATM cDNA construct and the selection marker that provides resistance to hygromycin.
6. pMEP4 is a plasmid that contains the EBV latent protein EBNA1 and uses a latent origin of replication (OriP), and therefore exists as an autonomously replicating episome after transfection. The stable, nonintegrative maintenance of EBV-based vectors in the presence of the selective antibiotic hygromycin B has been demonstrated for many cell generations ***(22)***. Indeed, stable expression levels of genes in EBV-based episomal vector have been observed for more than 1 yr when contstant hygromycin B selection is applied ***(23)***.
7. Do not prolong cultivation of A-T lymphoblastoid cells transfected with pMAT1, because there is a high propensity of A-T cell lines for gene rearrangements. Unfortunately it is not possible to freeze away and store these transfected lines, since they tend to lose their plasmids when rethawed, and very few cells survive in hygromycin-containing medium.

3.2.1. Induced Expression of ATM: Detection

1. Plate out stably transfected lymphoblastoid cells at 3×10^5/mL in 10-mL flasks and allow the culture to reach log phase (36 h) prior to adding 5 µ*M* $CdCl_2$ to induce ATM protein for a period of 16 h (*see* **Fig. 2,** top panel).
2. Harvest cells and lyse them in RIPA lysis buffer with proteinase and phosphatase inhibitors added (0.1 m*M* orthovanadate, 0.1 m*M* PMSF, 5 µg/mL leupeptin, and 1 µg/mL aprotinin).
3. Determine the protein concentration of extracts using the Bradford microassay (Bio-Rad).
4. Solubilize the protein extracts (80 µg) in 0.2 vol of 5X concentrated sample buffer and separate on a 5% low-crosslinked polyacrylamide gel (monomer to crosslinker ratio, 100:1) for transfer of ATM. The reason for the low percentage gel and low level of cross-linking is to allow efficient transfer of this high-molecular-weight protein (approx 380 kDa) from the gel to the membrane for subsequent immunoblotting.
5. After electrophoresis, transfer proteins to Hybond C nitrocellulose membranes (Amersham Biosciences) ***(21)***.
6. Block the filters in 5% skim milk for 60 min, then probe with ATM antibodies overnight, wash three times for 10 min in PBS–0.1% Tween-20 (PBS-T), incubate for 60 min with secondary antibody in 5% skim milk (PBS-T), then wash three times for 10 min in PBS-T, and visualize by the ECL method (Amersham).
7. Use densitometric scanning to quantify protein using Image Quant software (Molecular Dynamics).

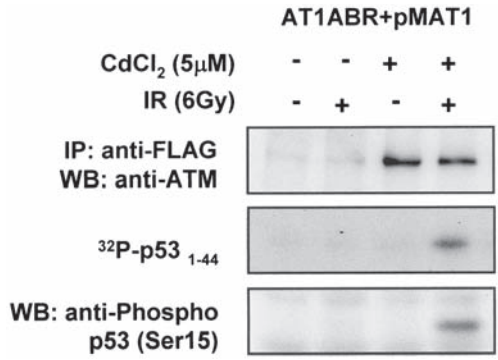


Fig. 2. Stable transfection of A-T lymphoblastiod cells (AT1ABR) with a full-length ATM cDNA construct (pMAT1). Expression of ATM protein was induced with $CdCl_2$ for 16 h before irradiation (6Gy; IR) and detected by immunoprecipitating (IP) ATM with anti-FLAG antibodies followed by immunoblotting (WB) with anti-ATM antibodies **(Top panel)**. In vitro ATM kinase activity was determined by immunoprecipitating ATM with anti-ATM antibodies and assaying for phosphorylation of GST-$p53_{1–44}$ substrate **(Middle panel)**. ATM kinase was determined in vivo by immunoblotting extracts with anti-p53 Ser-15 phosphorylation-specific antibody **(Lower panel)**.

3.2.2. Immunoprecipitation of ATM Protein for In Vitro Kinase Assay

1. Plate out stably transfected lymphoblastoid cells at 3×10^5/mL in 10-mL flasks and allow them to reach log phase (36 h).
2. Centrifuge between 10^7 and 1.5×10^7 cells at 3000*g*, wash once in PBS, and lyse in 1 mL of 20 m*M* HEPES buffer.
3. Carry out all subsequent steps in this procedure at 4°C (*see* **Note 2**).
4. Clear lysates of insoluble material by centrifugation at 5000*g* for 10 min and then pre-clear equivalent amounts of protein (0.5–1.0 mg) with 30 μL of Protein G sepharose beads (50% slurry in PBS).
5. Then incubate on ice for 2 h with 1–2 μg of ATM-specific antibodies. ATM-specific antibodies can be prepared in sheep according to standard immunization protocols.
6. Use GST-ATM2 fusion protein (ATM amino acids 250–522) in this case as antigen.
7. Obtain ATM-specific antibodies by immunizing sheep with GST-fusion proteins containing fragments of ATM protein as described in ***(16)***. Immunoprecipitating ATM antibodies from commercial sources can also be used (e.g., ATM Ab-3 from Oncogene Science–Calbiochem).
8. Precipitate the immune complexes with protein G (if sheep antibodies) and wash the resulting immunoprecipitates twice in lysis buffer, once in high-salt buffer, and once in kinase base buffer. It is important not to disturb the sedimented beads

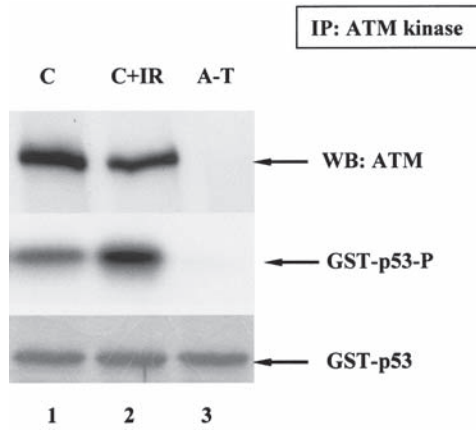


Fig. 3. ATM immunocomplex protein kinase activity. ATM protein was immunoprecipitated from control, control-irradiated, and A-T extracts. Protein kinase activity was determined using GST-p531-44 as substrate, and ATM protein was detected by immunoblotting.

during these stages. This may lead to loss of material and difficulty in making activity comparisons later in the procedure.

3.2.3. ATM Immunocomplex Protein Kinase Activity

1. Activate the kinase activity of ATM by exposing cells to ionizing radiation or other stimuli ***(19,24)***. Other stimuli include radiomimetric chemicals, H_20_2, and hormones such as insulin.
2. Determine in vitro activity using immunoprecipitated ATM bound to protein A/G beads and GST-$p53_{1–44}$ substrate (*see* **Fig. 3,** middle panel). Other substrates can be used in ATM protein kinase assays (e.g., BRCA1, NBS1, CHK2, BLM). Comparison is made between ATM immunoprecipitates from extracts from unirradiated and irradiated cells, thus providing a measure of the extent of activation by radiation.
3. As an additional control of specificity, process extracts from A-T cells in parallel, which should show no inducible kinase activity (**Fig. 3,** middle panel). Physiological status of the cells can influence the level of basal ATM kinase activity (*see* **Note 3**).
4. Wash immunoprecipitates of ATM twice in 10 m*M* HEPES, 50 m*M* NaCl, and 10 m*M* $MgCl_2$ prior to resuspending in kinase buffer: 10 m*M* HEPES (pH 7.4), 50 m*M* NaCl, 10 m*M* $MgCl_2$,10 m*M* $MnCl_2$, 1 m*M* DTT, 1 µg of GST-$p53_{1–44}$ and 10 µ*M* [γ-^{32}P] ATP (3000 Ci/mmol, Amersham) in a total volume of 20 µL.
5. Incubate for 30 min at 30°C and terminate by adding an equal volume of denaturing loading buffer.

6. Separate samples on 10% SDS-PAGE to identify GST-p53$_{1–44}$ or on 5% SDS PAGE for the autophosphorylation status of ATM.
7. Stain the gels with Coomassie blue, dry, and visualize incorporated ^{32}P by autoradiography (*see* **Note 4**).
8. Separate the reaction products by SDS-PAGE using a biphasic gel system similar to that described by Ziv et al. ***(25)***.
9. Use the 5% section of the separating gel for immunoblot analysis to estimate the amount of ATM present in the reaction. This will then allow for a comparison of ATM kinase activity in the different samples, in essence providing a specific activity. The amount of ATM kinase in the reaction can also be estimated from the relative intensity of Ig bands of ATM antibody used in the immunoprecipitation reaction. Both methods provide ATM kinase quantitation necessary for sample-to-sample comparisons.
10. Stain the 10–12% part of the gel with colloidal Coomassie G-250 to evaluate the p53 substrate in the kinase reaction, and subsequently dry onto a filter paper.
11. Expose dried gels to an x-ray film (Fuji Photo Film Co., Ltd.) with an intensifying screen at –80°C and estimate ATM kinase activity by measurement of the amount of ^{32}P incorporated into p53 substrate via densitometry scanning of the autoradiogram.
12. Perform direct measurement of ^{32}P incorporation into p53 substrate by excising the stained p53 band followed by liquid scintillation counting.

3.3. Functional Assay for Missense Mutations in ATM

There is accumulating evidence that some missense mutations in the ATM gene have the potential to interfere with the normal allele in A-T heterozygotes ***(26,27)***. Several authors have reported the presence of a number of rare missense variants in addition to common polymorphisms in breast cancer patients ***(28–31)***. What is uncertain is whether any of these rare variants affect the function of ATM and thus might be implicated in breast cancer. A functional assay for these ATM variants is described below.

3.3.1. Strategy

Missense changes are introduced into full-length ATM cDNA by a one-step site-directed mutagenesis protocol. These mutated cDNA's are transfected into both A-T and control cells to produce stable cell lines. The presence of a genuine missense change affecting ATM function would result in a failure to correct the cellular phenotype in an A-T cell line and might interfere with normal ATM function in control cells. A change that did not affect function would have the opposite effect in A-T and no effect in control cells.

3.3.2. Site-Directed Mutagenesis

The use of in vitro site-directed mutagenesis has become a powerful tool in the study of biological processes. The protocol described here for the introduc-

tion of mutations into ATM cDNA is a modification of the QuikChange™ Site-Directed Mutagenesis (QCM) kit developed by Stratagene (La Jolla, CA) ***(32,33)***. This method allows for the rapid introduction of mutations into sequences of interest using a pair of complementary mutagenesis primers to amplify the entire plasmid in a single PCR. Destruction of the parental template plasmid by *Dpn*I digestion, followed by transformation into *E. coli* cells allows introduction of the desired mutation with high efficiency (>80%) in as short as 24 h.

1. Set up a PCR mixture containing 75 ng of template plasmid DNA, 125 ng of each oligonucleotide, 300 μ*M* dNTPs, 3.75 U *PfuTurbo*™ DNA polymerase (Stratagene, La Jolla, CA), and the buffer supplied with the polymerase in a total volume of 50 μL.
2. Perform PCR under the following conditions: denaturation at 95°C for 2 min, followed by 16 cycles of denaturation at 95°C for 40 s, annealing at 58°C for 50 s, and extension at 68°C for 1.5 min/kb.
3. Then examine an aliquot of the PCR mixture using agarose gel electrophoresis to determine whether the correct-sized product is amplified.
4. Once the correct-sized PCR product is obtained, incubate the reaction mix (50 μL) with 30 U of *Dpn*I for 3 h at 37°C. This step is critical because it preferentially digests the methylated, non-mutated parental DNA strand and thereby increases the frequency of clones with the desired mutation ***(34)***.
5. Then ethanol precipitate the digested PCR product and introduce half of the resuspended pellet into 50 μL of competent Top 10® *E. coli* cells using standard transformation conditions (Invitrogen, Carlsbad, CA). To confirm the presence of the mutation and the integrity of the ATM cDNA, carry out DNA sequencing of these constructs.

3.3.3. Introduction of Specific Missense Changes into ATM cDNA

1. To test the method, use a 14-kb plasmid (pCAT1) and a 20.1-kb (pMAT1) plasmid, which have a 9.7-kb fragment containing the full-length ATM cDNA inserted into pCMV-Tag3a (4.3 kb, Statagene) and pMEP4 (10.3 kb, Invitrogen), respectively ***(21)***.
2. For mutagenesis use the primer set shown in **Table 3**. The four mutated sites correspond to sites predicted to alter the function of ATM. Three amino acids are altered in P1, which represents a C-terminal SKL sequence motif shown to be important in localizing ATM to peroxisomes ***(35)***. The other three sites (P2–P4) represent potential autophosphorylation sites in ATM. This approach of course can be employed to make alterations anywhere in the ATM cDNA.
3. To generate large PCR products (up to 20 kb) mutated at high efficiency, it is necessary to introduce several critical modifications to the QCM kit ***(33)***.
4. Although not critical for the efficiency of mutagenesis, the mutagenic primers are further purified by desalting. This option is inexpensive compared to the recommended purification by either HPLC or PAGE.
5. Increase the concentration of both the template DNA (75 ng) and dNTPs (300 μ*M*) to compensate for the larger size of the plasmids used.

Table 3
Oligonucleotide Primers Used in the Mutagenesis Experiments

Primer Pairs	Primers	Primer Sequence [a] (5'-3')
P1	ATMm30	CCAGGATGGAAA**t**CT**aa**G**c**TGTGATCTTCAGTATATG
	ATMm31	CATATACTGAAGATCACA**g**C**tt**AG**a**TTTCCATCCTGG
P2	ATMmP1F	GACATTATGAGAGCT**g**CTCAGGATAATCCG
	ATMmP1R	CGGATTATCCTGAG**c**AGCTCTCATAATGTC
P3	ATMmP2F	GGTGGTTCCCCTC**g**CTCAGCGAAGTGGTG
	ATMmP2R	CACCACTTCGCTGAG**c**GAGGGGAACCACC
P4	ATMmP3F	GAAGTGATGAGAAAC**g**CTCAGGAAACTCTG
	ATMmP3R	CAGAGTTTCCTGAG**c**GTTTCTCATCACTTC

[a] Bases that are lower case are designed point mutations in the ATM gene.

6. Increase the amount of *PfuTurbo* DNA polymerase from 1.5 U to 3.75 U and reduce the extension time from 2 min/kb to 1.5 min/kb owing to the size of the constructs and consequently the long extension times for PCR. Stratagene recommends an extension time of 2 min/kb for plasmids larger than 10 kb, but 1.5 min/kb is sufficient time to generate PCR products of 20 kb.
7. Use excess *Dpn* I (30 U) for a longer incubation time (3 h) than is suggested by the QCM kit (1 h) to ensure complete digestion of the parental DNA. This step is critical for providing higher efficiency of mutagenesis (85–100%) of large plasmids (20 kb).

3.3.4. Characterization of "Mutant" ATM cDNAs

1. Following the procedure described above, sequence plasmid DNA prepared from individual bacterial colonies and determine whether the 14-kb plasmid template efficiently generates PCR product with primer sets P1 and P4; a high percentage of the screened clones should contain the desired mutation (**Table 4**).
2. The 20.1-kb plasmid template should generate PCR products with all primer sets, and again a high percentage of the clones screened should contain the desired mutation (**Table 4**). Although the total number of colonies observed after transformation will be low, which is possibly due to the low yield of PCR products, the efficiency of mutagenesis should still be very high.
3. One drawback of mutagenesis on this scale is that it is necessary not only to confirm the presence of the introduced mutation(s) but also to sequence the full-length ATM cDNA to ensure that no other mutations were generated during PCR amplification. Sequencing one mutant clone of each of the four 20-kb constructs should be sufficient to reveal that no other mutations occur in the cDNA of ATM as a result of the PCR process, indicating that the proofreading ability of *PfuTurbo* DNA polymerase is sufficient for plasmids of this size.

Table 4
Generation of Mutant ATM cDNA Clones by In Vitro Mutagenesis

Primer Pairs	Template Size (kb)	Colonies (total)	Colonies screened	Mutagenesis efficiency
P1	14	174	6	100%
P4	14	236	6	100%
P1	20.1	37	6	83%
P2	20.1	45	6	83%
P3	20.1	24	6	100%
P4	20.1	31	6	100%

4. However, it is possible that sequence analysis may reveal the incorporation of single incorrect nucleotides in some of the mutants. Based on this, it is advisable to sequence at least one mutant clone to ensure intact cDNA and maintenance of the desired mutation.

4. Notes

1. As a source of mRNA control, lymphoblastoid cells are used. This is reverse transcribed into cDNA prior to amplification.
2. Amplify the 5' end of ATM cDNA using a forward primer 5'-AGCTCGAGAT CACTTAATGATCTGCTTATC-3' incorporating an Xhol site and extending from immediately downstream of the ATG initiation codon. The reverse primer (5'- CAACATGGTCAGGAAAAGGATC-3') corresponds to a sequence 4686 bp downstream of the ATG initiation codon of ATM.
3. Use the Expand High Fidelity PCR system to minimize misincorporations with PCR conditions (97°C for 3 min, 56°C for 2 min, and 70°C for 4 min [cycle 1] followed by 95°C for 30 s, 56°C for 1 min, and 70°C for 4 min up to cycle 32).
4. Other immunoprecipitation buffers can be used to measure ATM kinase activity. It seems, however, that universal IP (0.5% NP 40) and Triton X-100 up to 1% (e.g., ATR IP buffer) containing buffers severely diminish ATM kinase activity. This is most likely a result of the amount of detergent present.
5. The magnitude of the ATM kinase induction by radiation is in the two- to four-fold range. This appears to be influenced by the growth phase of the cells, with cells in stationary phase showing elevated basal activity. One should normally determine ATM kinase activities in mid-log-phase cells. ATM kinase activity can be readily assayed in a variety of cell types, including mouse tissue extracts *(27)*.
6. Storage of cell extracts is not recommended, as it results in significant decrease in ATM kinase activity. Incubation of immunoprecipitated ATM at room temperature or prolonged (overnight) storage of immunoprecipitates also diminishes ATM kinase activity.

Acknowledgments

The authors thank Dr. Yosef Shiloh for the λ Zap clone containing the partial 5.9 kb ATM cDNA 3' fragment. We acknowledge Dr. Ning Zhang, who produced the initial ATM cDNA construct (pMAT1). This work was supported by grants from the Australian NHMRC; the Australian Cancer Fund; the National Institute of Health, Washington; and the A-T Children's Project.

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12

Use of siRNA to Study the Function of MDC1 in DNA Damage Responses

Zhenkun Lou and Junjie Chen

Summary

Small interfering RNA (siRNA) technology has emerged as a powerful genetic tool to investigate gene function in mammalian cells. Here we use siRNA to study a mediator of DNA damage-checkpoint protein 1 (MDC1), previously known as Kiaa0170 or NFBD1, in DNA damage responses. We show that MDC1 siRNA specifically and efficiently down-regulates MDC1 expression, resulting in defective radiation-induced apoptosis in A549 cells. Transfection with siRNA-resistant MDC1 restores radiation-induced apoptosis. These findings suggest that siRNA can be a very useful tool for the exploration of gene function in mammalian checkpoint responses.

Key Words: siRNA; MDC1; DNA damage; apoptosis.

1. Introduction

To define the function of any given gene, the generation of knockout mice or a deficient cell line are obvious choices. However, both processes are time consuming and technically challenging. Recent breakthroughs in RNA interference have provided an additional tool for functional study in mammalian cells. First identified in plants ***(1)*** and *Caenorhabditis elegans* ***(2)***, double-stranded RNA (dsRNA) can cause gene silencing in many organisms ***(3,4)***. The prevailing model of how RNA silencing works is that the dsRNA is first converted to small RNAs (siRNA) in vivo by Dicer RNase, and the siRNA in turn causes the degradation of target mRNA by directing mRNA to a multicomponent nuclease complex ***(5)***.

Since long dsRNAs trigger antiviral responses in mammalian cells, transfection of cells with siRNA is one way to circumvent this problem. Elbashir et al. and Caplen et al. first successfully employed siRNA technology in mammalian cells ***(6,7)***. They used chemically synthesized 19 nt RNA duplexes with

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

2 nt 3' overhangs (dTdT), mimicking the siRNA generated in vivo by Dicer. The synthesized siRNA was used to transfect mammalian cells, resulting in target gene silencing. The development of siRNA technology in mammalian systems enables researchers to temporarily "knock down" gene expression, which provides a glimpse into gene function. Given the completion of the human genome sequence, this new technology will no doubt greatly accelerate the investigation of uncharacterized genes within the human genome.

One limitation of siRNA silencing is that the silencing effect is temporary. Normal gene expression usually resumes 4–5 d after siRNA transfection. The primary reason seems to be the dilution of siRNA due to cell proliferation. To overcome this limitation, plasmid- or virus vector-based expression of siRNA in mammalian cells has been developed ***(8–15)***, and successfully used to stably suppress gene expression in vivo ***(10,16)***.

Concern for the specificity of siRNA effects remains. Although the targeted sequence is used to search the database to ensure that no other gene contains the same sequence, one has to assume that the database is complete and that siRNA does not have any nonspecific effects. Thus, several approaches have been used by researchers to demonstrate the specificity of any given siRNA effect. Ideally, two or more independent siRNA sequences for the same target gene should be used. Similar biological phenotypes observed in response to these independent siRNAs can reduce the potential misinterpretation of nonspecific effects observed for a single siRNA sequence. Furthermore, the introduction of mismatches into the siRNA sequence should abrogate siRNA effects. Indeed, a single mismatch seems to be sufficient to abolish the siRNA effect ***(10)***, highlighting the specificity of siRNA silencing. Finally, reintroduction of target gene cDNA, which contains silent mutations that render it resistant to siRNA silencing, should reverse siRNA-induced effects. Thus, with proper controls, siRNA can be an extremely useful tool for the exploration of gene function in mammalian cells.

We have successfully used siRNA technology to investigate the function of a mediator of DNA-damage-checkpoint protein 1 (MDC1), previously known as Kiaa0170 or nuclear factor containing BRCT domain (NFBD1), in radiation-induced apoptosis. We showed that knocking down MDC1 expression using MDC1 siRNA resulted in defective radiation-induced apoptosis. Transfection with a second, different MDC1 siRNA had similar effects. But transfection with control siRNA, which contained mismatch mutations within the MDC1 siRNA sequence, abolished the siRNA-induced effects. Finally, expression of siRNA-resistant MDC1 restored radiation-induced apoptosis, confirming that the MDC1 siRNA-mediated effects are highly specific. These results suggest that siRNA technology can be a powerful and precise tool to study gene function in DNA-damage response pathways.

2. Materials

2.1. siRNA Transfection

1. A549 cells (American Type Culture Collection).
2. Six-well tissue-culture plate.
3. Small interfering RNAs (Xeragon), stored at –20°C at a concentration of 20 μ*M*.
4. Oligofectamine (Invitrogen), stored at 4°C, avoid freezing.
5. Opti-MEM reduced serum medium (Invitrogen).
6. RPMI medium.
7. Fetal bovine serum.
8. Trypsin.

2.2. Radiation-Induced Apoptosis

1. Trypsin.
2. PBS solution: 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4.
3. Fixation solution (methanol:acetone = 3:1), made fresh.
4. Hoechst solution (0.2 mg/mL), stored at room temperature in a foil-wrapped test tube.
5. Slides and coverslips.
6. Fluorescence microscope.

2.3. Transfection of siRNA-Resistant MDC1

1. pCDNA3 vector encoding siRNA-resistant MDC1 (MDC1*). siRNA-resistant constructs were generated by introducing a silent mutation at MDC1 coding region 70–75, from TTGAGG to CTTAGA.
2. GFP-expressiong vector: pIRES-EGFP (Clontech).
3. FuGENE™ 6 (Roche), stored at –20°C.
4. Flow cytometer.

3. Methods

3.1. Design of MDC1 siRNAs

The success of siRNA technology largely depends on the design of siRNA. However, currently there is no universal guideline as to the optimal siRNA design. As suggested by Tuschl and colleagues (http://www.mpibpc.gwdg.de/abteilungen/100/105/sirna.html), we observe the following guidelines in our siRNA design: (1) The target sequence is 50–200 bp downstream of the translation initiation site (AUG); (2) the ideal target sequence is AA(N19)TT, and the sense strand of siRNA is (N19)dTdT; (3) the GC content of the target sequence is about 50%; and (4) the target sequence should not be homologous to other genes in the database (*see* **Note 1**).

Because siRNA is a relatively new technology, for any given gene, several independent siRNAs are needed to ensure that the phenotypes observed are not owing to nonspecific effects by any given siRNA. For MDC1, we designed

four siRNAs within the region between 50 and 300 bp downstream of the starting codon. Two of them work very well (MDC1 siRNA1 and MDC1 siRNA2). We can achieve more than 90% down-regulation of endogenous MDC1 level as judged by immunofluorescence staining and Western blot analyses. Two other siRNAs only work to some extent, and lead to the decrease of MDC1 expression by approx 50%. The sense sequence for MDC1 siRNA1 is UCCAG UGAAUCCUUGAGGU*dTdT*; the sense sequence for MDC1 siRNA2 is ACAACAUGCAGAGAUUGAA*dTdT*. Furthermore, a control with four mismatches in the MDC1 siRNA1 sequence (U*U*CA*A*UAAAU*U*CUUGAGGU *dTdT*) is included in this study. The use of this mismatch siRNA ensures that phenotypes caused by MDC1 siRNA transfection are sequence specific. The siRNAs are chemically synthesized by Xeragon Inc., now part of QIAGEN (*see* **Note 2**).

3.2. siRNA Transfection

3.2.1. Choice of Cell Lines

HeLa cells are by far the best choice to study siRNA-induced silencing effects. More than 95% of the transfected cells show complete loss of MDC1 expression when transfected with siRNA, as judged by immunostaining. We also failed to observe any immuno-reactive band by anti-MDC1 western blotting in extracts prepared from MDC1 siRNA-transfected cells. However, for certain assays (e.g., apoptosis assay), HeLa cells may not be the best choice, given that p53 function is compromised in these cells. We also observed efficient siRNA effects in a number of other cell lines, including A549, BJ, and U2OS cells.

3.2.2. Cell Plating

We use six-well tissue culture plates to perform siRNA transfection. Cells are plated into six-well plates a day before transfection in complete medium (RPMI medium containing 10% FBS). For A549 cells, approx 1.5×10^5 cells are plated per well, and on the day of transfection, the cell density should be approx 30% (*see* **Note 3**).

3.2.3. siRNA Transfection

The conditions given below are for the transfection of one well in a six-well tissue-culture plate.

1. In a sterile microcentrifuge tube, mix 10 µL of 20 µ*M* siRNA with 160 µL OPTI-MEM medium without serum. The final concentration of siRNA (at **step 3** below) will be 200 n*M*.
2. In a separate microcentrifuge tube, mix 5 µL of oligofectamine with 25 µL serum-free OPTI-MEM medium.

3. After 10 min incubation at room temperature, combine the two solutions (a total of 200 µL), and incubate the mixture at room temperature for an additional 20 min.
4. Two min before the end of the 20-min incubation period, wash cell monolayer with 1 mL serum-free RPMI medium once, then add 800 µL serum-free RPMI per well.
5. Add the mixture from **step 3** to each well dropwise while gently rocking the plate (total volume will be 1 mL). Incubate the cells in CO_2 incubator for 4 h.
6. Add 500 µL of RPMI medium containing 30% fetal bovine serum to make a final serum concentration of 10%. Return cells to CO_2 incubator and culture for 20 h.
7. Perform a second siRNA transfection with the same siRNA by repeating **steps 1–6** (*see* **Note 4**).
8. 48 h after the start of the initial transfection, trypsinize the cells and transfer each culture to a 60-mm plate (in case of subsequent Western blot) or a six-well culture plate (in case of subsequent apoptosis assay). Culture the cells for an additional 24 h before performing any biological assays (*see* **Note 5**).
9. Perform immunostaining or Western blot analyses to verify the effect of MDC1 siRNA on MDC1 expression.

   As shown in **Fig. 1**, transfection of MDC1 siRNA effectively down-regulates MDC1 without affecting other proteins that we have examined (e.g., BRCA1, CHK2, or actin). However, transfection of a control siRNA that contains four mismatches in the MDC1 siRNA sequence does not affect MDC1 expression, suggesting the specificity of MDC1 siRNA (*see* **Fig. 1**). In addition, immunostaining with anti-MDC1 antibodies revealed that more than 90% of cells have marked reduction in MDC1 expression.

3.3. Radiation-Induced Apoptosis

1. 60 h after the initial transfection, irradiate the cells with 5 Gy of ionizing radiation (IR).
2. Return the cells to CO_2 incubator and culture for an additional 36 h.
3. Harvest the floating cells and trypsinize the remaining adherent cells. Combine both fractions.
4. Wash harvested cells once with 1X PBS.
5. Vortex cell pellets and at the same time add 1 mL fixing solution dropwise.
6. Add 30 µL of cell suspension to a glass microscope slide and leave to dry at room temperature for 10 min.
7. Add 20 µL of Hoechst staining solution and evaluate apoptotic cells under fluorescence microscope (*see* **Note 6**).

   As shown in **Fig. 2,** MDC1 siRNA, but not control siRNA with mismatch mutations, is able to abolish radiation-induced apoptosis. A second MDC1 siRNA shows similar results.

3.4. Transfection of siRNA-Resistant MDC1

The procedure in this section describes the expression of siRNA-resistant MDC1 (MDC1*) during siRNA transfection. This is an additional control that ensures that any biological effects observed in siRNA-transfected cells are a

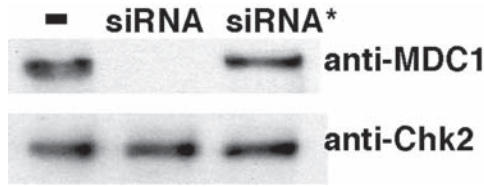

Fig. 1. MDC1 siRNA-inhibited MDC1 expression. A549 cells were mock-transfected or transfected with MDC1 siRNA or control siRNA (siRNA*) twice. Seventy-two hours after the initial transfection, cells were collected and immunoblotting experiments were performed with anti-MDC1 or -Chk2 antibodies ***(17)***.

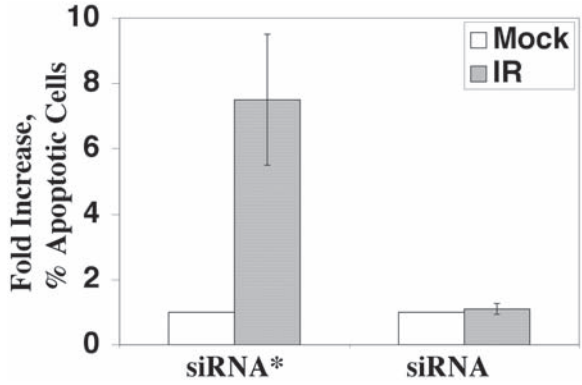

Fig. 2. A549 cells were transfected with MDC1 siRNA or control siRNA as described in **Subheading 3.2.3.**, then were left untreated or irradiated (5 Gy, IR). Thirty-six hours later, the cells were harvested and evaluated for apoptosis ***(17)***.

result of the down-regulation of the target gene. The conditions given here are for one well in a six-well tissue-culture plate.

1. Add 100 µL serum-free RPMI medium to a sterile microcentrifuge tube.
2. Add 5 µg of pcDNA3-MDC1* and 1 µg pIRES-EGFP (*see* **Note 7**).
3. Add 18 µL FuGENE 6 reagent and mix well by flicking the tube.
4. Incubate the mixture at room temperature for 20 min.
5. Add the above mixture to each well right after the addition of 30% serum during second siRNA transfection (*see* **Subheading 3.2.3., step 7**).
6. Return cells to CO_2 incubator and culture for another 36 h.
7. Trypsinize cells and sort GFP-positive cells by flow cytometry (*see* **Note 7**).
8. Irradiate the GFP-positive cells with 5 Gy IR and incubate the cells for 36 h. In parallel, mock-treat the GFP-positive cells as a control.
9. Collect the cells and evaluate apoptotic cells as described in **Subheading 3.3.**

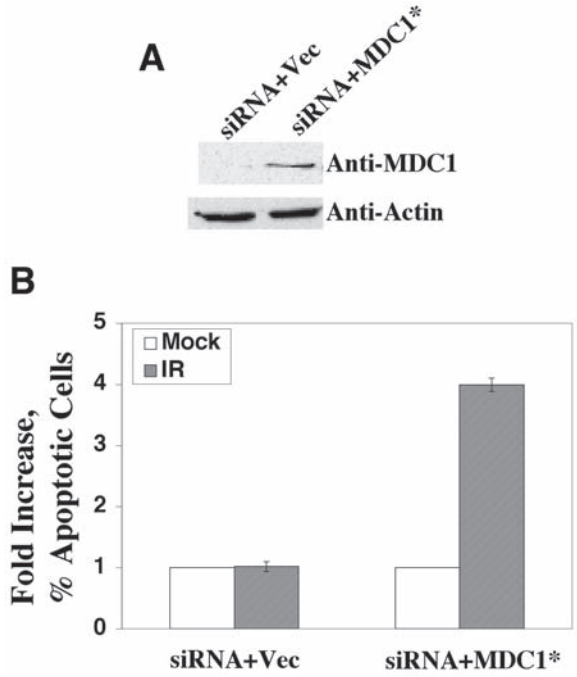

Fig. 3. A549 cells were transfected twice with MDC1 siRNA, and right after the second transfection, cells were transfected with pIRES-GFP together with either control pCDNA3 vector or pCDNA3 encoding siRNA-resistant wild-type MDC1 (MDC1*). Thirty-six hours later, cells were harvested and examined for MDC1 expression by Western blot **(A)**, or sorted for GFP-positive cells **(B)**. The GFP-positive cells were then either irradiated (5 Gy, IR) or mock-treated. Cells were evaluated for apoptosis 36 h later ***(17)***.

As shown in **Fig. 3,** cells transfected with siRNA-resistant MDC1(MDC1*) exhibited MDC1 expression after MDC1 siRNA transfection **(Fig. 3A),** while endogenous MDC1 expression was greatly inhibited in cells transfected with empty vector **(Fig. 3A)**. Furthermore, the expression of MDC1* restored radiation-induced apoptosis **(Fig. 3B)**.

4. Notes

1. Some people have used RNA secondary structure prediction programs such as Genebee (www.genebee.msu.su/services/rna2_reduced.html) to select the optimal targeting sequence—ideally, a sequence within a big loop (e.g., a sequence that does not base-pair with other sequences). In our experience, the success rate with siRNA selected by this method is similar to that with siRNA chosen according to the guidelines described in **Subheading 3.1.**

2. Besides chemically synthesized siRNA, there are products on the market that allow the generation of siRNA by in vitro transcription. Our preliminary experience is that the yield of siRNA generated by this method is low, and the siRNA appears to be more toxic than chemically synthesized siRNA (we observed more floating cells after tranfection).
3. Having evenly distributed cells and the right cell density (30%) is crucial for the success of siRNA transfection. Owing to the different plating efficiencies of various cell lines, investigators should determine the optimal number of cells plated on a six-well plate for any given cell line.
4. In some situations (e.g., when cell density is high or cells are unevenly plated), the transfection efficiency of siRNA is low. We observed that if we leave cells in 1 mL serum-free medium overnight (16 h) after the second siRNA transfection, we could greatly improve the transfection efficiency. 500 µL 30% serum is added the second day and cells are incubated for 8 h before being trypsinized for further functional assays. However, relatively more floating cells are observed using this protocol.
5. In our experience, siRNA works well 72–96 h after initial siRNA transfection. However, MDC1 is also effectively knocked down 48 h after one transfection. We choose two transfections to ensure MDC1 knock-down. For other proteins, investigators should determine the number of transfections and the timing of biological assays empirically.
6. We rated cells with fragmented nuclei as apoptotic cells. A total of 500 cells were rated in about 10 randomly selected fields across the slide. The identity of the sample is masked to ensure the objectivity of the rating. The results are expressed as fold of increase in the percentage of apoptotic cells when comparing irradiated cells to mock-irradiated control cells.
7. We only achieved low transfection efficiency of plasmid using FuGENE 6 reagent in siRNA treated cells. Normally, only 10% GFP-positive cells can be recovered after cell sorting. We have recently expressed siRNA-resistant MDC1 transiently using a Lenti-virus-based expression system. This appears to be a better alternative to the plasmid transfection and sorting protocol used in this study. Another way to improve expression is to establish stable cell lines expressing siRNA-resistant MDC1 using lentivirus or another expression system.

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13

Functional Analysis of APC-Cdh1

Tamotsu Sudo, Naoto T. Ueno, and Hideyuki Saya

Summary

To maintain genomic integrity against various kinds of genotoxic stress, cells have multiple checkpoints in the cell cycle. When one of the cell cycle events, such as DNA synthesis, DNA repair, and chromosomal segregation, has not been successfully completed, checkpoints will delay progression until the step is correctly accomplished, and only then will they relieve the arrest to allow the cell to move to the next phase. Cells lacking functional checkpoints display genomic aberrations, resulting in the acquisition of phenotypic changes of cancer cells.

Anaphase-promoting complex (APC) is activated by two regulatory proteins: Cdc20 and Cdh1. In yeast and *Drosophila*, Cdh1-dependent APC (APC-Cdh1) activity targets mitotic cyclins from the end of mitosis to the G1 phase. Loss of Cdh1 induces unscheduled accumulation of mitotic cyclins in G1, resulting in abrogation of G1 arrest caused by treatment with rapamycin, an inducer of $p27^{Kip1}$. Furthermore, Cdh1-deficient DT40 cells fail to maintain DNA damage-induced G2 arrest, and Cdh1-APC is activated by X-irradiation-induced DNA damage. Thus, activation of Cdh1-APC plays a crucial role in both cdk inhibitor-dependent G1 arrest and DNA damage-induced G2 arrest.

In light of the differences between normal and cancer cells, checkpoints can be ideal targets for developmental cancer therapeutics. In this chapter, we describe how to analyze three checkpoints (spindle assembly checkpoint, rapamycin-induced G1 checkpoint, and DNA damage-induced G2 checkpoint) in conjunction with cell synchronization.

Key Words: Cell cycle analysis; Cdh1; DT40; checkpoint; spindle assembly checkpoint; rapamycin; G2 checkpoint.

1. Introduction

To ensure the fidelity of genetic information and to precisely divide into two daughter cells, cells have checkpoint mechanisms that monitor the successful completion of various cell cycle events. Checkpoints make certain that events are completed correctly and in the proper order ***(1)***. When one cell cycle event has not been successfully completed, checkpoints will delay progression until the step is correctly accomplished, and only then will they relieve the arrest to

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

allow the cell to move to the next phase. Recent reports have clarified that checkpoints play a crucial role in maintenance of genome stability. Cells lacking functional checkpoints display genomic aberrations such as hyperploidy, aneuploidy, chromosome translocation, and fusion, which are hallmarks of cancer cells. Assessment of checkpoint functions in cancer cells is required for the development of novel cancer therapeutics.

Anaphase-promoting complex (APC) is activated by two regulatory proteins, Cdc20 and Cdh1 (Cdc20 homolog 1) ***(2)***. In yeast and *Drosophila*, Cdh1-dependent APC (Cdh1-APC) activity targets mitotic cyclins from the end of mitosis to the G1 phase ***(3,4)***. To investigate the function of Cdh1 in cell cycle checkpoints in vertebrate cells, we generated clones of chicken DT40 cells disrupted in their *Cdh1* loci. Loss of *Cdh1* induced retention and unscheduled accumulation of mitotic cyclins in late mitosis and G1 phase, resulting in prolonged mitotic duration and abrogation of G1 arrest caused by treatment with rapamycin, an inducer of $p27^{kip1}$. We also found that $Cdh1^{-/-}$ cells fail to maintain DNA damage-induced G2 arrest. Thus, activation of Cdh1-APC plays a crucial role in both Cdk inhibitor-dependent G1 arrest and DNA damage-induced G2 arrest ***(5)***.

2. Materials

1. Nocodazole (methyl-[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]-carbamate): soluble in DMSO. Stock solutions (1 mg/mL) are stable for up to 1 wk at 4°C or for 1 yr at –20°C.
2. Rapamycin: soluble in DMSO or methanol. Stock solutions (1 m*M*) are stable for 3 mo at –20°C.
3. Aphidicolin: soluble in DMSO, methanol, or ethanol. Stock solutions (1 mg/mL) are stable for 2 mo at –20°C.
4. Thymidine: soluble in H_2O. Stock solutions (1 *M*) are stable for 1 yr at –20°C.
5. 70% methanol: store at –20°C.
6. RNase A: store at –20°C.
7. Propidium iodine: store at 4°C.
8. Bromodeoxyuridine (BrdU): soluble in DMSO or 100 m*M* NaOH. Stock solutions (1 m*M*) are stable for 3 mo at –20°C.
9. Orcein: used in the form of a 1% solution in 45% acetic acid. Stock solutions are stable for 1 yr at room temperature.
10. PBS (phosphate-buffered saline): 137 m*M* NaCl, 2.7 m*M* KCl, 10.6 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4.
11. Blocking buffer: 1% bovine serum albumin in PBS.
12. Anti-BrdU antibody.
13. Anti-α tubulin antibody: store at 4°C.
14. Anti-MPM-2 antibody: stable for 2 yr at –20°C.
15. FITC-conjugated anti-mouse IgG: store at 4°C.

16. Lysis buffer: 20 m*M* Tris-HCl (pH 7.7), 100 m*M* KCl, 50 m*M* sucrose, 0.1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.5% Triton X-100, 20 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4, and a cocktail of protease inhibitors.
17. 4X SDS buffer: 8% SDS, 20% glycerol, 0.4 *M* Tris-HCl (pH 6.8), 0.05% bromophenol blue.
18. PBST: 0.1% Tween-20 in PBS.
19. Blocking buffer for Western blotting: PBST containing 5% nonfat powdered milk.
20. Anti-Rb antibody: store at 4°C.
21. Glycerol for mounting: 80% glycerol in PBS.

3. Methods

3.1. Spindle Assembly Checkpoint Induced by Spindle Inhibitors (Nocodazole)

The spindle assembly checkpoint ensures the fidelity of chromosome segregation in mitosis ***(6)***. The premature separation of sister chromatids leads to the loss or gain of chromosomes in daughter cells (aneuploidy), one of the hallmarks of human cancer. Nocodazole has the ability to depolymerize microtubules and destroy the mitotic spindle, leaving all kinetochores unoccupied. Therefore, it can be used to mimic the ectopic activation of the spindle assembly checkpoint (SAC). **Figure 1** shows the mitotic indices of various breast and ovarian cancer lines. MDA-MB231 and MCF-7 cells had a normal SAC response, resulting in an accumulation of cells with condensed chromosomes, characteristic of a sustained mitotic block. In contrast, SAC of Ovca432, Ovca433, and T47D cells was impaired, and there was no clear peak in mitotic index observed at any time point.

1. Seed the cells in appropriate petri dishes (we usually use 35-mm dishes) so that they are 30–50% confluent the day of treatment.
2. Twenty-four hours after seeding, change to fresh media containing 0.5 μg/mL nocodazole.
3. In our experiments, cells were harvested at 8 h intervals, until 48 h is reached. The time course is dependent upon the doubling time of each individual cell line. At the time of harvest, discard the medium without washing cells (*see* **Note 1**) and put a small drop of aceto-orcein stain on the dishes. Leave the stain on for 10 min at room temperature and place a coverslip on each dish.
4. Count the cells with mitotic condensed chromatin (*see* **Note 2**). Chromatin is visualized by staining cells with aceto-orcein.

3.2. Exit From Mitosis

Mitotic exit requires the down-regulation of the activity of cyclin B–CDK1 kinase. This down-regulation is owing to cyclin degradation by the anaphase-

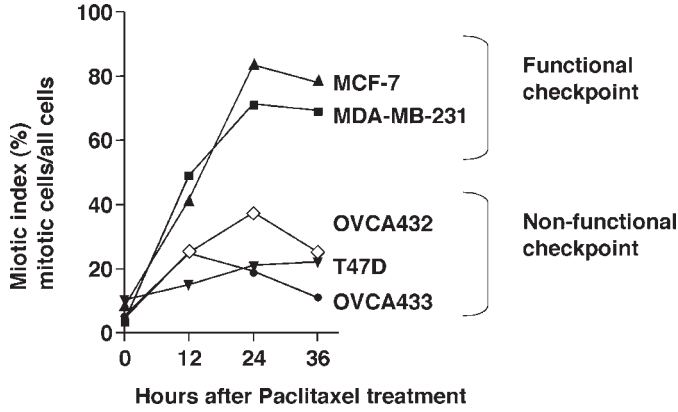

Fig. 1. Mitotic indices in cells having a functional or nonfunctional spindle assembly checkpoint. Breast cancer cell lines (MDA-MB231, MCF-7, and T47D) and ovarian cancer cell lines (Ovca432 and Ovca433) were incubated with 0.2 µg/mL nocodazole and harvested at each time point. Chromatin is visualized by staining the cells with aceto-orcein, with which mitotic condensed chromatin can be detected. Reproduced from **ref. *5***, with permission.

promoting complex (APC) activated by Cdc20 or Cdh1. Two major forms of the APC function during mitotic exit. Cdc20-activated APC recognizes proteins such as cyclin B and securin, initiating their ubiquitination during the metaphase/anaphase transition. The destruction of cyclin B is followed by the inactivation of CDK1 and the dephosphorylation of CDK1 targets, including the APC activator Cdh1. Cdh1-activated APC then maintains the ubiquitination-dependent destruction of several proteins during the completion of mitotic exit and G1. Anti-MPM-2 monoclonal antibody can recognize a phosphorylated epitope (serine/threonine) found in phosphoproteins that are phosphorylated at mitosis ***(9)***. The proportion of cells in the mitotic phase is also determined by FACS analysis using anti-MPM-2 antibody. In our experiments, we examined whether loss of Cdh1 affects the duration of the mitotic phase. Wild-type or *Cdh1*$^{-/-}$ cells were synchronized at metaphase by treatment with nocodazole for 10 h. At 45 min after release from metaphase block, approx 40–45% of *Cdh1*$^{-/-}$ cells remained in a mitotic state with positive MPM-2 staining, compared with only 20% MPM-2 positive in wild-type cells **(Fig. 2)**. These observations suggest that Cdh1-deficient cells delay exit from mitosis.

1. After incubation with nocodazole (*see* **Note 3**), wash cells at least three times with normal medium (*see* **Note 1**) and release with normal medium.
2. Harvest and rinse cells with PBS. Fix them in 70% methanol at –20°C for several hours (*see* **Note 4**).

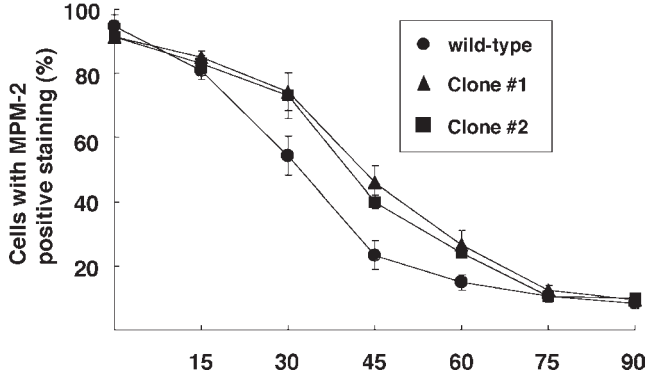

Fig. 2. Exit from mitosis with delay in *Cdh1*-deficient cells. DT40 cells were washed and released with normal medium after incubation with 0.5 μg/mL nocodazole for 10 h. Cells were immunostained with MPM-2 antibody and analyzed by FACS. The graph displays the percentage of positively stained cells at the time indicated in wild-type cells, $Cdh1^{-/-}$ clone 1, and $Cdh1^{-/-}$ clone 2 . The rate of positive stained cells was 100% at the time before release. Error bars show the standard error of the mean for three independent experiments. Reproduced from **ref. *5***, with permission.

3. After fixing, wash off methanol and incubate cells in blocking buffer (PBS + 1% BSA) at room temperature for 10 min to block nonspecific protein binding.
4. Wash cells three times in PBS and stain with MPM-2 antibody diluted with PBS for 1 h at room temperature.
5. After three washes with PBS, incubate cells with FITC-conjugated anti-mouse IgG diluted with PBS for 30 min at room temperature
6. After washing cells three times in PBS, resuspend them in 1mL of PBS.
7. Analyze with FACScan using Cell Quest software (Becton Dickinson).

3.3. G1 Arrest Induced by Rapamycin Treatment

3.3.1. Measuring G1 Arrest by FACS Analysis

During embryogenesis in *Drosophila, fzr*-deficient cells can overcome the $p27^{DAP}$-induced G1 arrest through unscheduled accumulation of mitotic cyclins, resulting in progression through an extra division cycle ***(3)***. $p27^{kip1}$, a vertebrate homolog of $p27^{DAP}$, inhibits the activity of cyclin E–Cdk2, leading to a block of the G1-S transition in vertebrate cells ***(7)***. Accordingly, we tested the possibility that Cdh1-deficient cells abrogate G1 arrest induced by the expression of $p27^{kip1}$. Rapamycin, which is known to activate $p27^{kip1}$ expression ***(8)***, can be used to induce G1 arrest. Bromodeoxyuridine (BrdU) provides us useful information about cell cycle distribution. Because BrdU is incorporated in place of thymidine only during S phase, each phase (G1, S, and G2/M)

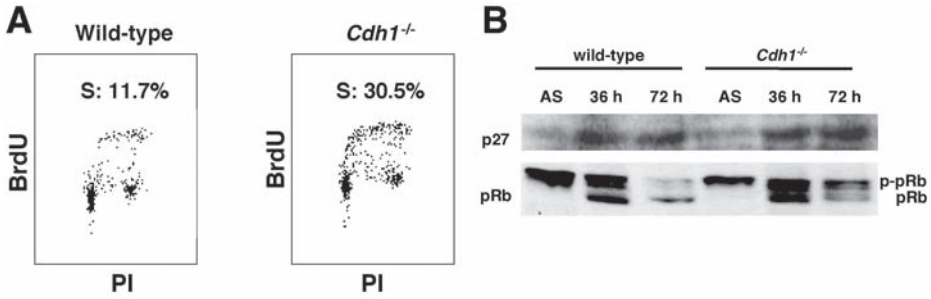

Fig. 3. **(A)** Abrogation of rapamycin-induced G1 arrest in *Cdh1*$^{-/-}$ cells. Wild-type and *Cdh1*$^{-/-}$ cells were treated with 100 n*M* rapamycin for 72 h. Cells were harvested and analyzed by FACS. Cells were pulse-labeled for 10 min before harvest, and subsequently stained with FITC-anti-BrdU to detect BrdU incorporation, and with propidium iodide to detect total DNA. **(B)** Expression of pRb in wild-type and *Cdh1*$^{-/-}$ DT40 cells treated with 100 n*M* rapamycin for 36 and 72 h. AS indicates extracts from asynchronously proliferating cells. Reproduced from **ref. *5***, with permission.

can be distinguished clearly. In our experiments, FACS analysis revealed that rapamycin treatment for 72 h induced G1 arrest in wild-type cells. In contrast, *Cdh1*$^{-/-}$ DT40 cells showed a significant decrease in the G1 fraction at 72 h of rapamycin treatment, and a corresponding increase in the number of cells having S-phase DNA content **(Fig. 3A)**.

1. Seed the cells so that they are 20% confluent on the day of treatment (it depends on the doubling time of each cell) in appropriate dishes (we usually use 10-cm dishes) or flasks, and incubate them with100 n*M* rapamycin (*see* **Notes 5** and **6**).
2. Add BrdU directly to the culture medium to achieve a final concentration of 10 µ*M* (*see* **Note 7**) and incubate the cells for 10 min in the CO_2 incubator at 37°C.
3. Harvest and fix the cells in 70% methanol at –20°C for several hours.
4. Centrifuge cells and aspirate the supernatant carefully.
5. Incubate in the following ways: (1) in 2 *N* HCl, 0.5% Triton X-100 for 30 min at room temperature; (2) in 0.1 *M* $Na_2B_4O_7{\cdot}10\ H_2O$ (pH 8.5) for 1 min; (3) with FITC-conjugated anti-BrdU antibody for 1 h at room temperature; (4) in 5 µg/mL propidium iodide (PI) in PBS (*see* **Note 8**). Between incubations, cells should be washed with PBS containing 2% FCS and 0.1% sodium azide.
6. Analyze the cells on FACScan using Cell Quest software to display fluorescence data as dot plots.

3.3.2. Measuring G1 Arrest by Analyzing Rb Phosphorylation

From the viewpoint of biochemical status, the expression level of phosphorylated pRb also provides useful information about G1 and S phase. pRb is well known to become phosphorylated during entry into S phase. In our experiments,

while pRb was not phosphorylated in wild-type cells treated with rapamycin, *Cdh1*$^{-/-}$ cells predominantly expressed the phosphorylated form **(Fig. 3B)**. In addition, *Cdh1*$^{-/-}$ cells expressing a human *Cdh1* transgene arrested at G1 in the presence of rapamycin, similar to wild-type cells **(Fig. 3B)**.

1. Seed the cells so that they are 20% confluent the day of treatment (it depends on the doubling time of each cell) in appropriate dishes (we usually use 10-cm dishes) or flasks and incubate them with100 n*M* rapamycin (*see* **Notes 5** and **6**).
2. At the time of harvest, discard medium and rinse the monolayer twice with 10 mL ice-cold PBS. Drain the PBS completely from the culture plate and add 500 µL of lysis buffer. With the use of a cell scraper, dislodge the cells from the plate.
3. Transfer the sample into 1.5-mL microcentrifuge tube in ice for 15 min.
4. Centrifuge the extracts at high speed at 4°C for 15 min.
5. Transfer the supernatants to a new microcentrifuge tube on ice. If the lysate is not to be used immediately, store at –70°C until needed.
6. Determine the protein concentration of each sample. Use your favorite protein determination assay kit and follow the manufacturer's instructions.
7. Mix each supernatant containing 20 µg of total proteins with 4X SDS buffer and denature at 95°C for 5 min.
8. After loading the samples onto a 6% polyacrylamide gel (the molecular weight of pRb is approx 105 kDa), run the gel.
9. Transfer the gel to a nitrocellulose membrane.
10. Incubate the membrane with PBST containing 5% milk for 1 h at room temperature or overnight at 4°C.
11. Incubate the nitrocellulose membrane for 1 h on a rocking platform with anti-Rb antibody diluted in PBST containing 5% milk.
12. Wash the nitrocellulose membrane three times in PBST for 10 min each on a rocking platform.
13. Incubate the membrane with HRP-conjugated secondary antibody diluted in PBST containing 5% milk for 40 min.
14. Wash the nitrocellulose membrane three times in PBST for 10 min each on a rocking platform.
15. Visualize the antigen by use of a chemiluminescense detection system.

3.4. Determine Mitotic Index After X-Irradiation

To clarify the function of the G2 checkpoint, cells should be exposed to X-irradiation during S phase. To synchronize at early S phase, DNA synthesis inhibitors can be useful agents. For the detection of cells abrogating the G2 arrest and entering into mitosis, mitotic indices were determined by staining of mitotic spindles with anti-α-tubulin antibody (*see* **Note 10**). In our experiments, following the treatment with 10-Gy X-radiation, the vast majority of wild-type cells appeared to arrest at the G2 phase until 6 h after irradiation, suggesting that wild-type cells have the damaged DNA-induced G2 checkpoint function

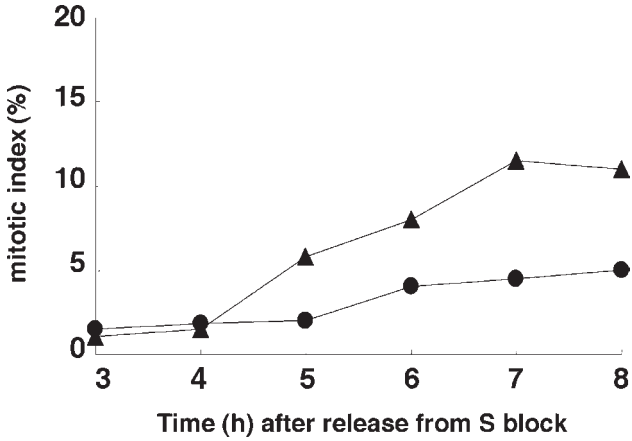

Fig. 4. *Cdh1*-deficient cells are unable to maintain G2 arrest after X-irradiation. After synchronizing at early S phase, cells were exposed to 10 Gy X-rays at 1 h (DT40) or 3 h (HeLa) after release from S block. For detection of cells abrogating the G2 arrest and entering into mitosis, mitotic indices were determined by staining of mitotic spindles with anti-α-tubulin antibody. Mitotic indices of wild-type cells (closed circle) and $Cdh1^{-/-}$ cells (closed triangle) were determined by staining cells with an anti-tubulin antibody. Reproduced from **ref. *5***, with permission.

(**Fig. 4**). However, substantially more $Cdh1^{-/-}$ cells entered mitosis relative to wild-type cells at 5 h after irradiation, indicative of a defect in the G2 checkpoint. These findings suggest that Cdh1 plays a crucial role in the damaged DNA-induced G2 checkpoint.

1. To synchronize DT40 cells at early S phase, incubate them with 1 μg/mL aphidicolin for 10 h (*see* **Note 6**). To synchronize HeLa cells at early S phase, incubate them with 2.0 m*M* thymidine for 24 h, release for 8 h, and then treat with 1 μg /mL aphidicolin for a further 16 h. (we usually use 35-mm dishes for both cell lines.)
2. After synchronization at early S phase, wash the cells three times and release them with normal medium.
3. Expose cells to 10-Gy X-rays 3 h after release from S block.
4. At the time of harvest (*see* **Note 9**), aspirate medium and add chilled 100% methanol for 10 min. In staining of DT40 cells, plate 100 μL of DT40 cells (5×10^5/mL – 10^6/mL) in culture medium using cytospin (500 rpm, 5 min) and mark border with Pap Pen™.
5. After washing fixed cells twice for 5 min each with PBS, incubate them in a blocking buffer for 1 h at room temperature.
6. After washing with PBS, incubate the cells with the antibody against α-tubulin in PBS overnight at 4°C (optimal antibody concentration should be determined by titration).

7. Wash with PBS three times for 5 min each.
8. Incubate with 1 µg/mL FITC-conjugated anti-mouse IgG in PBS for 1 h at room temperature.
9. Add coverslips with aqueous mounting medium or 80% glycerol. Use transparent nail varnish around the edges of the coverslip to seal the slide and prevent desiccation. Store slides in the dark.
10. Examine using a fluorescence microscope with appropriate filters.
11. Calculate the mitotic index (%) (number of cells in mitosis/total number of cells) (*see* **Note 11**).

4. Notes

1. Discarding of media and washing of cells with PBS should be performed very carefully, because mitotic cells are easy to float.
2. DNA staining using Hoechst 33342 dye can also be used. Condensed chromatin in mitotic cells shows highly intense staining with Hoechst 33342 dye.
3. As shown in **Fig. 1**, mitotic indices in MCF-7 and MDA-MB231 cells were decreased 36 h after exposure of nocodazole. It is well known that cells begin to enter the G1 phase without undergoing complete chromosome segregation and cell division after extended exposure to spindle inhibitors, even in cells having functional SAC. This phenomenon is termed *mitotic slippage*, representing failure to maintain cells in a mitotically arrested state, and is also called *adaptation **(10)***. Therefore, before the mitotic exit experiment, optimal exposure times of spindle inhibitors must be determined in each cell line.
4. Initially, cells are resuspended in 450 µL 1X PBS (final 30%). Then, 1050 µL of 100% methanol (final 70%) is added dropwise while vortexing at low speed.
5. In our experiments, G1 arrest occurred 48–72 h after treatment of rapamycin in DT40 cells. Cells should not be confluent before harvest.
6. Excessive exposure of cells to rapamycin and aphidicolin induces cell death. Optimal concentrations and exposure times of these drugs must be determined in different cell lines. We usually use 100 n*M* rapamycin for DT40 cells and 1 µg/mL aphidicolin for HeLa cells.
7. Do not wash cells just prior to incubation with BrdU. This will slow the growth of the cells during the incorporation phase of the procedure.
8. The final concentration of PI should be low (5 µg/mL) compared with single staining with PI (25µg/mL).
9. DT40 and HeLa cells can enter into mitosis 6 or 8 h after release from S phase respectively. In our experiments, cells were harvested at 2-h intervals, until 12 h is reached.
10. To avoid the morphological confusion between mitotic cells and apoptotic cells in the X-irradiation experiments, mitotic cells should be determined by counting cells with mitotic spindles. DNA staining using Hoechst 33342 dye is also used in conjunction with PI to distinguish between mitotic cells and apoptotic cells. Condensed chromatin in mitotic cells shows highly intense staining with Hoechst 33342 dye. PI is incorporated into dead cells only. Therefore, dead cells will be

stained with both PI and Hoechst 33342 dye, whereas mitotic cells will be stained with Hoechst 33342 dye only.

11. When cells have the functional G2 checkpoint, the mitotic index should not be increased.

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14

Purification of the Mitotic Checkpoint Complex, an Inhibitor of the APC/C From HeLa Cells

Valery Sudakin and Tim J. Yen

Summary

The mitotic checkpoint prevents cells with unaligned chromosomes from prematurely exiting mitosis by inhibiting the anaphase-promoting complex/cyclosome (APC/C) from targeting key proteins for ubiquitin-mediated proteolysis. Great efforts have been made recently to identify and characterize the factors involved in this checkpoint pathway. These studies will ultimately lead to a better understanding of cell cycle defects and chromosome instability. We report here a protocol for purification of an inhibitor of the APC/C from HeLa cells, called mitotic checkpoint complex (MCC). Our procedure is based on biochemical purification and characterization of the APC/C inhibitory activity from extracts of HeLa cells.

1. Introduction

The checkpoint pathway consists of three components that include a sensor that detects the presence of unaligned chromosomes, an effector or transducer that relays or amplifies the signal generated by the sensor, and an inhibitor that stops cells from exiting mitosis by inhibiting the APC/C until all chromosomes are properly aligned. Molecular mechanisms that explain the different segments of this checkpoint pathway remain to be established, but recent work has identified many components of the system. The mitotic checkpoint pathway is specified by a group of evolutionarily conserved genes that include *MAD1, MAD2, MAD3, BUB1*, and *BUB3* ***(1–12)***. The checkpoint is achieved by inhibiting the anaphase-promoting complex/cyclosome (APC/C), a multisubunit E3 ubiquitin ligase that targets key proteins whose destruction is necessary for sister chromatid separation and exit from mitosis ***(13–16)***.

Our current understanding about the mechanism by which the APC/C is inhibited by unaligned chromosomes has come primarily from studies of the MAD2 checkpoint protein ***(1,3,7,11,12,17–23)***. Although collectively these studies have shed considerable light on MAD2, it is still unknown whether

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

inhibition of APC/C is specified solely by MAD2 in vivo. In yeast and mammalian cells, MAD2 has been shown to interact with the APC/C through Cdc20, a protein that specifies substrate selectivity by the APC/C ***(22,24–28)***. One model postulates the checkpoint inhibition of the APC/C is achieved by the ability of Mad2 to sequester Cdc20 from recognizing its substrates. However, this model does not account for the necessity of other checkpoint proteins in this pathway. For example, complexes consisting of Mad3, Bub3, Cdc20, and Mad2 were identified in budding yeasts ***(29)***. In mitotic Hela cells, APC/C is associated with the hBUBR1 checkpoint kinase ***(8)***. This observation suggested that inhibition of APC/C in vivo might be achieved through more complex schemes. We recently reported the identification of a stable complex named the mitotic checkpoint complex (MCC), consisting of the proteins hBubR1, hBub3, Cdc20, and Mad2 ***(30)***.

We outline in this chapter a detailed biochemical approach, leading from a cell-free system to purified factor. Extracts prepared from HeLa cells are used to purify both the active APC/C and its inhibitor, the MCC. The two complexes have been separated by successive gel-filtration and ion-exchange chromatography. The MCC is further purified by an additional gel-filtration step. The APC/C activity and the ability of MCC to inhibit APC/C are assayed at every stage of the purification procedure by in vitro ubiquitination assays.

2. Materials

1. Nocodazole, 60 µg/mL in DMSO, stored at –20°C.
2. 45-mL high-pressure cell-disruption nitrogen bomb, Parr Instruments (item 4639).
3. Tank of liquid nitrogen.
4. Glass-glass Dounce tissue homogenizer, plunger "B," Wheaton, NJ.
5. Sorvall RT6000B refrigerated centrifuge, rotor H-1000B.
6. Sorvall refrigerated centrifuge 5B, rotors GS-3 and HB-4 swinging-bucket.
7. Beckman LB-70 ultracentrifuge, rotor 70.1 TI.
8. Disruption buffer: 40 m*M* HEPES (pH 7.2), 10 m*M* EDTA, 2 m*M* DTT, 5 µg/mL pepstatin, 10 µg/mL leupeptin, 5 µg/mL chymostatin.
9. Ubiquitination mix: 40 m*M* Tris-HCl (pH 7.6), 1 mg/mL rcm-BSA (bovine serum albumin, reduced, carboxymethylated), 1 m*M* DTT, 5 m*M* $MgCl_2$, 10 m*M* phosphocreatine, 50 mg/mL creatine phosphokinase, 50 µ*M* ubiquitin, 1 µ*M* ubiquitin aldehyde (Calbiochem or Sigma), 1 pmol of E1, 5 pmol of E2-C, and 1–2 pmol (approx 10^5 cpm) of ^{125}I-labeled cyclin B (13–91)/protein A (*see* **Note 1**).
10. SDS-PAGE apparatus.
11. Transfer apparatus.
12. PhosphorImager (Fujix).
13. Pharmacia Fast Performance Liquid Chromatography (FPLC) system.
14. Centricon-30 concentrators, Amicon.

15. FPLC columns: HiLoad Superdex 200 16/60, Superdex 200 HR 10/30, Superose 6 HR 10/30, Mono Q HR 5/5, Superdex 50 10/30.
16. FPLC gel filtration buffer, freshly prepared: 50 m*M* HEPES (pH 7.2), 250 m*M* KCl, 1m*M* DTT, 0.2 mg/mL rcm-BSA.
17. FPLC wash buffer: 50 m*M* HEPES (pH 7.2), 1 m*M* DTT, 10% glycerol.
18. Ammonium sulfate cut dilution buffer, freshly prepared: 20 m*M* Tris-HCl (pH 7.2), 1 m*M* DTT.
19. Ammonium sulfate cut dialysis buffer, freshly prepared: 20 m*M* Tris-HCl (pH 7.2), 1 m*M* DTT, 20% glycerol.
20. Buffer A (FPLC Mono Q), freshly prepared: 50 m*M* Tris-HCl (pH 7.2), 1 m*M* DTT.
21. Buffer B (FPLC Mono Q), freshly prepared: 50 m*M* Tris-HCl (pH 7.2), 1 *M* NaCl, 1 m*M* DTT.

3. Methods

3.1. Preparation of Extracts From HeLa Cells

Subheadings 3.1.1.–3.1.3. describe the preparation of the cell-free system from HeLa cells that is a source for biochemical purification of MCC and APC/C. Two alternative techniques have been employed for preparation of highly concentrated extracts (*see* **Note 2**): disruption of the cells in high-pressure nitrogen bomb (Parr Instruments) or in a glass-glass Dounce homogenizer (Wheaton, NJ, plunger "B"). All procedures in **Subheading 3.1.** have to be carried out on ice or in the cold room (4°C).

3.1.1. HeLa Cells

HeLa cells were purchased (as frozen washed pellets of the suspension culture) from the National Cell Culture Center (NCCC, Cellex Biosciences). The NCCC was supplied with the nocodazole stock to arrest cells in mitosis. Alternatively, cells can be grown in the laboratory at 37°C in flasks of 3-L volume. To prepare mitotic extracts, the cell suspension (concentration of approx 5×10^5 cell/mL) was supplemented with nocodazole to the final concentration of 60 ng/mL for 15–18 h. Untreated populations of asynchronously growing HeLa cells (approx 1×10^6 cells/mL) were used to prepare interphase extracts. In cases where cells must be harvested in the lab, the cell suspension is treated as follows:

1. Collect cells by spinning the suspensions in 500-mL bottles in Sorvall centrifuge, 200*g*, 10 min at 4°C.
2. Resuspend the cell pellets gently in equal volumes of ice-cold PBS; transfer the slurry into 50-mL Falcon plastic tube (entire 3-L suspension should be transferred into one 50-mL tube). Gentle pipetting can be used to break, resuspend, and transfer the pellets.
3. Wash cells three times in ice-cold PBS, 200*g*, and 5 min at 4°C.
4. Aspirate the media completely after the last wash.

3.1.2. Lysing Pelleted Hela Cells

This preparation requires approx 10–12 mL of packed cell pellet. We have found that 10 L of HeLa cells supplied by NCCC comes to approx 10 mL of cell pellet. In our lab, one 3-L suspension of HeLa cells blocked in mitosis (approx 1×10^6 cells/mL) will also yield a pellet of approx 10 mL. We found that procedures described in **Subheadings 3.1.2.1.** and **3.1.2.2.** can be used alternatively to disrupt the cells. **Subheading 3.1.3.** follows the cell disruption step.

3.1.2.1. Disrupting the Cells in Glass-Glass Homogenizer

1. Wash the homogenizer with disruption buffer and place it on ice for 10–15 min prior to use.
2. Dilute the cell pellet with an equal volume of the disruption buffer and transfer the slurry into the homogenizer.
3. Disrupt the cells with 10 firm strokes. Avoid foaming by applying moderate force to the piston during disruption!
4. Keep the homogenizer on ice for 10 min.
5. Repeat **step 3**.
6. Continue with **Subheading 3.1.3.**.

3.1.2.2. Disrupting the Cells in High-Pressure Nitrogen Bomb

1. Wash the bomb with disruption buffer and place it on ice for 10–15 min prior to use.
2. Dilute the cell pellet with an equal volume of the disruption buffer and transfer the slurry into the bomb.
3. Slowly bring the nitrogen pressure in the bomb to 1500 psi. Wait 2–3 min and adjust the pressure (the initial level of pressure will slightly drop owing to the absorption of nitrogen by the cell slurry).
4. Maintain the pressure for 30 min; keep the bomb on ice.
5. Very slowly and carefully release the pressure and collect crude extract into 50-mL Falcon tube **(Note 3)**.
6. Continue with **Subheading 3.1.3.**.

3.1.3. Preparation of High-Speed Extracts

The following section describes the preparation of high-speed (S100) extracts from HeLa cells using two successive steps of centrifugation. The resulting extracts (supplemented with glycerol to 10% final concentration) should contain no less than 15 mg/mL total protein.

1. Transfer the crude extracts into two 15-mL Corex glass tubes (pre-chill the tubes on ice for 10–15 min).
2. Spin the tubes in the Sorvall HB-4 swinging-bucket rotor at 10,000*g* for 15 min at 4°C.

3. Using a glass Pasteur pipet, carefully collect the intermediate layer (approx 10–12 mL), located between the thin white top lipid layer and the larger bottom dark layer of cellular debris, into a clean, prechilled 15-mL Corex tube. Be careful to avoid the crude debris! Some contamination with the upper lipid phase is unavoidable and is harmless.
4. Spin the intermediate layer in the Sorvall HB-4 swinging-bucket rotor at 10,000*g* for 15 min at 4°C.
5. Using a glass Pasteur pipet, transfer supernatant into a prechilled 30-mL Beckman ultracentrifuge tube. The two supernatants can be combined into one 30-mL tube.
6. Spin the tubes in the Beckman 70.1 TI fixed-angle rotor at 100,000*g* for 45 min at 4°C.
7. Collect supernatants, representing the high-speed (S100) extracts of HeLa cells, and supplement with glycerol to a final concentration of 10%. Unless the supernatant is being processed further immediately, transfer aliquots of 1–2 mL into prechilled microcentrifuge tubes and freeze in liquid nitrogen. Transfer the tubes to –80°C freezer for storage. Frozen extracts should be stable for at least 1 yr.

3.2. Purification of the Anaphase-Promoting Complex/Cyclosome From the HeLa Extracts

The following sections describe the preparation of active APC/C, which will be used to screen the inhibitory activity of MCC. This inhibitory assay is conducted at every step of the MCC purification procedure.

3.2.1. Fast-Performance Liquid Chromatography (FPLC) Purification of the APC/C

All procedures are carried out at 4°C. The typical profile of the APC/C activity on the Superdex 200 10/30 column is presented in **Fig. 1A**.

1. Equilibrate gel filtration column (either Superdex 200 or Superose 6 FPLC column can be used for this procedure) with the FPLC gel filtration buffer.
2. Load 0.5 mL (7–10 mg) of the S100 HeLa extract prepared from nocodazole-arrested HeLa cells onto the equilibrated column.
3. Elute the column with the FPLC gel filtration buffer at a flow rate of 0.5 mL/min, and collect 40 0.5-mL fractions.
4. Immediately upon the completion of the purification run, concentrate the fractions by using Centricon-30 concentrators. Wash every fraction in 1 mL of the FPLC wash buffer and concentrate again to final volumes of approx 50 µL.
5. Test 5% of each fraction for ubiquitination activity as described in **Subheading 3.2.2.**
6. Pool the peak of the APC/C activity, divide into small aliquots of 50–100 µL, and snap-freeze in liquid nitrogen **(Note 4)**.

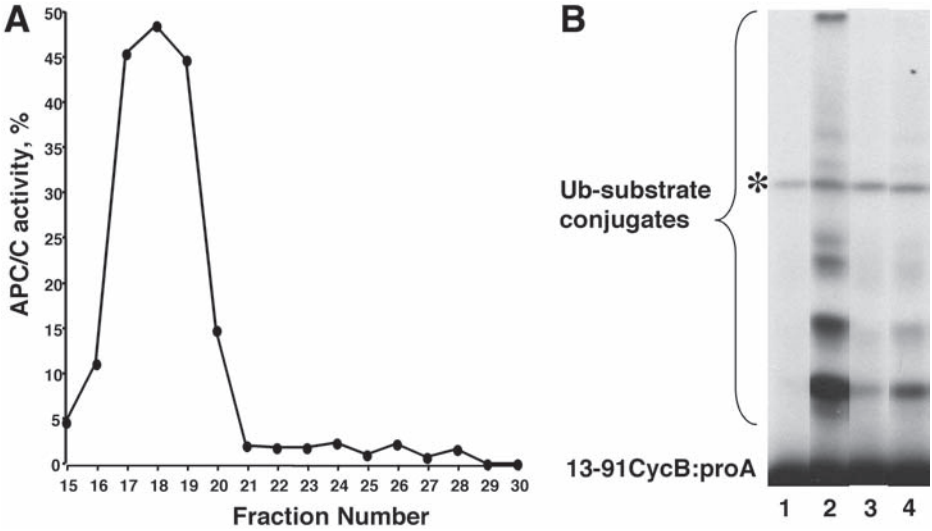

Fig. 1. Purification of the APC/C from HeLa extracts. **(A)** Identification of the APC/C activity peak in the fraction of the Superdex 200 HR 10/30 column. 10 mg of the extract prepared from nocodazole-arrested HeLa cells were loaded and eluted as described in **Subheading 3.2.2.** 5% of each fraction (2.5 μL) was tested for the ubiquitination activity as described in **Subheading 3.2.2.** The samples of the assay were separated on the SDS-PAGE and quantified on a PhosphorImager (*see* **Subheading 3.2.2.**). **(B)** Typical assay of the APC/C ubiquitination activity visualized by autoradiography. Identification of the MCC in HeLa extract. Purified mitotic APC/C was incubated for 30 min either alone (lane 2) or with fractions from the 300 to 500 kDa range of the Superose 6 HR 10/30 column (lane 4) and then assayed for ubiquitin ligase activity. The same column fractions were assayed without addition of APC/C to monitor contaminating APC/C activity (lane 3). A reaction containing only substrate served as a negative control (lane 1). Asterisk denotes an iodinated contaminant bacterial protein that is not a substrate for APC/C and was excluded from the quantification. Reproduced from ***(30)*** by copyright permission of The Rockefeller University Press.

3.2.2. APC/C Ubiquitination Assay

1. Supplement purified APC/C (*see* **Subheading 3.2.1.**) with MCC fractions (*see* **Subheading 3.3.**) in a final volume of 10 μL in microcentrifuge tubes, containing 2 μL of the ubiquitination mix.
2. Incubate for 30 min at 30°C.
3. Separate the reaction products by SDS-PAGE, dry the gel, and either expose it on film to visualize radioactive Ub-cyclin B conjugates (*see* **Fig. 1B**) or analyze results with a PhosphorImager (Fujix) to quantify the amount of ubiquitinated substrate. The APC/C activity is calculated as the percentage of the radiolabeled substrate converted into the high-molecular-weight ubiquitin–cyclin B conju-

gates. The total amount of the substrate is established as a sum of the high-molecular-weight conjugates and the nonconverted form (*see* **Fig. 1A**).

3.3. Purification of the Mitotic Checkpoint Complex From the HeLa Extracts

The following sections describe a series of biochemical procedures leading to the purification of the highly active, stable four-subunit APC/C inhibitory complex from crude HeLa extracts.

3.3.1. Ammonium Sulfate Cut of the Crude HeLa Extract

This section describes the enrichment of the MCC by ammonium sulfate cut of the crude HeLa extracts obtained from **Subheading 3.1.3.**. Pilot experiments (0–45%, 45–55%, and 55–85% saturation) should be performed on a small amount of the extract to verify the distribution of the protein of interest (*see* **Fig. 2**).

1. Prepare a saturated solution of $(NH_4)_2SO_4$. Dissolve 800 g of ammonium sulfate in 1000 mL of 20 m*M* Tris (pH 7.2). Heat the mixture slightly and stir until completely dissolved. Leave the solution at 20–25°C for 24 h. Transfer the supernatant into a new bottle (discard all crystallized ammonium sulfate) and keep it at 4°C for an additional 24 h. More ammonium sulfate will precipitate. Transfer the supernatant into a new bottle (discard all crystallized ammonium sulfate); keep at 4°C for an additional 24 h. Use the resulting supernatant as the saturated $(NH_4)_2SO_4$. Store the solution at 4°C over remaining ammonium sulfate crystals.
2. S100 extract subjected to the cut should contain 40–60 mg of total protein. Dilute the extract with ammonium sulfate cut dilution buffer (ASCDB) in 15-mL plastic tube to bring the final protein concentration to 10 mg/mL.
3. Calculate the volume of ammonium sulfate needed for 45% saturation (*see* **Note 5**). Add the $(NH_4)_2SO_4$ solution, mix gently, and keep on ice for 30 min. Spin the sample for 10 min at 6000*g*, 4°C. Keep the pellet on ice.
4. Record the volume of the supernatant and calculate the volume of $(NH_4)_2SO_4$ required to obtain 55% saturation (*see* **Note 5**). Add the $(NH_4)_2SO_4$ solution, mix gently, and keep on ice for 30 min. Spin the sample for 10 min at 6000*g*, 4°C. Keep the pellet on ice.
5. Record the volume of the supernatant and calculate the volume of $(NH_4)_2SO_4$ required to obtain 85% saturation (*see* **Note 5**). Add the $(NH_4)_2SO_4$ solution, mix gently, and keep on ice for 30 min. Spin the sample for 10 min at 6000*g*, 4°C. Keep the pellet on ice.
6. Upon the completion of the last centrifugation step, immediately dissolve the pellets in the smallest possible volume of ASCDB (usually 0.5–1.0 mL) and dialyze against 2 L of ammonium sulfate cut dialysis buffer at 4°C for at least 16 h. Spin the dialysis samples for 15 min at 10,000*g*, 4°C. Snap-freeze supernatants in liquid nitrogen (*see* **Note 6**). The protein concentration for the 0–45% fraction should be 20–25 mg/mL.

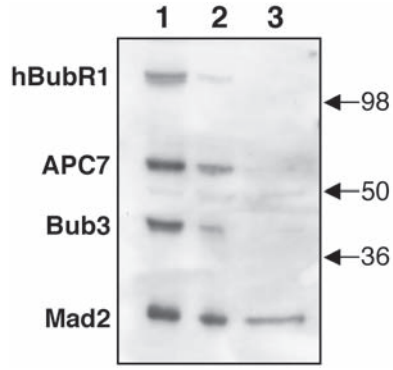

Fig. 2. Ammonium sulfate cut of the high-speed (S100) mitotic extract of HeLa cells. 30 mg of the extract were cut as described in **Subheading 3.3.1.**. 50 µg of each fraction was separated by 4–15% gradient SDS-PAGE and transferred to a membrane. The membrane was cut into four strips (arrows denote the cuts) and probed with appropriate MCC (hBubR1, Bub3, Mad2) and APC/C (APC7) antibodies (*see* **Note 7**). Lane 1: 0–45% fraction; lane 2: 45–55% fraction; lane 3: 55–85% fraction.

7. Load 50 µg of protein from each fraction on SDS-PAGE and perform a Western blot analysis to determine the distribution of APC/C and MCC subunits between the fractions. An example of such an experiment is presented in **Fig. 2**.

3.3.2. Purification of MCC on the HiLoad Superdex 200 16/60 FPLC Column

This section describes a large-scale purification of the MCC contained in the ammonium sulfate cut using the 130-mL gel filtration HiLoad Superdex 200 16/60 FPLC column. The maximal loading volume for this column is 2 mL (*see* **Note 8**).

1. Equilibrate the Superdex 200 16/60 column with two vols of FPLC gel filtration buffer.
2. Load 2 mL of the ammonium sulfate cut fraction 0–45% onto the column (*see* **Note 9**).
3. Elute the sample with the FPLC gel filtration buffer at a flow rate of 1.5 mL/min. Collect 40 1.5-mL fractions.
4. Immediately upon completion of the separation, concentrate the fractions approx 30-fold in Centricon-30, and then wash them with 10 vols of FPLC wash buffer. Concentrate the fractions again to a final volume of 50–100 µL.
5. Separate 5% of each fraction on SDS-PAGE and probe the FPLC profile with anti-hBubR1 antibodies to determine the migration of the hBubR1 peaks. MCC from mitotically blocked HeLa cells migrates as two stable complexes on gel filtration FPLC columns: a large mega-dalton APC/C–MCC complex and the

free MCC (400–500 kDa) ***(8,30)*** (*see* **Note 10**). Migration of the APC/C on this column can be verified by conducting the APC/C ubiquitination assay with 5% of each fraction. Pool the free MCC fractions and snap-freeze them in liquid nitrogen if not used immediately.

6. Check the APC/C inhibitory activity of the pooled MCC fractions. Incubate increasing amounts of the MCC fraction (1, 3, and 5 µL) with active APC/C for 30 min at 30°C and then determine the APC/C ubiquitination activity as described in **Subheading 3.2.2.**.

3.3.3. Purification of the MCC on the Anion Exchange Mono Q HR 5/5 FPLC Column

The following section describes further purification of the MCC on an ion-exchange column. A typical Mono Q profile of the MCC purification is presented in **Fig. 3** (the column fractions were separated by SDS-PAGE, transferred to membrane, and probed with the anti-MCC and anti-APC/C antibodies to determine the location of the complexes).

1. Equilibrate the MonoQ HR 5/5 column with buffer A.
2. Load the Superdex 200 MCC fraction (*see* **Subheading 3.3.2.**) onto the column (loading volume 0.5–2.0 mL).
3. Program an FPLC to elute the column with a shallow gradient of NaCl (200–500 m*M* salt over 30 min) using buffers A and B at a flow rate of 1 mL/min.
4. Collect 1-mL fractions. Immediately upon completion of the separation, concentrate and wash the fractions in Centricon-30 concentrators with the FPLC wash buffer.
5. Separate 5% of each fraction by SDS-PAGE and probe the Mono Q profile with the anti-hBubR1 antibody (*see* **Subheading 3.3.2., step 5** and **Note 10**). Pool the MCC-containing fractions and snap-freeze in liquid nitrogen as one aliquot of if not used immediately.
6. Check the APC/C inhibitory activity of the pooled MCC fraction. Incubate increasing amounts of the MCC fraction (1, 3, and 5 µL) with active APC/C for 30 min at 30°C, and then determine the APC/C ubiquitination activity as described in **Subheading 3.2.2.**

3.3.4. Purification of the MCC on the Superose 6 HR 10/30 Gel Filtration Column

The following section describes a final purification step of the MCC. The partly purified and enriched MCC fraction is purified further on a gel filtration column with high-resolution capabilities. The Superose 6 profile of MCC and its ability to inhibit an active APC/C are presented in **Fig. 4**.

1. Equilibrate the Superose 6 HR 10/30 column with two volumes of the FPLC gel filtration buffer.
2. Load 0.5 mL of the MCC fraction onto the column (*see* **Note 8**).

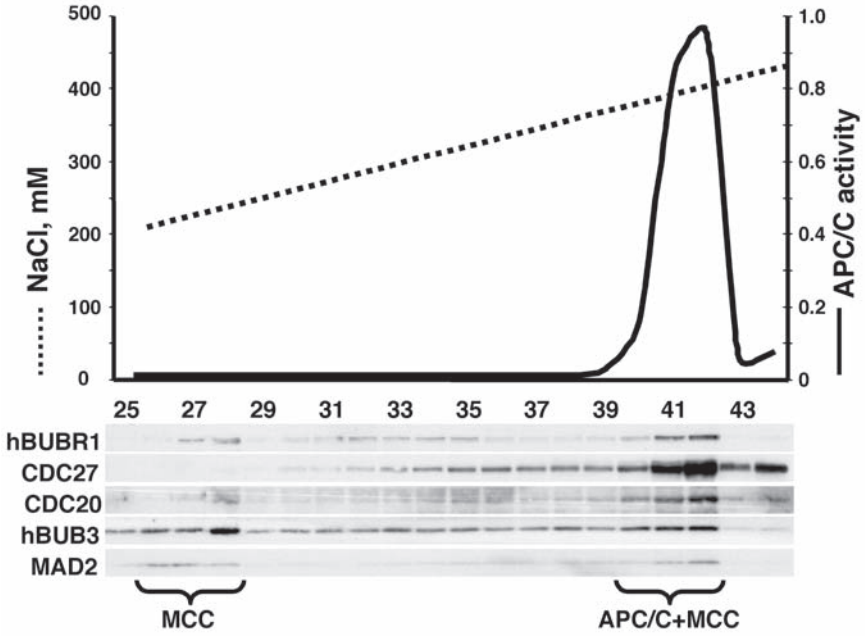

Fig. 3. The MonoQ anion exchange column profile of MCC and APC/C. Fractions were assayed for APC/C activity as described in the **Subheading 3.2.1.** (upper panel, solid line) and probed for the MCC subunits (hBubR1, hBub3, Cdc20, Mad2) and the APC/C subunit Cdc27 by Western blots (lower panels). Reproduced from ***(30)*** by copyright permission of The Rockefeller University Press.

3. Elute the sample with the FPLC gel filtration buffer at a flow rate of 0.5 mL/min. Collect 0.5-mL fractions.
4. Immediately upon completion of the separation, concentrate the fractions in Centricon-30 concentrators and wash them with 10 vols of FPLC wash buffer. Concentrate the fractions again to approx 50 µL.
5. Separate 5% of each fraction by SDS-PAGE and probe the FPLC profile with anti-hBubR1 antibodies to determine the migration of the MCC peaks. MCC migrates as a stable complex of 400–500 kDa ***(8,31)*** **(Note 10)**. In addition to the western analysis, assay the fractions for their ability to inhibit the APC/C-mediated ubiquitination. Pool the MCC fractions, divide the pooled purified MCC into small aliquots of approx 20–30 µL and snap-freeze them in liquid nitrogen. Purified MCC can be stored at –80°C for months without losing its inhibitory activity. Avoid repeating cycles of thawing–freezing (do not thaw the purified MCC more that two times).

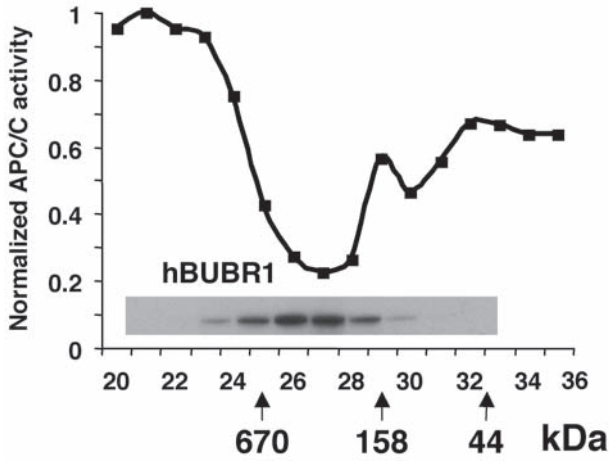

Fig. 4. MCC purified on the Superose 6 column. The elution profiles of hBubR1 (inset) and APC/C inhibitory activity from the Superose 6 column is shown. Arrows point to thyroglobulin (670 kDa), gamma-globulin (158 kDa), and ovalbumin (44 kDa), that served as migration standards. Reproduced from ***(30)*** by copyright permission of The Rockefeller University Press.

4. Notes

1. Sea urchin cyclin B derivative cyclin B (13–91)/protein A was obtained and iodinated as described previously ***(31)***. E1 is purified from human cells utilizing the method described in ***(32)***. Recombinant E2-C is expressed in bacteria as described in ***(33)*** and purified by gel filtration on Superdex 50 FPLC column.
2. It has been observed that the APC/C enzymatic and MCC inhibitory activities decay rapidly when protein concentrations of extracts are less than 10 mg/mL. The nitrogen bomb and glass-glass Dounce techniques were developed to produce high-concentration extracts to prevent the loss of activity.
3. If the pressure in the bomb is released too fast, the crude extract will be ejected from the vessel forcibly, which makes it very difficult to collect it carefully in a plastic tube. If using the Parr nitrogen bomb, please refer to the manual provided by the manufacturer.
4. The crude extracts can be kept at –80°C for at least 1 yr without any noticeable loss of enzymatic activity. It is highly recommended that the extracts be stored in small aliquots of 1–2 mL in order to prevent repeating thawing–freezing cycles. Do not thaw–freeze the extracts more than two times!
5. Calculate the volume of ammonium sulfate for three steps of precipitation (0–45%, 45–55%, and 55–85%) using the following: $X = Y \times V/1 - Y$, where X is the volume of saturated ammonium sulfate, Y is a desired final concentration of ammonium sulfate, and V is an initial volume of the sample undergoing the salt cut. For a 5-mL sample, the calculation will be: $X = 0.45 \times 5/(1 - .045) = 4.09$ mL

of the saturated ammonium sulfate for the first step. After completion of the first precipitation step, carefully record the volume of the supernatant transferred to a new tube. Usually it is 85–90% of the initial sample (extract plus ammonium sulfate). The supernatant already contains 45% of ammonium sulfate after the first step. This concentration needs to be increased by 10% to reach 55%. It is calculated as $X = 0.1 \times V_2 / (1 - 0.1)$ where V_2 is the volume of the supernatant after **step 1**. After completion of **step 2**, record the volume of the supernatant and calculate the volume of the saturated ammonium sulfate needed for **step 3** using the same equation (concentration of ammonium sulfate should be increased by 30% in the supernatant after **step 2** to reach the final 85%).

6. The samples of the ammonium sulfate cut should be kept in one aliquot (typically 0.5–1.0 mL) at –80°C. Usually three or four preparations will yield enough material for one fractionation on the Superdex 200 16/60 column (*see* **Subheading 3.3.2.** and **Note 8**).
7. Affinity purified antibodies against hBubR1, hBub3, and Mad2 were developed in our laboratory ***(30)***. Anti-APC7 antibody was a generous gift from Dr. J.-M. Peters ***(34)***. Final concentration of the primary antibodies in Western analysis was 1 μg/mL.
8. It is recommended to limit the loading volumes for gel filtration columns to not more than 2% of the column bed volume to prevent the increase of the so-called dead volume in the system. Maximal load volumes for gel filtration columns described in this chapter are 0.5 mL for Superose 6 HR 10/30 (26-mL bed volume), 0.5 mL for Superdex 200 10/30 (26-mL bed volume), and 2.0 mL for Superdex 200 16/60 (130-mL bed volume).
9. The capacity of the Superdex 200 16/60 column is around 20 mg of total protein per 1 mL of the matrix (there is 130 mL of total matrix). The usual concentration of the sample loaded is 20–25 mg/mL; therefore, overloading the column is not an issue for the particular procedure.
10. We recommend this Western analysis to map the MCC migration profile on your specific FPLC system and column. Once established, the MCC gel filtration profile can be safely used for further routine purification and pooling of the MCC-containing fractions.

Acknowledgment

VS is a Leukemia and Lymphoma Scholar. Work was supported by NIH GM44762, Core grants CA 75138, CA06927 and an Appropriation from the Commonwealth of Pennsylvania.

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15

Analysis of the Spindle-Assembly Checkpoint in HeLa Cells

Paul R. Andreassen, Dimitrios A. Skoufias, and Robert L. Margolis

Summary

The spindle-assembly checkpoint involves signaling at kinetochores, which leads to the arrest of mitotic progression in the absence of microtubule attachment or spindle tension. Here, we detail procedures for the analysis of the spindle-assembly checkpoint in adherent mammalian cells. These techniques focus on pharmacological approaches and immunofluorescence microscopy to verify the state of spindle assembly, kinetochore attachment of microtubules and spindle tension, chromosome positioning, and kinetochore signaling by the Mad2 or Bub1 checkpoint proteins. We also describe a bi-parameter flow cytometric assay, using either MPM-2 or anti-phospho-(Ser10)-histone H3 antibodies, for quantitating mitotic cells.

Key Words: Spindle checkpoint; metaphase checkpoint; immunofluorescence microscopy; flow cytometry; MPM-2; phospo-histone H3; Mad2; Bub1; centromere separation.

1. Introduction

The mitotic spindle checkpoint in mammalian cells is responsible for the integration of each chromosome into the bipolar spindle prior to the initiation of anaphase ***(1,2)***. In the absence of dynamic microtubules or bipolar attachment of the kinetochores on every chromosome ***(1,3)***, signaling at the kinetochore results in the maintenance of $p34^{cdc2}$ kinase activity, and the prevention of anaphase chromosome separation. More than one signaling mechanism appears to be in play at metaphase, as the Mad2 and Bub1/BubR1 checkpoint proteins are involved in discrete functions, imposing checkpoints following failure of either microtubule attachment or of spindle tension, respectively ***(3,4)***. Agents that interfere with microtubule assembly, such as nocodazole and vinblastine, can be utilized at concentrations that interfere with bipolar attachment of microtubules to kinetochores ***(3,5)*** or that specifically interfere with spindle tension in the presence of a fully formed mitotic spindle ***(3)***. The selec-

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

tion of the cell line utilized for analysis of the spindle-assembly checkpoint is critical. Many cell lines, whether rodent or human, transformed or primary, have a very limited capacity to maintain mitotic arrest in the absence of spindle assembly ***(6–10)***. HeLa cells present an ideal system because of a nearly indefinite mitotic arrest in the absence of spindle assembly ***(5,6)*** and because this cell line clearly manifests discrete spindle-assembly and spindle-tension checkpoints ***(3)***. The analysis described here can also be utilized in conjunction with dominant-negative mutants ***(11–13)***, or with siRNA-targeting checkpoint proteins, to suppress the function of the spindle-assembly checkpoint ***(14,15)***.

Here we will detail methods for analysis of the spindle-assembly checkpoint in HeLa cells. Our presentation will begin with cell culture for checkpoint analysis by immunofluorescence microscopy, and will examine pharmacological manipulation of the spindle-assembly checkpoint. Finally, we will describe aspects of analysis by immunofluorescence microscopy and bi-parametric flow cytometry. Immunofluorescence microscopy is utilized to verify multiple parameters, including the state of spindle assembly, metaphase chromosome alignment, microtuble attachment to kinetochores, spindle tension, kinetochore signaling, and timing of mitotic exit. Flow cytometry is utilized to quantitate mitotic arrest and to document mitotic exit. Given that many cells weakly maintain mitotic arrest and become tetraploid in the absence of spindle assembly ***(9,10)***, methods for quantitating mitotic arrest must be capable of distinguishing mitotic 4N cells from interphase cells that also have a 4N DNA content following exit from mitosis to tetraploid G1.

2. Materials

1. Circular glass coverslips, no. 1, 12-mm diameter (Bellco; Vineland, NJ).
2. Poly-L-lysine solution (Sigma; St. Louis, MO), stored at room temperature.
3. Ethanol, absolute.
4. Fine-tip forceps.
5. Nocodazole (methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate) (Sigma). 1 mg/mL stock in dimethylsulfoxide kept at –20°C.
6. 8% paraformaldehyde stock solution in PBS prepared from powder (Sigma). Add 2 drops of 1 *N* HCl/100 mL of solution and heat at 70°C until the solution becomes clear (about 5 min). Store the stock solution at 4°C (*see* **Note 1**).
7. Phosphate-buffered saline (PBS) solution: 136 m*M* NaCl, 2 m*M* KCl, 10.6 m*M* Na_2PO_4, 1.5 m*M* KH_2PO_4, pH 7.4. Working solution should be prepared fresh from a 20X stock solution (kept at RT) prior to use.
8. Triton X-100 (Sigma), 10% v/v solution kept at 4°C.
9. Antibody buffer (PBS containing 3% bovine serum albumin, 0.05% Tween-20, and 0.04% sodium azide), kept at 4°C.
10. Sodium azide (Sigma), 20% w/v solution kept at RT.
11. Bovine serum albumin (Sigma).

12. Tween-20 (Sigma), 10% v/v solution kept at 4°C.
13. Secondary antibodies (Jackson Immunoresearch; West Grove, PA): FITC-conjugated anti-human IgG, FITC-conjugated anti-rabbit IgG, FITC-conjugated anti-mouse IgG, and Texas Red-conjugated anti-mouse IgG antibodies. Antibodies cross-reacting with other species were depleted by the manufacturer.
14. Aluminum foil.
15. Precleaned microscope slides, (3" × 1" × 1 mm) (Fisher Scientific; Pittsburgh, PA).
16. Vectashield mounting medium (Vector Laboratories, Inc.; Burlingame, CA).
17. Kimwipes 1-Ply Wipers (Kimberly-Clark; Roswell, GA).
18. Nail polish, clear or with color.
19. Mouse anti-β-tubulin monoclonal antibody, clone 2.1 (Sigma).
20. Anti-centromere antibodies from human plasma, Cat no. HCT-0100 (Immunovision; Springdale, AR).
21. Rabbit anti-Mad2 antiserum (Babco; Richmond, CA).
22. Mouse anti-Bub1 monoclonal antibody (Chemicon; Temecula, CA).
23. Mouse MPM-2 monoclonal antibody (mitotic marker) (Dako; Carpinteria, CA).
24. Absolute methanol, HPLC-grade (Fisher Scientific; Fair Lawn, NJ).
25. RNase A (Sigma), 60 Kunitz Units/mL prepared in distilled H_20, boiled 20 min, and kept at –20°C.
26. Propidium iodide (Sigma), 5 mg/mL in distilled H_20, kept at –20°C.
27. Rabbit anti-phospho-(Ser10)-histone H3 antibody (mitotic marker) (Upstate Biotechnology, Lake Placid, NY).

3. Methods

The methods described below outline (1) cell culture of HeLa cells as optimized for immunofluorescence microscopy; (2) pharmacological induction of mitotic arrest; (3) various aspects of the spindle-assembly checkpoint as evaluated by immunofluorescence microscopy; and (4) bi-parametric flow cytometry in the analysis of the spindle-assembly checkpoint.

3.1. Culture of HeLa Cells for Analysis of the Spindle-Assembly Checkpoint

For analysis by flow cytometry, cells should be cultured on tissue culture treated plastic petri dishes for at least 24 h prior to treatment with vinblastine or nocodazole, such that cells are actively cycling. For immunofluorescence microscopy (*see* **Subheading 3.3.**), cells are grown on coverslips as detailed in **steps 1** and **2**.

1. Treat coverslips in 0.1 mg/mL poly-L-lysine for 30 min to promote the adhesion of cells to coverslips during mitosis. Rinse generously with distilled H_20, sterilize 30 min with 70% ethanol in a laminar flow hood, rinse with absolute ethanol, and allow to air dry. Coverslips can be kept indefinitely in a sterile container.
2. Using fine-tip forceps, place the desired number of coverslips in a culture dish and (re)plate cells at the desired density. Cells should be replated such that cells

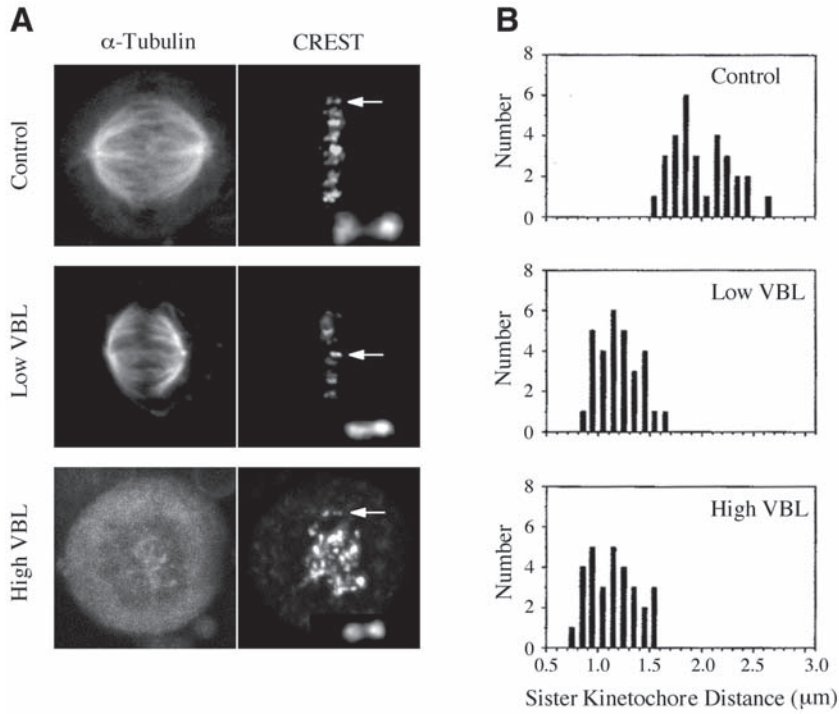

Fig. 1. Vinblastine has dose-dependent effects on mitotic spindle assembly and spindle tension. **(A)** In a control metaphase HeLa cell, sister kinetochore separation detected with anti-centromere antibodies is increased relative to the separation in a cell treated with 20 ng/mL vinblastine (low VBL) that has a bipolar spindle. By contrast, the mitotic spindle is completely disassembled at 1.5 μg/mL vinblastine (high VBL). Mitotic spindles are detected with anti-tubulin antibodies. Selected pairs of kinetochores are shown in an inset enlargement for each condition. **(B)** A comparison of kinetochore separation in 15 untreated control cells, 15 cells exposed to 20 ng/mL vinblastine and thus lacking spindle tension, and in 15 cells exposed to 1.5 μg/mL vinblastine and lacking microtuble attachment to kinetochores.

are subconfluent after 36–48 h (*see* **Note 2**). At that point, cells can be treated with anti-microtubule agents.

3.2 Pharmacological Manipulation of the Spindle-Assembly Checkpoint

The degree of spindle assembly can be modulated by exposure of cells to increasing concentrations of vinblastine **(Fig. 1A)**. At 20 ng/mL, HeLa cells

arrest with a bipolar spindle **(Fig. 1A)** ***(3)***. This concentration of vinblastine can be utilized to test specific effects upon spindle tension, since metaphase arrest occurs with intact microtubule attachment at kinetochores. At 1.5 μg/mL vinblastine, microtubules are completely depolymerized (**Fig. 1A**). This concentration should be chosen for general studies of the spindle-assembly checkpoint and for specific examination of checkpoints relating to the absence of microtubule attachment. Alternatively, nocodazole can be utilized at 10 ng/mL for arrest with a bipolar spindle or 400 ng/mL for complete spindle disassembly.

1. Treat cells with vinblastine for up to 16 h. Treatment for longer than 16 h can lead to cell death. It is best to use fresh stock aliquots of drug for reproducible results.
2. Since inactivation of gene products—for example, by the expression of dominant-negative mutants ***(13)***—can have effects upon spindle assembly itself, the level of microtubule and spindle assembly must be verified by immunofluorescence microsocopy (*see* **Subheadings 3.3.1.–3.3.2.**) for the particular concentration of vinblastine that is utilized.

3.3 Immunofluorescence Microscopy to Analyze Spindle-Assembly Checkpoint Function

This protocol can be utilized to evaluate microtubule assembly (*see* **Fig. 1**), chromosome alignment, kinetochore tension, and the kinetochore association of either Mad2 or Bub1 (*see* **Fig. 2**). Chromosome alignment is useful for determining the stage of mitotic arrest, effects of checkpoint failure upon chromosome segregation, and whether non-aligned chromosomes are present. Non-aligned chromosomes that lack bipolar spindle attachment are an important cause of metaphase checkpoint activation ***(3,16)***, and this checkpoint is accompanied by increased Mad2 and Bub1 binding to the non-attached kinetochores **(Fig. 2)** ***(3,17)***. At low concentrations of vinblastine or nocodazole, where all chromosomes have attained metaphase alignment but where spindle tension is inhibited, the distance between sister kinetochores is decreased relative to the distance in a normal metaphase (**Fig. 1B**) (*see* **Subheading 3.3.3.**). In cells which have attained microtubule attachment and metaphase chromosome alignment but which lack spindle tension, Bub1 but not Mad2 is strongly present on kinetochores **(Fig. 2)** ***(3)***.

3.3.1. General Protocol of Sample Preparation for Immunofluorescence Microscopy

1. Grow cells on coverslips as described above (**Subheading 3.1.**).
2. Following transfection with expression vectors (expression is generally optimal two days after transfection) or treatment with vinblastine, if any, fix cells for 20 min at 37°C (*see* **Note 3**) with 2% paraformaldehyde in PBS. Medium can be aspirated from the culture dish and fixative added directly, or coverslips can be transferred to a separate container for fixation.

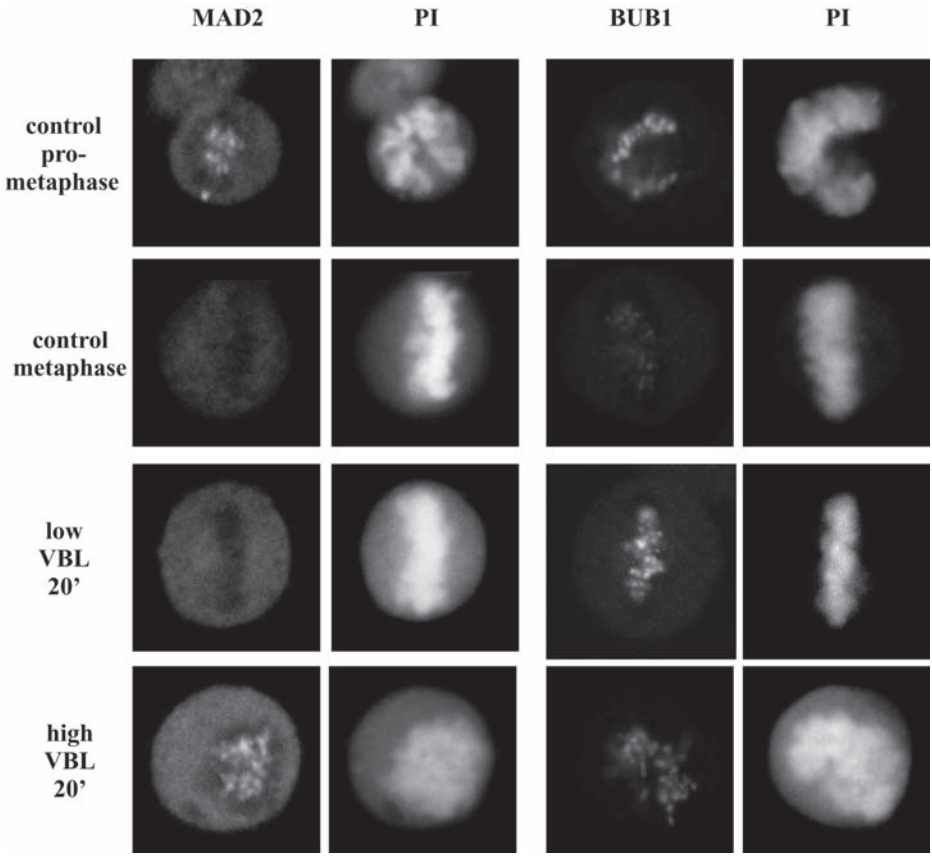

Fig. 2. Unattached kinetochores and the absence of spindle tension can be detected by immunofluorescence microscopy. Both Mad2 and Bub1 display strong signals at kinetochores in cells that lack bipolar attachment of microtubules either in control pro-metaphase cells or in cells treated with 1.5 µg/mL vinblastine for 20 min (high VBL 20'). By contrast, Bub1, but not Mad2, is increased at kinetochores in metaphase cells that lack spindle tension following treatment with 20 ng/mL vinblastine for 20 min (low VBL 20') relative to control metaphase cells.

3. Following fixation, place cells in PBS. At this point, cells can be stored at 4°C, with the addition of 0.04% sodium azide.
4. Permeabilize cells 3 min at RT with 0.2% Triton X-100 in PBS (*see* **Note 4**).
5. Rinse or wash with PBS.
6. Incubate with primary antibodies (*see* **Subheading 3.3.2.**) for 1 h at 37°C in antibody buffer. Place coverslips on a level sponge surface or on parafilm in a covered container, then apply 50 µL of diluted antibody solution to each coverslip.

7. Wash coverslips with PBS three times for 5 min per wash. Aspirate solutions from coverslips under vacuum using a fine pipet tip, and add 50 µL of PBS.
8. Dilute secondary antibodies 1:250 in antibody buffer and incubate at 37°C for 30 min. For double-label immunofluorescence microscopy, anti-tubulin antibody should be detected with Texas Red-conjugated anti-mouse IgG secondary antibody. Beginning with this step, protect cells from unnecessary exposure to ambient light. A convenient method is to cover the incubation chamber with aluminum foil.
9. Wash coverslips 5 min with PBS (as described above in **step 7**).
10. If visualization of DNA is desired, incubate with either DAPI (0.2 µg/mL) or propidium iodide (0.2 µg/mL) in PBS for 5 min. Peak excitations are at 461 nm and 617 nm for DAPI and prodium iodide, respectively.
11. Wash coverslips with PBS twice for 5 min per wash (as described above in **step 7**).
12. Mount coverlips on microscope slides by inverting over a drop (approx 10 µL) of Vectashield mounting medium. Aspirate excess PBS wash solution from coverslips prior to their inversion.
13. To remove any excess mounting medium from the slide, place a folded Kimwipe over the slide, and position an extra microscope slide over the slide that contains the coverslip, then apply light pressure.
14. Seal the coverslip on the slide with a light coat of nail polish at the edge. This will position the coverslip and improve preservation of the sample. Allow to dry for 5–10 min.
15. The sample can be immediately examined with a fluorescence microscope or can be stored indefinitely at –20°C.

3.3.2. Primary Antibodies for Immunofluorescence Microscopy

Detailed below are specific primary antibodies and their applications in the analysis of the spindle-assembly checkpoint.

1. Tubulin. Utilize for determination of the degree of spindle assembly and the state of mitotic progression. Dilute 1:400.
2. Centromere. Utilize to assess chromosome alignment and spindle tension at metaphase, and in double-label immunofluorescence with anti-tubulin antibodies to determine whether off-plate chromosomes lack microtubule attachment to either kinetochore. Dilute 1:100.
3. Mad2 and Bub1 (kinetochore-associated mitotic checkpoint proteins). Utilize to assay for activation of checkpoint signaling under conditions of non-attached centromeres (Mad2 or Bub1) or absence of spindle tension at metaphase (Bub1) **(Fig. 2)** ***(3)***. Dilute Mad2 and Bub1 antibodies 1:100.
4. MPM-2. Utilize to verify mitotic exit ***(6,10)***. Dilute 1:200.

3.3.3. Measurement of Sister Kinetochore Separation

Under conditions of spindle tension, sister kinetochores are more separated than in the absence of tension **(Fig. 1)** ***(3,4,17)***. Given that cells can arrest either without microtubule attachment to kinetochores ***(1,17)*** or without spindle

tension at attached kinetochores ***(3,4,17)***, this assay is employed to determine the nature of checkpoint arrest.

1. Set up cells for comparison of vinblastine-treated and untreated controls, as described in **Subheading 3.1.**. This analysis relies upon comparison to the kinetochore separation in untreated control cells. Alternatively, comparison can be made between untreated controls and cells either expressing a dominant-negative mutant or transfected with siRNA targeting a specific protein—for example, those expected to have an effect on the function of the mitotic apparatus ***(13–15)***.
2. Fix cells, as outlined in **Subheading 3.3.1., step 2**, and prepare for immunofluorescence microscopy using anti-centromere antibodies.
3. Using confocal microscopy or deconvolution microscopy, obtain defined z-plane images of kinetochore pairs from metaphase-aligned chromosomes. Utilizing restricted z-section images allows easier and more definite identification of sister kinetochore pairs.
4. Utilize image analysis software to measure the distance between the outer edges of each kinetochore that is a member of a resolvable pair.
5. Conduct these measurements for at least 15 cells.
6. Results can be displayed as a bar graph of the number of kinetochore pairs with a specified separation distance (in μ*M*). An example is given in **Fig. 1B**.

3.4. Quantitation of Mitotic Arrest by Flow Cytometry

While the mitotic index can be determined by visual counting with the aid of a microscope, quantitation of the percent mitotic cells using multi-parametric flow cytometry has several advantages. First, flow cytometry allows counts to be easily made in a large population. This leads to more accurate quantitation. Also, accuracy of the count improves, as quantitation by microscopy is subject to underestimation due to the fact that detached mitotic cells are not counted.

3.4.1. Preparation for Flow Cytometry

1. A minimum of 10^6 cells/sample are required. This number is approximately what is required to ensure that enough cells remain for analysis following antibody incubations and wash steps.
2. Harvest cells. For HeLa cells, collect culture medium as well and place in a 15-mL conical tube (*see* **Note 5**). Cells are trypsinized, and the dish is washed gently with the previously collected culture medium. Centrifuge 2 min at 300*g*.
3. For fixation, cells are first resuspended in 1 mL of PBS (*see* **Note 6**). To this add 9 mL of absolute methanol at –20°C, immediately cap, and mix by inversion several times. Incubate at –20°C for a minimum of 10 min. Alternatively, cells can be stored in fixative at –20°C indefinitely.
4. Allow cells to return to ambient temperature and collect by centrifugation at 300*g* for 2 min.
5. Wash each sample with 2 mL of PBS and collect cells by centrifugation at 300*g* for 2 min.

6. Add either MPM-2 or phospho-(Ser10)-histone H3 primary antibody for recognition of mitotic cells (*see* **Subheading 3.4.2.**). Resuspend cells in 400 µL of antibody diluted (1:200 for MPM-2 and 1:150 for phospho-histone H3) in antibody buffer (**Subheading 2., step 9**).
7. Incubate for 1 h at 37°C with gentle agitation.
8. Wash once with 2 mL PBS (centrifuge 2 min at 300*g*).
9. Add 400 µL secondary antibody (FITC-conjugated anti-mouse or anti-rabbit IgG antibodies for detection of MPM-2 and phospho-histone H3 primary antibodies, respectively). Dilute 1:500 in antibody buffer and incubate for 30 min at 37°C.
10. Prepare DNA labeling solution during incubation with secondary antibody. Add RNase A and propidium iodide to PBS at 30 Kunitz Units/mL and 50 µg/mL, respectively. Prepare 500 µL labeling solution for each sample.
11. Collect cells by centrifugation at 300*g* for 2 min.
12. To each sample, add 500 µL of labeling solution and incubate in a 37°C waterbath for 10 min.
13. Place samples on ice and cover with aluminum foil pending collection of flow cytometric data.

3.4.2. Selection of Primary Antibody for Flow Cytometric Quantitation of Mitotic Cells

Recognition of mitotic cells relies upon antibodies that detect mitosis-specific phospho-epitopes. Two antibodies are available that are suitable for this purpose. MPM-2 antibody recognizes phospho-antigens of a group of proteins that are specifically phosphorylated in mitosis ***(18)***. Another antibody that is useful recognizes the serine 10 phospho-epitope on histone H3, which is specific to mitosis ***(19)***. Both antibodies have been used for recognition of mitotic cells by flow cytometry ***(6,10,20–23)***. As shown in **Fig. 3**, results are comparable between the two antibodies. Since MPM-2 antibody recognizes a class of phosphorylated proteins, this antibody is preferable to the histone H3 antibody. The phospho-(Ser 10)-histone H3 epitope can potentially be uncoupled from the mitotic state in the case of direct interference with the specific phosphorylation of histone H3—for example, through suppression of Aurora B kinase activity ***(24,25)***.

3.4.3. Data Collection by Flow Cytometry

As shown in **Fig. 4A,** three data windows are required for collection of flow cytometric results for mitotic quantitation. These windows include a dot plot that allows gating to selectively analyze single cells, a histogram of DNA content, and a dot plot showing mitotic signal vs DNA content. Normally, 10^4 events are collected. The signal for the mitotic marker can be shown either on a logarithmic or linear scale (*see* **Fig. 4B**). Choice of scale is governed by the degree of signal separation in mitotic and non-mitotic cells, and by the degree

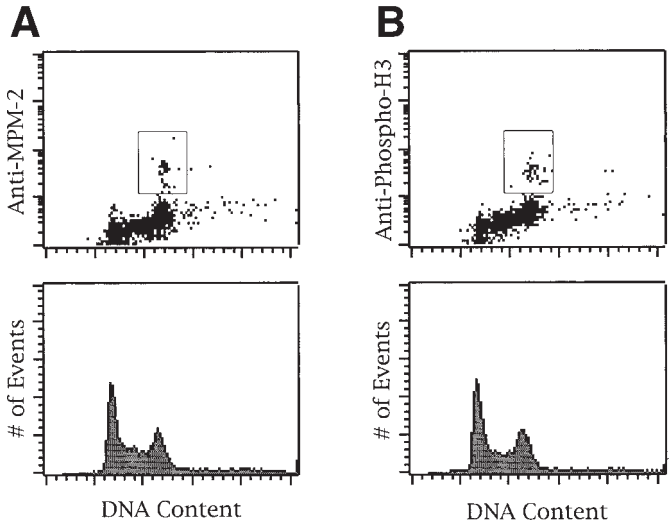

Fig. 3. Mitotic cells can be distinguished by MPM-2 or phospho-(Ser10)-histone H3 antibodies. Mitotic cells in untreated HeLa populations are detected by flow cytometry in dot plots with either **(A)** MPM-2 antibody or **(B)** phospho-(Ser10)-histone H3 antibody. For either MPM-2 signal or phospho-(Ser10)-histone H3 signal a logarithmic scale is shown. Only a subset of cells with 4N DNA content, detected with propidium iodide, have an elevated signal for either MPM-2 or phospho-(Ser 10)-histone H3 (indicated with a box). The corresponding histograms, displaying the number of cells with a particular DNA content, are shown below each dot plot and are equivalent for samples analyzed with either antibody.

of signal spread in positive cells. For HeLa cells, a logarithmic scale yields an optimal presentation.

4. Notes

1. The 8% paraformaldehyde stock solution should be used no longer than 30 d.
2. Mitotic HeLa cells detach relatively easily from coverslips, particularly after treatment with anti-microtubule agents. Maximum retention of mitotic cells on coverslips can be achieved by pre-treatment of coverslips with poly-L-lysine and by growth of cells on coverslips for 36 h or longer prior to manipulation.
3. Given the cold-lability of microtubules, for optimal preservation, fixative solution should be pre-warmed to 37°C, and fixation should be carried out at 37°C (in an incubator).
4. For the optimal retention of mitotic HeLa cells on coverslips, the permeabilization time should be controlled carefully (i.e., do not incubate beyond 3 min).

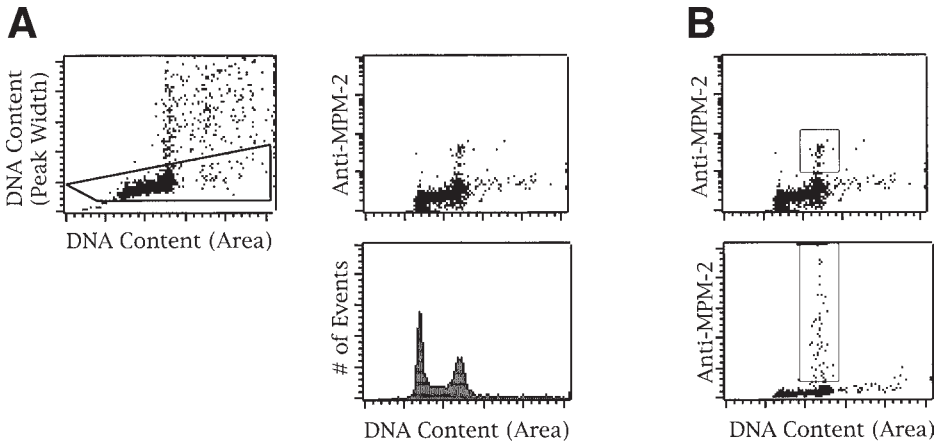

Fig. 4. Setup of data windows for flow cytometery. **(A)** Three windows are required for mitotic quantitation. Displays shown here were generated with a Becton-Dickenson FacsCalibur flow cytometer and CellQuest software. A dot plot with the signal peak width (FL2-W) "DNA Content (Peak Width)" vs the integrated peak intensity (FL2-A) "DNA content (Area)" for the propidium iodide signal is used to gate out aggregated cells (left column). The example has a box indicating selected singlet cells. Mitotic cells are detected in a dot plot of MPM-2 vs DNA Content (right column, above). A histogram displaying the number of cells with a particular DNA content is also shown (right column, below). For each display window, the ordinate value uses the same FL2-A setting based upon the integrated peak area. **(B)** Dot plots using either a logarithmic (top dot plot) or linear (bottom dot plot) scale for the MPM-2 signal are shown plotted vs. DNA content (FL2-A). In each dot plot, the mitotic cells are indicated with a box.

5. Mitotic cells treated with vinblastine or nocodazole readily detach from the culture dish. To ensure that all cells are included in the count, culture medium containing any detached cells is collected and pooled with cells collected by trypsinization.
6. To prevent cell aggregation, which will decrease the number of quantifiable cells and interfere with efficient data collection, cells are resuspended in PBS prior to exposure to methanol. Fixation with a final concentration of 90% methanol yields histograms with minimal peak broadening ***(26)***.

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16

Functional Analysis of the Spindle-Checkpoint Proteins Using an In Vitro Ubiquitination Assay

Zhanyun Tang and Hongtao Yu

Summary

The spindle checkpoint helps to ensure the fidelity of chromosome segregation during mitosis and meiosis. In response to sister chromatids not properly attached to the mitotic spindle, this checkpoint blocks the activity of a large ubiquitin protein ligase complex, called the anaphase-promoting complex (APC) or cyclosome. This chapter describes the detailed protocols of an in vitro ubiquitination assay reconstituted with purified APC, cofactors, other ubiquitination enzymes, and the spindle-checkpoint proteins. This assay is extremely useful in dissecting the biochemical functions of various spindle-checkpoint proteins.

Key Words: Ubiquitination; mitosis; chromosome segregation; spindle checkpoint; anaphase-promoting complex; APC; Cdc20; Mad2; BubR1.

1. Introduction

The genetic stability of an organism depends on accurate partitioning of the duplicated genetic materials between the two progenies during each cell division ***(1,2)***. Errors in chromosome segregation during mitotic or meiotic cell divisions result in aneuploidy (abnormal numbers of chromosomes), which leads to cancer or birth defects in mammals ***(3,4)***. The spindle checkpoint is a surveillance mechanism that halts the cell cycle in response to misaligned chromosomes, thus ensuring the fidelity of chromosome segregation during mitosis and meiosis ***(5,6)***. It has become increasingly clear that the spindle checkpoint detects sister chromatids not yet captured by microtubules, and those that lack tension exerted by the microtubules ***(5,6)***. Elegant experiments in mammalian cells have revealed that a single unattached kinetochore (protein complexes assembled around the centromeric DNA responsible for microtubule binding) within a cell is sufficient to activate the spindle checkpoint ***(7,8)***. For example, in PtK1 cells, laser ablation of the last unattached kineto-

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

chore relieves the delay of anaphase imposed by the checkpoint *(8)*. Thus, the lone unattached kinetochore must produce an inhibitory signal that diffuses away to block the cell cycle *(9)*.

The molecular components of the spindle checkpoint were initially identified in budding yeast *(10,11)*. Homologs of these checkpoint proteins were later found in vertebrates, including Mad1, Mad2, Mad3/BubR1, Bub1, Bub2, Bub3, and Mps1 **(Fig. 1)** *(5)*. Many of these proteins localize to kinetochores during mitosis in a transient and dynamic fashion—i.e., the kinetochore-bound pools of these proteins exchange rapidly with their cytosolic populations *(12–14)*. These observations suggest that the kinetochores play an essential role in generating checkpoint signals *(5)*. The target of the spindle checkpoint is a large ubiquitin ligase complex, called the anaphase-promoting complex (APC) or cyclosome *(15,16)*. APC, in complex with its substrate-recruiting factor Cdc20, tags securin with polyubiquitin chains to signal its destruction by the proteasome *(15,16)*. Degradation of securin relieves the inhibition of the separase, which then cleaves the Scc1 subunit of the cohesin complex *(1,2)*. The cleavage of Scc1 leads to the loss of cohesion between the sister chromatids and triggers their separation toward opposite poles of the cell *(1,2)*. Through inhibiting APC^{Cdc20}, the spindle checkpoint stabilizes securin, blocks the activation of separase, and prevents sister-chromatid separation until all chromatids have achieved proper attachment to the mitotic spindle *(5)*.

To understand how APC is inhibited by the spindle checkpoint, we and others have developed an in vitro ubiquitination assay reconstituted with purified recombinant enzymatic components and immuno-purified APC *(17–21)*. The effects of the addition of purified recombinant checkpoint proteins into this APC ubiquitination assay were then used to dissect the functions of the spindle-checkpoint proteins. We and others have recently discovered several such APC-inhibitory checkpoint signals **(Fig. 1)** *(19,20,22–24)*. First, both BubR1 and Mad2 bind directly to Cdc20 *(22,23)*. Binding of either BubR1 or Mad2 to Cdc20 is sufficient to inhibit the ligase activity of APC in vitro, presumably by interfering with the substrate-recruiting function of Cdc20 *(22,23,25,26)*. BubR1 and Mad2 possibly function in a single complex in vivo, since a mitotic checkpoint complex (MCC) containing BubR1, Bub3, Mad2, and Cdc20 has been characterized in several organisms *(27–30)*. The MCC inhibits APC more efficiently in vitro than do BubR1 and Mad2 alone *(22,27)*. Therefore, the checkpoint proteins form a complex signaling network to block the action of APC^{Cdc20}.

Despite the recent progress in this area, the mechanism by which the spindle checkpoint inhibits APC^{Cdc20} in response to a lone unattached kinetochore is still largely not understood. The in vitro reconstituted APC ubiquitination assay will undoubtedly provide more insights into this problem in the future. We now describe this important APC ubiquitination assay in detail.

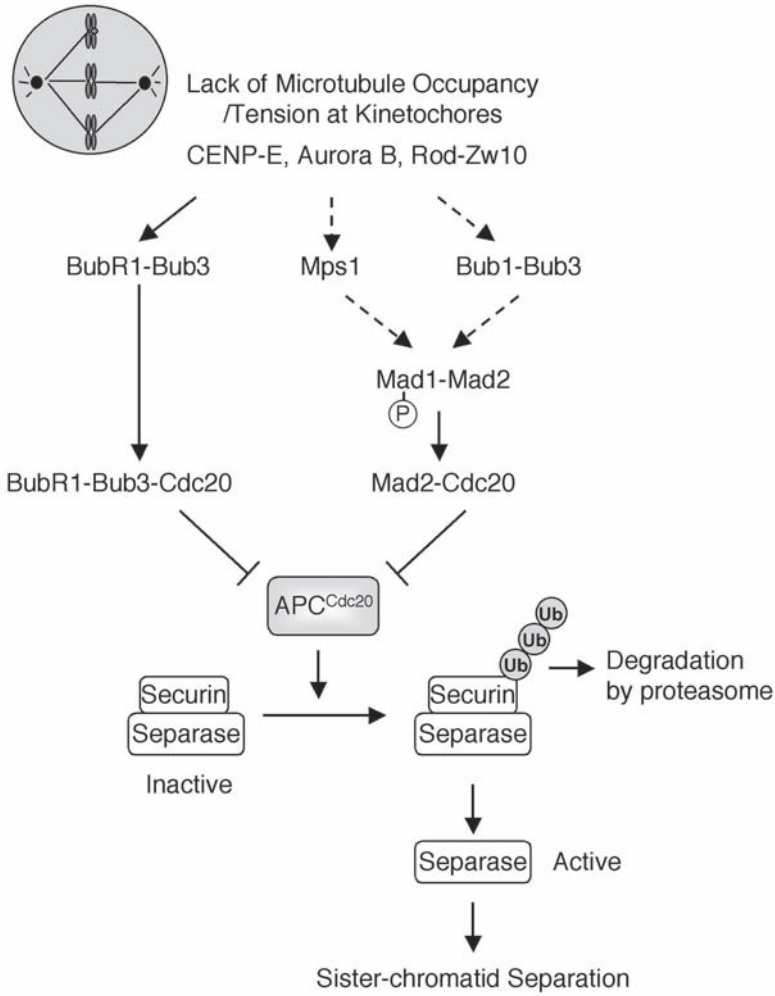

Fig. 1. Molecular mechanism of the spindle checkpoint. To maintain chromosome stability, chromosome segregation occurs only after all sister chromatids have achieved proper attachment to the mitotic spindle. The anaphase-promoting complex (APC) initiates chromosome segregation by ubiquitinating securin, an inhibitor of separase. Degradation of the ubiquitinated securin leads to the activation of separase, which then cleaves the Scc1 subunit of cohesin. The loss of cohesion between the sister chromatids permits their separation and subsequent partition into the two daughter cells. In response to misaligned sister chromatids, the spindle checkpoint establishes an intricate signaling network to block the activity of APC. Inhibition of APC causes stabilization of securin, inhibition of separase, and delay in sister-chromatid separation.

2. Materials

2.1. Preparation of Recombinant Cyclin B, Securin, E1, and E2 (UbcH10) Enzymes in Bacteria

1. Turbo Pfu polymerase (Stratagene).
2. dNTP (Invitrogen), stock concentration 25 m*M* each nucleotide, store at –20°C.
3. Human fetal thymus marathon-ready cDNA library (Clontech).
4. Restriction enzymes (New England Biolabs).
5. T4 DNA ligase (New England Biolabs).
6. pET-28a vector (Novagen).
7. DH5α competent cells (Invitrogen).
8. QIAprep Miniprep kit (Qiagen).
9. QIAquick Gel Extraction Kit (Qiagen).
10. BL21(DE3)pLysS competent cells (Novagen).
11. LB-broth medium (Difco).
12. Isopropyl-β-D-thiogalactoside (IPTG) (Roche).
13. Ni^{2+}-NTA agarose (Qiagen), store at 4°C.
14. Protease inhibitor cocktail: pepstain A, leupeptin, chymostatin (Sigma), stock concentration 1000X: 10 mg/mL each inhibitor dissolved in DMSO, aliquot and store at –20°C.
15. Lysozyme (Sigma), store at –20°C.
16. PD-10 Column (Amersham).
17. Lysis buffer: 50 m*M* Na phosphate, 300 m*M* NaCl, 10 m*M* imidazole, 0.1% Triton X-100; adjust pH to 8.0 using NaOH. Immediately before use, add 1X protease inhibitor cocktail.
18. Wash buffer: 50 m*M* Na phosphate, 300 m*M* NaCl, 20 m*M* imidazole; adjust pH to 8.0 using NaOH.
19. Elution buffer I: 50 m*M* Na phosphate, 300 m*M* NaCl, 50 m*M* imidazole; adjust pH to 8.0 using NaOH.
20. Elution buffer II: 50 m*M* Na phosphate, 300 m*M* NaCl, 100 m*M* imidazole; adjust pH to 8.0 using NaOH.
21. Storage buffer: 50 m*M* Tris-HCl (pH 7.7), 100 m*M* KCl, 1 m*M* DTT, 10% glycerol. Store at 4°C.
22. SDS sample buffer (2X), 40 mL: Mix 2.9 g SDS, 0.4 g Tris base, 12 mL glycerol, 40 mg bromophenol blue, and 620 mg DTT. Add H_2O to 40 mL. Rotate end-over-end to allow SDS to dissolve. Aliquot and store at –20°C.

2.2. Baculoviral Expression of Cdc20 and the Spindle-Checkpoint Proteins in Insect Cells

1. Luria agar (Difco).
2. Gentamicin (Invitrogen), store at room temperature.
3. Bluo-gal (Invitrogen), store at –20°C.
4. Tetracycline (Sigma), store at 4°C.
5. Kanamycin (Sigma), store at room temperature.

6. pFastBac histidine-tagged expression vector (Invitrogen).
7. Fetal bovine serum (Invitrogen), store at –20°C.
8. Sf9 insect cells (ATCC).
9. Hink's TNM-FH insect medium supplemented Grace's (JRH Biosciences), store at 4°C.
10. Pluronic F-68 (Invitrogen), store at room temperature.
11. Sf-900 II SFM (Invitrogen), store at 4°C.
12. Lysis buffer: 50 m*M* Tris-HCl (pH 7.7), 150 m*M* KCl, 0.1% Triton X-100, 1X protease inhibitor cocktail.
13. Wash buffer: 50 m*M* Tris-HCl (pH 7.7), 300 m*M* KCl, 10 m*M* imidazole.
14. Elution buffer I: 50 m*M* Tris-HCl (pH 7.7), 300 m*M* KCl, 50 m*M* imidazole.
15. Elution buffer II: 50 m*M* Tris-HCl (pH 7.7), 300 m*M* KCl, 100 m*M* imidazole.
16. Storage buffer: 50 m*M* Tris-HCl (pH 7.7), 100 m*M* KCl, 1 m*M* DTT, 10% glycerol.

2.3. Immuno-Purification of APC From Xenopus Egg Extracts or HeLa Cell Lysates

1. Affi-gel protein A agarose (Bio-Rad), store at 4°C.
2. Sodium borate (Sigma).
3. Dimethyl pimelimidate (Sigma), store at –20°C.
4. Ethanolamine (Sigma), store at room temperature.
5. Interphase *Xenopus* egg extracts, store at –80°C.
6. Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 2 m*M* L-glutamine (Invitrogen) and 100 units/mL each of penicillin and streptomycin (Invitrogen). Store at 4°C.
7. HeLa S3 cells (ATCC).
8. Trysin-EDTA (Invitrogen), store at –20°C.
9. Thymidine (Sigma). Dissolve in H_2O at a concentration of 200 m*M* (100X). Store at –20°C.
10. XB buffer: 10 m*M* HEPES (pH 7.7), 100 m*M* KCl, 0.1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 50 m*M* sucrose.
11. Wash buffer: XB containing 500 m*M* KCl and 0.5% NP-40.
12. HeLa cell lysis buffer: 50 m*M* Tris-HCl (pH 7.7), 150 m*M* KCl, 1 m*M* DTT, 0.5 μ*M* okadaic acid, 0.5% NP-40, 1X protease inhibitor cocktail, 10% glycerol.

2.4. APC Ubiquitination Assay

1. Bovine ubiquitin (Sigma). Dissolve in water at 10 mg/mL, aliquot, and store at –80°C.
2. Mouse anti-Myc monoclonal antibody (9E10) (Roche), store at 4°C.
3. Anti-mouse Ig, horseradish peroxidase-linked whole antibody from donkey (Amersham), store at 4°C.
4. Tween-20 (Bio-Rad).
5. ECL Western blot detection reagents (Amersham), store at 4°C.
6. Energy Mix (20X), 100 mL: 3.827 g phosphocreatine (150 m*M* final concentration) (Sigma), 1.102 g ATP (20 m*M*) (Sigma), 0.4 mL of 0.5 *M* EGTA (pH 7.7)

(2 m*M*), 2 mL of 1 *M* $MgCl_2$ (20 m*M*), adjust pH to 7.7 with 1 *N* NaOH, aliquot, and store at –80°C.

3. Methods

3.1. Preparation of Recombinant Cyclin B, Securin, E1, and E2 (UbcH10) Enzymes in Bacteria

3.1.1. Construction of the Bacterial Expression Vectors for Ubiquitination Enzymes and APC Substrates

1. Amplify the coding regions of human cyclin B1 (residues 1–102), securin, E1, and E2 (UbcH10) enzymes from human fetal marathon-ready cDNA library by standard polymerase chain reactions (PCR) using Turbo Pfu polymerase with primers that anneal to the 5'- and 3'-ends of these cDNAs and contain the appropriate restriction sites (*see* **Note 1**).
2. Analyze the PCR products by electrophoresis on a 1% agarose/EtBr gel. Excise the bands on the gel corresponding to the correct size of the genes. Extract the DNA from the gel slices by the QIAquick Gel Extraction Kit per manufacturer's protocol.
3. Digest the pET28 vector and the purified DNA fragments with the appropriate restriction enzymes. Repeat **step 2**.
4. Ligate the gene inserts into pET28 using standard protocols.
5. Transform DH5α competent cells with the ligation mixture. Plate 100 µL of each transformation reaction directly onto separate LB agar plates containing 50 µg/mL of kanamycin. Incubate overnight at 37°C.
6. Pick up several single bacterial colonies and transfer into 3 mL LB medium supplemented with 50 µg/mL of kanamycin. Grow at 37°C overnight with shaking at 250 rpm. Isolate plasmids using QIAprep Miniprep kit per manufacturer's protocols.
7. Verify the identity of the recombinant plasmids first with restriction digestion, then by DNA sequencing.

3.1.2. Expression and Purification of Cyclin B, Securin, E1, and E2 (UbcH10) Enzymes in Bacteria

1. For protein expression, transform the pET28 constructs into BL21(DE3)pLysS-competent cells (*see* **Note 2**).
2. Inoculate a single bacterial colony into 100 mL LB medium containing 50 µg/mL of kanamycin and 30 µg/mL of chloramphenicol. Incubate overnight (preferably for less than 16 h) at 37°C with shaking at 250 rpm.
3. Transfer 10 mL of the overnight cell culture to 1 L LB medium in the presence of 50 µg/mL of kanamycin and 30 µg/mL of chloramphenicol.
4. Grow the cells at 37°C with vigorous shaking until an OD_{600} of 0.6, which typically takes approx 3 h. Take a 50-µL sample and store on ice. Induce recombinant protein expression by the addition of 1.0 m*M* IPTG (final concentration) and

incubate the culture at 25°C with shaking at 250 rpm for an additional 4 h. Take another 50-µL sample.

5. Spin down the cells at 2000*g* for 30 min at 4°C. Discard supernatant and resuspend the cells in lysis buffer. Use 20 mL of lysis buffer for 1 L of bacteria culture. Freeze immediately in liquid nitrogen and store at –80°C until protein purification.
6. Spin down the samples taken before and after addition of IPTG in a microcentrifuge. Aspirate away supernatant. Add 50 µL SDS sample buffer. Check for expression of the recombinant proteins using 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) followed by Coomassie blue staining.
7. For protein purification, thaw the cells for 30 min in cold water. Add lysozyme to 0.1 mg/mL (final concentration) and 1X protease inhibitor cocktail. Incubate on ice for 30 min.
8. Sonicate on ice for 2 min, until the lysate is clear. Centrifuge the lysate at 30,000*g* for 30 min at 4°C. Pass the lysate through a 0.45 µm filter.
9. Wash Ni^{2+}-NTA slurry three times with the lysis buffer. Add 3 mL of the 50% Ni^{2+}-NTA slurry to the cleared lysate from 1 L of cell culture. Mix gently by shaking end-over-end for 60 min at 4°C.
10. Load the mixture of the lysate and the Ni^{2+}-NTA beads on an empty column. Wash the column twice with wash buffer (at least 15 mL for each wash).
11. Elute the proteins bound to the Ni^{2+}-NTA beads twice with elution buffer I (2.5 mL each time) and twice with buffer II (2.5 mL each time). Collect the four elutions in four separate tubes.
12. Check the purities and yields of the proteins. Analyze 5 µL of each of the four elution fractions by 12.5% SDS-PAGE and Coomassie blue staining. If necessary, further purify the eluted proteins on gel filtration or ion exchange chromatography (*see* **Note 3**).
13. Change the buffer of only the fractions that contain the recombinant proteins (as judged by the SDS-PAGE analysis) into the storage buffer using PD10 columns (*see* **Note 4**). Aliquot and freeze proteins in liquid nitrogen. Store at –80°C.

3.2. Baculoviral Expression of Cdc20 and the Spindle-Checkpoint Proteins in Insect Cells

3.2.1. Construction of Baculoviral Expression Vectors Encoding Cdc20 and Spindle-Checkpoint Proteins

Clone the coding regions of human Cdc20 and the spindle-checkpoint proteins including Mad1, Mad2, Bub1, BubR1, Bub3, and Mps1 into the pFastBacHT vectors (Invitrogen) as described in **Subheading 3.1.1.**

3.2.2. Expression of Cdc20 and the Spindle-Checkpoint Proteins in Sf9 Cells

1. Prepare and amplify the recombinant baculoviruses using the Bac-to-Bac system (Invotrogen) per manufacturer's protocols with one important exception. For the

amplification of the baculoviruses, we normally harvest the viral supernatant until the infected Sf9 cells are lysed (typically 5–7 d post-infection), instead of 48 h post-infection. The lysis of the cells increases the titer of the viruses.

2. Grow Sf9 cells in Grace's medium supplemented with 10% FBS and 1% Pluronic F-68.
3. Take a 50-µL sample of the Sf9 cell culture at a density of 2×10^6 cells/mL. Spin down the 50-µL culture sample at 500*g* for 10 min. Discard supernatant. Store the cell pellet at –80°C.
4. Infect Sf9 cells at a density of 2×10^6 cells/mL with recombinant baculoviruses at a multiplicity of infection (MOI) of 5 to 10. We typically use 500 mL of Sf9 cell culture for each protein expression.
5. Incubate at 27°C for 48 h with shaking at 120 rpm.
6. Take a 50-µL sample and store on ice. Harvest the rest of the infected cells by centrifugation at 500*g* for 10 min at room temperature.
7. Discard the supernatant, wash the cells once with PBS, repeat **step 5**, and discard PBS. Store at –80°C.
8. Spin down the 50-µL culture sample taken at **step 6** at 500*g* for 10 min. Aspirate the supernatant. Add 40 µL SDS sample buffer each to the samples taken before (at **step 3**) and after viral infection and boil for 5 min. Load 15 µL for SDS-PAGE analysis followed by Coomassie blue staining (*see* **Note 5**).

3.2.3. Purification of Cdc20 and the Spindle-Checkpoint Proteins in Sf9 Cells

1. Thaw the cells for 30 min in cold water.
2. Add 25 mL of lysis buffer to the cell pellet from 500 mL of Sf9 cell culture. Vortex and incubate on ice for 30 min.
3. Sonicate mildly on ice for 1–2 min.
4. Spin the cell lysate at 30,000*g* for 30 min at 4°C. Collect the supernatant and pass the supernatant through a 0.45-µm filter.
5. Wash Ni^{2+}-NTA slurry three times using lysis buffer.
6. Add 3 mL of 50% Ni^{2+}-NTA slurry to the cleared lysate. Mix gently and incubate with end-over-end shaking for 1 h at 4°C.
7. Load the mixture onto a column. Wash twice with wash buffer (15 mL for each wash).
8. Elute the bound proteins twice with elution buffer I (2.5 mL each elution) and twice with elution buffer II (2.5 mL each elution). Collect the four elutions in four separate tubes (*see* **Note 3**).
9. Check the purity and yield of the proteins by 12.5% SDS-PAGE with Coomassie blue staining. If necessary, further purify the eluted proteins with gel filtration or ion exchange chromatography (*see* **Note 6**).
10. Change buffer into the storage buffer using PD10 columns (*see* **Note 4**). Aliquot and freeze proteins in liquid nitrogen. Store at –80°C.

3.3. Immuno-Purification of APC From Xenopus Egg Extracts or HeLa Cell Lysates

3.3.1. Coupling of Anti-APC3 (Cdc27) Polyclonal Antibody to Affi-Prep Protein A Beads

1. Wash Affi-Prep protein A beads twice with PBS. In general, bind approx 1 mg of purified antibody per mL of solid beads. For crude serum, use a volumetric ratio of 1:1. The antibody coupling protocol is essentially the same as described in ***(31)***.
2. Add 1 mL of crude rabbit anti-APC3 (Cdc27) polyclonal antibody serum to 1 mL of solid Affi-Prep protein A beads.
3. Incubate at room temperature for 1 h with gentle rocking.
4. Wash the beads twice with 10 vols (approx 10 mL) of 200 m*M* sodium borate (pH 9.0) by centrifugation at 200*g* for 2 min.
5. Resuspend the beads in 10 vols of 200 m*M* sodium borate (pH 9.0). Remove the equivalent of 10 µL of beads (approx 100 µL slurry), spin down this bead sample, discard the supernatant, and add 30 µL 2X SDS sample buffer. Store this sample at room temperature for later processing.
6. To the bulk of the antibody beads, add dimethyl pimelimidate (solid) to a final concentration of 20 m*M*.
7. Incubate at room temperature for 30 min with end-over-end shaking. Remove the equivalent of 10 µL of beads, spin down this bead sample, discard the supernatant, and add 30 µL 2X SDS sample buffer. Store this sample at room temperature for later processing.
8. Stop the reaction by washing the bulk of the beads once with 10 mL of 200 m*M* ethanolamine (pH 8.0). Then add 10 mL of 200 m*M* ethanolamine (pH 8.0) and incubate for 2 h at room temperature with end-over-end shaking.
9. Wash beads once with 10 mL of 100 m*M* glycine (pH 2.5) and then quickly wash beads three to four times with PBS (pH 7.4) (10 mL each wash).
10. After the final wash, resuspend the beads in PBS with 0.02% NaN_3. Store at 4°C.
11. Check the efficiency of coupling by boiling the two samples of beads taken before and after coupling in 2X SDS sample buffer. Analyze both samples by 12.5% SDS-PAGE with Coomassie blue staining. Good coupling is indicated by the presence of the Ig heavy chain band (at around 55 kD) in the "before coupling" but not in the "after coupling" lanes.

3.3.2. Immuno-Purification of APC From Xenopus Egg Extracts

1. Prepare *Xenopus* egg extracts as described in ***(32)***.
2. Incubate the desired amount of anti-APC3 (Cdc27) protein A beads with 10 vols of interphase *Xenopus* egg extract for 2 h at 4°C with gentle end-over-end shaking. Do not vortex the mixture (*see* **Note 7**).
3. Spin down beads at 6000 rpm in a microcentrifuge for 30 s. Discard the supernatant. Wash the anti-APC3 beads five times with 10 vols of XB buffer containing 500 m*M* KCl and 0.5% NP-40, and twice with 10 vol of XB only.

4. Add 2 vol of 0.1 mg/mL purified human Cdc20 protein (from **Subheading 3.2.3.**) and ten vol of XB buffer to the interphase APC beads. Incubate for 1 h at room temperature with gentle rocking.
5. After incubation, wash the APC beads twice with 10 vol of XB and assay immediately for cyclin B ubiquitination activity (*see* **Note 8** and **Subheading 3.4.**).

3.3.3. Immuno-Purification of APC From HeLa Cell Lysates

1. Grow 10 150-mm plates of HeLa cells in DMEM growth medium.
2. When cells have grown to 80% confluency, add thymidine to a final concentration of 2 m*M* for 18 h.
3. Wash the cells twice with warm PBS. Then culture the cells in fresh medium without thymidine for 8 h.
4. Incubate the cells with thymidine at 2 m*M* final concentration for another 18 h.
5. Wash cells twice with warm PBS again, transfer into fresh medium, and harvest at various time points (e.g., 0, 2, 4, 6, 8, 10, 12, 14 h). Harvest one plate of cells per time point. Store the cell pellets at –80°C.
6. Thaw the cell pellets in cold water. Add four cell pellet vols of HeLa cell lysis buffer. Vortex briefly and incubate on ice for 30 min. Lyse the cells by passing the lysate through a 22-gage needle several times.
7. Spin the lysate at top speed in a microcentrifuge for 1 h at 4°C. Transfer the supernatant to fresh tubes and discard the pellet.
8. Incubate the anti-APC3 (Cdc27) beads with 10 vols of the supernatant of HeLa cell lysate for 2 h at 4°C.
9. Wash the APC beads five times with 10 vol of XB containing 500 m*M* KCl and 0.5% NP-40, and twice with 10 vol of XB.
10. Add 2 vol of 0.1 mg/mL purified human Cdc20 protein (from **Subheading 3.2.3.**) and 10 vol of XB buffer to the HeLa APC beads. Incubate for 1 h at room temperature with gentle rocking.
11. After incubation, wash the APC beads twice with 10 vol of XB and assay immediately for cyclin B ubiquitination activity **(Subheading 3.4.)**.

3.4. APC Ubiquitination Assay

3.4.1. Ubiquitination Assay With Xenopus or HeLa APC

1. Prepare ubiquitination reaction mixture: 1X energy mixture, 150 μ*M* bovine ubiquitin, 5 μ*M* Myc-tagged N-terminal fragment of human cyclin B1, 5 μ*M* human E1, 2 μ*M* UbcH10. For assays with securin as the substrate, replace cyclin B1 with 5 μ*M* Myc-tagged securin protein in the ubiquitination mixture (*see* **Note 9**).
2. Add 5 μL of the ubiquitination mixture to each reaction containing 4 μL of the APC beads (1:1 slurry in XB buffer) prepared as described in **Subheadings 3.3.2.** and **3.3.3.**. Incubate for 1 h at room temperature with gentle rocking.
3. Quench the reactions with 2X SDS sample buffer, and analyze by SDS-PAGE followed by immunoblotting with mouse anti-Myc antibody. The activity of APC can be quantified by the intensity and molecular weight of the cyclin-ubiquitin conjugates (*see* **Fig. 2**).

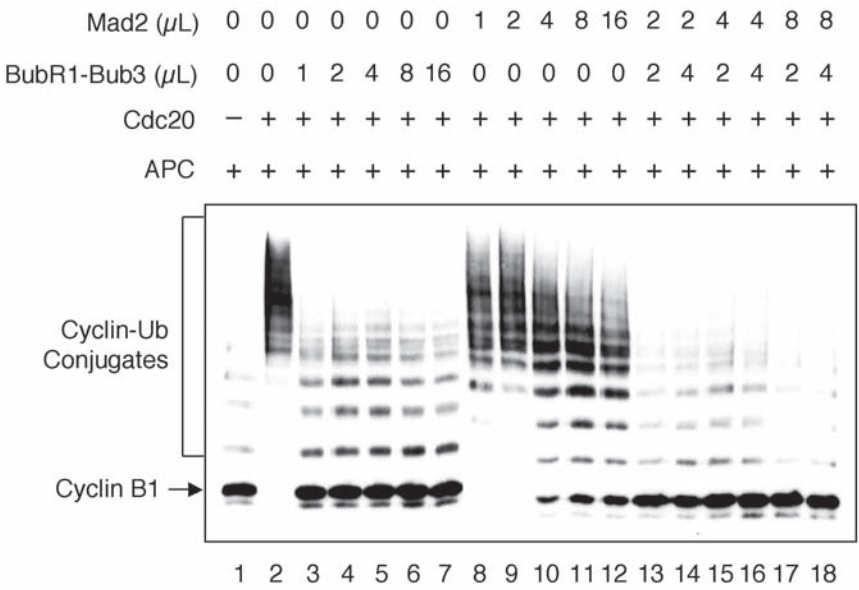

Fig. 2. BubR1 and Mad2 cooperate to inhibit APC^{Cdc20}. APC was isolated from mitotic *Xenopus* egg extracts and incubated with recombinant human Cdc20 protein, in the presence of buffer (lane 2) or varying amounts of recombinant bacterially expressed Mad2 oligomer and BubR1–Bub3 from Sf9 cells (lanes 3–18). The ubiquitination activity of APC was assayed with a Myc-tagged N-terminal fragment (residues 1–102) of human cyclin B1. The reaction mixtures were separated by SDS-PAGE and blotted with the anti-Myc antibody. The positions of the cyclin B1 substrate and the cyclin B1–ubiquitin conjugates are labeled. For the ease of labeling, the volumes of the Mad2 and the BubR1–Bub3 proteins are indicated instead of the molar concentration. Addition of 1 μL of Mad2, Bub1–Bub3, or BubR1–Bub3 is equivalent to 50 n*M* of each protein.

3.4.2. Effect of the Spindle-Checkpoint Proteins on the APC Ubiquitination Activity

1. Purify interphase *Xenopus* or HeLa APC as described in **Subheadings 3.3.2.** and **3.3.3.**.
2. Set up the checkpoint inhibition assay reactions as follows: Aliquot the same amount of Cdc20 (5 μL of 0.1 mg/mL Cdc20) to each reaction tube. Add different amounts of the checkpoint proteins (e.g., Mad2 or the BubR1–Bub3 complex). Use Cdc20 alone and XB alone as positive and negative controls, respectively. Add XB to make up the volume of all reactions to 50 μL and mix thoroughly (*see* **Note 10**).

3. Incubate these protein mixtures at room temperature for 1 h.
4. Add 4 µL of the APC beads (1:1 slurry in XB buffer) to each inhibition assay reaction mixture. Incubate at room temperature for 1 h with gentle rocking.
5. Wash twice with 400 µL of XB and assay immediately for cyclin B ubiquitination activity as described in **Subheading 3.4.1.**.

4. Notes

1. The length and GC ratio of gene-specific primers are important for successful PCR. When the Tm of the primer is over 70°C, touchdown PCR is recommended. Touchdown PCR can significantly improve the specificity of many PCR reactions. It involves using an annealing/extension temperature that is several degrees (typically 3–10) higher than the Tm of the primers during the first several PCR cycles. The annealing/extension temperature is then reduced to the primer Tm for the remaining PCR cycles.
2. We normally use the host strain BL21(DE3)pLysS for protein expression. BL21 has the advantage of being deficient in both *lon* and *ompT* proteases. The lDE3 lysogen encodes the T7 polymerase that is required for the transcription of the T7-promoter-driven pET vectors. The BL21(DE3)pLysS strain also carries the pLysS plasmid that encodes T7 lysozyme. T7 lysozyme is a potent inhibitor of T7 polymerase. Therefore, the background expression of pET vectors in the BL21(DE3)pLysS strain in the absence of IPTG is further suppressed by the low levels of T7 lysozyme. This is especially beneficial for the expression of proteins that are toxic for the bacteria.
3. Elution buffers containing 50 or 100 m*M* imidazole are sufficient to elute the majority of proteins from the Ni^{2+}-NTA beads. We have found that the proteins eluted using 50 or 100 m*M* imidazole typically contain less aggregated species and exhibit better biological activities. Proteins eluted at higher concentrations of imidazole (e.g., 250 m*M* used in many protocols) contain a higher percentage of the aggregated forms. Furthermore, higher concentrations of imidazole have adverse effects on the stability of certain proteins and should be avoided. For E1, UbcH10, securin, and the N-terminal fragment of cyclin B1 expressed in bacteria, the yields are in the range of 1–10 mg/L of culture with better than 90% purity after a single Ni^{2+}-NTA affinity column. Because the bacteria do not possess any components of the ubiquitination machinery, this purity is typically enough for all subsequent assays. However, if the purity of these proteins is less than 80%, further purification is necessary. Owing to their relatively small molecular weights, we find that UbcH10, securin, and the N-terminal fragment of cyclin B1 can be effectively purified with a Superdex-75 gel filtration column (Amersham).
4. Dialysis can be used instead of PD columns to exchange the buffer of the eluted proteins. However, we have found that for certain proteins, such as Cdc20, BubR1, and Bub1, extended presence of these proteins (e.g., several hours) in the imidazole-containing buffers causes the precipitation of significant portions of these proteins. It is thus desirable to change the buffers of these proteins using the PD10 columns, which takes minutes to complete.

5. The expression of the checkpoint proteins should be apparent as judged by Coomassie staining. If the expression of the protein is not apparent, perform western blotting using specific antibodies against each protein to verify expression. However, if the expression of the recombinant protein cannot be detected by Coomassie staining, its yield will very likely be less than satisfactory. Prior to subsequent purifications, the expression of the proteins should then be optimized by further amplifying the viruses to increase their titers and/or by initiating fresh Sf9 cell cultures for protein expression.
6. For the expression of Cdc20 and the spindle-checkpoint proteins in Sf9 cells, the yield and purity after a single Ni^{2+}-NTA column are typically lower as compared to the recombinant proteins expressed in bacteria. Among these proteins, Cdc20 and Mad1 are the most difficult cases. The yield is typically around 0.5 mg per 500 mL Sf9 cell culture, with the purity in the 50–70% range. In addition, as a result of their low solubilities, the concentration of Cdc20 and Mad1 should be kept at 0.1–0.2 mg/mL. The recombinant Cdc20 and Mad1 proteins also fractionate poorly on both gel filtration and anion exchange columns. Further purification of Cdc20 and Mad1 is not recommended. For Mad2, Bub1, BubR1, Bub3, and Mps1, the yield is typically in the range of 1–5 mg per 500 mL Sf9 cell culture, and the purity is around 80% after the Ni^{2+}-NTA column. These proteins can be further purified by gel filtration chromatography. We typically use a Superdex-75 column (Amersham) for Mad2 purification and a Superose-6 column (Amersham) for Bub1, BubR1, Bub3, and Mps1. It should be noted that functional Mad2 protein can also be expressed in bacteria.
7. For each ubiquitination reaction, we use 2 μL of solid APC beads. Therefore, the total volume of anti-APC3 beads used in this step is determined by the total number of ubiquitination reactions. However, we typically use a minimum of 40 μL of anti-APC3 beads and 400 μL of interphase *Xenopus* egg extract for the immunoprecipitation in a 500 μL Eppendorf tube. If more ubiquitination reactions are needed, volumes of anti-APC3 beads and *Xenopus* extract can be scaled up accordingly. Mix the beads and the extracts by gently tapping the tubes with fingers. Do not vertex to mix the contents. The *Xenopus* egg extracts contain complex organelles that may be disrupted by strong mechanical force. This will perturb the cell cycle status of these extracts and may lead to the loss of APC activity.
8. APC can also be isolated from the mitotic *Xenopus* egg extract and substituted for the interphase *Xenopus* APC in the ubiquitination assay. To prepare mitotic *Xenopus* egg extracts, add Δ90 clam cyclin B (a mutant form of cyclin B lacking the N-terminal 90 residues) to interphase *Xenopus* egg extract and incubate for 90 min at room temperature. The mitotic extracts can then be aliquoted and stored at –80°C. Preparation of mitotic APC beads is the same as that of the interphase APC beads.
9. In the APC ubiquitination assays, ^{35}S-labeled cyclin B and securin translated in vitro in reticulocyte lysate can also be used instead of the purified Myc-tagged recombinant proteins produced in bacteria. In this case, the coding regions of

cyclin B and securin are cloned into expression vectors containing the SP6 or T7 promoter (e.g., pCS2 or pCDNA3 vectors), which are then translated in vitro using TNT SP6 or T7 Quick Master Mix (Promega). Add 1 µL of the in vitro translated cyclin B or securin proteins to each ubiquitination reaction. The reaction mixture is then separated by SDS-PAGE and analyzed by autoradiography using x-ray film or by a PhosphorImager.

10. In addition to Cdc20, Cdh1 can also activate the APC. APC^{Cdc20} ubiquitinates securin, which is required for proper chromosome segregation. APC^{Cdh1} mediates the degradation of cyclin B1, which is required for mitotic exit. Unlike APC^{Cdc20}, APC^{Cdh1} is not directly inhibited by the spindle-checkpoint proteins Mad2 and BubR1 ***(23)***. Thus, APC^{Cdh1} can be used as an important control for the reconstituted checkpoint inhibition assay. To assay the activity of APC^{Cdh1}, Cdh1 can be added to the interphase *Xenopus* or HeLa APC instead of Cdc20. The Cdh1 protein can be expressed and purified from Sf9 insect cells using a strategy similar to the expression of Cdc20.

Acknowledgments

This work is supported by the National Institutes of Health (GM61542), the Packard Foundation, the Burroughs Wellcome Fund, and the Robert A. Welch Foundation (I-1441). H. Y. is the Michael L. Rosenberg Scholar in Biomedical Research.

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Analysis of Checkpoint Responses to Histone Deacetylase Inhibitors

Heather Beamish, Robyn Warrener, and Brian G. Gabrielli

Summary

Cell cycle checkpoints respond to a wide range of stresses to prevent compromise to the integrity of the cell. The best studied checkpoints are those induced by genotoxic agents that cause DNA damage. Histone deacetylase inhibitors not only increase the acetylation state of chromatin histones, but they also perturb the cell cycle, causing both G1 and G2 phase arrests, the latter by initiating a checkpoint response. In this chapter we will describe the analysis of the histone deacetylase inhibitor-sensitive G2 checkpoint using synchronized cell populations.

Key Words: Histone deacetylase inhibitor; G2 checkpoint; acetylation; histone; mitosis.

1. Introduction

Cell cycle checkpoints are mechanisms that respond to a diverse range of stresses that could ultimately compromise cell integrity. Histone deacetylase inhibitors (HDACIs) are a class of drugs that block the deacetylation of proteins, the best characterized being the chromatin histone proteins, although an increasing number of nonhistone proteins have also been shown to be modified by acetylation ***(1)***. HDACI treatment of cells often results in perturbations of the cell cycle, notably cell cycle arrests in G1 and G2 phase. The G1 arrest resulting from HDACI treatment can be in part ascribed to transcriptional up-regulation of the Cdk2 inhibitor $p21^{Waf1/Cip1}$, a direct result of increased histone acetylation affecting transcription factor accessibility to the promoter of p21 ***(2)***. On the other hand, the mechanism by which HDACI treatment imposes the G2 phase arrest is not clear, but a checkpoint response is initiated as opposed to the transcriptional up-regulation of an inhibitor of CDK activity ***(3)***. Activation of the G2 checkpoint is dependent on the concentration of HDACI used ***(2,4)***. Many immortalized and tumor cell lines are defective for this checkpoint, but are competent to impose a G2 checkpoint

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

arrest in response to other agents, such as DNA-damaging drugs, indicating that this checkpoint is distinct from other G2 checkpoint mechanisms ***(3)***. A consequence of failure of this checkpoint following HDACI treatment is that cells undergo an aberrant mitosis and often fail cytokinesis, resulting in cells with tetraploid DNA which have in fact exited mitosis and entered the subsequent cell cycle. In this chapter, we will outline methods for analyzing the effects of HDACI treatment on the cell cycle, primarily examining the G2 checkpoint response.

2. Materials

1. Dulbecco's modified Eagle's medium (DMEM; GIBCO), supplemented with 10% (v/v) donor calf serum.
2. Trichostatin A (TSA; Sigma-Aldrich).
3. Sodium butyrate (NaBu; Sigma-Aldrich): 20 m*M* stock solution in H_2O, make freshly before addition.
4. Azelaic bishydroxamic acid (ABHA) (generously synthesized by Mike West, Institute of Molecular Biosciences, University of Queensland), dissolved in DMSO at a concentration of 100mg/mL.
5. Thymidine: 100 m*M* stock solution in PBS. This will last for 1 wk at 4°C, stock solutions are maintained at –20°C.
6. 2-deoxycytidine: 10 m*M* stock solution in PBS, store at 4°C for 10 d.
7. Hydroxyurea: 400 m*M* stock solution in PBS, for long-term storage keep at –20°C.
8. Propidium iodide (PI): 1 mg/mL stock solution in H_2O, store at 4°C in the dark to prevent photo-bleaching.
9. RNase A: 10 mg/mL stock solution in H_2O, store at –20°C. To inactivate any contaminating DNase present, heat the stock solution to 95°C for 10 min.
10. 4',6-diamidino-2-phenylindole (DAPI): 1 mg/mL stock solution in H_2O, store at 4°C in the dark to prevent photo-bleaching.
11. Bromodeoxyuridine (BrdU): 5 m*M* stock solution in H_2O, store at 4°C.
12. Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody (Becton Dickson). Keep at 4°C in the dark to prevent photo-bleaching.
13. BrdU detection buffer: 0.25% (v/v) Tween-20, 2.5 mg/mL BSA, 0.1 mg anti-BrdU-FITC (Becton Dickson). This buffer should be made when required and be kept in the dark to prevent bleaching of the FITC-conjugated antibody.
14. Antibody to mitotic-specific phosphoproteins: MPM-2 antibody (DAKO Corporation, M3514, 0.3 mg/mL).
15. Flow cytometer.
16. 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich).
17. Microplate reader.
18. NETN buffer: 20 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, 100 m*M* NaCl, 0.5% (v/v) Nonidet P-40, store at 4°C. Immediately before use add 5 µg/mL of each of

leupeptin, aprotinin, and pepstatin, 1 m*M* PMSF (phenylmethylsulfonyl fluoride; 100 m*M* stock in isopropanol; store at –20°C), 10 m*M* NaF (sodium fluoride, stock is 1 *M,* store at –20°C), and 0.1 m*M* Na_3V0_4 (sodium orthovanadate; stock is 20 m*M*, store at –20°C).

19. 5X SDS sample buffer: 0.313 *M* Tris-HCl (pH 6.8), 10% (w/v) SDS (sodium dodecyl sulfate), 50% (v/v) glycerol, 0.5 *M* DTT (dithiothreitol), 0.05% (w/v) bromophenol blue.
20. Polyacrylamide gel electrophoresis equipment.
21. Western blot transfer equipment.
22. 0.2 µ*M* PVDF membrane (NEN Life Science Products).
23. Ponceau S (Sigma).
24. PBS/Tween: 0.05% (v/v) Tween-20 in PBS. Store at room temperature.
25. Blotto: 5% (w/v) dried skimmed milk powder in PBS/Tween. Make fresh, store at 4°C.
26. Protein A–sepharose (Roche).
27. RC buffer: 20 m*M* Tris-HCl (pH 7.4), 15 m*M* $MgCl_2$, 1 m*M* DTT, 0.2 m*M* ATP, 5 µCi [γ^{32}P] ATP, 15 µg histone H1. This buffer contains the radioisotope γ^{32}P; therefore, all work with this buffer should be carried out in a suitably designated area. Strict guidelines for exposure, handling, and waste disposal should be outlined in the radiation safety plan for the laboratory in question.
28. Acrylamide gel staining buffer: 40% (v/v) methanol, 10% (v/v) acetic acid, 0.5 % (w/v) Comassie blue. Acetic acid is corrosive; aliquot the stock solution in a fume hood.
29. Acrylamide destaining buffer: 40% (v/v) methanol, 10% (v/v) acetic acid. Acetic acid is corrosive; aliquot the stock solution in a fume hood.
30. PhosphorImaging system (Molecular Dynamics).
31. Rehydration buffer: 0.1% (v/v) Tween-20 and 3% (w/v) BSA in PBS.
32. Vectorshield (Vector Laboratories).
33. Immunofluorescent microscope.

3. Methods

The methods below outline the analysis of the HDACI-initiated G2 checkpoint in both synchronized and asynchronously growing cells. Two cell lines will be described: the human cervical cancer cell line, HeLa, an example of a cell line with a defective HDACI-sensitive G2 checkpoint; and primary cultures of neonatal foreskin fibroblasts (NFF), which have a functional checkpoint arrest. Three different HDACIs will be mentioned: sodium butyrate (NaBu), trichostatin A (TSA), and azelaic bishydroxamic acid (ABHA).

3.1. Cell Lines and Culture Conditions

Mycoplasma-free HeLa and NFFs are cultured in complete DMEM (containing 10% [v/v] donor calf serum) at 37°C in the presence of 5% CO_2.

3.2. Cell Synchrony

Generally, drug treatment experiments are performed on asynchronously cycling cells, disregarding any cell cycle phase-specific drug effects. However, in the case of HDACIs, very significant differences are observed, depending on which phase of the cell cycle the drugs are administered in (*see* **Note 1**). The use of synchronized cells helps to overcome this problem. It also permits more refined analysis of the cell cycle phase progression, particularly progression through G2/M, than can be obtained using asynchronously growing cultures, which are normally composed of 60% G1-phase cells.

3.2.1. HeLa Cell Synchrony

HeLa cells are synchronized using a double thymidine block release protocol as described below.

1. To block cells in late G1/early S phase, trypsinize cells and replate at 50% confluence. Twenty-four hours later, remove medium and replace with prewarmed complete DMEM supplemented with 2.5 m*M* thymidine for 16 h.
2. Release cells from the block with three washes of prewarmed DMEM (without donor calf serum).
3. Trypsinize and reseed the cells into 10-cm-diameter dishes at a density of 1×10^6 cells per dish, followed by the addition of prewarmed complete medium supplemented with 24 μ*M* thymidine and 24 μ*M* 2-deoxycytidine (*see* **Note 2**).
4. After 8 h, block the cells again by the addition of 2.5 m*M* thymidine directly into the medium. Incubate for a further 16 h.
5. Release the cells from the block with three washes of prewarmed DMEM (without donor calf serum), followed by the addition of prewarmed complete medium supplemented with 24 μ*M* thymidine and 24 μ*M* 2-deoxycytidine.

   HeLa cells normally enter mitosis within 10 h after release from the second thymidine block with 60–80% synchrony using this protocol (*see* **Note 3**).

3.2.2. Fibroblast Culture Synchrony

One to two million NFF cells are seeded into 10-cm-diameter dishes, and then synchronized using a single hydroxyurea block. This synchronizes cells in late G1/early S phase.

1. Treat exponentially growing NFFs with 2 m*M* hydroxyurea for 24 h; the hydroxyurea can be added directly into the medium on the plate.
2. Release the cells from the hydroxyurea arrest with three washes of prewarmed DMEM (without donor calf serum), followed by the addition of prewarmed complete media.

This normally results in up to 50% of cells cycling synchronously through S phase and into mitosis by 12–14 h after release (*see* **Note 4**).

3.3. HDACI Treatment of Cells

1. Treat cells with varying concentrations of HDACIs (*see* **Note 5**) (*see* **Subheading 3.11.**), either immediately after release from the second thymidine or single hydroxyurea block (G1 phase) or within 4–8 h post-release (S-G2 phase).
2. Harvest floating and attached treated and untreated cells by trypsinization at various times after release.
3. Pellet the cells by centrifugation at 5000*g* in a microfuge. Wash the cell pellet twice with PBS. At this stage, the cell pellet can be divided, and one-third fixed for FACS analysis (*see* **Subheading 3.4.**) and two-thirds set aside for biochemical analysis (*see* **Subheadings 3.8.** and **3.9.**).

3.4. Flow Cytometric Analysis of the DNA Content of Cells

One-dimensional flow cytometric analysis is used to examine the cell cycle phase distribution of cell populations. It is particularly useful in analyzing cell cycle arrest in synchronized cell populations, where the transit of cells through cell cycle phases can be followed by analyzing DNA content at successive time points after release from the synchrony arrest.

1. Treat cells (normally 0.5–1 $\times$ 10^6), harvest and pellet as in **Subheading 3.3.**, wash twice with PBS, and fix in 70% ethanol at –20°C. Fixed cells may be stored for up to 2 wk at –20°C before analysis.
2. Wash fixed cells twice with PBS. Care should be taken when aspirating the PBS, as the cell pellet is fragile at this stage, and can easily be lost.
3. Stain nuclear DNA with a solution of PI (4 µg/mL) and RNase A (400 µg/mL) in 500 µL PBS for 15 min at room temperature in the dark.
4. Wash the cells three times in PBS, and then filter them through fine silk gauze (35 µm) to remove cell clumps.
5. Analyze the single cell suspension by flow cytometry, exciting the cells at 488 nm, and measuring the PI intensity through a 600 nm wavelength filter on a FACScalibur (Becton Dickinson) using CellQuest and ModFit data analysis software. Analyze at least 10^5 cells for each DNA distribution.

ModFit allows the analysis of the diploid and subdiploid populations, the former representing the viable population and the latter the nonviable cells. Nonviable cells are normally reported as the debris in the ModFit analysis. A G2 checkpoint arrest will be detected as a stable accumulation of cells in a G2/M peak at a time when the untreated control cells have progressed through mitosis into the subsequent G1 phase. This will be apparent as a decrease in the G2/M population and an increase in the G1 peak.

3.5. Bromodeoxyuridine (BrdU) Labeling

This method selectively labels S phase cells, allowing their passage through mitosis to be monitored without the need for drug arrest synchronization, avoid-

ing any potential artifacts caused by synchronization. Cells are pulse-labeled with the thymidine analog BrdU, which is incorporated into the newly synthesized DNA during S phase. DNA containing BrdU can be identified by using a fluorescently tagged antibody specifically against BrdU.

1. Pulse-label exponentially growing cells (1×10^7) with 10 µ*M* BrdU for 2 h. Wash the cells on the dish twice with pre-warmed PBS to remove the unincorporated BrdU, followed by addition of prewarmed complete media.
2. Immediately treat the cells with HDACIs for up to 24 h. Harvest all cells both floating and attached, wash with PBS, and resuspend the cell pellet in 300 µL PBS. Fix the cells with dropwise addition of 700 µL 100% ethanol at –20°C to a final concentration of 1×10^6/100 µL.
3. Following fixation, resuspend the cells in 2 *M* HCl in 0.5% (v/v) Triton X-100. Incubate for 30 min at room temperature. Pellet cells gently, by centrifugation at 3300*g* for 5 min.
4. Neutralize the cells by resuspending the cell pellet in 0.1 *M* sodium tetraborate (pH 8.5) for 5 min at room temperature.
5. Detect BrdU incorporation by staining with FITC-conjugated anti-BrdU monoclonal antibody in 400 µL BrdU detection buffer supplemented with PI (2 µg/µm) to counterstain the DNA. Incubate in the dark for 30 min at room temperature.
6. Wash the cells three times with PBS and filter them through fine silk gauze (35 µm) to produce single-cell suspensions.
7. Analyze the stained cells by two-dimensional flow cytometry. Excite cells at 488 nm and collect green fluorescence from FITC through a 514 nm filter, indicating the amount of incorporated BrdU. Measure PI fluorescence though a 600 nm wavelength filter. Use electronic compensation to prevent contamination of the PI channel by the FITC fluorescence. Plot FITC fluorescence intensity (FL-1) on the Y axis and PI staining intensity (FL-3) on the X axis.

   In untreated cells, the S phase BrdU-stained population will have progressed to the G2/M peak by 6 hr post-labeling. By 24 h there should be a population of BrdU-stained cells in the G1 and S phases, representing cells that have undergone mitosis (**Fig. 1**, upper panels). If HDACI treatment results in a G2/M arrest, then the BrdU-positive cells (those cells in S phase at the time of treatment) will remain in the G2/M peak at 24 h, while in the case of a G1 arrest, the BrdU positive cells will appear only in the G1 peak (**Fig. 1**, lower panels). These cells will have been in S phase when treated with HDACI, passed through G2/M, and arrest in the subsequent G1 phase.

3.6. MPM-2 Staining FACS Analysis

To determine the percentage of cells in M phase by FACS analysis, the specific mitotic phospho-epitope MPM-2 can be used. This will identify the proportion of mitotic cells in a PI-stained G2/M peak.

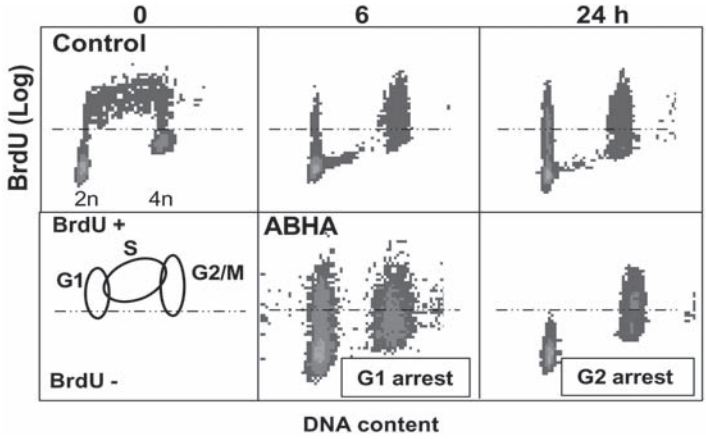

Fig. 1. FACS analysis of BrdU staining. Fibroblasts were pulse labeled with BrdU for 2 h, then either harvested or chased with fresh medium for 6 or 24 h. For the ABHA treated cells, HeLa cells (G1 arrest, *see* **Note 12**) or NFF (G2 arrest) were labeled with BrdU as above, and then treated with 100 µg/mL ABHA for 24 h. The mask shows the positions of the G1, S, and G2/M phase BrdU-labeled populations. Cells not labeled with BrdU would appear below the dotted line.

1. Treat either exponentially growing cells or synchronized cells with HDACIs and harvest at various times after treatment.
2. Fix cells in –20°C 70% ethanol for 2 h. Wash with PBS and incubate with anti-MPM-2 antibody diluted 1/200 in PBS for 1 h at 4°C. Wash cells twice with PBS and incubate with species-specific FITC- labeled secondary antibody and PI (2 µg/mL) to counter-stain the DNA, for 30 min at 4°C in the dark.
3. Wash cells twice with PBS and counterstain the DNA with PI (2 µg/mL) for 30 min at 4°C in the dark. Filter the cells through a fine silk gauze (35 µm).
4. Analyze MPM-2-positive cells using bivariate flow cytometry with Cell Quest and Modfit software (Becton Dickinson).

Mitotic cells have a 4N DNA content by PI staining and are positive for MPM-2 staining, whereas G2-phase cells have a 4N DNA content by PI staining and no MPM-2 staining.

3.7. MTT Cell Proliferation Assay

MTT (3-[4, 5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide) measures mitochondria activity, which is a good marker of cell viability and measures proliferation.

1. Seed cells into 96-well plates at a density of $2–5 \times 10^3$ per well, 24 h prior to the addition of HDACIs.

2. On day 0 (the day HDACIs are added) and for the following 3–4 d, measure the cell viability and proliferation in untreated control and HDACI-treated cells. Add MTT to the culture media at a final concentration of 0.5 mg/mL and incubate the plates for 4 h at 37°C.
3. Precipitate the insoluble formazan product by centrifugation of the plate, followed by aspiration of the supernatant (*see* **Note 6**).
4. Dissolve the formazan crystals in 100 µL DMSO with gentle shaking at room temperature. This routinely takes 5 min, but if a large amount of formazan crystals form, then a longer time is required to dissolve all the crystals.
5. Measure absorbance at 570 nm using a microplate reader.

3.8. Immunoblotting for Acetylated Histones

As a measure of inhibition of histone deacetylases, the level of histone acetylation can be determined by immunoblotting, using either a generic anti-acetyl lysine antibody or acetylation site-specific antibodies.

1. Pellet and lyse synchronized and treated cells in 500 µL of NETN buffer on ice for 30 min.
2. Determine the protein levels using bicinchoninic acid according to the manufacturer's instructions (BCA; Pierce Chemical Co.) using bovine serum albumin (BSA) as a standard.
3. To 20 µg of protein lysate diluted to 20 µL in NETN, add 5 µL 5X SDS sample buffer and boil for 5 min.
4. Resolve samples on a 12.5% SDS-PAGE, and transfer to a PVDF membrane using semi-dry Western blotting equipment according to manufacturer's instructions. Assess sample loading by brief Ponceau S (Sigma) staining of the membranes. The histone proteins stain as prominent bands between 15 and 25 kDa. Remove Ponceau S staining by washing membrane three times for 5 min in PBS/Tween.
5. Block the membrane in Blotto for 1 h. Detect acetylated histone levels with either generic or acetylation site-specific antibodies, and the appropriate horseradish-peroxidase-conjugated secondary antibody using enhanced chemiluminescent (ECL; NEN) detection.

This method analyzes the whole cell lysate, including the chromatin histones which are not extracted using normal detergent and low-salt buffers. With the generic anti-acetyl lysine antibodies, other acetylated nonhistone proteins are also detected. **Fig. 2** shows acetylation of α-tubulin in response to ABHA and TSA, but not NaBu treatment.

3.9. Immunoprecipitation and Cyclin B/Cdc2 Kinase Assay

Cyclin B/Cdc2 is activated at the G2/M transition. Its activity is essential for entry into mitosis, and the inactivation and destruction of cyclin B is required for exit from mitosis ***(5)***. Thus, measurement of the activity of cyclin B/Cdc2

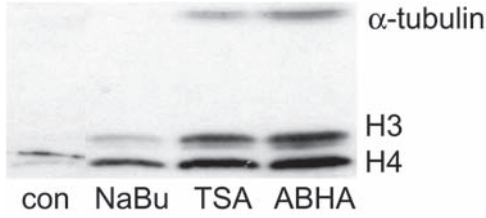

Fig. 2. Whole-cell lysates of HeLa cells treated for 24 h with either 10 m*M* sodium butyrate (NaBu), 100 ng/mL TSA, or 100 µg/mL ABHA. Lysates were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane, immunoblotted with an anti-acetyl lysine antibody (Cell Signalling Ac-K-103), and then developed with enhanced chemiluminescence staining. The blot shows acetylated histone H3 and H4 and acetylated α-tubulin (*see* **Note 13**).

and the abundance of the cyclin B protein by immunoblotting are excellent indicators of entry into and exit from mitosis. The measurement of cyclin B/Cdc2 activity is particularly useful when used in conjunction with synchronized cell cultures, as it provides a biochemical measure of transit through mitosis. Cyclin B/Cdc2 activity is determined by the immunoprecipitation of the complex followed by an in vitro kinase assay performed on histone H1, which is its in vivo substrate. Flow cytometry can provide only an indirect measure of G2/M transition, and may be inaccurate, especially if a high proportion of cells fail cytokinesis. The latter may result in cells with 4N DNA content, which will be detected in a G2/M peak, although these cells are actually in G1 phase. The failure of cytokinesis is common in HDACI treated cells.

1. Resuspend and lyse treated cells ($1–5 \times 10^6$) on ice for 30 min in 1 mL NETN buffer supplemented with 0.15 *M* NaCl.
2. Remove cellular debris by centrifugation at 16,000*g* for 10 min at 4°C.
3. Determine the protein concentration of the lysates (*see* **Subheading 3.8.**) transfer 1 mg to a fresh microfuge tube.
4. Incubate the precleared cell lysate (*see* **Note 7**) with anti-cyclin B antibody (0.5 µg) prebound to 30 µL 50% suspension of protein A–sepharose (Roche) (*see* **Note 8**), for 3 h at 4°C with constant mixing. Wash precipitates three times with NETN, once with 20 m*M* Tris-HCl (pH 7.4), 1 m*M* DTT.
5. In an approved area for radioactive work, resuspend the precipitates in 30µL RC buffer. Incubate at 30°C for 15 min. Add 10 µL 5X SDS sample buffer and boil for 5 min. Run samples on a 10% PAG, stain with acrylamide staining buffer for 30 min with rocking, and destain with acrylamide destain buffer to determine the even loading of histone H1.
6. Quantitate histone phosphorylation using a PhosphorImaging system (Molecular Dynamics).

7. In addition to the immunoprecipitation of the cyclin B complex and kinase assay as performed previously, a 20μg sample of each lysate should be prepared for immuno-blotting. Add 5X SDS sample buffer to the lysate and boil (*see* **Subheading 3.8.**). These samples can be kept at –20°C for 2 wk before running on a PAG to determine the levels of cell cycle regulatory proteins cyclin B1 and Cdc2. The level of cyclin B1 should increase as cells enter mitosis, and decline on exit. The level of Cdc2 remains constant through the cell cycle and acts as a convenient loading control.

3.10. Immunofluorescent Staining

One of most dramatic consequences of a defect in the HDACI-sensitive G2 checkpoint is the appearance of cells undergoing an aberrant mitosis following HDACI treatment (*see* **Note 9**). To examine this phenotype, immunostaining of fixed cells is performed.

1. Seed exponentially growing cells onto coverslips (*see* **Note 10**). To obtain a higher proportion of cells undergoing mitosis, these cells can be synchronized as described in **Subheading 3.2.**
2. Treat cells with HDACI, and harvest the cells on the coverslips at various times after treatment. Wash cells with PBS, fix with –20°C 100% methanol, and store at –20°C in methanol until required. The optimal time for harvesting the synchronized cultures is when the control and HDACI-treated samples both display a high proportion of the rounded mitotic phenotype, which normally occurs 8–12 h after synchrony release. With asynchronous cultures, a high proportion of mitotic cells are evident 16–24 h after drug treatment.
3. Air-dry coverslips, and rehydrate with a drop of rehydration buffer for 1 h at room temperature.
4. Stain the cells with anti-α-tubulin (1:1000 in rehydration buffer; Amersham), for 1 h at room temperature in a humidified chamber (*see* **Note 11**).
5. Wash coverslips three times for 15 min with PBS containing 0.1% dried skimmed milk powder, then incubate with an appropriate fluorochrome-conjugated secondary antibody and DAPI (5 μg/mL), to stain the DNA, for 20 min in the dark in a humidified chamber.
6. Wash coverslips three times with PBS containing 0.1% dried skimmed milk powder, once with PBS, and then mount by inverting onto a glass slide on a drop of Vectorshield mounting solution to preserve the fluorescence.
7. Store coverslips in the dark at 4°C, or analyze immediately with a immunofluorescence microscope. Determine the number of aberrant mitotic cells with each treatment (*see* **Note 9**).

3.11. Determining the Effective Dose of HDACI

We have used a range of different HDACIs to examine the cell cycle effects of these drugs. In each case, it is important to define the concentration of drug required to produce a G2/M effect—either G2 arrest in cells with an intact

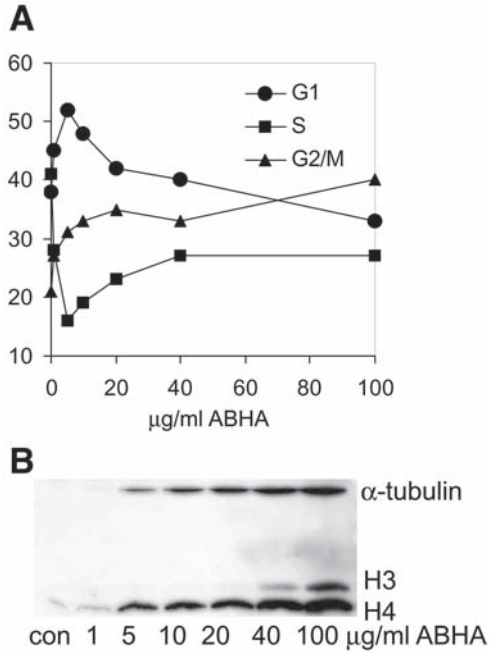

Fig. 3. Titration of ABHA with DG75 Burkitts lymphoma cells._ Asynchronously growing cells were treated with the indicated concentrations of ABHA for 24 h and then harvested. A portion of each sample was fixed for FACS analysis of the DNA content **(A)**, and the remainder was lysed and immunoblotted with anti-acetyl lysine antibody **(B)**. The levels of acetylated histone H3 and H4, and acetylated tubulin are shown.

HDACI-sensitive checkpoint or aberrant mitosis in cells defective for this checkpoint control. For example, a low dose of ABHA, which produces only a relatively small increase in histone acetylation in DG75 Burkitts lymphoma cells, produces a very strong accumulation of these cells in G1 phase and loss of the S phase population. In contrast, at higher doses, which produce substantial histone acetylation, the accumulation of these cells in G2/M is more prominent **(Fig. 3)**. To examine the effective dose of HDACI, the following protocol should be used.

1. Treat asynchronous cell cultures with a range of doses of HDACI (*see* **Note 5**).
2. Harvest at 4, 8, and 24 h after treatment. Determine histone and nonhistone protein acetylation by immunoblotting (*see* **Subheading 3.8.**).
3. In addition, determine the effect on the cell cycle by analyzing cells harvested at 24 h by flow cytometry (*see* **Subheading 3.4.**).

At low doses of HDACIs, a G1 arrest is often observed without any G2/M effects. This is in part a consequence of the transcriptional up-regulation of the CDK inhibitor $p21^{WAF1/CIP1}$ ***(2)***. Higher doses are required to observe G2/M effects.

4. Notes

1. When 100 µg/mL ABHA is added to cells in the S phase, a large proportion of the cells will undergo an aberrant mitosis, and subsequently within 24 h a large proportion are dead. If the same amount of ABHA is added to cells in the S phase of the cell cycle and removed in the G2 phase, then cell death does not occur. In addition, 100 µg/mL ABHA added in G2 phase does not produce an aberrant mitosis and causes little cell death ***(6)***. Similar results have been observed for additional HDACIs tested.
2. Thymidine depletes the deoxycytidine triphosphate pool, and in this way induces a G1 block in the cell cycle. The inhibitory action of thymidine is alleviated by removal of the high concentration of thymidine and the addition of 2-deocytidine to the medium, which appears to be the rate limiting factor.
3. HeLa cells are the best characterized cell line for use with a double block release protocol. Many cell lines will not successfully exit the second arrest, or if they do, they will have a reduced level of synchrony. For most cell lines, an overnight block with 2 m*M* hydroxurea produces a robust G1/S arrest that is readily reversed upon removal of the drug, resulting in populations which progress into mitosis with 50% synchrony. Synchrony with all these procedures is improved if the tissue-culture hood or room in which the release is performed is also prewarmed to 37°C.
4. Primary fibroblast cultures are difficult to synchronize, and after release a variable proportion of the population often remains arrested in G1. This does not provide any difficulties in interpreting data, as it is effectively a noncycling population that remains constant throughout the experiment. A higher degree of synchrony can be obtained with NFF if they are first synchronized by serum starvation with 0.5% serum for 48 h, then stimulated to enter the cell cycle with 10% serum readdition, then blocked with 2 m*M* hydroxyurea for 24 h, and then released from this arrest by removal of the drug as described. The drawback with this method is that the proportion of noncycling cells is often higher than after the single hydroxyurea block.
5. All HDACIs examined appear to have similar effects on the cell cycle. Stability is a problem for both TSA, with a half-life of 8 h, and NaBu. In addition, NaBu has a number of additional effects on the cell; therefore other HDACIs are used in preference. Typical concentration ranges for HDACIs used on Hela and NFF cells are shown in **Table 1.**
6. To aspirate the supernatant from the plate, a multi-channel pipet is used to remove approx 90% of the total volume in each well.
7. Preclearing a lysate removes all proteins present which will bind nonspecifically to the protein A or G sepharose used to purify the antibody of interest. This is

Table 1
Typical Concentration Ranges for HDACIs Used on Hela and NFF Cells

ABHA	10–200 µg/mL
TSA	100–500 ng/mL
NaB	10–20 m*M*
SBHA (Suberohydroxamic Acid)	50–200 µg/mL
Oxamflatin	5–20µ*M*
Depsipeptide	0.5–2.0 µ*M*

done by first incubating the lysate with 30 µL of protein A/G–sepharose for 30 min with rocking at 4°C. The protein A/G is removed by centrifugation of the lysate at 8000*g* for 1 min in a microfuge, and transfer the supernatant to a fresh microfuge tube for the subsequent immunoprecipitation.

8. To prebind the antibody to the protein A–sepharose, take 0.5 µg anti-cyclin B antibody and mix it with 30 µL 50% protein A–sepharose. Incubate with rocking for 20 min at 4°C, pellet the complex by centrifugation at 5000*g* in a microfuge, wash with an equal volume of PBS, and remove the supernatant. Repeat the washing step three times.

The decision to use protein A or protein G to pull down the antibody of interest will depend on the species of animal in which the antibody was raised and which subclass of immunoglobulin it is. Protein A binds well to antibodies raised in humans, pigs, rabbits, guinea pigs, and mice, while protein G binds antibodies generated in humans, horses, cows, pigs, sheep, goats, rabbits, hamsters, guinea pigs, rats, and mice.

Protein A/G–sepharose is typically stored in 20% ethanol; the ethanol will need to be removed before addition into a cellular lysate. This is done by washing the protein A/G–sepharose five times in a large volume of PBS. The washed protein A/G–sepharose can be stored at 4°C for 10 d.

9. Aberrant mitosis refers to a mitotic cell which is abnormal in appearance (**Fig. 4** and **ref. *6***). Normally, chromosomes congress to the metaphase plate, with a very distinctive spindle cap on either side of the midline. HDACI-treated mitotic cells contained well-formed spindles, but DNA staining shows chromosomes failing to migrate to the midline of the cell. Staining of the kinetochores can also be carried out to demonstrate the failure of the chromosomes to migrate to the midline.
10. Owing to the variation in quality of coverslips, all coverslips are precleaned before use, to ensure that cells will adhere to them. Coverslips (10 mm) are first boiled in water for 20 min, then rinsed in 100% ethanol, and allowed to air dry. When dry, they are packaged into tubes and autoclaved before use.
11. As the volumes involved in the immunofluorescent staining of coverslips are low, a humidified chamber is used. This should be a dark box with a lid. Line the

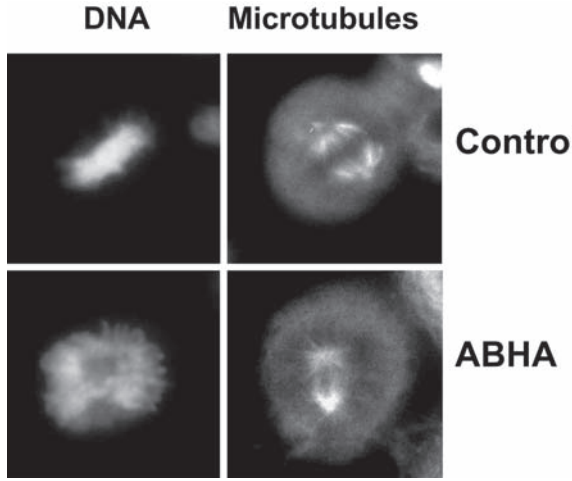

Fig. 4. Aberrant mitosis phenotype. Cells attempting mitosis in the presence of ABHA undergo aberrant mitosis. HeLa cells synchronized by a double thymidine block and then released and treated without (upper panels) or with 100 µg/mL ABHA (lower panels), cells were fixed as the cultures entered mitosis. Cells were stained for DNA and microtubules. The normal congression of condensed chromosomes at metaphase is clearly observed with the DNA staining. The ABHA-treated mitotic cells contained well formed spindles, but DNA staining shows chromosomes failing to migrate to the midline of the cell.

bottom of the box with blotting paper, and wet it with PBS. Take a length of parafilm large enough to place all the coverslips on, and place on the blotting paper. Mark the parafilm with the identification of each coverslip, to prevent a mix-up of the samples. Use this chamber for the rehydration and the incubations with the primary and secondary antibodies. Place the coverslips cell-side down on a drop of the buffer/solution in question for each step. For washing, transfer the coverslips to a 24-well plate cell-side up.

12. In NFF cells, a G2/M checkpoint is activated in the presence of the HDACI, ABHA. In HeLa cells this checkpoint is not activated, and the cells progress through the cell cycle into G1 phase. In the figure of the BrdU-Hela cells, there are a significant proportion of cells still in the G2/M or 4N peak after 24 h of treatment. This peak corresponds to cells which have gone through mitosis but have failed cytokinesis; therefore, they are in fact G1-phase cells.
13. Differential acetylation of the H3 and H4 histone can be seen when cells are treated with different HDACIs. At present it is unclear whether this is a result of the inhibitors having differential effects, or whether it is a result of the relative instability of TSA and NaBu compared to ABHA.

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18

Biochemical Analysis of the Cell Cycle and Cell Cycle Checkpoints in Transiently Transfected Cells After Collection With Magnetic Beads

Xiaofen Ye, Maxim Poustovoitov, Hidelita Santos, David M. Nelson, and Peter D. Adams

Summary

It is frequently necessary to examine the biochemical effects of ectopically expressed proteins or short hairpin (sh) RNA-mediated protein knock-down in intact cells. Plasmids that direct the expression of ectopic proteins or shRNAs can be conveniently introduced into cells by transient transfection of plasmid DNAs. However, most protocols used for the transient transfection of plasmid DNAs introduce the foreign DNA into only a minority of the total cells. Therefore, to investigate the biochemical effects of the foreign DNA it is necessary to purify the transfected cells away from the untransfected cells. This can be easily achieved by cotransfection of a plasmid encoding the cell surface marker protein CD19 or CD20, followed by immunopurification of the CD19- or CD20-expressing cells with magnetic beads coated with an anti-CD19 or anti-CD20 antibody. The purified cells can be used for a wide range of biochemical analyses, including protein extraction for Western blot and immunoprecipitation, RNA extraction for Northern blot, and DNA and chromatin extraction for nuclease digestion. Since the CD19/CD20 cell surface marker approach can be readily combined with analysis of cell cycle distribution of propidium-iodide-stained cells, it is straightforward to simultaneously determine the biochemical and cell cycle effects of an ectopically expressed or knocked-down protein.

Key Words: Transient; transfection; magnetic; bead; sorting.

1. Introduction

Analysis of the cell cycle, cell cycle checkpoints, and many other areas of cell biology frequently requires investigation of the biochemical effects of an overexpressed protein. More recently, with the advent of RNA interference (RNAi) technology, it has become possible to examine the biochemical effects of RNAi-mediated knock-down of a protein ***(1)***. RNAi technology allows loss-

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

of-function phenotypes to be characterized without the need to go through a time-consuming homologous recombination-mediated gene knock-out procedure. Such biochemical analyses are necessary to determine the molecular function of a protein in vivo.

One of the most straightforward ways to ectopically express a protein of interest in a cell is through transient transfection of a plasmid that directs expression of that protein. Recently, plasmid vectors have been developed which direct expression of shRNAs (short hairpin RNAs) that trigger knock-down of the corresponding endogenous proteins ***(2–5)***. In either case, the plasmid DNA can be introduced into the cells by transient transfection, using, for example, calcium phosphate or a liposome-based approach. However, the ability to examine the biochemical consequences of ectopic expression or knock-down of the protein in question is limited by the fact that most transient transfection procedures introduce plasmid DNA into only 5–30% of the cell population. Thus, even in a readily transfectable cell line, such as U2OS, any biochemical parameter analyzed in the total cell population reflects the state of primarily the untransfected cells. To overcome this problem, other approaches for gene and protein delivery have been developed that introduce the protein into a higher proportion of the cultured cells, such as recombinant adenoviruses ***(6)***, retroviruses ***(7)***, and protein fusions to cell-penetrating peptides ***(8)***. Frequently, cells successfully infected with a retrovirus are selected or purified through use of a retrovirus-encoded drug-resistance gene or GFP (green fluorescent protein). Each of these approaches has its own characteristic advantages and disadvantages. For example, adenoviruses can infect almost 100% of the cells on a plate, but their construction can be time-consuming.

As one approach to this problem, we and others have utilized a cell surface marker to identify the transiently transfected cells **(Fig. 1)** ***(9–14)***. In this method, the plasmid of interest, directing expression of an ectopic protein or shRNA, is cotransfected with another plasmid that encodes the cell-surface marker CD19 or CD20. These proteins are normally expressed only on B-cells, but are efficiently routed to the cell surface of many other cell types when ectopically expressed. Thus, it serves as a marker of the transfected cells that can be detected in either live or fixed cells. In fixed and permeabilized cells, detection of the cell surface marker with fluorescent-conjugated anti-CD19 or anti-CD20 antibodies has been widely used in conjunction with propidium iodide staining of DNA to determine the cell cycle distribution of transfected cells ***(15,16)***. More recently, this approach has been applied to viable, unfixed cells to physically purify the transfected CD19/CD20-expressing cells away from the untransfected nonexpressing cells, using anti-CD19/CD20-coated magnetic beads and/or fluorescence-activated cell sorting (FACS). In this way, it is quite straightforward to obtain a relatively pure population of viable,

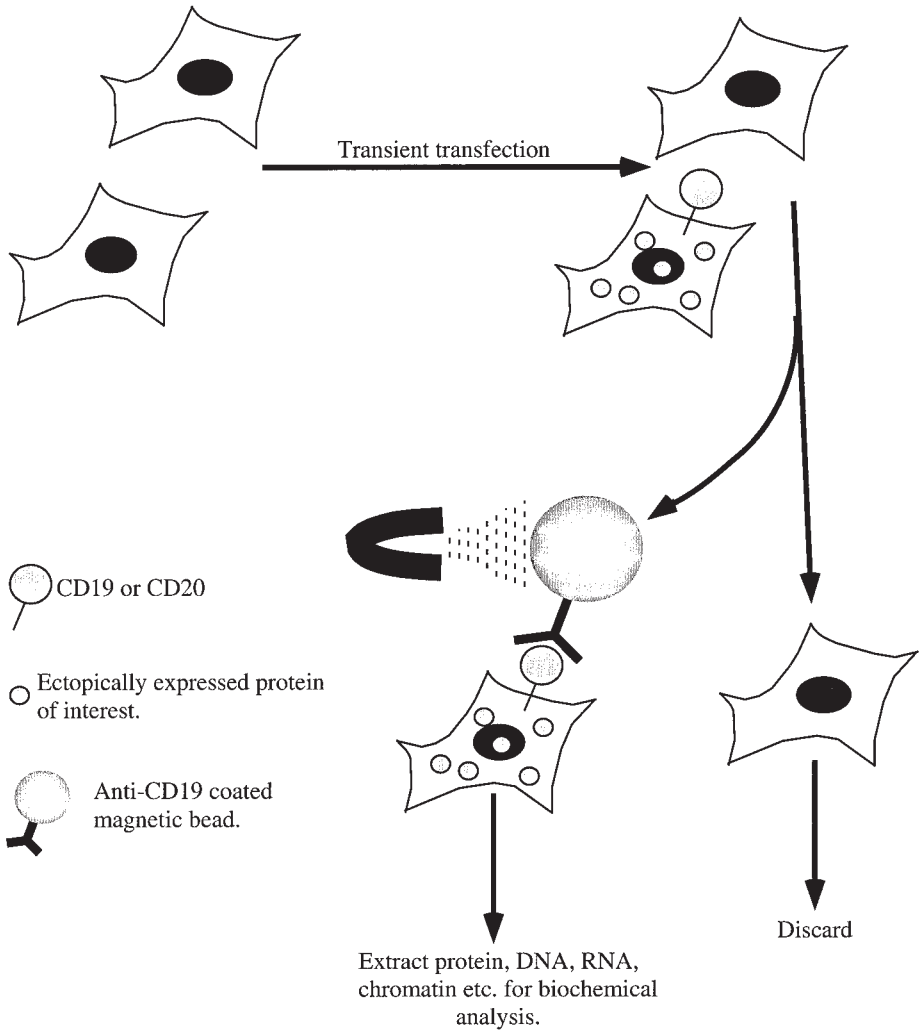

Fig. 1. Magnetic bead sorting of transfected cells. Cells are transiently transfected with plasmids encoding the cell surface marker CD19 or CD20, and the protein of interest. Thirty-six hours after transfection, the cells are collected and incubated with magnetic beads coated with an anti-CD19 or anti-CD20 antibody. A magnet is used to immunopurify the CD19- or CD20-expressing cells, which are subsequently processed for biochemical analysis.

unfixed transfected cells from a mixed population of transfected and untransfected cells. These purified cells are amenable to analysis by most common biochemical approaches.

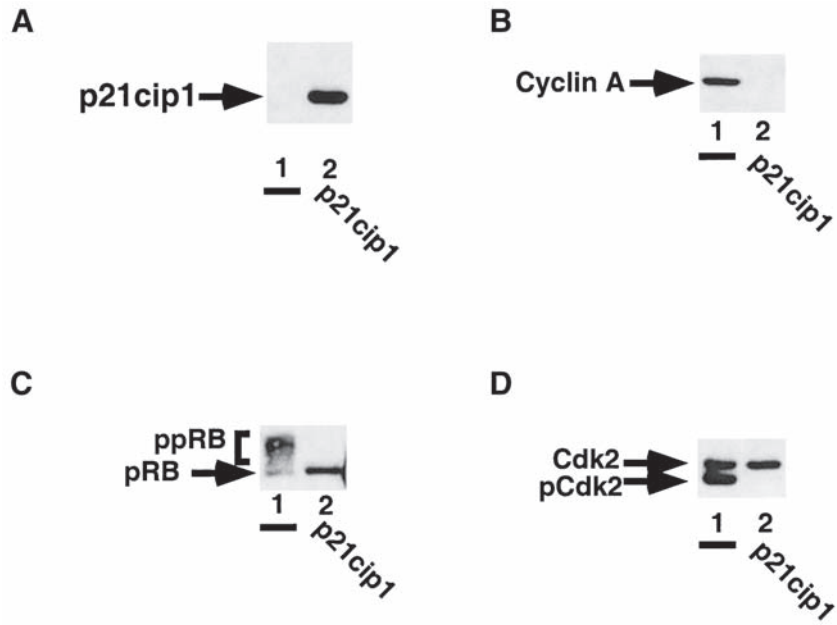

Fig. 2. Analysis of cyclin A expression, Cdk2 phosphorylation, and pRB phosphorylation in transiently transfected magnetic bead-sorted cells. U2OS cells were transiently transfected with plasmids encoding the cell-surface marker, CD19, in the absence (lane 1) or presence (lane 2) of a plasmid encoding p21cip1. The CD19-expressing cells were immunopurified with anti-CD19-coated magnetic beads. Whole cell lysates were prepared, fractionated by SDS-PAGE, and analyzed by western blot with antibodies to p21cip1 **(A)**, cyclin A **(B)**, pRB **(C)**, and cdk2 **(D)**. Hyperphosphorylated pRB (ppRB) migrates as a series of lower-mobility bands and Cdk2 phosphorylated on T160 (pCdk2) migrates as a single higher-mobility band. Ectopic expression of p21cip1 induces G1/S arrest and this is accompanied by repression of cyclin A expression and dephosphorylation of pRB and Cdk2. Reproduced from **ref. *12***, with permission.

A major advantage of this approach is its speed and simplicity. Moreover, since the CD19/CD20 cell surface marker can be readily combined with analysis of cell cycle distribution of propidium iodide-stained cells, it is straightforward to simultaneously determine the biochemical and cell cycle effects of an ectopically expressed or knocked-down protein. The major limitation of this approach is the relatively small number of cells that can easily be obtained. Despite this limitation, using anti-CD19-coated magnetic beads as the sole method of immunopurification, we have obtained sufficient transfected U20S cells to extract RNA for northern analysis, proteins for immunoprecipitations and Western blots, and chromatin for nuclease analysis (**Figs. 2** and **3**) ***(11–13)***.

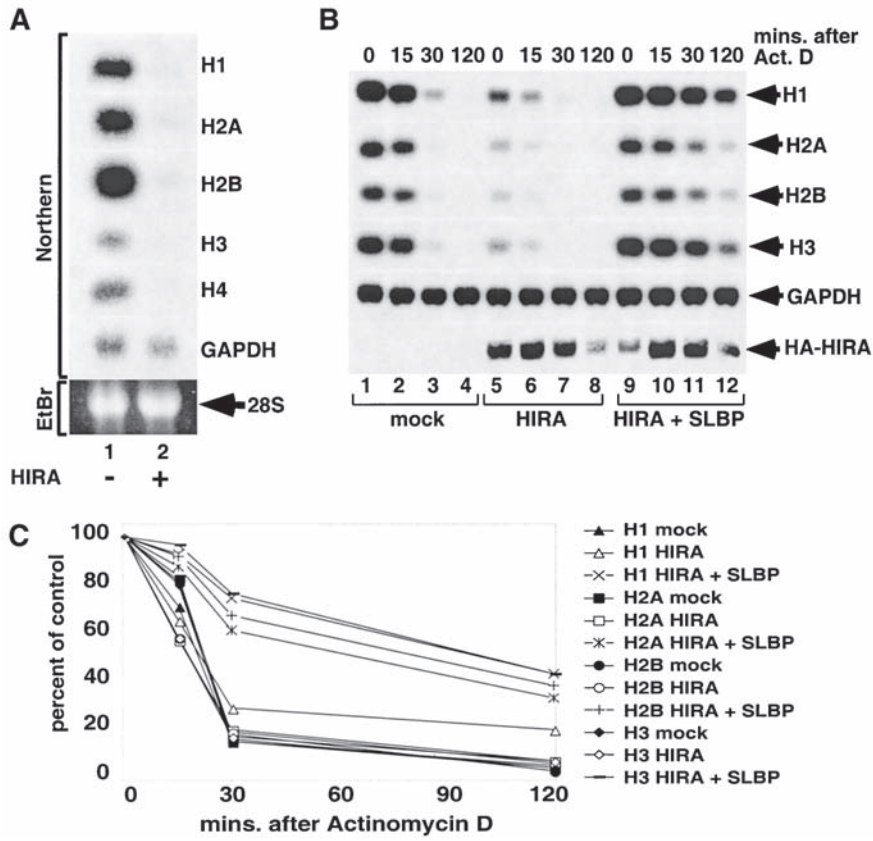

Fig. 3. Analysis of histone mRNA abundance and stability in transiently transfected magnetic bead sorted cells. **(A)** U2OS cells were transiently transfected with plasmids encoding the cell surface marker, CD19, in the absence (lane 1) or presence (lane 2) of a plasmid encoding human repressor of histone transcription, HIRA ***(12)***. The CD19 expressing cells were immunopurified with anti-CD19-coated magnetic beads. Total RNA was prepared, fractionated by gel electrophoresis and analyzed by northern blot to detect expression of histone H1, H2A, H2B, H3, H4, and GAPDH mRNAs. **(B)** U2OS cells were transiently transfected with a plasmid encoding CD19 in the absence (lanes 1–4) or presence (lanes 5–12) of a plasmid encoding human HIRA and the absence (lanes 1–8) or presence (lanes 9–12) of a plasmid encoding a histone mRNA binding protein, stem loop binding protein (SLBP) ***(12)***. Thirty-six hours after transfection the cells were treated with 10 μg/mL actinomycin D and at the indicated intervals harvested and immunopurified with anti-CD19 coated magnetic beads. Total RNA was prepared and analyzed by northern blot to determine expression of the indicated mRNAs. An aliquot of the immunopurified cells was lysed in Laemmli sample buffer and analyzed by western blot with an anti-HA antibody to detect expression of HA-HIRA. **(C)** A PhosphorImager was used to quantitate the mRNAs detected in **(B)**. *(continued)*

2. Materials

1. U2OS cells grown in DME supplemented with 10% (v/v) FBS in a humidified 37°C, 10% (v/v) CO_2 incubator (or most other readily transfectable cell types grown under appropriate conditions).
2. 2.5 *M* $CaCl_2$. Store at –20°C.
3. 2X BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffered saline (BBS): 50 m*M* BES (pH 6.95), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4.
4. 5% (v/v) CO_2, 37°C, humidified incubator.
5. Transfection-quality supercoiled plasmid DNA (double-banded cesium chloride purity or equivalent).
6. PBS (phosphate buffered saline): 137 m*M* NaCl, 2.7 m*M* KCl, 1.4 m*M* KH_2PO_4, 10 m*M* Na_2HPO_4 (pH 7.3).
7. 0.25% (w/v) trypsin/1 m*M* EDTA solution.
8. PBS + 0.3% (w/v) BSA (bovine serum albumin) (pH 7.3).
9. Anti-CD19-coated magnetic beads (Dynal, Norway) (pre-washed three times in PBS + 0.3% (w/v) BSA prior to use).
10. Magnet (Dynal, Norway).
11. Laemmli sample buffer: 50 m*M* Tris-HCl (pH 6.8), 100 m*M* DTT, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol.

3. Methods

The methods below describe a transfection protocol that efficiently cotransfects the plasmid of interest and the plasmid encoding CD19 **(Subheading 3.1.)**, and the protocol for harvesting and magnetic bead purification of the transfected cells **(Subheading 3.2.)**.

3.1. Transfection of U2OS Cells

U2OS cells are routinely grown and transfected in DME + 10% (v/v) FBS in a humidified 37°C incubator with 10% (v/v) CO_2 (*see* **Note 1**). They are transfected at 20–60% confluence by the method of Chen and Okayama ***(17)*** (*see* **Note 2**). All transfection reagents should be sterilized by filtration through a 0.22 μm sterile filter membrane.

1. Four hours prior to transfection, remove the medium from each 10-cm plate of cells and replace with 9 mL of fresh medium (*see* **Note 3**). Return the cells to a humidified 37°C incubator with 10% (v/v) CO_2.

Fig. 3. *(continued from previous page)* The values are expressed as a percent of the control zero minutes after addition of actinomycin D (untreated). Ectopic expression of HIRA represses the steady-state abundance of histone mRNAs but does not affect the mRNA half-life. Coexpression of the histone mRNA binding protein SLBP stabilizes the histone mRNAs and restores the steady-state mRNA abundance. Reproduced from **ref. *12***, with permission.

2. Dilute the required amount of 2.5 *M* $CaCl_2$ to 250 m*M* and aliquot 0.5 mL per transfection to separate 15-mL tubes.
3. Add supercoiled plasmid DNA to a total of 30 µg. For example, in the experiment shown in **Fig. 2**, 2 µg of pCMV CD19 and 28 µg of pRCCMV or pRCCMVp21cip1 was added.
4. Add 0.5 mL of 2X BBS dropwise by dripping slowly from a 1-mL pipet vertically down the center of the tube (1–2 drops per second). Do not mix. Wait 15 min. At this time, the precipitate should be barely visible to the naked eye (*see* **Note 4**).
5. Use a 1-mL pipet to blow air bubbles through the solution to mix the precipitate. Distribute the mixture dropwise into the medium evenly over the plate of cells.
6. Rock plates very gently and place in humidified 37°C incubator with 5% CO_2 (v/v) overnight (*see* **Note 5**).
7. On the following morning remove the medium, replace with 10 mL of fresh medium and return to humidified 37°C incubator with 10% CO_2 (v/v).

3.2. Harvesting Cells

In experiments where a protein is being ectopically expressed, the cells are usually harvested approx 36 h after transfection. Where the goal is to use a plasmid-encoded shRNA to produce knock-down expression of an endogenous protein, we have found that more efficient knock-down is generally achieved 60 h after transfection (*see* **Note 6**).

1. Wash the cells with warm PBS and trypsinize by incubation with 1 mL of 0.25% (w/v) trypsin/L m*M* EDTA solution for 3 min at 37°C (*see* **Note 7**). Add 9 mL of ice-cold DME + 10% (v/v) FBS and repeatedly aspirate with a pipet to disrupt clumps of cells. Visual inspection of the cells through a microscope should confirm that they are predominantly single cells with few doublets or clumps.
2. Transfer the cells to a 15-mL tube and pellet them by centrifugation for 3 min at 200*g* at 4°C. Aspirate buffer, leaving approx 50µL, and resuspend cell pellet in this remaining solution. Add 10 mL of PBS + 0.3% (w/v) BSA at 4°C, invert tube to wash cells, pellet again by centrifugation, aspirate the supernatant, resuspend the cells in the remaining 50 µL of buffer, and add 950 µL of PBS + 0.3% (w/v) BSA at 4°C (*see* **Note 8**). Determine the number of cells/mL by counting with a hemocytometer.
3. Transfer the cells to Eppendorf tube. Add required volume of anti-CD19-coated magnetic beads. Typically, we add 1.2×10^7 beads per plate of cells. Assuming that at the time of harvesting, one 60% confluent, 10-cm plate of U2OS contains 2×10^6 cells and the transfection efficiency is 30%, this would mean 0.7×10^6 target cells per plate, and the bead:target cell ratio is approx 17:1. Rotate at 4°C for 15 min (*see* **Notes 9** and **10**).
4. Place in magnet until all beads are pulled down. Invert tube holder/magnet a few times to get beads and cells out of the liquid meniscus.
5. While tube is still in magnet, aspirate wash buffer. Remove tube and add 1 mL of PBS + 0.3% (w/v) BSA at 4°C and, using a blue tip, very gently resuspend cells. Take care not to damage the cells.

6. Repeat **steps 4** and **5** four times.
7. View cells through microscope. All remaining cells should have a "rosette" of beads around them. Cells without are probably untransfected. Perform extra round of sorting if necessary (*see* **Note 11**).
8. Process the immunopurified cells further as desired. Typically, we extract RNA for Northern blots or protein for immunoprecipitations and Western blots, or chromatin for nuclease digestion ***(12,13)***, according to standard protocols.

4. Notes

1. The use of a cell surface marker to sort for transfected cells has been successfully applied to a number of different cell types, including SAOS2, MG63 and U2OS osteosarcoma cells, Rat1a fibroblasts, C33A cervical carcinoma cells, T98G glioblastoma cells, J82 bladder carcinoma cells, MCF7 breast carcinoma cells, VA13 (human diploid fibroblasts transformed with SV40 virus), NIH3T3 cells, and C2C12 myoblasts ***(10,15,18–21)***. This list is not meant to be exhaustive.
2. This protocol requires that every cell that expresses CD19 also expresses the protein of interest. Thus, it depends upon efficient cotransfection of the plasmids encoding the CD19 cell surface marker and the protein of interest. Efficient cotransfection is achieved with the calcium phosphate transfection protocol described here. However, it should not automatically be assumed that other transfection protocols reliably cotransfect both plasmids. Most reports using this method have used calcium phosphate as the transfection reagent ***(9–14)***.
3. The number of cells that should be transfected and immunopurified obviously depends upon the transfection efficiency of the cells and the number of cells required for the final assay. Typically, the transfection efficiency of U2OS cells is approx 20–30% of the total cells on the plate. Thus, sufficient immunopurified cells are derived from a single transfected plate for Western blotting of moderately abundant proteins, such as pRB, Cdk2, and cyclin A (**Fig. 2**). However, when working with cells that transfect relatively poorly (transfection efficiency of 5% or less) the number of plates transfected should be increased accordingly.
4. The pH of the BBS is critical to obtain a good transfection efficiency. Normally, we prepare three batches of 500 mL of BBS of pH 6.93, 6.95, and 6.97. A preliminary test is carried out by mixing $CaCl_2$, DNA, and BBS, as described in **Subheading 3.1.** At the end of **step 4**, the precipitate should be barely visible to the naked eye. A batch of BBS that gives such a precipitate in this assay will usually work well. However, this is normally confirmed by transfection of cells with plasmid-encoding GFP and visualization by fluorescence microscopy.
5. In the original description of this transfection method by Chen and Okayama, it was reported that transfection efficiency was greatest when the cells were incubated overnight in 2–4% CO_2 ***(17)***. We have found that 5% CO_2 also works well. We have not directly compared incubation at 5% and 10% CO_2, although Chen and Okayama found that this greatly decreased the efficiency of transfection ***(17)***.

6. shRNA-mediated knock-down targets the mRNA rather then the protein. Therefore, the rate at which the protein level is reduced depends upon the rate of degradation of the protein. An unstable protein with a short half-life will be knocked down more quickly than a stable protein with a long half-life.
7. Adequate trypsinization of the cells is necessary to generate single cells prior to immunopurification. However, excessive trypsinization could conceivably destroy the cell-surface epitope that is recognized by the anti-CD19 antibody. By adhering to the trypsinization conditions described in **Subheading 3.2.**, we have avoided this potential problem.
8. Addition of BSA to the PBS prevents cells being lost during the wash steps through their sticking to the sides of the tube.
9. The number of beads added is in large excess over the number of cells. Consequently, variations in the number of cells, owing to changes in cell density or cell type, do not affect the efficiency of the sorting process.
10. A crude measure of transfection efficiency is obtained at **step 3** of **Subheading 3.2.** When the anti-CD19-coated magnetic beads are added to the suspension of CD19-expressing cells, visible cell clumping occurs within a few minutes. The difference between cells transfected with and without the plasmid-encoding CD19 should be readily apparent. If not, it suggests that the transfection efficiency is low and relatively few immunopurified cells will be obtained at **step 7** of **Subheading 3.2.**
11. It is generally necessary to perform a western blot of an extract from the immunopurified cells to confirm that efficient ectopic expression or protein knock-down has been achieved. An aliquot of the immunopurified cells obtained in **Subheading 3.2.** should be boiled in Laemmli sample buffer and fractionated by SDS-PAGE for this purpose.

Acknowledgments

This work was supported by grants RO1 GM62281 and DAMD17-02-1-0726 to PDA. PDA is a Scholar of the Leukemia and Lymphoma Society. **Figures 2** and **3** are reproduced from reference ***12*** with permission from the American Society for Microbiology.

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Analysis of DNA Repair and Chromatin Assembly In Vitro Using Immobilized Damaged DNA Substrates

Jill A. Mello, Jonathan G. Moggs, and Geneviève Almouzni

Summary

Significant advances have been made in identifying a complex network of proteins that could play a role in the repair of DNA damage in the context of chromatin. Insights into this process have been obtained by combining damaged DNA substrates with mammalian cell-free systems that contain both DNA repair and chromatin assembly activities. The methods described in this chapter provide a powerful approach for the detection of proteins recruited during the recognition and repair of DNA lesions, including repair proteins and chromatin-associated factors. Substrates for the recruitment assay consist of damaged DNA that is immobilized on magnetic beads. A human cell-free system that supports both DNA repair and chromatin assembly is incubated with the immobilized DNA-damaged substrates, and proteins associated with the DNA are then isolated and subjected to analysis. We present here protocols for preparing bead-linked DNA substrates containing different types of lesions, for the reaction of the damaged DNA with cell-free systems, and for the subsequent analysis of proteins that are recruited to the immobilized damaged DNA substrates.

Key Words: DNA damage; repair; chromatin; immobilized DNA; in vitro.

1. Introduction

The DNA of a eukaryotic cell experiences thousands of daily insults from both environmental and endogenous sources, resulting in numerous types of DNA damage that must be repaired in order to maintain genomic stability ***(1)***. The recognition, removal, and repair of DNA lesions in the eukaryotic genome require repair enzymes to operate within the complex chromatin environment of the nucleus. Chromatin is a nucleoprotein complex consisting of a basic repeating unit known as the nucleosome. A single nucleosome contains two turns of DNA wrapped around a core histone octamer comprising the histones H2A, H2B, H3, and H4 ***(2)***. Nucleosomes represent the first level of compaction in chromatin, restricting access to enzymes involved in DNA metabolism

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

(3). In addition to these basic components, linker histones and a variety of nonhistone proteins are incorporated to generate a fully functional genome within a higher-order chromatin structure *(4)*. Maintenance of this complex chromatin structure is critical to ensure regulated DNA metabolism and proper epigenetic inheritance *(3)*. This position of chromatin at the crossroads of DNA metabolism places it as an ideal sensor to participate in checkpoint controls.

The repair of DNA damage in the context of chromatin has been proposed to involve the sequential steps of accessing the DNA lesion within chromatin, repairing the DNA lesion, and restoring the canonical chromatin structure, referred to as the "access, repair, restore" model *(5,6)*. In recent years, significant advances have been made in identifying a complex network of repair proteins, chromatin remodeling factors, and chromatin assembly factors that could play a role in this process (*see* **refs. *6–8***). In order to coordinate the repair of DNA damage with the remodeling and maintenance of chromatin structures, a fine tuning of the functions of these protein factors is required. Significant mechanistic insights into how this process is operating at the nucleosomal level have been obtained by combining damaged DNA substrates with mammalian cell-free systems that contain both DNA repair and chromatin assembly activities *(9–11)*. The methods described in this chapter provide a powerful approach for the detection of proteins recruited during the recognition and repair of DNA lesions, including repair proteins and chromatin-associated factors. Substrates for the recruitment assay consist of damaged DNA that is immobilized on magnetic beads. DNA damage can be introduced with UV-C light (UV_{254nm}) to induce predominantly cyclobutane pyrimidine dimers and 6-4 photoproducts, DNA lesions that are repaired by the nucleotide excision repair pathway. Alternatively, DNase I can be used to enzymatically introduce single-strand DNA breaks that are repaired by the base excision repair pathway. In addition, ionizing radiation (^{137}Cs) can be used to induce more complex damage into the DNA, including single-strand breaks. A human cell-free system that supports both DNA repair and chromatin assembly is incubated with the immobilized damaged DNA substrates, and proteins associated with the DNA are then isolated and subjected to analysis. This experimental strategy is summarized in **Fig.1**. Discussed here are optimized conditions for preparing bead-linked DNA substrates containing these different types of lesions, for the reaction of the damaged DNA with cell-free systems, and for the subsequent analysis of proteins that are recruited to the immobilized damaged DNA substrates. A detailed description of the preparation of the human cell-free extracts is available elsewhere *(12)* and is therefore not included in these protocols. It should also be noted that this assay can be relatively easily adapted to additional DNA-damaging agents and alternative cell-free systems.

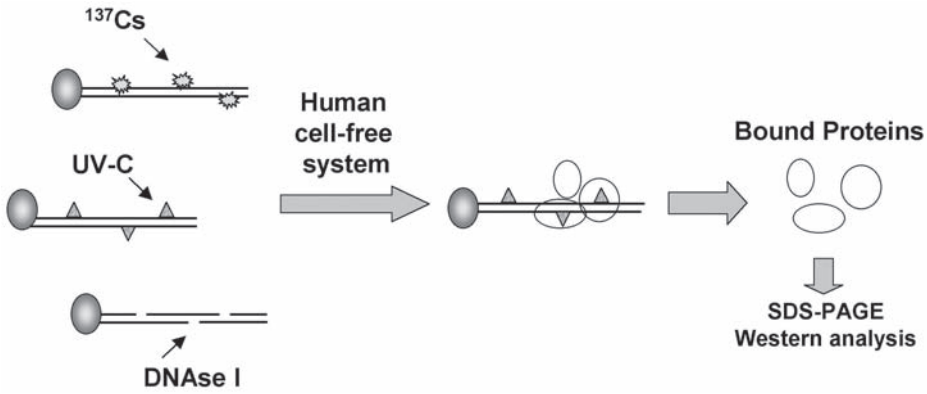

Fig. 1. Schematic representation of assay for DNA repair and chromatin assembly factors recruited during DNA damage processing. Magnetic-bead-linked DNA substrates are prepared, and lesions in DNA created using UV-C, ^{137}Cs, or DNase I as indicated. These magnetic-bead-linked DNA substrates (or undamaged duplex DNA as a control) are then incubated in a human cell-free system that is competent for both DNA repair and chromatin assembly. Specific proteins that bind to this DNA can be detected by SDS-PAGE, coupled to Western blotting or silver staining.

Examples of experimental data obtained with the recruitment assay during chromatin assembly associated with a variety of DNA excision repair mechanisms ***(11,13)*** are shown in **Fig. 2**. Proteins associated with the damaged DNA can be monitored over time to gain mechanistic insights into the order in which various proteins participate in DNA damage processing and chromatin remodeling. The approach described herein can be further adapted by using damaged DNA that has been preassembled into chromatin in order to analyze proteins potentially involved in chromatin rearrangements during the recognition of DNA lesions ***(14)***.

2. Materials

2.1. Immobilizing Linearized DNA on Paramagnetic Beads

1. pUC19 plasmid (New England Biolabs, Beverly, MA) transformed into *E. coli* strain DH5α (BRL).
2. Qiagen plasmid purification maxi-kit.
3. TE, pH 8.0 : 10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA (pH 8.0).
4. 10X TBE stock solution: 108 g Tris base, 55 g boric acid, 40 mL 0.5 *M* EDTA (pH 8.0), dissolved in deionized water to a final volume of 1 L.
5. Agarose (ultra pure, Sigma) for a minigel: 0.8% agarose, 0.25 mg/mL ethidium bromide running buffer (1X TBE, 0.25 mg/mL ethidium bromide).

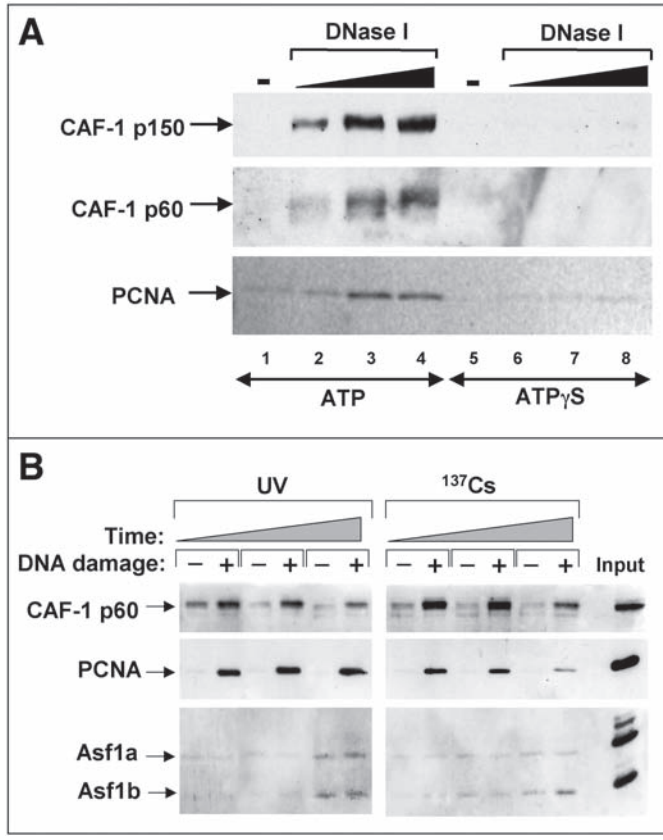

Fig. 2. **(A)** Co-recruitment of PCNA (proliferating cellular nuclear antigen) and CAF-1 (chromatin assembly factor-1) p150 and p60 subunits to bead-linked DNA substrates containing different amounts of DNase I-induced single-strand breaks (lanes 2–4). The replacement of ATP by ATPγS (a nonhydrolyzable analog of ATP) inhibits DNA excision repair, and thus prevents the recruitment of both PCNA and the two largest subunits of CAF-1 (lanes 6–8). **(B)** Recruitment of PCNA and the CAF-1 p60 subunit to bead-linked DNA substrates containing DNA lesions induced by ionizing radiation (^{137}Cs) and UV_{254nm} irradiation. By contrast, the Asf1 (anti-silencing function) proteins, Asf1a and Asf1b, are not specifically recruited to the damaged DNA.

6. Restriction enzyme Xma1 (NEB).
7. Restriction enzyme EcoRI (NEB).
8. Klenow polymerase (exo-free) (Boehringer Mannheim).
9. Biotin-14-dATP (Boehringer Mannheim) 0.4 m*M* stored at –20°C.
10. α-thio-dNTPs (Boehringer Mannheim) 10 m*M* stored at –20°C.
11. Sephadex G50 column (Boehringer Mannheim).

12. 2X wash/storage buffer: 10 m*M* Tris-HCl (pH 7.5), 2 *M* NaCl, 1 m*M* EDTA.
13. 10 mL Dynabeads M-280 in suspension at about 6.7×10^8 beads/mL (Dynal SA, Oslo, Norway).
14. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 1.4 m*M* KH_2PO_4, 0.01 *M* Na_2HPO_4 (pH 7.4).
15. PBS containing 1mg/mL BSA and 0.05% NP40.
16. 5 *M* ammonium acetate.

2.2. Induction of DNA Lesions in Bead-Linked DNA

1. Bead-linked DNA.
2. Buffer A: 40 m*M* HEPES (pH 7.8), 40 m*M* KCl, 0.05% NP40.
3. UV-C 254 nm lamp.
4. Ice-cold steel block.
5. Vilber Lourmat VLX-3W UV dosimeter.
6. Siliconized petri dishes.
7. 2X wash/storage buffer: 10 m*M* Tris-HCl (pH 7.5), 2 *M* NaCl, 1 m*M* EDTA.
8. Buffer B: 10 m*M* HEPES (pH 7.6), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* EGTA, 10% glycerol.
9. DNase I (stock solution of 10 units/µL in buffer B; stored at –20°C) (Boehringer Mannheim).
10. 0.5 *M* EDTA.
11. ^{37}Cs-source γ-irradiator (IBL 637 irradiator, CIS Biointernational).

2.3. Reaction of Bead-DNA Substrates With Human Cell-Free Extracts

1. Buffer B: 10 m*M* HEPES (pH 7.6), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.5 m*M* EGTA, 10% glycerol.
2. Cytosolic extract derived from HeLa cells (*see* **Note 1**).
3. Nuclear extract derived from HeLa cells (*see* **Note 1**).
4. Buffer A: 40 m*M* HEPES (pH 7.8), 40 m*M* KCl, 0.05% NP40.
5. Buffer A containing 1 mg/mL BSA.
4. 5X reaction buffer: 25 m*M* $MgCl_2$, 200 m*M* HEPES-KOH (pH 7.8), 2.5 m*M* dithiothreitol (DTT, Sigma), 200 m*M* phosphocreatine (di-Tris salt, Sigma), 100 µ*M* each of dGTP, dATP, dTTP, 40 µ*M* dCTP (dNTPs 100 m*M* lithium salt solutions, Boehringer Mannheim). The 5X reaction buffer can be prepared in advance, aliquoted, and stored at –80°C.
5. 100 m*M* ATP (Pharmacia) aliquoted and stored at –80°C. Note that each aliquot should be used only once.
6. Creatine phosphokinase (Boehringer): 2.5 mg/mL in H_2O aliquoted and stored at –80°C. Each aliquot is used only once.
7. 1X Laemmli buffer: 60 m*M* Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 m*M* DTT, 0.001% bromophenol blue.
8. Reaction mix: 10 µL 5X reaction buffer, 2 µL ATP (final concentration of 4 m*M*), 2 µL creatine phosphokinase (final concentration of 4 µg), 20 µL cytosolic extract (200 µg proteins), 4 µL nuclear extract (20 µg), 12 µL H_2O.

3. Methods

A scheme of the general experimental strategy is summarized in **Fig. 1**. Substrates for the recruitment assay consist of damaged DNA that is immobilized on magnetic beads. Distinct damaging agents can be used in order to obtain different type of lesions. The bead-linked DNA substrates can then be used to assay the binding of various proteins. As a source of protein, a human cell-free system that supports both DNA repair and chromatin assembly is incubated with the immobilized damaged DNA substrates. Proteins associated with the DNA can then be isolated and subjected to analysis.

3.1. Immobilizing Linearized DNA on Paramagnetic Beads

We describe here how to immobilize linearized DNA on paramagnetic beads. The conditions are chosen in order to bind DNA at a density that should not be too high in order to avoid steric hindrance and remain accessible to the various DNA-damaging agents. This is critical to obtain an equal distribution of lesions (on average) over all the DNA molecules and ensure reproducibility in the experiments.

3.1.1. Preparation of Biotinylated Linear DNA

1. Purify pUC19 DNA from bacteria using a Qiagen maxi plasmid purification kit. Resuspend the collected plasmid in TE pH 8.0 and determine the DNA concentration by measuring the optical density at 260 nm (OD_{260} of 1 = 50 mg/mL). Check the quality of the DNA preparation to verify that it is nick-free DNA (less than 5% nicked form) by running a 200-ng aliquot on a 0.8% agarose minigel containing 0.25 mg/mL ethidium bromide (*see* **Note 2**).
2. Linearize 40 µg pUC19 with XmaI and verify that it is completely digested by running 200 ng on a 0.8% agarose minigel.
3. Add an equal volume 5 *M* ammonium acetate and 2 vols of cold ethanol to precipitate the DNA, place at –20°C for 30 min, spin at maximum speed in a cooled microcentrifuge, wash the pellet with cold 70% ethanol, air dry, and resuspend in 40 µL TE.
4. Digest the linearized plasmid with EcoRI to generate a 5' overhang at one end.
5. Precipitate and resuspend as in **step 3**.
6. Fill in the 5' overhang with Klenow polymerase using a final concentration of 50 µ*M* biotin-14-dATP, as well as 200 µ*M* α-thio-dCTP, 200 µ*M* α-thio-dTTP, and 200 µ*M* α-thio-dGTP, typically in a 60-µL vol reaction (*see* **Note 3**).
7. Remove the unincorporated dNTPs and the small restriction fragment by passing the DNA over a Sephadex G50 column. The eluted, biotinylated DNA (usually 60 µL can be recovered) can be used immediately or frozen and stored for later experiments.

3.1.2. Coupling Biotinylated Linear DNA to Dynal M-280 Beads

For the coupling reaction we usually work at a ratio of 0.0625 pg of DNA per bead.

1. Take an aliquot of 960 µL dynabead M-280 suspension from stock solution and place in a 1.5-mL microtube. Concentrate the beads using a magnetic rack (*see* **Note 4**), then remove the liquid with a pipet and discard. Wash the dynabeads by resuspending in 300 µL PBS containing 1 mg/mL BSA and 0.05% NP40. Repeat this procedure two times with 300 µL 2X wash/storage buffer (*see* **Note 4**).
2. The coupling of linearized DNA to Dynabeads is carried out here by adding 40 µg of DNA to the bead suspension (here in 300 µL). The reaction can be scaled up or down as required (*see* **Note 5**).
3. Dilute 40 µg biotinylated DNA to a final volume of 1340 µL with 2X wash/storage buffer. Resuspend the washed, concentrated beads in this DNA solution. Incubate on a rotating wheel for 16 h at room temperature.
4. Concentrate beads using the magnetic rack (*see* **Note 4**), discard supernatant (save a 10 µL aliquot) and wash beads twice with 2X wash/storage buffer. The bead-linked DNA can be stored up to 1 mo in this same buffer at 4°C.
5. Run a 10 µL sample of the supernatant on an agarose gel alongside 300 ng linearized pUC19 to verify efficient binding to the beads. No DNA should be visible in the supernatant if efficient binding has been achieved.

3.2. Induction of DNA Lesions in Bead-Linked DNA

The DNA damage can be introduced on the immobilized DNA using different methods which will result in the formation of distinct type of lesions. These different types of substrates can be useful to distinguish binding activities which can be lesion specific. UV-C light (UV_{254nm}) is used to induce predominantly cyclobutane pyrimidine dimers and 6-4 photoproducts, DNA lesions that are repaired by the nucleotide excision repair pathway (NER). DNase I can be used to enzymatically introduce single-strand DNA breaks that can be repaired by the base excision repair pathway (BER). In addition, ionizing radiation (^{137}Cs) can be used to induce more complex damage into the DNA, including single-strand breaks.

3.2.1. UV-C Radiation

1. Prewarm a UV 254 nm lamp for at least 15 min before irradiation. Place a steel block on ice under the lamp at a distance of approx 10 cm. Measure the UV fluency at the position of the block with a Vilber Lourmat VLX-3W dosimeter. A typically achieved UV fluency is 5–10 $J/m^2/s$. For UV protection the manipulation is carried out in a chamber; gloves and glasses should be used.

2. Resuspend the bead-linked DNA to be irradiated (typically 10 µg) in 500 µL of buffer A, and then transfer it to a siliconized petri dish. Place the petri dish on the ice-cold steel block and remove the lid to begin irradiation. Agitate the petri dish every 20 min during the irradiation to resuspend the beads until the desired dose is achieved. For example, to achieve a dose of 50,000 J/m^2 (5 J/cm^2), irradiate the bead-linked DNA at 8 J/m^2/s for 105 min. Treat the control, nonirradiated DNA similarly on ice, but without the irradiation.
3. Following irradiation, recover the bead-linked DNA from the petri dish and transfer to a fresh 1.5-mL tube. Concentrate the beads using the magnetic rack as before; discard the supernatant. Wash the petri dish with 500 µL 2X wash/storage buffer and add this suspension to the recovered and concentrated beads. Concentrate the beads again, wash twice with 300 µL 2X wash/storage buffer, and store in the same buffer at 4°C.

3.2.2. DNase I

1. To determine the optimal conditions for DNase I treatment that will produce single-strand but not double-strand breaks, a titration should first be carried out on circular pUC19. Dilute the stock of DNase I (10 U/µL) to 0.1 U/µL in buffer B just before use. To titrate the DNase I digestion, dilute 1 µg plasmid DNA to 65 µL in buffer B, add 0.65 units of DNase I, and incubate at 25°C over a range of time (for example 2, 4, and 6 min) or in the absence of DNase I as control. 20-µL aliquots corresponding to each time point are removed and transferred to a tube containing EDTA to 100 m*M* final concentration to stop the reaction. Analyze the products on an agarose gel. Choose conditions that yield the greatest number of nicked circular molecules but no linear molecules. This is usually obtained within the time range indicated above.
2. To induce single-strand breaks in immobilized DNA, concentrate 10 µg of bead-linked DNA (approx 25% from **step 3** in **Subheading 3.1.2.**) and resuspend in 650 µL buffer B. Digest DNA with 6.5 units DNase I for the amount of time determined above. Stop reactions with EDTA as above, wash DNA with 2X wash/storage buffer, and store in this same buffer at 4°C.

3.2.3. Ionizing Radiation

1. Resuspend the bead-linked DNA to be irradiated (typically 10 µg) in 500 µL of buffer A, and then transfer to a siliconized petri dish as described above for UV irradiation.
2. Place the plate in a ^{137}Cs-source γ-irradiator and irradiate at 4.4 Gy/min for 30 min to achieve a dose of 75 Gy. Treat control DNA similarly but without irradiation during the same amount of time.
3. Recover bead-linked DNA as at 4°C.

3.3. Reaction of Bead-DNA Substrates With Cell-Free Extracts

Set up reactions by mixing the bead-linked DNA with a human cell-free system derived from HeLa cells (*see* **Note 1**). Standard reactions of 50 µL

contain the following: 600 ng bead-linked DNA, 200 µg proteins from the cytosolic extract, 20 µg of proteins from the nuclear extract, 40 m*M* HEPES (pH 7.8), 5 m*M* $MgCl_2$, 0.5 m*M* DTT, 4 m*M* ATP, 20 µ*M* each of dGTP, dATP, dTTP, 8 µ*M* dCTP, 40 m*M* phosphocreatine, and 5 µg creatine phosphokinase.

1. Prior to the incubation, wash the bead-linked DNA once in buffer A containing 1 mg/mL BSA, and once in buffer A alone.
2. Prepare the reaction mix and preheat at 37°C for 5 min.
3. Aliquot the bead-linked DNA to tubes, concentrate the beads, and remove the supernatant.
4. Add 50 µL preheated reaction mix to each tube and incubate at 37°C.
5. Remove 15-µL aliquots at 2, 5, and 15 min. Terminate the reaction of the aliquots by concentrating the beads on a magnet and immediately removing the supernatant, and then washing beads five successive times with 500 µL buffer A.
6. Resuspend the concentrated beads in 30 µL 1X Laemmli buffer and elute the bound proteins by heating to 95°C for 5 min. Samples can then be stored at –20°C until ready to analyze.
7. Load samples onto an SDS-polyacrylamide gel of appropriate percentage for proteins of interest.
8. Detect bound proteins by silver staining, or transfer gel to a membrane and carry out Western blot analysis for proteins of interest (*see* **Fig. 2** and **Note 6**).

4. Notes

1. HeLa cell-free extracts usually used in our assays were prepared from adherent HeLa cells grown in plates as described in ***(12)***. These extracts were largely characterized for their competence in repair of DNA damages and chromatin assembly assays ***(12)***. Other extract preparations can potentially be used, but their abilities to repair DNA damage and assemble chromatin should then be tested.
2. DNA produced by this method should migrate as a single band corresponding to the supercoiled form; if a significant amount of nicked form (which migrates more slowly than the supercoiled form ***(18)***) is present, the DNA can be further purified on sucrose gradients ***(15)***.
3. The incorporation of phosphothioate bonds using α-thio-dNTPs is carried out to protect the 3'-end from possible exonuclease activity during the incubation with cell-free extract (*see* **Subheading 3.3.**).
4. Follow reference ***(16)*** for general handling of beads and for using the magnetic tube racks. Note that beads should never be allowed to dry out. Beads can be stored at 4°C, and should not be frozen.
5. These binding conditions, which do not yield a high quantity of DNA per bead, were specifically chosen to obtain an optimal induction of DNA lesions at this recommended DNA:bead ratio. Although higher levels could be achieved, we found these were not optimal for functional assays, owing to steric hindrance.
6. Several variations can be introduced into the experimental protocol in order to garner further mechanistic insight into the interplay between DNA repair and

chromatin assembly/remodeling. For example, in one variation to this experiment, damaged DNA was reconstituted into nucleosomal organization by salt dialysis using pure histones prior to carrying out the recruitment assay ***(14)***. In another example of this application, the provided protocol can be extended to the identification of novel repair/chromatin remodeling proteins by first separating recruited proteins by SDS-PAGE as described above (*see* **Subheading 3.3.**), and then carrying out tryptic digests of eluted polypeptides and mass spectrometry analysis for protein identification. Finally, given that other researchers have successfully used bead-linked DNA substrates to assay RNA polymerase II-mediated transcription ***(17)***, it may be possible to adapt the assay described here to gain insights into the interplay between DNA repair, chromatin remodeling, and transcription.

Acknowledgments

The authors thank Dr. J.-P. Quivy for advice regarding the use of Dynal beads and D. Roche for many insightful suggestions. This work was supported by la Ligue Nationale contre le Cancer (Equipe labellisée la Ligue), Euratom (FIGH-CT-1999-00010 and FIGH-CT-2002-00207), the Commissariat à l'Energie Atomique (LRC no. 26) and RTN (HPRN-CT-200-00078 and HPRN-CT-2002-00238).

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Analyzing Cell Cycle Checkpoints After Ionizing Radiation

Bo Xu and Michael B. Kastan

Summary

Several methods to measure cell cycle perturbation after exposure to ionizing radiation are presented in this chapter. These methods include the G1 checkpoint assay by 5' bromodeoxyuridine (BrdUrd) labeling followed by flow cytometric analysis, the S-phase checkpoint assay by measuring DNA synthesis in response to ionizing radiation, and the G2/M checkpoint assay by quantitating histone H3 phosphorylation after ionizing radiation.

Key Words: Ionizing radiation; cell cycle checkpoints; ATM; histone H3 phosphorylation; radio-resistant DNA synthesis; flow cytometric analysis; DNA content; mitosis.

1. Introduction

Among the mechanisms developed by eukaryotic cells to optimally respond to DNA damage, such as the damage caused by ionizing radiation (IR), are cell cycle arrests or checkpoints. These perturbations of cell cycle progression presumably enhance genetic stability and limit tumorigenesis in the organism. Elucidating the mechanism of cell cycle checkpoints has obvious importance for understanding how tumors start, and for improving tumor prevention and treatment.

After IR, the cell cycle can potentially arrest at several stages, including at G1 phase or during S phase (known as G1 and S phase checkpoints, respectively), to prevent the cell from progressing through DNA replication, or before mitosis (the G2/M checkpoint), to prevent aberrant segregation of damaged chromosomes ***(1)***. Measurement of cell cycle perturbation after ionizing radiation can be achieved by fixing or permeabilizing cells and staining them with a DNA-binding dye, such as propidium iodide (PI). The histogram data from flow cytometric analysis will yield the percentage of cells in the G1 (2N DNA content), S (between 2N and 4N DNA content), and G2/M (4N DNA content)

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

phases of the cell cycle. Although some inferences about the movement of cells through the cycle may be drawn, the information gained is essentially static. For example, it is not known whether a cell with S-phase DNA content is actually synthesizing DNA; also, assessment of the progression of cells from G2 into mitosis is difficult because both G2 and mitotic cells have 4N DNA content and thus are not simply distinguishable from each other by PI staining. Several methods have been adopted to study IR-induced cell cycle checkpoints. Of these methods, dual-color flow cytometric analysis to study the G1/S checkpoint and the G2/M checkpoint, and $^{14}C/^{3}H$ thymidine incorporation assay to study the S-phase checkpoint are described in this chapter.

2. Materials

2.1. The G1 Checkpoint Assay: BrdUrd Labeling

1. 5'-bromodeoxyuridine (BrdUrd).
2. Pepsin.
3. 1% BSA/0.25% Tween-20/PBS buffer.
4. FITC-conjugated anti-BrdUrd antibody (Becton Dickinson).
5. Propidium iodide (Sigma).
6. RNase A (Sigma) .

2.2. The S-phase Checkpoint Assay

1. Thymidine [2-^{14}C] (NEC-156, NEN).
2. Thymidine [methyl-^{3}H] (NET-027Z, NEN).
3. Whatman™ GF/C filters (Whatman).
4. Millipore™ 1225 sampling manifold (Millipore).
5. Ecolite™ scintillation fluid (IGN).
6. Scintillation counter (Beckman).

2.3. The G2/M Checkpoint Assay: Phospho-Histone H3 Staining

1. Anti phospho-histone H3 (rabbit polyclonal IgG, Upstate Biotechnology).
2. Goat anti-rabbit FITC-conjugated antibody (Jackson ImmunoResearch Laboratories).
3. Propidium iodide (Sigma).
4. RNase A (Sigma).

3. Methods

3.1. The G1 Checkpoint Assay: BrdUrd Labeling

Dynamic information about cell cycle progression can be obtained by labeling cells with 5'-bromodeoxyuridine (BrdUrd), which is incorporated into DNA in place of thymidine. Detection of BrdUrd in the DNA allows the fraction of cells in the S phase to be enumerated and, if samples are taken at different time intervals, also gives information about the G1/S cell cycle kinetics after ionizing

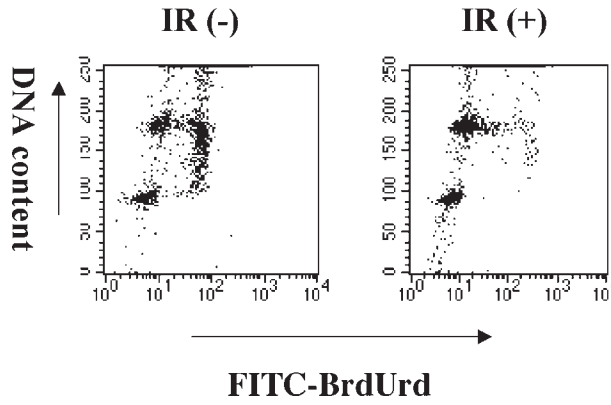

Fig. 1. Flow cytometric profiles of cell cycle distribution before treatment or 16 h after 6 Gy of IR. Staining for DNA content (propidium iodide, *y-axis*) and for BrdUrd (FITC-BrdUrd, *x-axis*) is presented.

radiation ***(2,3)***. After the application of a pulse label, monoclonal antibodies that react specifically with BrdUrd reveal those cells that are in S phase. Counterstaining with propidium iodide shows the cell cycle **(Fig. 1)** (*see* **Note 1**).

The methods described below outline (1) treatment; (2) pulse labeling; (3) fixation; (4) BrdUrd staining; (5) propidium iodide (PI) staining; and (6) flow cytometric analysis (*see* **Note 2**).

3.1.1. Treatment

1. Treat cells with ionizing radiation in exponential phase. All radiation treatments should be carried out using a γ-radiator or an x-ray generator. Cells should be irradiated at room temperature in 25m^2 tissue-culture dishes when cultures are 85% confluent. Cells will receive either a mock treatment for control or 1–10 Gy, respectively. After irradiation, cultures will be returned to 5% CO_2/37°C incubation until harvesting at the time points required.

3.1.2. Pulse Labeling

1. After incubation for 8, 16, or 24 h, add 10 μ*M* BrdUrd into medium. Incubate for an additional 30 min at 37°C.
2. Harvest cells by trypsination. Transfer cells into 15-mL conical tubes. Wash twice with PBS to remove unincorporated BrdUrd.

3.1.3. Fixation

1. Centrifuge cells at 1500*g* at 4°C for 5 min. Aspirate supernatant.
2. Rinse cells in PBS and vortex.
3. Centrifuge cells again at 1500*g* at 4°C for 5 min. Aspirate supernatant.

4. Add 600 µL PBS and pipet to mix well.
5. *Very slowly*, add 1.4 mL 100% methanol to the tube while gently vortexing. This will result in a final concentration of 70% methanol (*see* **Note 3**).
6. Store at –20°C for up to 48 h.

3.1.4. BrdUrd Staining

1. Centrifuge methanol fixed cells at 1500*g* for 5 min. Aspirate supernatant.
2. Suspend the pellet in 1 mL of 2 *N* HCl plus 0.2 mg/mL of pepsin.
3. Incubate exactly 30 min at room temperature.
4. Add 3 mL of 0.1 *M* sodium tetraborate (pH 8.5) to neutralize HCl.
5. Centrifuge at 2000*g* for 5 min. Aspirate supernatant.
6. Wash with 1% BSA/PBS.
7. Centrifuge at 2000*g* for 5 min. Aspirate supernatant.
8. Wash with 1% BSA/0.5%Tween-20/PBS.
9. Centrifuge at 2,000*g* for 5 min. Aspirate supernatant.
10. Dilute FITC-conjugated anti-BrdUrd antibody with 1%BSA/0.5% Tween-20/PBS. 1:20 dilution is suggested.
11. Suspend the cells with 100 µL diluted FITC-conjugated anti-BrdUrd antibody.
12. Incubate in the dark for 30 min at room temperature.
13. Wash once with PBS. Centrifuge and aspirate supernatant.

3.1.5. Propidium Iodide (PI) Staining

1. Stain with 25 µg/mL of propidium iodide and 0.1 mg/mL RNase A. Resuspend the cells in total volume of 500 µL PI/RNase A solution and transfer to 5-mL snap-cap tubes. When transferring, pipet through a very thin filter paper (40 µm mesh) to get rid of clumps that might clog the flow cytometer.
2. Incubate at room temperature in the dark for 20–30 min.

3.1.6. Flow Cytometric Analysis

1. Acquire data through a Becton Dickinson flow cytometer.
2. Analyze data using Cell Quest software.

3.2. The S-Phase Checkpoint Assay: RDS Assay

The activation of the S-phase checkpoint represents a transient decrease of DNA synthesis, as measured by [^{3}H] thymidine incorporation, in very early time points (usually within an hour) after DNA damage. The absence of IR-induced S-phase arrest, referred to as radioresistant DNA synthesis (RDS), has been previously reported from cells with defective ATM ***(4,5)***, BRCA1 ***(6)***, or NBS1 ***(7)***. The assay for studying the S-phase arrest, here called the RDS assay, is done as follows. Cells in the logarithmic phase of growth are pre-labeled with [^{14}C] thymidine, thus providing an internal control for cell number by allowing normalization for total DNA content of samples. Thirty to forty-five minutes after irradiation, cells are pulse labeled with [^{3}H] thymidine. The samples are mea-

sured by counting ^{3}H and ^{14}C in a liquid-scintillation counter. The resulting ratios of ^{3}H counts per minute to ^{14}C counts per minute, corrected for those counts per minute that are the results of channel crossover, are a measure of DNA synthesis.

The methods below will outline (1) ^{14}C-thymidine prelabeling; (2) treatment; (3) ^{3}H-thymidine labeling; (4) fixation; and (5) scintillation counting (*see* **Note 4**).

3.2.1. ^{14}C-Thymidine Prelabeling

1. Seed 1×10^{6} cells into T-25 flasks. An additional control flask is needed for each cell line.
2. Replace medium after 24 h with medium containing 10 nCi/mL ^{14}C-thymidine.
3. Incubate the cells for 24 h (*see* **Note 5**).

3.2.2. Treatment

1. Replace the ^{14}C-thymidine-containing medium with regular medium (*see* **Note 6**). Incubate at 37°C for 24 h.
2. Irradiate the cells with 0, 5, 10, or 20 Gy. Set aside the control cells (*see* **Note 7**).
3. Incubate for 30 min (*see* **Note 8**).

3.2.3. ^{3}H-Thymidine Labeling

1. Pulse label the cells by adding 2.5 µCi/mL ^{3}H-thymidine.
2. Incubate at 37°C for 15 min. While waiting, harvest the control (^{14}C only).

3.2.4. Fixation

1. Remove ^{3}H medium and wash once with 2 mL of cold PBS.
2. Trypsinize cells for 1–2 min at room temperature.
3. Add 5 mL PBS and centrifuge at 1200*g* for 5 min. Remove supernatant.
4. Wash again with PBS. Remove supernatant completely. Store on ice.
5. Fix cells in 70% methanol. *Very slowly*, add 2 mL 70% methanol to the tube while gently vortexing.
6. Store at 4°C for up to 48 h.

3.2.5. Scintillation Counting

1. Set up labeled tubes in scintillation counter racks.
2. Set up the filter apparatus. Apply a vacuum. Place Whatman GF/C filters on each of the 12 spots using forceps. Place the lid on and clamp it down. Close the vacuum.
3. Wet the filters by adding 0.5 mL of 70% methanol.
4. Pour the fixed cell suspension through the filters.
5. Rinse sequentially with 1 mL of 70% and 95% methanol. Leave on apparatus and air-dry the filters.
6. Using forceps, transfer filters to the scintillation vials. Add 5 mL scintillation fluid and place caps on the tubes. Wipes of surrounding areas should be performed to test for contamination.
7. Measure vials in a scintillation counter.

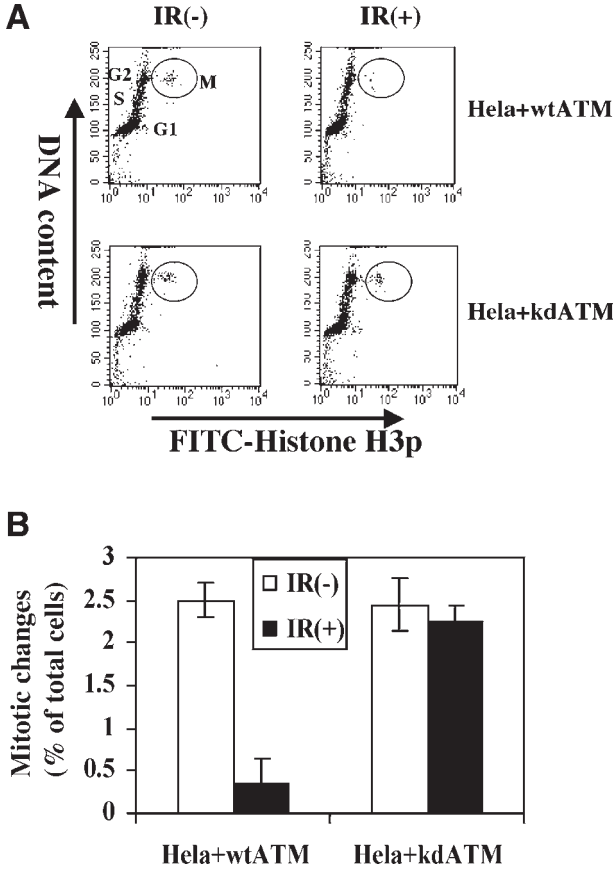

Fig. 2. Flow cytometric profiles of cell cycle distribution before treatment or 1 h after 6 Gy of IR. Shown are Hela cells transfected with wtATM or kdATM. Staining for DNA content (propidium iodide, *y-axis*) and for histone H3 phosphorylation (FITC-histone H3p, *x-axis*) is presented. The mitotic cell population is circled and labeled M. **(B)** quantitative analysis of the flow cytometric data in **(A)**. Shown is the ratio (irradiated:unirradiated) of the percentage of cells in mitosis.

3.3. The G2 Checkpoint Assay: Phospho-Histone H3 Staining

Since both G2 and mitotic cells have 4N DNA content and thus are not easily distinguishable from each other by propidium-iodide staining, rapid assessment of cell cycle progression from G2 into mitosis must utilize specific markers to recognize mitotic cells. Histone H3 is phosphorylated exclusively during mitosis; therefore, an antibody that specifically recognizes the

phosphorylated form (at serine 10) of histone H3 can be used in a flow cytometric assay ***(6,8,9)***. Co-staining of cells with PI to assess DNA content and an anti-phospho-histone H3 antibody demonstrates that the mitotic cells can be distinguished from G_2 cells in the 4N population of cells **(Fig. 2)**.

The methods described below outline (1) treatment; (2) fixation; (3) permeabilization; (4) phospho-histone H3 staining; (5) propidium iodide (PI) staining; and (6) flow cytometric analysis (*see* **Note 9**).

3.3.1. Treatment

1. Plate cells into T-25 flasks, incubate for 24 h (*see* **Note 10**).
2. Irradiate cells in exponential phase (*see* **Note 11**).
3. After 60 min incubation (*see* **Note 12**), rinse cells with PBS, aspirate PBS, and add 0.5 mL trypsin. Incubate at 37°C for 2 min.
5. After cells are dislodged, add 5 mL PBS to the trypsin. Rinse cells off the side of the flask. Pipet liquid and cells into a 15-mL tube (*see* **Note 13**). Use a cell scraper if necessary to harvest all cells.

3.3.2. Fixation

1. Centrifuge cells at 1200*g* at 4°C for 5 min. Aspirate supernatant.
2. Suspend cells in 5 mL PBS.
3. Centrifuge cells again at 1200*g* at 5°C for 5 min. Aspirate supernatant.
4. Add 600 µL PBS and pipet to mix well.
5. *Very slowly*, add 1.4 mL 100% ethanol to the tube while vortexing (to achieve a final concentration of 70% ethanol) (*see* **Note 14**).
6. Store at –20°C for up to 48 h.

3.3.3. Permeabilization

1. Centrifuge the ethanol-fixed cells at 1200*g* at 4°C for 5 min. Aspirate supernatant.
2. Rinse with 2 mL of PBS (twice). Centrifuge and aspirate supernatant.
3. Add 1 mL of 0.25% Triton/PBS.
4. Keep on ice for 10 min.

3.3.4. Phospho-Histone H3 Staining

1. Centrifuge at 1500*g* for 5 min and aspirate the supernatant.
2. Add 100 µL of the anti-phospho-histone H3 antibody in 1% BSA/PBS (final concentration is 10 µg/mL).
3. Incubate at room temperature for 3 h.
4. Rinse with 2 mL 1% BSA/PBS. Centrifuge at a higher speed of 2000*g* for 5 min and aspirate supernatant.
5. Dilute the secondary antibody (goat anti-rabbit FITC-conjugated antibody) 1:30 with 1% BSA/PBS. Wrap diluted stock containers in aluminum foil because the FITC antibody is light sensitive.
6. Add 100 µL of diluted secondary antibody into cells. Incubate at room temperature in the dark for 30 min.

3.3.5. Propidium Iodide (PI) Staining

1. Wash with PBS (twice). Centrifuge at 2000*g* for 5 min and aspirate supernatant.
2. Suspend cells with 500 µL PI/RNase A (final concentration is 0.25 µg/mL for PI and 0.1 mg/mL for RNase A) solution and transfer to 5-mL snap-cap tubes. When transferring, filter through 40 µm mesh to remove clumps that might plug the FACS flow stream.
3. Incubate at room temperature in the dark for 20–30 min.

3.3.6. Flow Cytometric Analysis

1. Acquire data through a Becton Dickinson flow cytometer.
2. Analyze data using Cell Quest software.

4. Notes

1. Pulse-labeling cells with BrdUrd can also be used to follow the S-phase cell progression after ionizing radiation. In this case, cells should be prelabeled with 10 µ*M* BrdUrd at the time of irradiation ***(8)***. Cells will be harvested at different time points after irradiation and stained with an anti-BrdUrd antibody. The BrdUrd-positive cells will be gated and the cell cycle distribution could be displayed by PI staining. For a detailed method, please see **ref. *8***.
2. In general, most of the cultured mammalian cell lines are suitable for the assays described in this chapter. Since IR-induced G1 is a p53-dependent process, tumor cell lines with proficient and deficient p53, such as MCF-7 and H1299 cells, can serve as good responders/controls to start with.
3. To avoid cell clumping, methanol should be added drop by drop, while votexing gently. If green fluorescence protein (GFP) is used as a marker for transfected cells, para-formaldehyde (PFA) should be used for fixation. This step, however, adversely affects PI staining and dramatically reduces the overall yield of cells available for FACS (in part by requiring extra centrifugation and in part by altering cell surface properties so as to cause aggregation in the presence of PI). To limit the degradation of peak resolution on the PI histogram, lower concentrations of PFA (0.25%) and shorter exposure times (5 min) should be applied.
4. In general, most of the cultured mammalian cell lines are suitable for the assay. Simian virus 40–transformed human fibroblast lines from a healthy A-T heterozygote or from a patient with A-T (GM0637 or GM9607, respectively; NIGMS Human Mutant Cell Repository) are good for positive and negative controls. The GM637 will show an intact S-phase response, while the GM9607 will display the RDS phenotype.
5. For transient transfection, cells will be transfected using lipofectamine reagents. After transfection, the medium will be incubated with ^{14}C-thymidine-containing medium for 24 h. The ^{14}C-thymidine-containing medium is then replaced by regular medium, and the cells will be incubated at 37°C for 4 h before irrradiation.
6. One wash with PBS is needed to remove residual ^{14}C-thymidine.
7. Timing is critical for dose-responsive RDS assays. If cells are to be irradiated for different doses, in order to finish irradiation at same time, we place the cells that

are to be irradiated 20 Gy into the radiation chamber first and irradiate them for 10 Gy, and then we add the cells that are to be irradiated for 10 Gy, and irradiate both groups of cells for additional 5 Gy. Finally, we put the cells that are to be irradiated for 5 Gy and irradiate all groups of cells for 5 Gy.

8. Most cell lines with active S-phase checkpoint display 50% inhibition of DNA synthesis 30 to 45 min after 10 Gy irradiation. However, the earliest time point to see the down-regulation of DNA synthesis varies among cell lines.
9. Most of the cultured mammalian cell lines, except some heterogeneous human fibroblastic cell lines such as GM0637, are suitable for the assay. HeLa cells can be used as positive controls, while GM9607 (ATM–/–) or HCC1937 (BRCA1–/–) cells can be used as negative controls. HeLa cells are especially useful as they have high transfection efficiency, thus allowing genetic manipulations.
10. At least 2×10^6 cells are needed for the histone H3 staining.
11. The G2/M checkpoint is a dose-independent process. We usually radiate cells at 6 Gy.
12. The typical G2/M checkpoint occurs at very early time points (usually within an hour); therefore 1 to 2 h after irradiation is the best harvest time.
13. Most dead cells float in the medium. Mitotic cells round up and detach, and so should be in the medium as well. However, we have not seen good evidence for this when we used FACS to observe cells in the medium compared with the adherent cells from the same plate.
14. To avoid cell clumping, ethanol should be added drop by drop while vortexing gently.
15. With regard to fixation: If green fluorescence protein (GFP) is used as a marker for transfected cells, para-formaldehyde (PFA) should be used for fixation. This step, however, adversely affects PI staining and dramatically reduces the overall yield of cells available for FACS (in part by requiring extra centrifugation and in part by altering cell surface properties so as to cause aggregation in the presence of PI). To limit the degradation of peak resolution on the PI histogram, lower concentration of PFA (0.25%) and shorter exposure times (5 min) should be used.

Acknowledgments

We thank Jim Houston and Diane Woods for their technical assistance.

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21

FACS-Based Detection of Phosphorylated Histone H3 for the Quantitation of Mitotic Cells

William R. Taylor

Summary

The G2 checkpoint blocks cells from entering mitosis when DNA is damaged, and helps to protect the integrity of the genome. Tumor cells contain mutations that can inactivate checkpoints, and the inactivation of the G2 checkpoint can induce genomic instability and alter cellular responses to chemotherapeutic agents that damage DNA. The traditional method to assess whether the G2 checkpoint is normal is to microscopically count mitotic cells. A method using the fluorescence-activated cell scanner (FACS) is described, based on the presence of an intranuclear antigen present only in mitotic cells, detected using a specific, commercially available antibody. Cell staining and FACS analysis can be done in a single day, making this a rapid and reliable method to quantitate mitotic cells for various applications.

Key Words: Checkpoint; cell cycle; mitosis.

1. Introduction

Progression through the cell cycle is a highly regulated process. When conditions are not right, the cell cycle is halted to prevent extensive damage to the genome ***(1)***. A major point of arrest in response to DNA damage is in the G2 phase of the cell cycle ***(2)***. Several features of the cell cycle are important to consider when analyzing the G2 checkpoint. Upon completion of G2, typical mammalian cells enter mitosis, which is a short phase of the cell cycle (approx 1 h) compared to the entire cell cycle (approx 24 h). Therefore, even in rapidly growing populations, mitosis is a relatively rare event; only approx 4% of the cells are in mitosis at any one time. Thus, it is essential to have sensitive methods to detect mitotic cells. Also, when DNA is damaged, cells that are in mitosis are capable of progressing through to G1, but entry of new cells into mitosis from G2 is rapidly blocked. Since there are no specific markers for cells blocked in G2, the feature commonly used to assess G2 arrest is the depletion

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

of mitotic cells after DNA damage ***(3)***. DNA damage can also block cells in S phase and G1. Therefore, to obtain the most reliable measurement of G2 arrest, analyses should be carried out soon after DNA is damaged (approx 1–2 h). If longer time points are analyzed, the interpretation of the arrest point will be less certain. For example, if cells lack a G2 arrest but retain an S-phase arrest, the depletion of mitotic cells will still occur, but only after the cells that are in G2 have completed mitosis.

The traditional method to measure the depletion of mitotic cells is to treat cells with a hypotonic buffer, fix them, drop them onto slides, and microscopically count mitotic figures (for example, *see* **ref. *4***). Since mitosis is normally a rare event, many cells must be assessed, making this a very laborious method. Here we describe a sensitive method to count mitotic cells using the FACS. Cells are stained with an antibody that recognizes an antigen present only in mitotic cells, combined with propidium iodide to stain DNA. Two-dimensional FACS scanning allows the differential quantitation of G2 and mitotic cells. Several labs are currently using this method, and several antibodies to different mitotic markers have been used ***(5,6)***. We originally used an antibody to a highly phosphorylated form of histone H1b that was prepared by the Allis lab ***(7,8)***. Antibodies to MPM-2 antigens present in mitotic cells have also been used ***(5)***. The commercial availability and specificity of antibodies to histone H3 phosphorylated at serine 10, which is present only in mitotic cells, has made FACS-based detection of mitosis more popular ***(9)***. The method we provide is based on our experience and on a recently published study using the phospho-H3 antibody ***(6)***. Since FACS-based measurement of mitotic cells eliminates the dreaded visual counting of thousands of cells, it is likely to replace the traditional method faster than you can say mitosis.

2. Materials

1. Phosphate buffered saline (PBS): 1.06 m*M* KH_2PO_4, 155.17 m*M* NaCl, 2.96 m*M* Na_2HPO_4.
2. PBSBA: PBS containing 1% bovine serum albumin (w/v) and 0.02% sodium azide (w/v). Can be stored at 4°C for at least 1 mo.
3. PBS containing 0.25% (v/v) Triton X-100.
4. Rabbit antiserum against histone H3 phosphorylated on serine 10 (Upstate Biotechnology).
5. Goat anti-rabbit antibody labeled with fluorescein isothiocyanate (FITC) (Sigma).
6. Cellular DNA Flow Cytometric Analysis Reagent Set (Roche), which contains: propidium iodide solution (500 µg/mL) and RNase (DNase-free solution, 200 units/mL).
7. Blue Max™ Jr. 15-mL polypropylene conical tube (17 × 120 mm, Falcon).
8. 5-mL polystyrene round-bottom tubes (12 × 75 mm, Falcon).
9. BD Falcon Tube with Cell Strainer Cap (12 × 75 mm, Falcon).

10. Standard aspiration flask.
11. Trypsin-EDTA (Gibco-BRL).

3. Methods

1. Cells are grown on a single 10-cm tissue-culture plate per sample at approx 70% confluency. When seeding cells, it is important to prepare an extra plate that will be used as a negative control (*see* **Note 1**).
2. Add 1 mL 100% ethanol to a 5-mL round-bottom tube. Place at –20°C for use in **step 5** below.
3. Collect the cells by trypsinization. Mitotic cells have a tenuous attachment to the culture plates. Therefore, decant the tissue culture medium from a 10-cm plate into a Blue Max™ Jr. 15-mL tube to save any mitotic cells that are floating in the medium. Release the cells attached to the plate by adding 1 mL trypsin-EDTA and add the cells to the appropriate 15-mL Falcon tube. Collect the cells by centrifugation at 450*g* for 20 min.
4. Wash the cells by resuspending the pellet in 5 mL PBS and collect them by centrifugation at 450*g* for 20 min.
5. Fix cells in 70% ethanol at –20°C as follows: Resuspend the cell pellets from **step 4** with 400 µL PBS using a P1000 Pipetman. Add the 400-µL cell suspension dropwise to the 1-mL cold ethanol (prepared in **step 2**) while gently vortexing the ethanol. Fix the cells for at least 1 h at –20°C. Fixed cells can be stored at –20°C for a few days (probably much longer).
6. Transfer the fixed cells to a 1.5-mL Eppendorf tube and centrifuge at 1400*g* for 7 min at 4°C. Carefully remove the supernatant solution by aspiration (*see* **Note 2**).
7. Resuspend the cell pellet with 1.0 mL PBS. Centrifuge at 1400*g* for 7 min. Remove the supernatant as above.
8. Resuspend cells with 1.4 mL PBS + 0.25% Triton X-100. Incubate on ice for 15 min.
9. Centrifuge at 1400*g* for 7 min. Remove the supernatant.
10. Resuspend the cell pellet in 100 µL PBSBA containing 0.75 µg anti-phosphorylated histone H3. Incubate for 2–3 h at room temperature. Invert tubes every 15 min or rock gently for the entire period.
11. Centrifuge at 1400*g* for 7 min. Remove as much of the supernatant as possible without disrupting the pellet (*see* **Notes 2 and 3**).
12. Add 1.3 mL PBSBA. Invert to mix.
13. Centrifuge at 1400*g* for 7 min. Remove the supernatant.
14. Resuspend pellet in 100 µL PBSBA containing fluorescein-labeled goat anti-rabbit antibody (1:200). Incubate for 30 min at room temperature in the dark with gentle mixing as described in **step 10**.
15. Add 1.3 mL PBSBA. Invert to mix.
16. Centrifuge at 1400*g* for 7 min. Remove the supernatant.
17. Resuspend in 500 µL PBS.
18. Add 5 µL RNase, DNase-free, mix by inversion, and incubate at 37°C for 30 min (*see* **Note 5**).

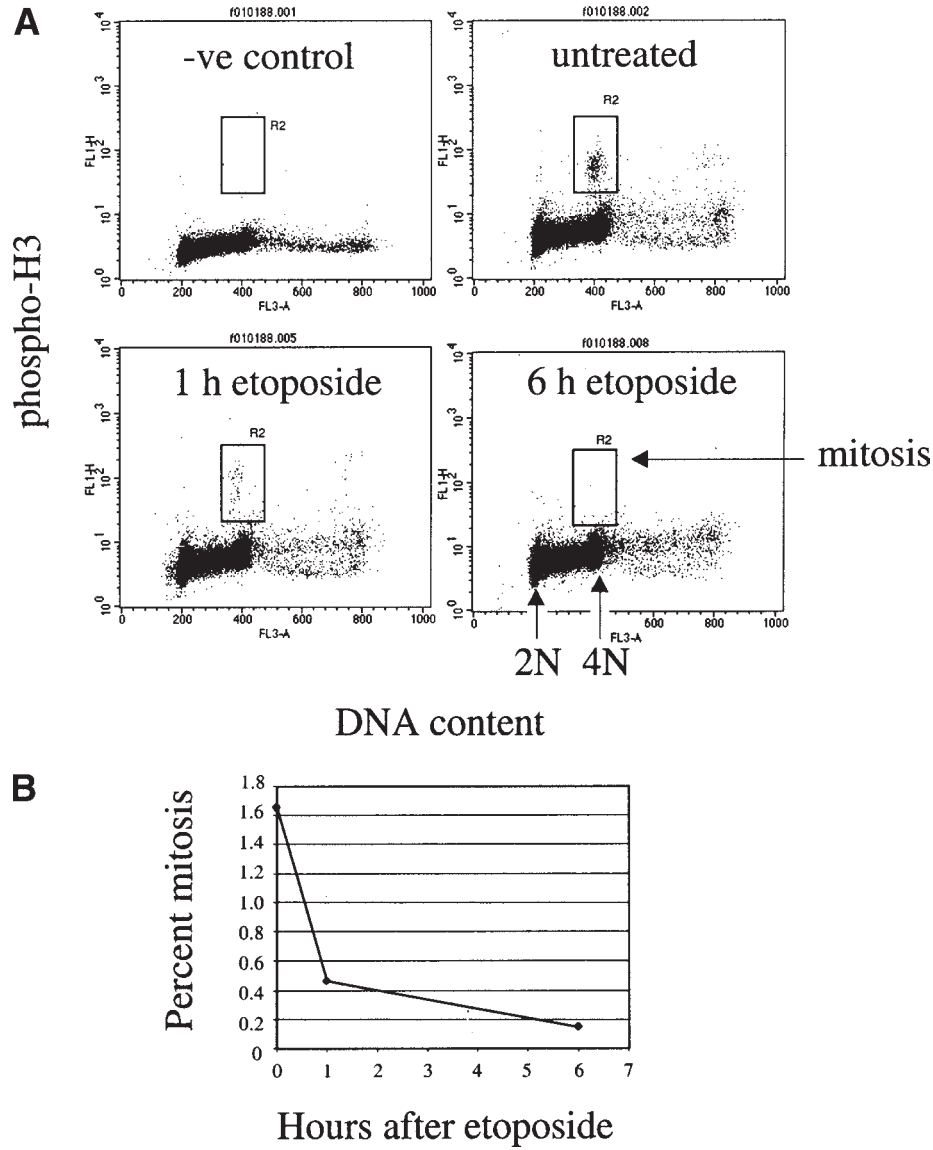

Fig. 1. Analysis of the G2 checkpoint. HT1080 fibrosarcoma cells untreated or exposed to 10 μ*M* etoposide were analyzed. **(A)** Two-dimensional scatter plots of phosphorylation of histone H3 as a function of DNA content. The negative control ("–ve control") (*see* **Note 1**) corresponds to cells not incubated with the primary antibody, but exposed to all other steps of the procedure. The rectangle (R2) encompasses cells that have a 4N content of DNA and high levels of histone H3 phosphorylation, and therefore that are in mitosis. **(B)** The percent of cells in mitosis before and after treatment with etoposide (*see* **Note 6**). *(Continued on next page)*

19. Cool on ice for a few seconds and add 100 µL propidium-iodide solution (*see* **Note 5**). Mix by inversion and incubate on ice for 15 min.
20. Analyze by FACS using two-parameter scanning within 6 h of staining (*see* **Note 4**). By plotting FL1-H (FITC) versus FL3-A (propidium iodide), mitotic cells with 4N DNA and high levels of phosphorylated histone H3 can be detected. Typical results are shown in **Fig. 1**.

4. Notes

1. A negative control sample is essential in setting gates to find cells that are positive for the phospho-histone H3 antigen. For this purpose, a parallel plate of the same type of cells should be treated just as the experimental sample, following all the steps above *except* for adding the primary antibody (**step 10**). Instead, add 100 µL of PBSBA. Carry out all other steps, including staining with the anti-rabbit FITC antibody and propidium iodide, as described. With rapidly growing cells, the mitotic population is easily detectable, and a positive control is not necessary. However, if no mitotic cells are detected, the staining and flow cytometry steps should be tested using a cell line that is known to grow rapidly—for example, HT1080 or HeLa.
2. The entire method involves many centrifugation and resuspension steps. Every time the supernatant is aspirated, some cells are lost. To reduce the loss of cells, attach a 200 µL pipet tip to a vacuum tube but do not submerge the tip into the bottom of Eppendorf tube near the pellet. Instead tilt the tube and allow the supernatant to drain down into the vacuum tip. It is not necessary to remove all of the supernatant, and we usually leave approx 50 µL in the tube so as not to disrupt the pellet. Also, the pellet becomes more and more fragile with every manipulation. Therefore, care should be taken so that the pellet does not become dislodged and lost during aspiration.
3. We have found that we can omit **step 11** and still obtain good separation between background staining and the positive staining of mitotic cells. However, it is possible that other batches of antibodies may produce higher background staining, and **step 11** will help to remove more of the residual primary antibody solution and increase the signal-to-noise ratio. If background remains a problem, an additional wash can be added by repeating **steps 12** and **13**. However, when **step 12** is repeated, the cell suspension should be rocked for 10 min at room temperature to remove additional antibody that is nonspecifically bound before proceeding to **step 13** and completing the rest of the protocol.
4. Ethanol fixation can lead to cell clumping, which can interfere with the operation of the FACS. To remove clumps, cell suspensions are first filtered using Falcon tubes with a 35 µm nylon mesh incorporated in the cap (*see* **Materials**, item 9). Since mitotic cells are relatively rare, it is advisable to analyze 20,000 cells per

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Fig. 1. *(Continued)* Scanning was performed with a Becton, Dickinson FACScan (San Jose, CA), and data were analyzed with Cell Quest 3.3 software (San Jose, CA).

sample. Cell doublets and dead cells are excluded from the analysis by gating, and thus it will be necessary to collect approx 10 to 15% more events to acquire enough cells for analysis.

5. For convenience, we use a reagent set from Roche which contains separate RNase, and propidium iodide solutions. The individual reagents are available from other suppliers and could be used instead.
6. In rapidly growing HT1080 cells, we find approx 2% are in mitosis, based on the detection of high levels of phosphorylated histone H3. Based on the relative length of mitosis (approx 1h) compared to the total length of the cell cycle (approx 24 h), we expect approx 4% of the cells to be in mitosis. However, 4% is an estimate based on the length of mitosis and the entire cell cycle. Therefore, this estimate will be affected by how the beginning and end of mitosis is defined. Thus, using a quantitative method to detect a defined mitotic marker may provide a more consistent measure. However, since histone H3 dephosphorylation starts during anaphase and before mitosis is complete (defined by completion of cytokinesis), the actual number of cells in mitosis may be underestimated using histone H3 phosphorylation compared to other morphological markers ***(10)***. This fact does not adversely affect the utility of the FACS method for comparing populations of cells, but should be kept in mind when interpreting the data with respect to the overall process of mitosis.

Acknowledgments

I thank George Stark and Cathy Stanko for critical review of this manuscript, and Cathy Stanko and Dolly Klingman for help with flow cytometry. This work was supported by grant GM49345 from the National Institutes of Health.

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22

Analysis of Cell Cycle by Flow Cytometry

Piotr Pozarowski and Zbigniew Darzynkiewicz

Summary

Described are four widely used procedures to analyze the cell cycle by flow cytometry. The first two are based on univariate analysis of cellular DNA content following cell staining with either propidium iodide (PI) or 4',6'-diamidino-2-phenylindole (DAPI) and deconvolution of the cellular DNA content frequency histograms. This approach reveals distribution of cells in three major phases of the cycle (G_1 vs S vs G_2/M) and makes it possible to detect apoptotic cells with fractional DNA content. The third approach is based on the bivariate analysis of DNA content and proliferation-associated proteins. The expression of cyclin D, cyclin E, cyclin A, or cyclin B1 vs DNA content is presented as an example. This approach allows one to distinguish, for example, G_0 from G_1 cells, identify mitotic cells, or relate expression of other intracellular proteins to the cell cycle position. The fourth procedure relies on the detection of 5'-bromo-2'-deoxyuridine (BrdU) incorporation to label the DNA-replicating cells.

Key Words: Flow cytometry; cellular DNA content; cyclins; BrdU incorporation; mitosis.

1. Introduction

A variety of flow or cytometric methods to analyze the cell cycle progression have been developed over the past three decades. These methods can be grouped into three categories:

1. In the first are the methods that rely on a single time point ("snapshot") cell measurement. This analysis may be either univariate, generally based on measurement of cellular DNA content alone ***(1)***, or multivariate (multiparameter), when in addition to DNA content another cell attribute is measured ***(2–4)***. The additional attribute is generally a metabolic or molecular feature that correlates with a rate of cell progression through the cycle or is a marker the cell proliferative potential or quiescence. The single time-point measurement reveals the percentage of cells in G_1 vs. S vs. G_2/M, but it does not provide information on cell cycle kinetics. The duration of each phase, however, can be estimated from the percentage of cells in this phase if the length of the cell cycle (or the doubling time of cells in the culture) is known.

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

2. In the second group are the methods that rely on time-lapse measurements of cell populations synchronized in the cycle, or whose progression through the cycle was halted by the agent arresting them at a specific point of the cycle. These methods reveal kinetics of cell progression through the cycle. An example of such methods is the stathmokinetic approach, where cells are arrested in mitosis, e.g., by vinblastine or colcemide, and the rate of cell entrance into mitosis ("cell birth" rate) is estimated from the slope representing a cumulative increase in percentage of mitotic cells as a function of the time of the arrest ***(5)***.
3. Methods of the third category are based on the detection of incorporation of the thymidine analog 5'-bromo-2'-deoxyuridine (BrdU), often combined with DNA content measurements. They may be either single time-point measurements or use the time-lapse strategy. The incorporated BrdU is detected either cytochemically, based on the use of the DNA dyes, such as Hoechst 33258, whose fluorescence is quenched by BrdU ***(6)***, or immunocytochemically, using fluoresceinated BrdU-antibodies ***(7)***. The time-lapse measurement of the cohort of BrdU-labeled cells allows one to estimate their rate of progression through different points of the cell cycle ***(8)***.

Only a few methods are presented in this chapter. In **Subheadings 3.1.** and **3.2.**, two methods are presented that rely on the univariate analysis of cellular DNA content. The first utilizes propidium iodide (PI) as the DNA fluorochrome and requires blue light as the excitation source (e.g., 488 nm argon ion laser). Because PI also stains double-stranded RNA, the latter is removed by the addition of RNase A to the staining solution. In the second protocol, the UV-light-excitable 4',6'-diamidino-2-phenylindole (DAPI) is used as a DNA-specific fluorochrome. Cell staining with DAPI is simpler, as it does not require incubations with RNase, and is generally preferred when a cytometer that has a UV light excitation source is available. In **Subheading 3.3.**, the analysis of cellular DNA content, combined with the expression of proliferation-associated proteins, is described. The method is illustrated by demonstrating the characteristic expression of cyclins D1, E, A, or B1 in normal cells, but this approach may be used to measure the expression of other intracellular antigens in relation to the cell cycle position. The immunocytochemical detection of incorporated BrdU, combined with the analysis of DNA content, is presented in **Subheading 3.4.**. More detailed descriptions of these and other methods, their modifications and applicability to different cell systems, as well as advantages and limitations, are provided in numerous chapters of the methodology books specifically devoted to the cell cycle and cytometry ***(9–15)***.

2. Materials

1. Flow cytometer. A variety of models of flow cytometers of different makers can be used to measure cell fluorescence following staining according to the procedures listed under **Subheading 3.1.**. The manufacturers of the most common flow

cytometers are Becton Dickinson Immunocytometry Systems, Beckman/Coulter Inc., DACO/Cytomation, and PARTEC GmbH.
2. The software used to deconvolute the DNA content frequency histograms, to estimate the proportions of cells in the respective phases of the cycle, is available from Phoenix Flow Systems and Verity Software House (*see* **Note 1**).
3. Centrifuge that can accommodate 5-mL tubes.
4. PI staining solution: 0.1% (v/v) Triton X-100, 10 µg/mL PI (Molecular Probes, Inc.), and 100 µg/mL DNase-free RNase A in PBS.
5. PBS (phosphate buffered saline, e.g. Dulbecco PBS): 136.9 m*M* NaCl, 2.68 m*M* KCl, 8.1 m*M* Na_2HPO_4, 0.9 m*M* $CaCl_2$, 0.49 m*M* $MgCl_2$.
6. DAPI staining solution: 0.1% (v/v) Triton X-100 and 1 µg/mL DAPI (Molecular Probes, Inc.) in PBS.
7. Monoclonal or polyclonal antibodies (Abs) applicable to cell cycle analysis, including cyclin Abs (provided, e.g., by DACO Corporation, Sigma Chemical Co., Upstate Biotechnology Incorporated, B.D. Biosciences/PharMingen, and Santa Cruz Biotechnology, Inc.).
8. Cell permeabilizing solution: 0.25% Triton X-100, 0.01% sodium azide in PBS.
9. Rinsing solution: 1% bovine serum albumin (BSA), 0.01% sodium azide in PBS.
10. DNA denaturation buffer: 0.1 m*M* Na-EDTA in 1 m*M* Na-cacodylate; adjust pH to 6.0. To make 0.2 *M* stock solution of cacodylate buffer, dissolve 42.8 g $Na(CH_3)_2$ $As_2{\cdot}3H_2O$ in 100 mL H_2O, take 50 mL of this solution, add to it 29.6 mL of 0.2 *M* HCl, and adjust volume to 200 mL with H_2O.
11. Diluting buffer: 0.1% Triton X-100, 0.5% (w/v) BSA in PBS.
12. 0.2 *M* phosphate buffer, pH 7.4 (mixture of 81 vol of 0.2 *M* Na_2HPO_4 with 19 vol of 0.2 *M* KH_2PO_4).

3. Cellular DNA Content Measurement

3.1. Cell Staining With PI

1. Suspend approx 10^6 cells in 0.5 mL of PBS. Vortex gently (approx 5 s) or gently aspirate several times with a Pasteur pipet to obtain a mono-dispersed cell suspension, with minimal cell aggregation.
2. Fix cells by transferring this suspension, with a Pasteur pipet, into centrifuge tubes containing 4.5 mL of 70% ethanol, on ice. Keep cells in ethanol for at least 2 h at 4°C. Cells may be stored in 70% ethanol at 4°C for weeks.
3. Centrifuge the ethanol-suspended cells for 5 min at 300*g*. Decant ethanol thoroughly.
4. Suspend the cell pellet in 5 mL of PBS, wait approx 30 s and centrifuge at 300*g* for 5 min.
5. Suspend the cell pellet in 1 mL of PI staining solution. Keep in the dark at room temperature for 30 min, or at 37°C for 10 min.
6. Transfer sample to the flow cytometer and measure cell fluorescence. Maximum excitation of PI bound to DNA is at 536 nm, and emission is at 617 nm. Blue (488 nm) or green light lines of lasers are optimal for excitation of PI fluorescence.

Emission is measured using the long-pass 600- or 610-nm filter (for data acquisition, interpretation, and possible pitfalls, *see* **Notes 1–5**).

3.2. Staining With DAPI

1. Suspend the cell pellet in 1 mL of DAPI staining solution. Keep in the dark, at room temperature, for 10 min.
2. Transfer the sample to the flow cytometer and measure cell fluorescence. Maximum excitation of DAPI bound to DNA is at 359 nm, and emission is at 461 nm. For fluorescence excitation, use the available UV light laser line at the wavelength nearest to 359 nm. When a mercury arc lamp serves as the excitation source, use a UG1 excitation filter. A combination of appropriate dichroic mirrors and emission filters should be used to measure cell fluorescence at wavelengths between 450 and 500 nm (for data acquisition, interpretation, and possible pitfalls, *see* **Notes 1–5**).

3.3. Cellular DNA Content and Expression of Proliferation-Associated Proteins

1. Fill 5-ml polypropylene tubes with 4.5 mL of methanol (or 70% ethanol; *see* **Note 6**). Keep tubes on ice.
2. Suspend 1–2 × 10^6 cells in 0.5 mL of PBS. Fix the cells by transferring this suspension with a Pasteur pipet into the ice-cold methanol tube. Keep cells in the fixative at –20°C at least overnight (the cells can be stored in the fixative at – 20°C for days).
3. Centrifuge at 300*g* for 3 min. Resuspend the cell pellet in 5 mL PBS. Keep for 5 min at room temperature. Spin at 200*g* for 5 min.
4. Resuspend the cells in 0.5 mL of the permeabilizing solution. Keep at room temperature for 5 min. Centrifuge as above.
5. Resuspend cell pellet in 100 µL of the rinsing solution that contains the primary Ab. Follow instructions supplied by the vendor regarding the final titer of the supplied antibody (0.5–1.0 µg of the Ab per 10^6 cells suspended in 100 µL is generally optimal). Incubate 60 min at room temperature with gentle agitation or overnight at 4°C (*see* **Notes 6** and **7**).
6. Add 5 ml of the rinsing solution. Centrifuge at 300*g* for 5 min.
7. Use the isotype immunoglobulin as a negative control (*see* **Note 7**). Process as in **steps 5** and **6**.
8. Resuspend cells in 100 µL of rinsing solution that contains the fluoresceinated secondary Ab, generally at a final 1:20 to 1:40 dilution. Incubate at room temperature for 30–60 min, gently agitating.
9. Add 5 mL of the rinsing solution, centrifuge at 300*g* for 5 min.
10. Suspend the cell pellet in 1 mL PBS containing 5 µg of PI and 100 µg of DNase-free RNase A. Keep in the dark at room temperature for 1 h.
11. Transfer the cells to the flow cytometer. Use blue light (488 nm laser line) for fluorescence excitation. Measure cell fluorescence in green (FITC, 530 ± 20 nm) and red (PI, >620 nm) light wavelength.

12. Analyze the data (*see* **Notes 7–9**).
    Note: If the primary Ab is fluorochrome-tagged, skip **steps 8** and **9**.

3.4. Detection of Incorporated BrdU and Cellular DNA Content

3.4.1. Thermal Denaturation of DNA

1. Incubate the cells with 10–50 µ*M* BrdU in the dark (*see* **Note 10**).
3. Fix the cells in suspension in 70% ethanol.
4. Centrifuge the cells ($1–2 \times 10^6$) at 200*g* for 5 min, resuspend cell pellet in 1 mL of diluting buffer containing 100 µg/mL of RNase A, and incubate at 37°C for 30 min.
5. Centrifuge cells (300*g*, 5 min) and suspend cell pellet in 1 mL of ice-cold 0.1 *M* HCl containing 0.1% Triton X-100. After 1 min, centrifuge the cells again. Drain thoroughly and resuspend in 5 mL of DNA denaturation buffer. This step is designed to dissociate histones from DNA in order to increase DNA susceptibility to denaturation.
6. Centrifuge the cells again and resuspend the cell pellet in 1 mL of DNA denaturation buffer.
7. Heat cells at 90 or 95°C for 5 min, then place on ice for 5 min (*see* **Note 11**).
8. Add 5 mL of diluting buffer and centrifuge at 300*g* for 5 min.
9. Drain well and suspend the cells in 100 µL of BrdU Ab dissolved in diluting buffer, for 30 min at room temperature (follow the instructions provided by the supplier regarding the dilution, time, and temperature of incubation with anti-BrdU).
10. Add 5 mL of diluting buffer and centrifuge as above.
11. Suspend the cells in 100 µL of FITC-labeled goat anti-mouse IgG (dissolved in diluting buffer), and incubate for 30 min at room temperature. If the primary BrdU Ab was FITC-labeled, skip this step.
12. Add 5 mL of diluting buffer, centrifuge, drain, and resuspend the cells in 1 mL of this buffer containing 5 µg/mL of PI.
13. Measure the BrdU-associated green fluorescence and DNA-associated red fluorescence as described in **Subheading 3.3., step 12** (*see* Note 10)**.**

3.4.2. Denaturation of DNA by HCl

1. Follow **steps 1–4** as described above for thermal denaturation of DNA (**Subheading 3.4.1.**).
2. Centrifuge the cells (300*g*, 5 min) and resuspend cell pellet in 1 mL of 2 *M* HCl (*see* **Note 11**). After 20 min at room temperature, add 5 mL of PBS, centrifuge, and drain well.
3. Resuspend the cells in 5 mL of 0.2 *M* phosphate buffer at pH 7.4, to neutralize any traces of the remaining HCl.
4. Centrifuge the cells at 300*g* for 5 min.
5. Follow **steps 8–13** as described above for thermal denaturation of DNA (**Subheading 3.4.1.**).

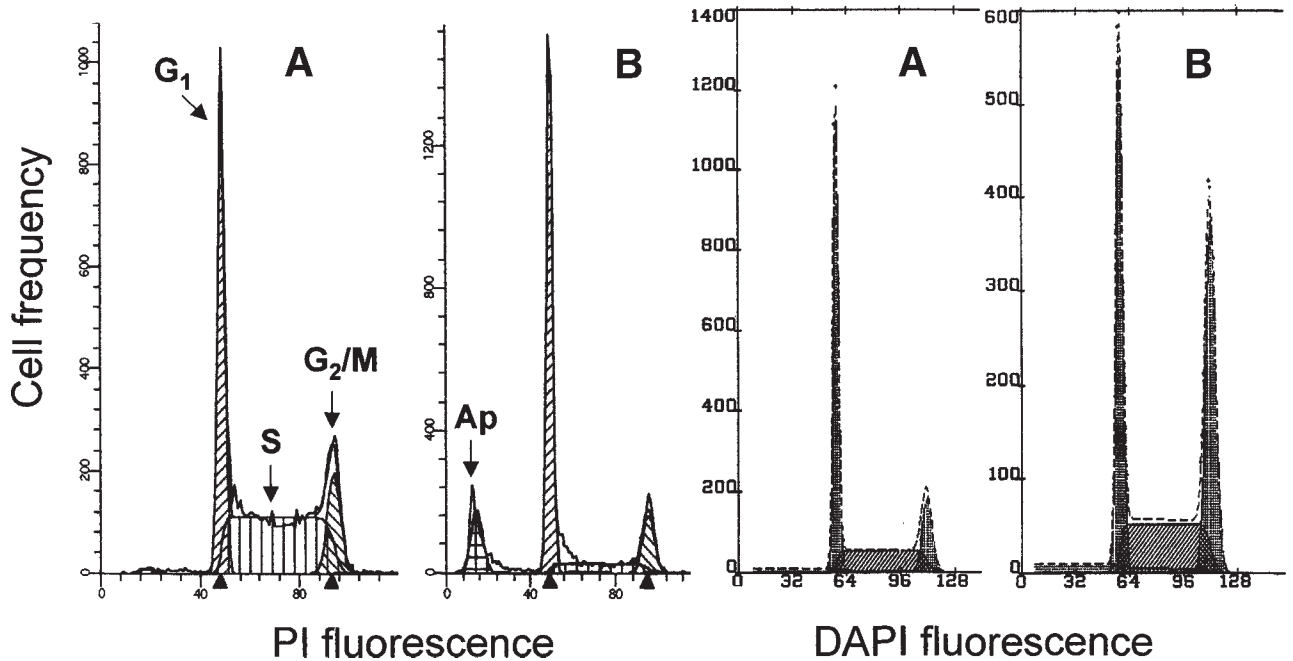

Fig. 1. Typical DNA content frequency histograms representing cells from the untreated cultures **(A)** and from the cultures treated with the drugs that affect the cell cycle distribution and induce apoptosis **(B)**. The cells were stained with PI or DAPI, according to the presented protocols, respectively. Fluorescence of the PI-stained cells was measured using FACScan (Becton Dickinson Immunocytometry Systems) and ModFit deconvolution software. DAPI flourescence was measured using EPICS ELITE cytometer (Beckman/Coulter, Inc.) and the histograms were plotted by the MultiCycle (Phoenix Flow Systems) program. Both software programs provide the estimate of percentage of cells with fractional DNA content (apoptotic cells: Ap) and cells in G_1, S, and G_2/M phases of the cycle.

4. Notes

1. The data acquisition software of most flow cytometers/sorters allows one to record the fluorescence intensities (the integrated area of the electronic pulse signal) of 10^4 or more cells per sample. The data are presented as cellular DNA content frequency histograms **(Fig. 1)**. The data analysis software packages ***(16)*** that deconvolute the frequency histograms to obtain percentage of cells in $G_{0/1}$, S, and G_2 + M are either included with the purchase of the flow cytometer or are commercially available from other vendors, as listed in **Subheading 2.**. For details of the procedures of deconvolution of DNA content frequency histograms, see **ref. *16***.
2. All cells in G_1 have a uniform DNA content, as do cells in G_2/M; the latter have twice as much DNA as G_1 cells. Under ideal conditions of DNA staining, the fluorescence intensities of all G_1 or G_2/M cells are expected to be uniform, and after analog-to-digital conversion of the electronic signal from the photomultiplier (representing their fluorescence intensity), to have uniform numerical values, respectively. In practice, however, the G_1 and G_2 cell populations are represented on frequency histograms by peaks of various width. The coefficient of variation (CV) of the mean value of DNA-associated fluorescence of the G_1 population (width of the peak) is a reflection of the accuracy of DNA content measurement, and should not exceed 6%. Improper staining conditions, instrument mis-adjustment, and the presence of a large number of dead or broken cells, will all result in high CV of the G_1 cell populations.
4. Apoptotic cells often have fractional DNA content due to the fact that the fragmented (low MW) DNA undergoes extraction during the staining procedure. Some cells also lose DNA (chromatin) by shedding apoptotic bodies. Thus. only a fraction of the DNA remains within apoptotic cells. They are then represented on the DNA content frequency histograms by the "sub-G_1" peak **(Fig. 1)**. Commercially available software packages to deconvolute DNA histograms are able to identify and quantity the "sub-G_1" cell population.
5. If the duration of the cell cycle (or the cell doubling time) is known, the length of each phase can be estimated from the percentage (fraction) of cells in that phase. For example, during the exponential phase of cell growth, the duration of G_1 (T_{G1}) can be calculated from the equation:

$$T_{G1} = [T_C \times \ln(f_{G1} + 1)]/\ln 2$$

   where T_C is duration of the cell cycle and f_{G1} is the fraction of cells in G_1. T_C, with some approximation, equals the cell doubling time in cultures that can be estimated from growth curves.
5. The critical steps for immunocytochemical detection of intracellular proteins are cell fixation and permeabilization. The fixative is expected to stabilize the antigen *in situ* and preserve its epitope in a state where it remains reactive with the Ab. The cells have to be permeable to allow access of the Ab to the epitope. The choice of the optimal fixative and permeabilizing agent vary, primarily depending on the intracellular antigen, less on the cell type. General strategies of cell fixation, permeabilization, and stoichiometry of antigen detection are discussed

by Jacobberger ***(17)***. Cold methanol appears to be optimal for the detection of D-type cyclins. For cyclins E, A, and B1, 70% cold ethanol is equally good.

6. Another critical point is the choice of a proper Ab. Often, the Ab applicable to immunoblotting fails in immunocytochemical applications, and vice versa. This may be the result of differences in the *in situ* accessibility of the epitope or differences in the degree of denaturation of the antigen on the immunoblots, compared to that within the cell. Some epitopes may not be accessible *in situ* at all, while the accessibility of others may vary, depending on their functional state—for example, owing to chemical modifications (e.g., phosphorylation or acetylation) or steric hindrance. Since there is strong homology between different cyclin types, cross-reactivity also may be a problem. Because commercially available Abs may differ in specificity, degree of cross-reactivity, and so on, it is essential to provide detailed information (vendor and the hybridoma clone number) of the reagent used in the study. Although the isotypic IgG is commonly used as a negative control, some IgG preparations may show reactivity to various cell constituents. The optimal negative control would be the "knock-out" cell line (if available), where the gene coding for the studied protein has been deleted.
7. Expression of proliferation-associated proteins varies during the cell cycle **(Fig. 2)** and is often different in cycling and quiescent cells. The most widely used markers are the proliferating cell nuclear antigen (PCNA) ***(4)***, the antigen detected by Ki-67 antibody ***(3)***, and cyclins D, E, A, and B ***(2)***.
8. Cyclins are the key components of the cell cycle progression machinery ***(18)***. The timing of expression of several cyclins—in particular cyclins D, E, A, and B—during unperturbed growth of normal cells is discontinuous, occurring as discrete sections of the cell cycle **(Fig. 2)**. This periodicity in cyclin expression provides cell cycle landmarks that can be used to subdivide the cell cycle into several subcompartments, additional to the subdivision into four major phases ***(2)***. Furthermore, bivariate analysis of cyclin expression vs. DNA content makes it possible to discriminate between cells having the same DNA content but residing in different phases of the cycle, such as between G_2 and M cells (based on differences in cyclin A content), or between G_2 diploid and G_1 tetraploid cells (based on differences in expression of cyclins E and/or B1). Also, G_0 cells, lacking expression of D-type cyclins or cyclin E, can be distinguished from G_1 cells, which are cyclin D and/or cyclin E positive. Strategies for the use of cyclins as additional markers of the cell cycle position are discussed elsewhere ***(2)***. It should be noted that some tumor cell lines, or normal cells when their cell cycle progression is perturbed, show unscheduled expression of cyclins D, E, and B1; i.e., the G_1 cyclins (e.g., cyclin E) are expressed during G_2/M and the G_2/M cyclins (cyclin B1) during G_1 ***(2)***.
9. There is often a need to identify mitotic cells in order to estimate the mitotic index, e.g., to assess the effectiveness of the drugs that disrupt microtubules, arresting cells in mitosis. The most convenient immunocytochemical method to detect mitotic cells appears to be the one that utilizes an Ab that is specific to histone H3 phosphorylated on Ser-10 (H3-P) ***(19)***. Because histone H3 is phos-

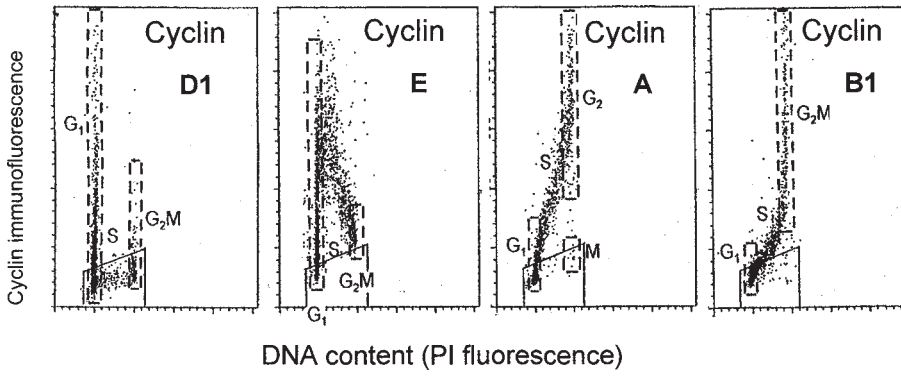

Fig. 2. Bivariate distributions (scatterplots) showing the characteristic pattern of expression of cyclins D1, E, A, and B1 vs DNA content in normal, nontumor cells, processed as described in the protocol ***(2)***. Cyclin D1 was measured in exponentially growing human normal fibroblasts, while cyclins E, A, and B1 were measured in mitogen-stimulated human lymphocytes ***(2)***. The boundaries of G_1 and G_2/M populations are marked by the dashed lines. The trapezoid windows show the level of the unspecific background fluorescence, which is measured separately using the isotypic, irrelevant Ab. It is evident that cyclin D1 is expressed by a fraction of G_1 cells; the cells entering and progressing through S, and most cells in G_2/M are cyclin D1 negative. Cyclin E is maximally expressed by the cells entering S, and its level drops during the progression through S. Cyclin A is expressed by cells in S phase and maximally by G_2 cells; mitotic cells (post-prometaphase) are cyclin A negative. Cyclin B1 is expressed by late S cells, and maximally in G_2 and M.

phorylated during prophase, and dephosphorylated late in telophase, the "time window" of detection of mitosis by this Ab spans these two mitotic stages. Sigma Chemical Co. (monoclonal) and Upstate Biotechnology, Inc. (polyclonal) offer the histone H3-P-specific Abs. The methodology of cell staining and fluorescence measurement is similar to that described above for the analysis of DNA content and proliferation-associated proteins. Optimal cell fixation, however, requires a brief (15 min) pretreatment with 1% formaldehyde (in PBS, on ice) followed by post-fixation in 70% ethanol

10. Different BrdU concentrations and different incubation times may be used depending on the cell type and design/aim of the experiment (pulse, pulse followed by time-lapse analysis, or cumulative BrdU labeling over long periods of time) (**Fig. 3**). Time-lapse analysis of the population of pulse-BrdU-labeled cells as it moves through G_2, M, and G_1 reveals the kinetics of cell cycle progression. Continuous or pulse time-lapse labeling with BrdU allows one to estimate a variety of cell cycle kinetic parameters: applications of this methodology and mathematical analysis of the data are discussed elsewhere ***(8)***. Some cell types/lines may be overly sensitive to BrdU, and their cell cycle may be perturbed at higher concentrations

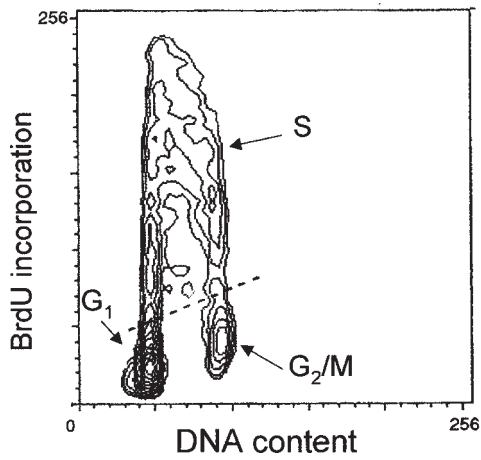

Fig. 3 Bivariate distribution (contour map) of cellular DNA content and BrdU incorporation. The cells were incubated with BrdU for 30 min, fixed, and DNA was denatured by 2 *M* HCl, The incorporated BrdU was detected by BrdU mAb, and DNA was counterstained with PI, as described in the protocol.

and longer exposure times. A control (BrdU-untreated cells) is needed for a comparison to exclude such a possibility and also as a negative control.

11. The critical step in this procedure is induction of partial DNA denaturation by heat or acid, to obtain an optimal balance between denatured (single-stranded, accessible to BrdU Ab) and nondenatured (stainable with PI) sections of DNA. Because DNA susceptibility to denaturation varies depending on chromatin structure (cell type), pilot studies should be done to find optimal conditions for a particular cell type by testing different temperatures of DNA denaturation (80–100°C) or different strengths of HCl to induce DNA denaturation (1–4 *M*). The denaturation step often results in cell damage and may lead to a significant cell loss. Use of silanized tubes during centrifugations may reduce cell loss.

Acknowledgment

Supported by NCI RO1 CA28 704.

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23

Analyzing Markers of Apoptosis In Vitro

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Summary

Cell death by apoptosis was first identified based on morphological changes reproduced with great fidelity in cells of widely different origin when exposed to a death stimulus. These changes include condensation of the cytosol and the nuclear chromatin, blebbing of the plasma membrane, and cell fragmentation into corpses that are engulfed by neighboring cells. Apoptotic cells demonstrate various levels of DNA fragmentation and exposed phosphatidylserine on the outer leaflet of their plasma membrane. Most apoptotic pathways converge on the mitochondria, inducing the disruption of the mitochondrial trans-membrane potential and the release of soluble molecules from mitochondrial inter-membrane space. One of these molecules is cytochrome c, which, in the cytosol, activates proteases of the caspase family. This chapter suggests methods to identify these characteristic morphological and biochemical events, and cell-free systems that can be used to identify the molecular pathways leading to the death phenotype.

Key Words: Apoptosis; DNA fragmentation; caspases; annexin V binding; microscopy; flow cytometry; cell-free systems.

1. Introduction

1.1. Cell Death by Apoptosis

Apoptosis is an orderly and synchronized death process which is characterized morphologically by condensation of the cytosol and the nuclear chromatin, and blebbing of the plasma membrane, ending with cell fragmentation into corpses that are engulfed by neighboring phagocytic cells ***(1)***. Biochemically, apoptotic cells expose phosphatidylserine on the outer leaflet of their plasma membrane ***(2)*** and demonstrate DNA fragmentation ***(3)***. In mammal cells, this process involves two main signaling pathways. One implicates the death receptors at the plasma membrane level, while the other is regulated by the Bcl-2 family of proteins, including pro-apoptotic and anti-apoptotic proteins.

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

These pathways converge at the level of caspase activation, and could be connected, at least in some cell types, through the protein Bid, a BH3-only member of the Bcl-2 family ***(4)***. At the level of the mitochondria, which are the primary sites of action of Bcl-2 proteins, apoptosis can be associated with the disruption of the mitochondrial trans-membrane potential and the release of soluble molecules from mitochondrial inter-membrane space ***(5)***. These soluble molecules include the electron shuttle cytochrome c, which, in the cytosol, activates the caspase cascade by inducing oligomerization of the adapter molecule Apaf-1 in the presence of ATP, thus generating a complex known as apoptosome, in which caspase-9 is activated ***(6)***. Then, caspase-9 triggers the catalytic maturation of caspase-3 and other caspases, which induce the cell to dismantle ***(7)***. These post-mitochondrial events are tightly regulated by heat shock proteins ***(8)*** and some members of the IAP (inhibitors of apoptosis proteins) family (XIAP, c-IAP1, c-IAP2), which inhibit caspase-9, -3, and -7 ***(9)***. Other soluble molecules released from the mitochondria include apoptosis inducing factor (AIF), a flavoprotein that triggers nuclear chromatin condensation; Smac/DIABLO, which interacts with and neutralizes caspase inhibitors of the IAP family of proteins; HtrA2/Omi, a serine protease that contains an XIAP-interacting N-terminus similar to that of Smac/DIABLO; and endonuclease G, which induces DNA fragmentation ***(5)***. Over time, the damage to mitochondria progressively becomes irreversible, due to impaired mitochondrial respiratory function. All these morphological and biochemical events can be used experimentally to identify apoptotic cells.

1.2. Morphological Identification of Apoptosis

Apoptosis was initially recognized in histological studies of ischemic liver injury. Besides necrosis, characterized by cell swelling, rupture of intracellular organelles, and plasma membrane disruption, another type of death was observed in scattered cells. These latter cells demonstrated shrinkage, plasma membrane blebbing, and nuclear chromatin condensation, and then converted into small round masses of cytoplasm that often contained specks of condensed nuclear chromatin and closely packed intact organelles, surrounded by a nondisrupted membrane. These small masses were subsequently engulfed and digested by resident tissue cells. Such morphological changes were reproduced with great fidelity in cells of widely different lineages exposed to a wide range of death stimuli. Cell death that occurs during normal development revealed a similar morphological pattern. This newly defined type of cell death was called *apoptosis* ***(1)***. Morphological changes remain a gold standard for apoptosis identification. A simple, cheap, and accurate method to identify these changes in a cell culture is nuclear chromatin staining with a cell-permeable, DNA-binding fluorochrome such as Hoechst 33342 or Hoechst 33258 ***(10)***.

1.3. DNA Fragmentation

Apoptosis is associated with the fragmentation of nuclear DNA, which occurs at two levels. First, DNA loop excision generates high-molecular-weight fragments ranging from 30 to 50 kbp. Secondly, internucleosomal DNA fragmentation typically generates low-molecular-weight fragments that are multiples of 180–200 bp. This fragmentation requires detachment of proteins that prevent nuclease access to DNA in living cells, including topoisomerase II, which is involved in the caspase-independent generation of high-molecular-weight fragments ***(11)***, lamin B, which binds matrix attachment regions (periodicity: 30–50 kbp), and histone H1, which is spaced every 180 to 200 bp ***(3)***. Internucleosomal DNA fragmentation involves CAD (caspase-activated DNase), also known as DFF40 (DNA fragmentation factor 40), a magnesium-dependent endonuclease which migrates in the nucleus when its inhibitor (ICAD/DFF45) is cleaved by caspase-3, and generates double-strand breaks with 3'-hydroxyl ends ***(12)***, and endonuclease G, which is released from the mitochondria ***(5)***. Other involved nucleases with a less defined role include Ca^{2+}/Mg^{2+} endonucleases such as DNase I and DNase γ, and cation-independent endonucleases such as DNase II and L-DNase II, a protease inhibitor that becomes a nuclease when cleaved ***(13)***. Numerous assays have been developed to assess the nuclease involved in apoptosis, including chromatin-independent assays (plasmid degradation and radioactive gel assays) that examine the degradation of naked DNA, and chromatin structure-dependent assays (nuclear autodigestion and HeLa nuclei assay) that recapitulate the genomic destruction observed in vivo ***(14)***. DNA fragmentation can be used to identify apoptosis by widely used techniques such as pulsed-field gel electrophoresis, conventional agarose gel electrophoresis, and terminal transferase-mediated dUTP nick end-labeling (TUNEL) ***(15)***. Here, we will focus on methods allowing quantification of apoptotic DNA fragmentation ***(16)***.

1.4. Plasma Membrane Changes

Phosphatidylserine (PS) is a phospholipid that under normal conditions resides almost exclusively in the inner leaflet of the plasma membrane. Apoptosis is associated with an outward movement of PS in the plasma membrane, as a consequence of either inhibition of an ATP-dependent aminophospholipid translocase or activation of a scramblase. Externalization of PS contributes to the recognition of apoptotic bodies by phagocytic cells ***(17)***. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. When coupled to a fluorochrome, this protein can be used as a sensitive probe for detecting PS exposure on the outer leaflet of the plasma membrane ***(2)***. Translocation of PS to the cell surface occurs also during necrosis, which, contrary to apoptosis, is associated with the disruption of the

plasma membrane. To distinguish apoptosis from necrosis, annexin V must be used in conjunction with a dye exclusion test, which establishes plasma membrane integrity, e.g., by using propidium iodide, which requires plasma membrane permeability to stain nuclear DNA ***(18,19)***.

1.5. Mitochondrial Events Associated With Apoptosis

Mitochondria are essential to cell life as they produce ATP, the source of metabolic energy. Alterations in mitochondrial membrane function are critically involved in Bcl-2-regulated pathways to apoptosis ***(5)***. The mitochondrial transmembrane potential can be disrupted early in the death process, which suppresses the electrochemical gradient required for ATP synthetase activity in the oxidative phosphorylation pathway ***(20)***. Most importantly, the outer membrane becomes permeable to soluble proteins, leading to the release of molecules from the inter-membrane space into the cytosol ***(5)***, and to increased production of reactive oxygen species, which in turn oxidize the inner membrane cardiolipin ***(21)***. These events can be analyzed to identify and quantify mitochondrial alterations associated with apoptosis. The electrochemical gradient across the mitochondrial membrane, referred to as the mitochondrial membrane potential ($\Delta\psi_m$, usually 120–180 mV), drives lipophilic cation accumulation in the mitochondrial matrix following the Nernst equation ***(22)***. Thus, the $\Delta\psi_m$ dissipation associated with apoptosis can be detected and measured by using various cationic fluorochromes and cytofluorometry or fluorescence microscopy. The translocation of soluble inter-membrane proteins can be detected by either subcellular fractionation and immunoblotting or immunofluorecence microscopy. The production of reactive oxygen species can be measured by flow cytometry.

1.6. Caspase Activation

Cysteinyl proteases of the caspase family ($n = 11$ in humans) play an essential role in most apoptotic pathways that have been studied in mammal cells. These enzymes are synthesized as inactive zymogens that must be cleaved at specific aspartate sites to be activated.

This cleavage releases an N-terminal prodomain and separates a large from a small subunit. The large subunit, which contains the cysteine of the active site in a conserved QACXG sequence, heterodimerizes with the small one, and the active enzyme is a tetramer made of two heterodimers with two active sites. These proteases have an absolute requirement for aspartic acid in the P_1 position of the scissile bond, and their specificity is determined by the P_4-P_1 tetrapeptide sequence. Somewhat artificially, caspases have been classified as upstream "activator" enzymes (e.g., caspase-8, -9, -10) with a long prodomain that permits their recruitment by means of adapter molecules and downstream

effector caspases with a short prodomain (e.g., caspase-3, -6, -7) that cleave intracellular proteins. Schematically, caspase-9 is activated when recruited by the adapter molecule Apaf-1 in the Bcl-2-regulated pathway to apoptosis, whereas caspase-8 (or -10) is recruited by the adapter molecule FADD in the death receptor pathway ***(23)***. Identification of caspase activation/activity can be used as a biochemical marker for apoptosis identification. One of the commonly used methods to identify caspase activity is to measure the ability of cell extracts to cleave synthetic peptide substrates that mimic the peptide sequence specifically recognized by a studied enzyme ***(24)***.

1.7. Cell-Free Systems for Apoptotic Pathways Analysis

Cell-free systems, which had proven to be invaluable for the study of a number of cellular events such as mitosis, have been largely used to dissect and order the molecular events involved in apoptosis, e.g., to distinguish premitochondrial, mitochondrial, and post-mitochondrial events ***(25)***. For example, the ability of cytochrome c to activate a caspase cascade when released from the mitochondria was demonstrated by adding the electron shuttle molecule to cytosolic extracts in the presence of ATP ***(6)***. Then, cell-free systems helped to clarify the ordering of the caspase cascade downstream of the mitochondria ***(7)*** and, more recently, to demonstrate that caspase-2 acted upstream of mitochondria in etoposide-induced cell death ***(26)***. We describe the preparation of various cell-free extracts to be used for the dissection of apoptotic pathways.

2. Materials

2.1. Morphological Identification of Apoptosis

1. 1X PBS (phosphate-buffered saline) 0.02 *M* (pH 7.4): 154 m*M* NaCl, 4 m*M* KH_2PO_4, 12 m*M* Na_2HPO_4.
2. Bisbenzimide Hoechst No.33342 trihydrochloride (Sigma, St Louis, MI), diluted in distilled water for stock solution (2–10 mg/mL) and stored at –20°C for up to 6 mo (light sensitive). Bisbenzimide Hoechst No33258 trihydrochloride can be used as an alternative.
6. Fluorescence microscope equipped with filters for UV excitation (<350 nm).

2.2. DNA Fragmentation

1. [2-^{14}C]-thymidine, 59 mCi/mmol (Amersham, Piscataway, NJ).
2. Omnifix 60-mL syringes (VWR International, Poole, UK).
3. Swinnex Filtration Support 25 mm (VWR International).
4. Vinyl/acrylic copolymer filters (polyvinylindene fluoride filters, 0.65-µm pore size; Metricel membrane, Gelman Science, Ann Arbor, MI).
5. Lysis buffer LS10: 0.2% sodium sarkosyl, 2 *M* NaCl, 0.04 *M* EDTA pH 10.0.
6. 0.02 *M* EDTA (pH 10.0).

7. 1 *M* HCl solution.
8. 0.4 *M* NaOH solution.
9. 1X PBS (*see* **Subheading 2.1.**, item 1).
10. Scintillant liquid (e.g., OptiPhase "HiSafe," Perkin Elmer, Boston, MA).

2.3. Plasma Membrane Changes

1. Binding buffer: 10 m*M* HEPES/NaOH (pH 7.4), 140 m*M* NaCl, 2.5 m*M* $CaCl_2$. Store at 4°C.
2. Fluorescein isothiocyanate (FITC)-labeled annexin V (BD PharMingen, San Diego, CA). Store at 4°C in the dark.
3. Propidium iodide (PI) (BD PharMingen). PI is toxic: wear gloves and exercise caution. PI is light sensitive and should be kept in the dark and stored at 4°C.
4. 1X PBS (*see* **Subheading 2.1.**, item 1). Store at 4°C.
5. Flow cytometer with appropriate filters.

2.4. Mitochondrial Events Associated With Apoptosis

1. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Eugene, OR). Stock solution (10 m*M*) in DMSO. Aliquot and store at –20°C in the dark.
2. CCCP (carbonyl cyanide *m*-chlorophenyl-hydrazone; Sigma, St. Louis, MO). Stock solution (20 m*M*) in DMSO. Aliquot and store at –20°C.
3. DMSO (dimethylsulfoxide) (Sigma).
4. 1X PBS (*see* **Subheading 2.1.**, item 1).
5. Protease inhibitor cocktail tablets. Stock solution is 25X (one tablet in 2 mL distilled water) (Roche, Mannheim, Germany).
6. Hypotonic lysis buffer: 250 m*M* sucrose, 20 m*M* HEPES, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* dithiothreitol.
7. Trypan blue solution: mix 4 vols of 2 mg/mL trypan blue in distilled water with 1 vol of 42.5 mg/mL NaCl.
8. Dounce homogenizer with a teflon pestle.
9. Flow cytometer with appropriate filters.

2.5. Caspase Activation

1. Lysis buffer: 150 m*M* NaCl, 50 m*M* Tris-HCl (pH 8.0), 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate. Store at 4°C.
2. Reaction buffer: 100 m*M* HEPES (pH 7.0), 1 m*M* EDTA, 0.1% CHAPS, 10% glycerol, 20 m*M* dithiothreitol. Store at –20°C.
3. Synthetic substrates (short peptide sequences, usually four to seven amino-acids) are conjugated to either a chromophore such as *p*-nitroanilide (*p*-NA; emission 405 nm) or a fluorochrome such as 7-amino-4-methylcoumarin (AMC: excitation, 360 nm; emission 450 nm) or 7-amino-4-trifluoromethyl coumarin (AFC: excitation, 400 nm; emission, 505 nm). For example, DEVD is a consensus cleavage site for caspase-3 and caspase-7, and DEVD-*p*NA or DEVD-AFC is used to measure the activity of these enzymes in cell extracts (Calbiochem, San Diego, CA).
7. Spectrophotometer and fluorimeter or microtiter plate reader.

2.6. Cell-Free Systems for Apoptotic Pathways Analysis

1. Buffer A: 5 m*M* HEPES (pH 7.4), 5 m*M* $MgCl_2$, 150 m*M* NaCl, 1 m*M* KH_2PO_4, 1 m*M* EGTA, 1 m*M* Na_3VO_4. Store at 4°C.
2. 1X PBS (*see* **Subheading 2.1.**, item 1), without Ca^{2+} or Mg^{2+}.
3. PMSF (phenylmethylsulfonyl fluoride, Sigma) 50 m*M* in dimethylsulfoxide. Store at –20°C.
4. Buffer B: Buffer A containing 10% glycerol, 0.3% Triton X-100, 0.1 m*M* PMSF, and 0.15 UI/mL aprotinin. Store at 4°C.

3. Methods

3.1. Morphological Identification of Apoptosis

The method can be used for cells in suspension and adherent cells detached with trypsin. Labeling is performed without fixation.

1. Wash 10^5 cells in 1X PBS and collect the cells by centrifugation at 500*g* for 10 min.
2. Suspend the cells in 500 µL Hoechst 33342-containing 1X PBS (final concentration is approx 2–10 µg/mL) (*see* **Note 1**).
3. After a 30-min incubation in the dark at 37°C, centrifuge the cells at 500*g* for 5 min, suspend in 20 µL 1X PBS, and analyze immediately using a fluorescent microscope.

Illumination of DNA-bound Hoechst 33342 produces a blue-light fluorescence. Viable cells have a round nucleus with a homogeneous labeling. An early feature of apoptosis is the appearance of brightly stained chromatin condensed along the nuclear membrane (half-moon shape). Nuclear chromatin subsequently appears as bright blue beads in a fragmented nucleus **(Fig. 1)** (*see* **Note 2**).

3.2. DNA Fragmentation

A filter-binding assay that allows quantification of DNA fragmentation is described ***(16)***.

1. Label DNA by incubating exponentially growing cells at an initial concentration of 5×10^5/mL in standard culture medium containing [2-^{14}C]-thymidine (0.02 µCi/mL) or [*methyl*-^{3}H]-thymidine (0.1 µCi/mL) for two doubling times.
2. Chase free radioactivity by overnight incubation of cells in isotope-free medium.
3. Expose cells to apoptotic stimuli for various times.
4. Use a trypan blue exclusion test to determine the percentage of cells with plasma membrane disruption (late apoptosis or necrosis).
5. Load 0.5×10^6 cells, suspended in 10 mL ice-cold 1X PBS, onto protein-absorbing filters through a 50-mL syringe **(Fig. 2)**.
6. Wash once with an additional 10 mL ice-cold 1X PBS. Recover the culture medium and the wash in a scintillation tube (fraction 1).
7. Induce cell lysis with 5 mL cell lysis buffer.
8. Wash filters with 10 mL of 0.02 *M* EDTA (pH 10.0) to elute fragmented DNA.

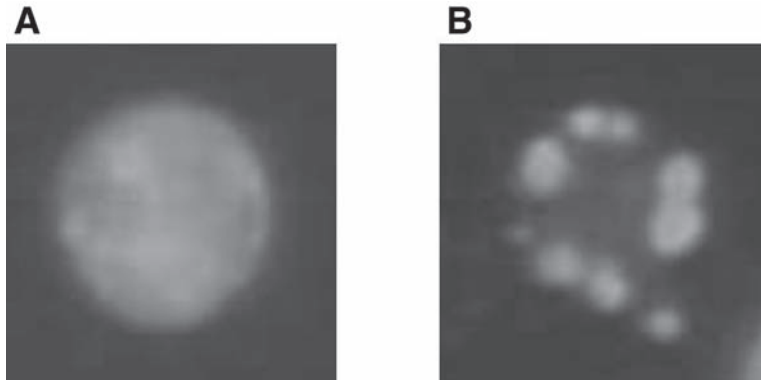

Fig. 1. Identification of apoptotic cells by Hoechst 33342 staining of nuclear DNA. **(A)** Healthy cells show a diffuse staining of their nuclei. **(B)** Apoptotic cells demonstrate a condensed and fragmented nuclear chromatin.

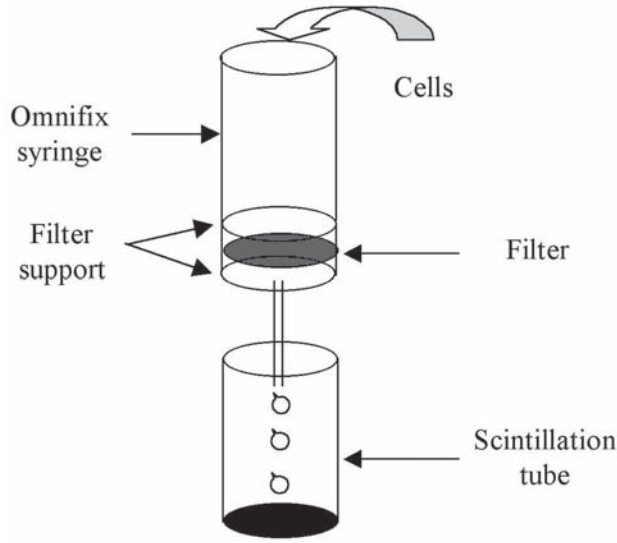

Fig. 2. The filter-binding assay. A protein-absorbing filter is screwed onto a 50-mL syringe. Cells in ice-cold 1X PBS are loaded on the filter before washing and lysis. Fractions are collected in scintillation tubes.

9. Collect lysis and wash pooled fractions in a scintillation tube (fraction 2).
10. Recover nonfragmented DNA, which remains on the filter, by incubation of the filter with 10 mL of 1 *M* HCl at 65°C for 45 min. Neutralize HCl with the same volume of 0.4 *M* NaOH for 45 min at room temperature (fraction 3).

11. Determine DNA fragmentation by counting the radioactivity contained in each fraction by liquid scintillation spectrometry. Results can be expressed as the percentage of disintegrations per min (dpm) in fractions 1 and 2 divided by total intracellular dpm (fraction 1 + 2 + 3). The percentage of dpm in fraction 2 divided by total intracellular dpm (2 + 3) indicates true apoptosis and eliminates late apoptosis or necrosis.

    A simpler variant of this method can be used that includes cell lysis and centrifugation in lysis buffer (13,000*g* for 20 min at room temperature) to separate fragmented (dpm in the supernatant) from nonfragmented (dpm in the pellet) DNA ***(27)*** (*see* **Note 3**).

3.3. Plasma Membrane Changes

1. Collect 1×10^6 cells by centrifugation at 500*g* for 5–10 min at 18–24°C.
2. Wash twice in 500 µL cold 1X PBS.
3. Suspend the cell pellet in 100 µL binding buffer.
4. Add FITC-annexin V (final concentration is 1 µg/mL) and PI (final concentration is 1 µg/mL) for a 10-min incubation in the dark at room temperature.
5. Collect the cells by centrifugation (500*g* for 5–10 min).
6. Wash the cells once in 300 µL binding buffer, centrifuge again, and suspend in 400 µL binding buffer.
7. Flow cytometry analysis must be performed within 1 h. A flow cytometer equipped with a 15-mW, 488-nm argon ion laser allows collection of the green fluorescence (FL1) through a 530/30 band pass filter and the red fluorescence (FL3) through a 650 long pass filter.
8. Perform data acquisition with CellQuest software (Becton Dickinson, San Jose, CA) with the *x*-axis (FL1) of the dot plot reflecting log FITC-annexinV fluorescence and the *y*-axis (FL3) log PI fluorescence. This analysis discriminates between intact cells (FITC–/PI–, lower left quadrant), apoptotic cells (FITC+/PI–, lower right quadrant), and necrotic (and late apoptotic) cells (FITC+/PI+, upper right quadrant) **(Fig. 3)** (*see* **Note 4**).

3.4. Mitochondrial Events Associated With Apoptosis

3.4.1. Detection of Alterations in the Mitochondrial Transmembrane Potential ($\Delta\psi_m$)

JC-1 forms monomers at low concentrations (green fluorescence, Ex_{485nm}/Em_{527nm}) and aggregates (red fluorescence, Ex_{535nm}/Em_{590nm}) at higher concentrations. In cells undergoing apoptosis, the dissipation of $\Delta\psi_m$ decreases the concentration of the dye in the mitochondria. Thus, a red, punctuate labeling becomes a green diffuse staining ***(28)***. These changes can be measured by flow cytometry and visualized by fluorescence microscopy.

1. Keep a total of 1×10^6 cells in 0.5 mL 1X PBS or culture medium until staining.
2. Prepare a working dilution of JC-1. Mix 2 µL stock solution with 1 mL 1X PBS, yielding a final concentration of 20 µ*M*.

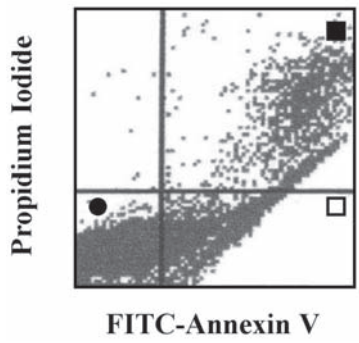

Fig. 3. Flow cytometry analysis of Annexin V/PI-labeled cells. Healthy cells are negative for both PI and annexin V (black circle). Apoptotic cells are PI negative and annexin V positive (white square). Late apoptotic and necrotic cells are positive both for PI and annexin V (black square).

3. Add appropriate amounts to the cell suspension. The final concentration should be 1 μ*M*. To avoid cytotoxicity, the organic solvent concentration should not exceed 1% v/v.
4. Incubate for 30 min at 37°C under 5% CO_2 protected from light.
5. Centrifuge the cells at 500*g* for 5 min and resuspend in 1 mL ice-cold 1X PBS.
6. Return to ice and analyze within 10 min by flow cytometry.
7. Using a FACScan (Becton Dickinson), monitor JC-1 in FL1 (green monomers: emission 527 nm) vs FL-2 (red fluorescence: emission 590 nm) **(Fig. 4)**. The ratio of green and red fluorescence provides an estimate that is relatively independent of mitochondrial mass.
8. As a positive control, treat the cells for 20 min with the protonophore CCCP (which causes complete disruption of the $\Delta\psi_m$) at a 100 μ*M* final concentration. The staining profile obtained with CCCP in different experimental conditions might be identical (*see* **Note 5**).

3.4.2. Detection of the Translocation of Soluble Mitochondrial Inter-Membrane Proteins

The translocation of soluble mitochondrial proteins from the mitochondria to the cytosol upon apoptotic stimuli can be detected in subcellular fractions by immunoblot. This technique requires a large quantity of cells (60×10^6 per experimental condition) ***(29)***.

1. Wash the cells twice in ice-cold 1X PBS.
2. Centrifuge at 500*g* at 4°C for 5 min.
3. Add an equal volume of ice-cold hypotonic lysis buffer (which has been prepared immediately prior the experiment and supplemented by adding 1X protease inhibitors).
4. Transfer the cell suspension into a homogenizer and keep on ice for 30 min. Thereafter, cell homogenization is induced by a first series of 25 strokes.

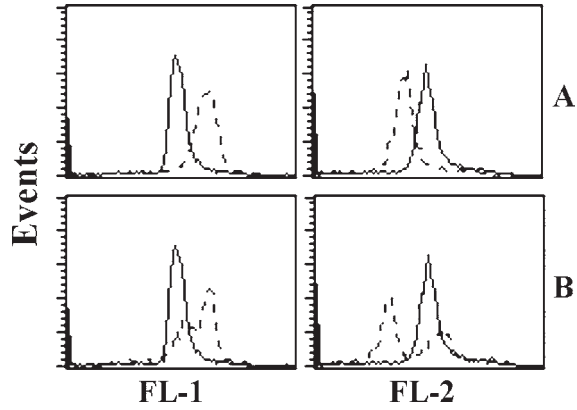

Fig. 4. Detection of $\Delta\psi_m$ decrease by using JC-1 probe. A typical JC-1 staining of healthy cells is shown as solid lines. Dissipation of $\Delta\psi_m$ under CCCP **(A)** or pro-apoptotic **(B)** treatment (dashed lines) entails a shift from the JC-1 aggregates (decrease in fluorescence measured in FL-2) to JC-1 monomers (increase in fluorescence measured in FL-1).

5. Remove a small aliquot of cells and check the percentage of trypan blue–stained cells: strokes are repeated until there is uptake of trypan blue in approx 50% of cells (a more extensive lysis would induce organelle damage).
6. Transfer the cell homogenate to a 1.5-mL tube and centrifuge at 750*g* for 10 min at 4°C.
7. Carefully collect the supernatant and centrifuge at 750*g* for 10 min at 4°C. These steps discard nuclei and non-lysed cells (the last pellet could be used for nuclear extraction).
8. Carefully collect the supernatant and centrifuge at 10,000*g* for 25 min at 4°C.
9. Suspend the pellet (which represents the fraction enriched in mitochondria) in 100 µL hypotonic lysis buffer and store at –80°C until analysis.
10. Centrifuge the supernatant (which can be stored at –80°C) at 100,000*g* for 60 min at 4°C. Collect the supernatant (S100 fraction), which constitutes the cytosolic fraction. In addition, dilute the pellet, which is the microsomal fraction, in 100 µL of hypotonic buffer.
11. Determine the protein concentration in all fractions. Store at –80°C until Western blot analysis of the expression of soluble inter-membrane mitochondrial proteins (*see* **Note 6**).

3.5. Caspase Activation

1. Pellet 3×10^6 cells by centrifugation.
2. Wash twice in ice-cold 1X PBS.
3. Resuspend in 100 µL lysis buffer for 30 min at 4°C.
4. Centrifuge at 10,000*g* for 20 min at 4°C to pellet cellular debris and DNA.

5. Measure the protein concentration in this supernatant using a Lowry quantification assay. The supernatant contains cellular proteins, including caspases, either in an inactive or an active form.
6. Incubate 50 µg proteins in 50 µL reaction buffer in the presence of 100 µ*M* chromogenic or fluorogenic substrate for 1 h at 37°C.
7. Quantify the cleavage of a chromophore such as *p*-NA by spectrophotometry at 400–405 nm.
8. Quantify the release of AMC and AFC, which emit a fluorescence (green fluorescence at 450 nm and yellow-green fluorescence at 505 nm, respectively), by fluorimetry.

   In all cases, the signal intensity is proportional to the amount of cleaved substrate, indicating the activity of the caspase that specifically recognizes the peptide sequence. Two methods can be used for measuring caspase activity: the measurement can be done once after a 60-min incubation at 37°C using a water-jacketed sample compartment, or repeated every min for a 30-min incubation time. The enzyme activity is expressed in pmol/min/10^6 cells or pmol/min/mg of protein ***(30)*** (*see* **Note 7**).

3.6. Cell-Free Systems for Apoptotic Pathways Analysis

A cell-free system can be used to identify cytoplasmic pathways involved in nuclear DNA fragmentation associated with apoptosis. Nuclei from untreated cells whose DNA has been labeled with [2-^{14}C]-thymidine (0.02 µCi/mL) or [*methyl*-^{3}H]-thymidine (0.1 µCi/mL) (*see* **Subheading 3.2.**) are incubated for 30 min with Triton-soluble (cytoplasmic) extracts from unlabeled cells exposed to an apoptotic stimulus ***(16)*** before measuring DNA fragmentation. Additional chemicals (e.g., caspase inhibitors, purified heat shock proteins, and so on) can be tested for their ability to modulate the pathways leading to DNA fragmentation.

1. Harvest labeled and unlabeled cells by centrifugation at 500*g* for 5 min.
2. Wash twice with ice-cold PBS 1X with neither Ca^{2+} nor Mg^{2+}.
3. Suspend the cells in a 1.5-mL Eppendorf tube at a density of 3×10^6 cells/mL in ice-cold buffer A, containing 10% glycerol (v/v) and 0.1 m*M* PMSF.
4. Centrifuge at 500*g* for 5 min at 4°C.
5. Incubate the cells on ice for 10 min at a density of 10^7 cells/mL in buffer B.
6. Centrifuge at 2000*g* for 10 min at 4°C.
7. Unlabeled cells: discard the pellet, collect the supernatant (Triton-soluble extracts), and determine its protein concentration.
8. Labeled cells: discard the supernatant and wash the pellets (labeled nuclei) twice in buffer B without Triton X-100.
9. Immediately incubate the labeled nuclei from 1×10^6 untreated cells for 30 min at 37°C in the presence of 500 µL Triton-soluble extracts from 5×10^6 untreated or treated cells. Chemicals can be added to Triton-soluble extracts before incubation with labeled nuclei in an attempt to modulate the DNA fragmentation pathways.

10. Measure DNA fragmentation by using the filter elution assay (*see* **Subheading 3.2.**) or, more simply, by centrifugation in buffer A (13,000*g* for 20 min at room temperature) before counting the radioactivity in the supernatant (fragmented DNA) and the pellet (non-fragmented DNA) ***(27)*** (*see* **Note 8**).

4. Notes

1. Hoechst 33342 or Hoechst 33258 can be added directly to adherent cells in a culture dish and to tissues embedded in paraffin blocks ***(31)***. Other dyes such as DAPI (generally used at 1 µg/mL), a cell-permeable fluorescent minor groove-binding probe for DNA (Sigma), acridine orange alone (if low concentrations are used) or in a mixture with ethidium bromide (100 µg/mL each; excitation at 488 nm) can be used as well ***(32,33)***. In contrast, trypan blue and propidium iodide, which stain cells with disrupted membranes, are not good dyes for apoptosis identification ***(34)***.
2. Electronic microscopy is the most sensitive and specific technique for morphological identification of apoptosis. This time-consuming procedure may be used when the cell death process remains doubtful after having used simpler techniques. Membrane blebbing, nuclear pyknosis, and cell fragmentation can be visualized on hematoxylin/eosin-stained cells or tissue sections using conventional light microscopy, but apoptotic cells are not easily distinguishable from other cells with condensed chromatin, such as mitotic cells in telophase. A time-lapse examination of apoptosis can also be performed to study the chronology of morphological changes during apoptosis, including shrinkage, budding, and phagocytosis in individual cells ***(34,35)***.
3. Several other methods can be used to identify apoptosis-associated DNA fragmentation. The electrophoresis of DNA in ethidium bromide-stained agarose gels identifies internucleosomal DNA fragmentation as a ladder pattern (in contrast, random DNA cleavage in necrotic cells has a smear appearance) ***(36)***. Pulsed-field gel electrophoresis (PFGE) is used to identify large DNA fragments (20 Kb–10 Mb) when the cells do not demonstrate internucleosomal DNA fragmentation ***(37)***. The TUNEL (terminal dUTP nick-end labeling) method, which is based on the addition of labeled deoxyribonucleotides at the 3' OH ends of DNA fragments, is used to detect DNA breaks in tissue samples by standard ***(38)*** and electronic microscopy ***(39)***. Lastly, the DNA diffusion assay allows identification of apoptotic DNA fragmentation in single cells ***(40)***.
4. Annexin V/propidium iodide analysis is a fast, simple, and sensitive method to detect PS externalization, and various kits are commercially available. Cells must not be fixed prior to incubation with FITC–annexin V. Similarly, fixation is not recommended after FITC–annexin V labeling, as the fluorescence may be quenched. The binding of annexin V to externalized PS can be measured by other techniques, including confocal laser scanning ***(41)*** and electron microscopy ***(42)***. Annexin V can be labeled with other fluorochromes, biotin, and radioligands ***(41)***. The annexin V affinity assay, which has found widespread use to measure apoptosis in vitro, has been recently modified to measure apoptosis in vivo, both

in animal models and in humans ***(43)***. Merocyanine 540, a fluorescent dye, may be used as an alternative or complement to annexin V ***(44)***.

5. JC-1 staining can be analyzed by fluorescence microscopy using standard filters after staining of adherent cells. Labeling of nuclear chromatin with Hoechst 33342 can be performed simultaneously. Several other lipophilic cationic probes, including chloromethyl-X-rosamine (CMXRos), 3,3' dihexyloxacarbocyanine iodide ($DiOC_6$[3]), tetramethylrhodamine methyl ester (TMRM), and rhodamine 123 (all can be purchased from Molecular Probes), can be used to measure $\Delta\psi_m$ collapse **(Table 1)** ***(22)***. All these probes must be used carefully, as they can be cytotoxic and interact with various cellular proteins. CMXRos is a potent photosensitizer, which can induce death of cells imaged by laser scanning confocal microscopy ***(45)***. JC-1, rhodamine 123, and TMRM reduce mitochondrial respiration and can be exported by the plasma membrane P-glycoprotein involved in multiple drug resistance (MDR). Both the P-glycoprotein and the permeability transition pore of the mitochondria are affected by cyclosporine A, whereas cyclosporine H (1.6 μ*M*) inhibits P-glycoprotein more specifically ***(46)***. $DIOC_6$(3) stains endoplasmic reticulum when used at high concentration (>5 μ*M*) ***(28)***. Simultaneous staining with a fluorochrome-associated antibody (Ab) can be done by coupling a phycoerythrin-associated Ab with $DiOC_6$(3), or a fluorescein isothiocyanate-labeled Ab with TMRM ***(22)***, but such a simultaneous staining is not compatible with CMX-Ros ***(47)***.
6. Cytochrome c translocation can be detected by fluorescent microscopy, using a specific Ab and a weak nonionic detergent such as digitonin (190 μg/mL) to permeabilize the plasma membrane without altering intracellular membranes whose cholesterol content is lower ***(46)***. The punctuated staining pattern that characterizes healthy cells is replaced by a cytosolic diffuse pattern of staining (as a control, use an anti-mitochondrial HSP70 Ab; ABR, Golden, CO). A commercially available kit, based on a sandwich enzyme immunoassay, can be used to quantify cytochrome c release (R&D Systems, Minneapolis, MN) ***(48)***. Reactive oxygen species produced in excess as a consequence of mitochondrial membrane permeabilization can be detected by using a dihydroethidine probe, a molecule that is oxidized by superoxide anion to become ethidium bromide, thus emitting a red fluorescence, or nonyl acridine orange, which interacts stochiometrically with non-oxidized cardiolipin ***(22,49)***.
7. Detection of caspase activity by measuring the ability of cell extracts to cleave synthetic peptides is a quantitative, fast, and sensitive method ***(50)***, but its specificity remains low, as several caspases usually cleave a given substrate ***(51)***. A procedure to specifically determine the activity of a given enzyme is the fluorometric immunosorbent enzyme assay (FIENA), which works by capturing the caspase of interest from cell lysates with a specific monoclonal antibody, followed by fluorometrically measuring the enzyme activity after adding a peptide substrate. Briefly, 2×10^6 cells are lysed in a dithiothreitol (DTT)-containing solution. A 96-well plate is coated with anti-caspase-3 mAb at 4°C for 12 h, excess mAb is removed, and unspecific binding blocked. Samples containing

Table 1
Cationic Fluorochrome Currently Used in $\Delta\psi_m$ Disruption Detection

Dye	Stock solution	Final concentration	Incubation time	Emission (color)
CMX-Ros	1 m*M* in DMSO	100 n*M*	15–20 min	630 nm (red)
DiOC6(3)	40 m*M* in DMSO	20 n*M*	15–20 min	530 nm (green)
TMRM	1 m*M* in DMSO	150 n*M*	15–20 min	575 nm (orange)
Rhodamine 123	10 m*M* in MeOH	1-10 m*M*	30 min	529 nm (green)

caspase-3 are incubated for 2 h in the wells before the addition of Ac-DEVD-AMC as a substrate. Caspase-3 activity is quantified using a fluorometer at an excitation wavelength of 360 nm and an emission wavelength of 450 nm ***(52)***. Anti-caspase-3-coated microtiter plates are commercially available (Roche, Mannheim, Germany) for such a procedure. Several other methods can be used to detect caspase activation in vitro. Maturation of these enzymes requires their proteolytic cleavage, which can be easily detected by immunoblotting using antibodies to demonstrate either the disappearance of the enzyme proform or the appearance of the cleavage fragments ***(53)***. Antibodies that specifically recognize the active form of these enzymes can be used for immunofluorescence analysis ***(54)***. Immunoblot identification of the cleavage of intracellular proteins—for example, cleavage of the 116 kDa poly(ADP-ribose)polymerase, or PARP, into an 85 kDa C-terminal fragment—can be used as a signature of caspase activation ***(54)***, and the ability of cell-permeant peptides that inhibit caspases with various (and always relative) specificity can help to identify the enzyme involved in the process under investigation ***(23,51)***.

8. A variety of other methods have been described to purify cell fractions, e.g., to isolate nuclei in the absence of detergent ***(55)***, to purify mitochondria that can be added to S-100 extracts ***(56)***, and to prepare cytoplasmic extracts with and without mitochondria and cell fragments ***(25)***. Combinations of these various extracts can be used to screen, for example, molecules that directly target the mitochondria and induce the outer membrane permeabilization.

   The S100 fraction described in **Subheading 3.4.2.** can be used as a cytosolic fraction to analyze the post-mitochondrial events associated with apoptosis. For example, to determine whether the caspase cascade can be activated by cyto-

chrome c in a studied cell type, bovine cytochrome c (50 µg/mL) can be added to 50 µL of S100 fraction (prepared from 1×10^8 cells) in the presence of dATP (final concentration: 1 m*M*) before measuring caspase activation ***(57)***.

Acknowledgments

Our group is supported by a special grant from the Ligue Nationale Contre le Cancer and grants from its departmental committees (Nièvre, Haute-Marne, Côte d'Or, Saône et Loire).

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24

Analysis of Telomerase Activity and Telomere Function in Cancer

Katrina E. Gordon and E. Kenneth Parkinson

Summary

Telomeres are the structures at the ends of chromosomes, composed of repetitive sequences and associated proteins, which cap chromosome ends to maintain genomic stability. These structures are maintained by the enzyme complex telomerase in germ cells and some stem cells, but are absent in the majority of somatic cells. The consequence of this lack of telomerase in normal somatic cells is the shortening of the telomeric repeat, which results in a limited replicative life span. However, in cancer cells, which grow indefinitely, telomerase activity has been detected in a large number of different cancer cell types. This has lead to a great deal of interest in establishing techniques to measure telomerase activity, telomere length, and telomere function in both normal and cancer cells/tissue. Here we describe the TRAP (telomeric repeat amplification protocol) assay, a technique to measure telomerase activity in cells, TRF (terminal restriction fragment length) analysis to estimate telomere length, and both the anaphase bridge index and the frequency of dicentric chromosomes as indicators of telomere dysfunction.

Key Words: Telomeres; telomerase activity; telomere length; telomere dysfunction.

1. Introduction

One of the hallmarks of cancer cells is their unlimited proliferative capacity. Understanding how this is achieved has been a key question in cancer biology for many years. The discovery of the enzyme complex telomerase ***(1,2)*** and telomerase activity in 85–95% of human cancers ***(3)*** has led to a great deal of interest in studying telomere function and telomerase activity. This work has focused on determining the role of telomerase in maintaining functional telomeres at the ends of chromosomes, thus bypassing the normal replicative lifespan of somatic cells during the development of cancer ***(4,5)***.

Here we describe assays used to measure telomerase activity ***(6)***, telomere length ***(7)***, and telomere dysfunction in tumor cells ***(8–13)***.

From: *Methods in Molecular Biology, vol. 281: Checkpoint Controls and Cancer, Volume 2: Activation and Regulation Protocols*
Edited by: Axel H. Schönthal © Humana Press Inc., Totowa, NJ

2. Materials

1. Tissue or cell pellets.
2. Trapeze Kit: (Appligene Oncor Lifescreen).
3. Restriction enzymes and buffers: Hinf I (Roche), Rsa I (Roche), and One-Phor-all buffer (Amersham Pharmacia).
4. Oligonucleotide primers.
5. Perfect hybridization solution (Sigma).
6. Histological stains: hematoxylin and eosin.
7. Light microscope with ×100 objective.
8. Pulse field gel electrophoresis.
9. Polyacrylamide gel electrophoresis equipment.
10. PhosphoImager and software or densitometer and quantification software.
11. Trapeze kit (Intergen) containing: 1X CHAPS lysis buffer, 10X TRAP reaction mix, 50X dNTP mix, TS primer, PCR grade water. Store aliquots of these reagents at –20°C. TSR8 control template, store at –20°C. Control cell pellet (10^6 cells), resuspend in 200 µL lysis buffer. Aliquot and store at –80°C for up to 1 yr (*see* **Note 1**).
12. *Taq* polymerase.
13. PBS (phosphate buffered saline): 137m*M* NaCl, 2.7 m*M* KCl, 10.6 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4.
14. Protein determination assay—for example, the Pierce BCA (bicinchoninic acid) protein assay.
15. 5X TBE (Tris-borate EDTA): 54 g Tris-base, 27.5 g boric acid, 20 mL 0.5 *M* EDTA (pH 8) in a final volume of 1 L of H_20.
16. γ^{32} P-ATP (3000Ci/mole, 10 mCi/mL).
17. T4 polynucleotide kinase and 10X kinase buffer (for example, from Roche).
18. 10% polyacrylamide stock: 100 mL 40% polyacrylamide solution (19:1), 40 mL 5X TBE in a final volume of 400 mL of H_2O.
19. For a 10% gel, mix the following: 24.5 mL 10% polyacrylamide stock, 0.25 mL 10% APS (ammonium persulphate), and 25 µL TEMED.
20. Loading dye (native): 5 mL glycerol, 2 mL 1.25% bromophenol blue, 2 mL 1.25% xylene cyanol, 1 mL 0.5 *M* EDTA (pH 8), in a final volume of 10 mL with distilled H_20.
21. Gel fixing solution: 50% methanol, 0.5 *M* NaCl, 40 m*M* sodium acetate (pH 4.2).

3. Methods

The following methods describe (1) the semi-quantification of telomerase activity in cell/tumor extracts; (2) the estimation of telomere lengths based on the average telomere restriction fragment (TRF) length; and (3) the assessment of telomere dysfunction based on the frequency of dicentric chromosomes and anaphase bridges.

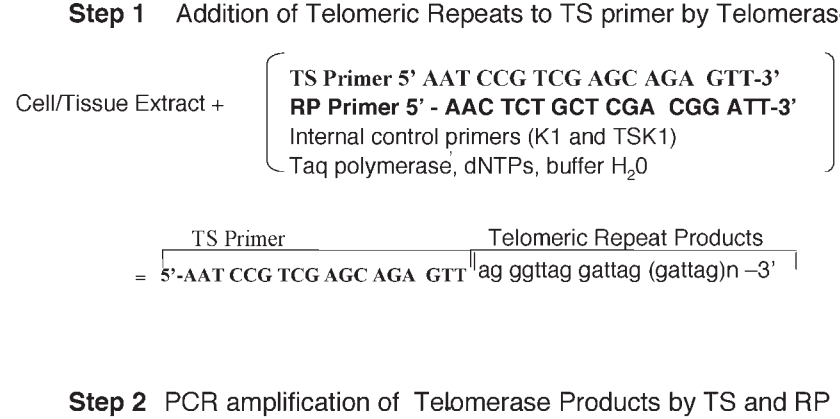

Fig. 1. Schematic representation of the TRAP assay. The assay is performed in two steps. In the first step, cell or tissue extracts are incubated with an end-labeled synthetic telomere end (TS primer) for 30 min at 30°C. If telomerase activity is present in the extract, then TTAGGG repeats are added to the labeled TS primer. The second step involves PCR amplification of these extended products. These amplified products can then be separated by electrophoresis (*see* **Subheading 3.1.4.**) to generate the characteristic 6-bp ladder observed in telomerase-positive samples.

3.1. Telomeric Repeat Amplification Protocol (TRAP) Assay (Intergen)

The most widely used method for measuring telomerase activity is the highly sensitive PCR-based TRAP assay ***(6)***. It has been estimated that 0.1% of telomerase-positive cells within a sample can be detected using this method. An overview of the assay is shown in **Fig. 1**. The following subheadings describe (a) preparation of cell extracts and controls; (b) performing the assay, (c) typical results, and (d) semi-quantification of telomerase activity in cell extracts.

3.1.1. Preparation of Cell Extracts (see **Note 2**)

1. Wash cell pellets to be tested once with PBS. Pellet again by spinning at 12,000*g* in a microfuge at 4°C for 5 min and carefully removing PBS.
2. Resuspend cell pellets (10^5–10^6 cells) in 200 µL 1X CHAPS lysis buffer (*see* **Note 3**).
3. Incubate on ice for 30 min.

4. Microfuge samples at 12,000*g* at 4°C for 20 min.
5. Retain the supernatant and determine the protein concentration. Use duplicate samples, 10 µL of each supernatant in a standard protein assay—for example, Pierce BCA protein assay.
6. Freeze remaining extract on dry ice and store aliquots at –80°C for up to 1 yr.

3.1.2. Performing TRAP Assay

A number of controls must be set up to ensure that telomerase activity is being measured in the sample and to avoid false positive or negative results.

3.1.2.1. Preparation of Controls

The following controls must be set up each time the TRAP assay is performed: (1) heat-inactivated cell-extract control; (2) a known positive telomerase extract control; (3) internal PCR control; (4) negative extract control; (5) a quantitation control. These are described in detail below.

1. Heat-inactivated cell-extract control. Telomerase is a heat-sensitive enzyme; therefore, each cell extract to be tested should have a duplicate heat-treated and nonheated sample. This control is performed to ensure that the TRAP ladder present in the non-heat-treated sample is specifically a result of telomerase activity (*see* **Note 4**). Incubate an aliquot of the extract (5 µL) at 85°C for 10 min. Store on ice.
2. Positive telomerase extract control. A known telomerase-positive extract should be incorporated into each assay to ensure that the assay has worked. This can be carried out using the cell pellet supplied in the TRAP kit, or alternatively a known telomerase-positive cell line—for example, HeLa. Resuspend the cell pellet from the kit in 200 µL 1X CHAPS lysis buffer and spin at 12,000*g* in a microfuge at 4°C. Aliquot and store at –80°C. Dilute this extract 1:20 with 1X CHAPS lysis buffer and use 2 µL in the assay (approx 500 lysed cells). Run one positive control per assay.
3. PCR amplification control. An internal PCR amplification control is incorporated into the assay to ensure that the PCR step has not failed. Included in the TRAP primer mix (*see* master mix below) are two primers—K1 and TSK1—that produce a 36-bp PCR product in each of the samples when the PCR amplification step is successful (*see* **Note 5**) (**Figs. 1** and **2**).
4. PCR contamination control. To ensure the absence of cross-contamination of the components of the assay, a negative control should always be included, where the cell extract is replaced with 2 µL 1X CHAPS lysis buffer. No TRAP ladder should be present in this lane, indicating that there is no cross-contamination of the different components that make up the assay.
5. Telomerase quantitation control template (*see* **Note 6**). Each kit contains a control template, which can be used as a telomerase standard so that telomerase activity can be compared between samples on different gels. To use this stan-

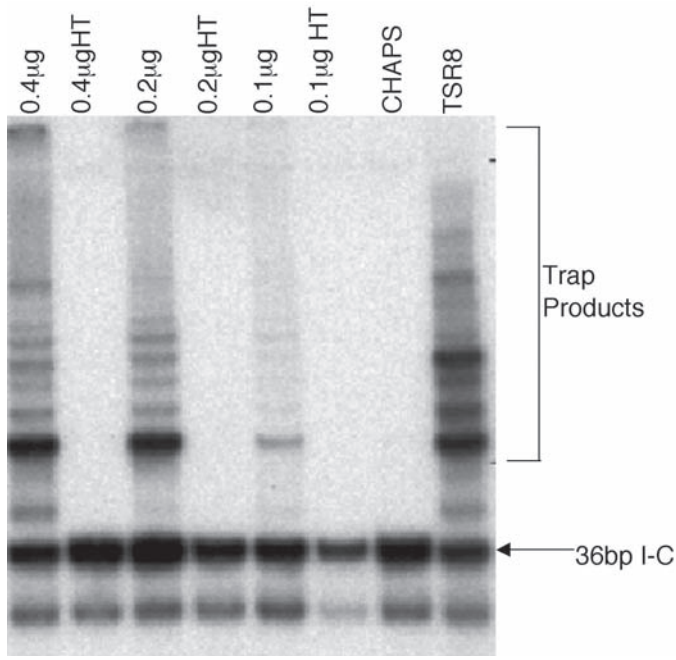

Fig. 2. Typical TRAP assay gel. Serial dilutions of the cell extract were made and run alongside heat-treated (HT), negative (CHAPS), and positive (TSR8) controls. For a valid assay the follow criteria should be observed: The 36-bp internal standard should be visible only in the primer/dimer PCR control lane and the heat-treated samples, and no ladder. The telomerase positive control lane should contain the 36-bp internal control (I-C) band and a ladder of products from 50 bp increasing by 6 bp for each rung of the ladder (*see* **Note 8**).

dard, set up a tube with 1 µL of TSR8 control template plus 1 µL CHAPS buffer instead of cell extract in the assay.

3.1.2.2. Radioactive End Labeling of TS Primer (*see* Note 7)

Make up a master mix for the samples to be assayed. Remember that for each extract to be assayed, you should have a heat-treated and non-heated sample. In addition, the controls outlined above should be included.

For 10 reactions, mix the following: 2.5 µL γ^{32} P-ATP, 10 µL TS primer, 2.0 µL 10X kinase buffer, 0.5 µL T4 PNK (10 units/µL) and 5.0 µL distilled H_20. Incubate for 20 min at 37°C, immediately followed by 5 min at 85°C. Add the total 20 µL of this labeled primer (^{32}P-TS primer) to the TRAP assay mix (*see* **Subheading 3.1.2.3.**).

3.1.2.3. TRAP Assay

1. To set up the TRAP assay, a master mix is made containing the following components (for 10 reactions): 50 µL 10X TRAP reaction buffer, 10 µL 50X dNTP mix, 20 µL ^{32}P-TS primer, 10 µL TRAP primer mix, 4µL *Taq* polymerase (5 units / µL) and 386 µL distilled H_20.
2. Aliquot 48 µL of this master mix into pre-labeled thin-walled RNase free PCR tubes.
3. Add either 2 µL of the non-heat-treated or heat-treated cell extract to the appropriate tubes.
4. Set up the other controls as follows: for the telomerase-positive control add 2 µL of the positive control extract, for the negative/PCR-contamination control add 2 µL 1X CHAPS instead of any extract, and for the quantitative control add 1 µL TSR8 (control template) + 1 µL CHAPS buffer.

3.1.3. PCR Amplification

1. 30°C for 30 min (1 cycle).
2. 95°C for 30 s and 59°C for 30 s (30 cycles each).

3.1.4. PAGE and Data Analysis

1. Add 10 µL of loading dye to each tube and run the 25-µL samples on a 10% non-denaturing PAGE gel with 0.5X TBE running buffer.
2. Load non-heat-treated and heat-treated samples in alternate lanes. Load the TSR8 quantitation control in the last lane to minimize carryover into adjacent lanes.
3. Run gel at 400 V for approx 1.5 h or until the xylene cyanol is 3/4 down the gel and the bromophenol blue has just run off the bottom of the gel.
4. Fix the gel in the fixing solution for 20 min and transfer to Whatman paper.
5. Dry the gel and visualize reaction products using a PhosphoImager or expose to film (*see* **Note 8**).

3.1.5. Semi-Quantification of Telomerase Activity

Where possible it is important to perform a standard curve on each of the samples being assessed, to ensure measurements are made within the linear range of the assay (*see* **Note 9 and Fig. 3**).

For quantification, the following measurements are taken:

1. The signal is measured from the area corresponding to the TRAP ladder products in all samples, including non-heat-treated (X) and heat-treated lanes (Xo) and from the CHAPS lysis buffer control (Ro) and the TSR8 quantitation control (R).
2. The signal is measured from the internal standards (S-IC) in the non-heat-treated samples (C) and the TSR8 quantitation control (C_R).
3. The amount of telomerase products is then calculated using the following formula:

$$\text{TPG (units)} = [(X\text{-}Xo)/C]/[(R\text{-}Ro)C_R] \times 100 \text{ (if 0.1 mole =1µL of TSR8 is used)}$$

TPG= total products generated

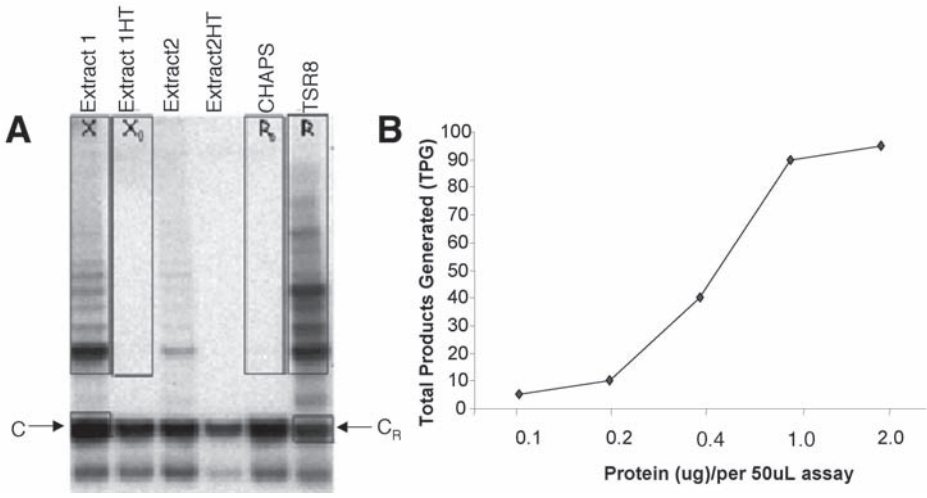

Fig. 3. Calculation of TRAP activity and generation of standard curve. **(A)** The intensity of the signal in the boxed areas was measured and the total products generated (TPG) for each reaction were calculated as described above (*see* **Note 9**). **(B)** Typical standard curve obtained from serial dilution of a cell extract with telomerase activity.

3.2. Terminal Restriction Fragment (TRF) Length Analysis

Terminal restriction fragment length analysis gives an estimate of telomere length. Genomic DNA is cut with restriction enzymes that recognize DNA sequences outside the telomeric repeat region and subtelomeric region (*see* **Fig. 4**). These restriction fragments are then resolved on a standard agarose gel or by using pulse field gel electrophoresis. It is important to note that the TRF length measurement consists of both the telomeric (TTAGGG) repeats and the adjacent subtelomeric repeat region **(Fig. 4)** and is therefore an overestimate of actual telomere length.

3.2.1. Isolation and Digestion of Genomic DNA

1. Prepare genomic DNA (gDNA) and measure its concentration and purity using standard protocols ***(14)*** (*see* **Note 10**).
2. Set up the following digest: 4 µg gDNA (*see* **Note 11**), 2 µL Hinf I (=80 units), 2 µL Rsa I (=80 units), 5 µL 10X restriction digest buffer (Gibco React 1 or Amersham Pharmacia One-phor-all buffer) and make up the volume to 50 µL with distilled H_20. Incubate the digest overnight at 37°C.

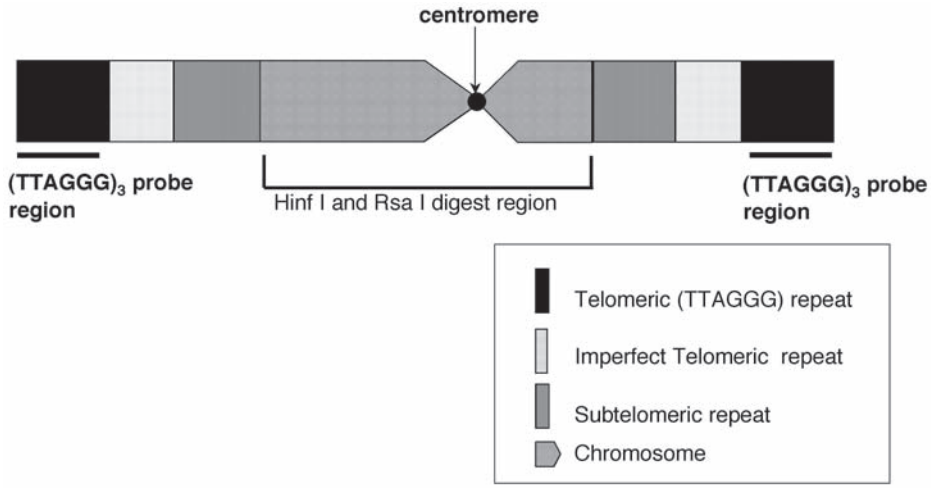

Fig. 4. Schematic of the telomere structure. Telomeric probe hybridization region is shown, and the Hinf I and Rsa I digest region.

3.2.2. Separation of Terminal Restriction Fragments (TRFs)

Human telomeric lengths have been estimated to range from 23 kb to 3 kb ***(13,15)***. Standard agarose gel electrophoresis or pulse-field gel electrophoresis can be used to separate digestion products.

1. A 0.7% agarose gel run under standard conditions gives sufficient separation within the 0.8–10 kb range.
2. Alternatively, if the bulk of the TRF lengths are within the 9–23 kb range, then a 1% pulse-field gel gives better separation.
3. Good size markers for this range include {1}/Hind III (range 23.1 kb–2 kb), 1-kb (Invitrogen), and 5-kb ladders (Biorad).
4. Running conditions will depend on the gel apparatus used. Typical running conditions for an 18 cm × 20 cm agarose gel are 50 V for 20 h. For an 18 cm × 20 cm pulse-field gel (CHEF DR II, Biorad), the following conditions were used: 6 V, 1 s initial switch, 6 s final switch, 16 h run time at 14°C.
5. Under these running conditions, the 2-kb DNA marker is approx 1 cm from the bottom of the gel.
6. Photograph the gel with a ruler alongside to provide a positional reference for the DNA ladders.

3.2.3. DNA Transfer

1. Transfer the DNA to membrane (e.g., Hybond [Amersham Pharmacia] or Zeta probe [Biorad]) using standard southern blotting techniques ***(16)***.
2. Mark the wells and crosslink the DNA to the membrane either by using a UV crosslinker or baking at 65°C for 1 h.

*3.2.4. Labeling Probe (*see ***Note 12****)*

1. TRF oligonucleotide primer consists of $(TTAGGG)_3$.
2. To end-label the oligo, mix the following: 1 µL TRF oligo (100 ng), 1 µL T4 polynucleotide kinase, 2 µL 10X buffer, 5 µL γ^{32} P-ATP, 11 µL distilled H_2O. Incubate at 37°C for 30 min.

3.2.5. Hybridization and Washes

1. Prehybridize the blot for 15 min at 42°C in Perfect Hybridization (Perfect Hyb, Sigma) or any hybridization solution suitable for southern blot hybridization.
2. Hybridize blot either overnight or for 4 h at 42°C (if using Perfect Hyb).
3. Washes: two 20-min washes in 6X SSC/1% SDS at 42°C; two 20-min washes in 3X SSC/0.1% SDS at 42°C.
4. Wrap membrane in saran wrap and expose to PhosphoImager/film.

3.2.6. Calculation of Mean TRF Length **(7)** *(*see ***Note 13****)*

An estimate of TRF length can be obtained by comparing the size of the signal smear in each lane with the molecular weight markers (*see* **Fig. 5**). To obtain quantitative measurements, the TRF length for each sample is estimated by calculating the mean TRF length. This analysis can be carried out using a PhosphoImager or densitometric scan of an autoradiogram.

The mean TRF length of each sample is calculated by integrating the signal intensity above background over the entire TRF distribution as a function of the TRF length, using the following formula:

$$L = \Sigma(OD_i \times L_i)/\Sigma\,(OD_i)$$

OD_i = signal intensity at position i on the gel

Li = TRF length at position i on the gel

1. Divide the scanned image into a grid consisting of X columns (= number of samples) and multiple rows (typically around 30 rows), as shown in **Fig. 6**.
2. Use two rows above and two rows below the signal smear to calculate the average background (bkgd).
3. Subtract the background from the OD of each grid box to give the signal owing to the TRFs at this position (*see* **Fig. 6** and the example on the following page).

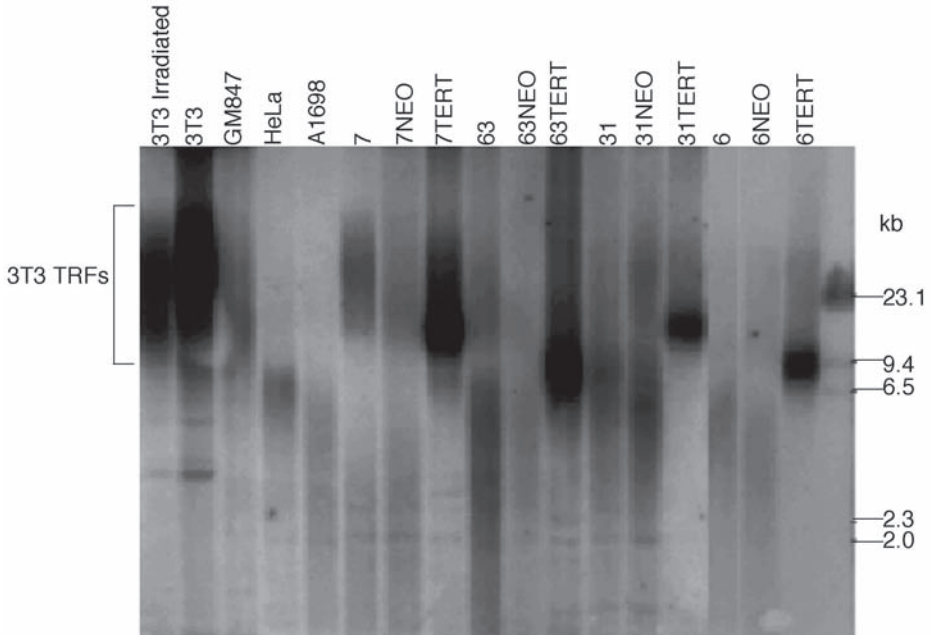

Fig. 5. Terminal restriction fragment (TRF) length blot with a variety of cell lines. The TRF length of genomic DNA isolated from cells were analyzed along with GM847 (skin fibroblast), HeLa (cervical adenocarcinoma cell line), and A1698 cells (bladder carcinoma cell line) TRF control lines (GM847 = 23 Kb, HeLa = 6 Kb, and A1698 = 3 Kb). Modified from reference ***13***.

Example of mean TRF length calculation:

Background (bkgd) = the average ODi of the first and last 2 boxes in each column

$$bkgd = (2 + 2 + 2 + 2)/4 = 2$$

$_i$ denotes the box numbers 1...n (n = number of grid boxes in column).

OD_i = density reading from grid box i after adjustment for background.

Li = the size (kbp) of a band at the centre of grid box i, relative to markers.

From the example in **Fig. 6** above:

$$L = \Sigma(OD_i \times L_i)/\Sigma\ (OD_i) = 1121.5/111 = 10.1\ kb = \text{Mean TRF length}$$

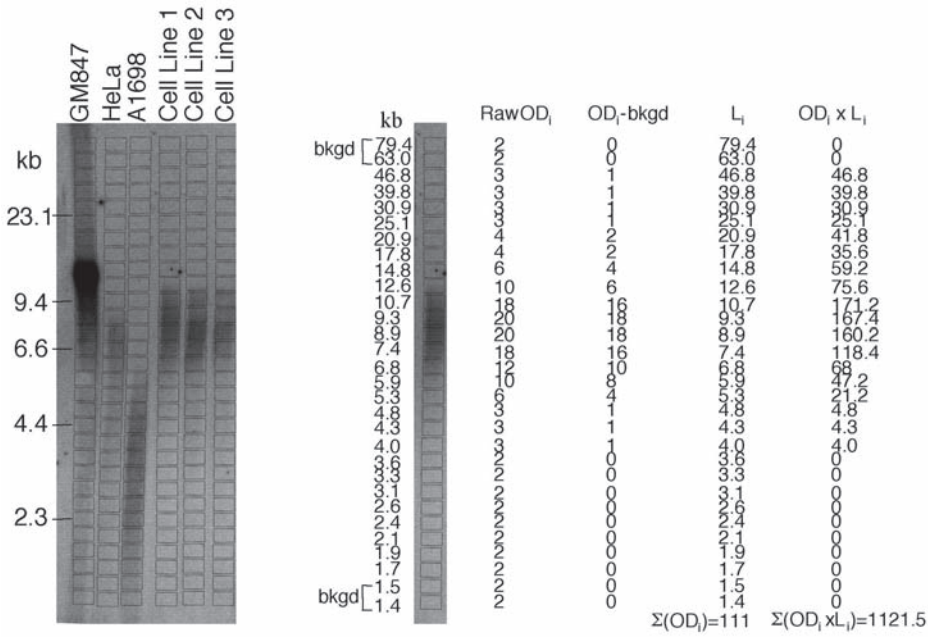

Fig. 6. Example of mean TRF length calculation. Each lane is divided into a number of boxes (typically 30) and the size in kb at the middle of each box extrapolated from the size markers that have been used. The optical density reading for each box is measured and corrected for background and the mean TRF length calculated (*see* **Subheading 3.2.6.**).

3.3. Frequency of Dicentric Chromosomes and Anaphase Bridges As a Correlate of Telomere Dysfunction and Genomic Instability

There is accumulating evidence to suggest that short, dysfunctional telomeres can contribute to increased genomic instability resulting in complex chromosomal rearrangements and aneuploidy in a number of human cancers ***(9)***. The critical loss of telomeric sequences from the ends of the chromosomes results in uncapped chromosome ends, which are highly recombinogenic and result in fusions creating dicentric chromosomes and anaphase bridges (*see* **Note 14**) during mitosis (*see* **Fig. 7**).

In **Subheadings 3.3.1.** and **3.3.2.** we describe two methods of assessing telomere function and genomic instability: (1) analysis of the number of dicentric chromosomes, created by end-to-end fusions in metaphase spreads; and (2) examination of the anaphase bridge index (ABI) of cells or tissue sections.

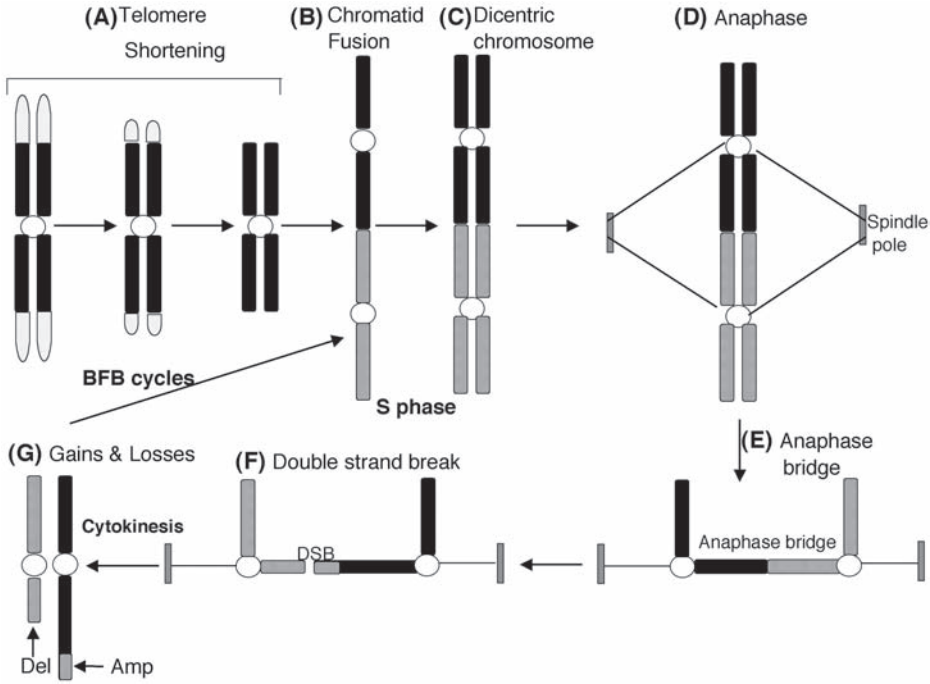

Fig. 7. Telomeric erosion and the generation of dicentric chromosomes and anaphase bridges. As cells divide in the absence of telomerase, telomere shortening **(A)** occurs, exposing the ends of the chromosomes. These ends are thought to be recognized as double-strand breaks (DSB) by the DNA repair machinery DNA and are fused to form a dicentric chromosome—e.g., fusion of two sister chromatids (black and spotted pattern) **(B)**. The dicentric chromosome is replicated during S phase **(C)** and results in anaphase bridging during segregation in mitosis **(D and E)**. The dicentric chromosome breaks when pulled to opposite spindle poles, resulting in a double-strand break (DSB) **(F)**, and causes changes in gene dosage (amplifications = Amp, and deletions = Del) **(G)**. The broken chromosome must be repaired again, and can enter a breakage fusion breakage (BFB) cycle and facilitate the genomic instability associated with cancer.

3.3.1. Frequency of Dicentric Chromosomes in Metaphase Spreads

Dicentric chromosomes can be formed by the fusion of two sister chromatids, which can be visualized by preparing metaphase spreads of colcemid-arrested cells using standard procedures ***(8)***.

3.3.2. Frequency of Anaphase Bridges In Vitro and In Vivo

An anaphase bridge is defined as an anaphase where two-thirds of the distance between the separating poles is spanned by a bridge ***(12)***. They can be

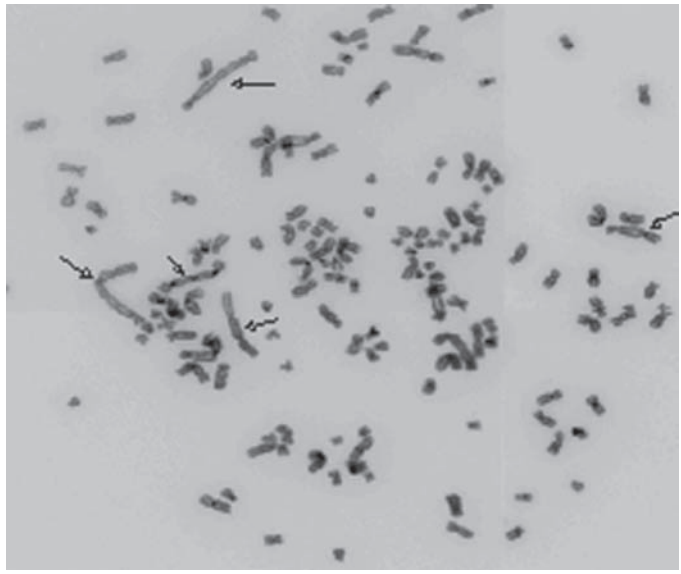

Fig. 8. Typical dicentric chromosomes observed in metaphase spreads from a head and neck squamous cell carcinoma (HN-SCC) cell line. Arrows indicate typical dicentric chromosomes. Taken from **ref. *13***.

formed during mitosis, when dicentric chromosomes fail to separate properly **(Fig. 8)** and have been shown to be a good correlate of telomere dysfunction in vitro and in vivo. **Figure 9** shows a typical bridge observed in a HN-SCC culture **(A)** and a normal anaphase in the same culture **(B)**. In addition, if the corresponding tissue biopsy is available, it can also be examined for the presence of anaphase bridges **(C)**, indicating that telomere dysfunction occurred in vivo prior to culturing.

3.3.2.1. Identification and Anaphase Bridge Scoring

1. Pre-fix cells in ice-cold methanol at –20°C for 20 min and air dry.
2. Fix 5 min in 10% formalin.
3. Prepare tissue samples using standard histological procedures.
4. Stain tissue culture sections or cells with hematoxylin and eosin using standard histological techniques and examine under a light microscope using ×100 magnification.
5. Score the number of nuclei with anaphase bridges, which span 2/3 of the distance between the two poles, and the number of anaphase nuclei ***(12)***. The results are often expressed as the ABI (anaphase bridge index):

ABI = (Number of nuclei with anaphase bridges)/(Total number of anaphase nuclei)

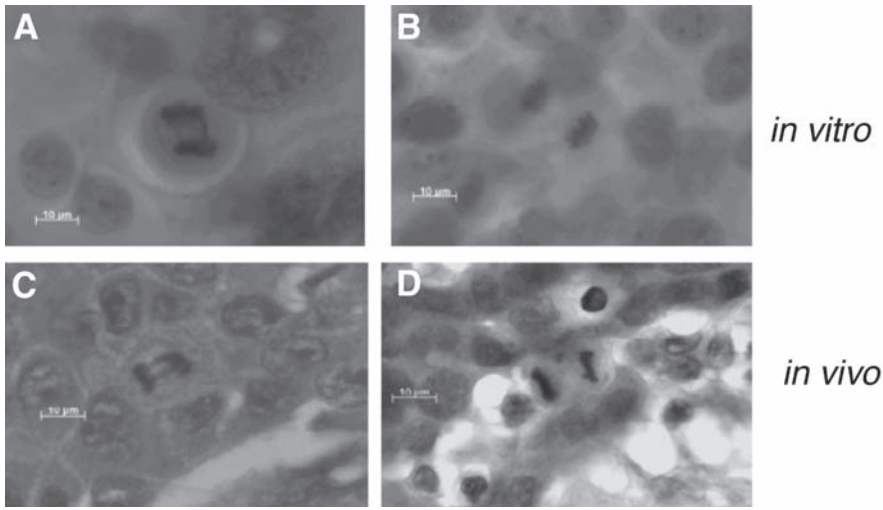

Fig. 9. Anaphase bridging and normal anaphases in vitro and in vivo induced by telomere attrition. **(A)** Anaphase bridge. **(B)** Normal anaphase in tissue culture cells. **(C)** Anaphase bridge. **(D)** Normal anaphase in the corresponding tissue biopsy.

6. Ideally, the ABI should be scored by two independent examiners, and where possible >25 anaphases and a minimum of 10 anaphases should be scored.

4. Notes

1. Due to the TRAP assay's sensitivity to RNase and PCR artifacts, it is important to have segregated areas to prepare cell/tissue extracts, prepare component aliquots and set up reaction mixes, keep positive control (TRS8 and positive extract) separate from reaction components and test extracts, and perform PCR amplification and analysis.
2. Telomerase is a ribonucleoprotein, which uses an RNA template to synthesize telomeric repeats; therefore ensure that RNase-free conditions are adhered to.
3. The cell pellet supplied in the TRAP kit can be used as a telomerase-positive cell line, or a known telomerase-positive cell line such as HeLa cell extracts can be used.
4. There should be no TRAP ladder present in the heat-treated extract sample; this indicates that the TRAP ladder present in the non-heated sample is specifically a result of telomerase activity.
5. The PCR amplification control serves as a control for the successful amplification in each reaction and can be used to monitor the presence of inhibitors of *Taq* polymerase, which can lead to false negative results.
6. TSR8 control template is an oligonucleotide consisting of the TS primer extended with 8 telomeric repeats. This control can be used as a standard for estimation of processivity and telomerase activity of the enzyme.

7. Alternatively, a nonradioactive method, for example Sybr Green (Molecular Probes), can be used; however, in our experience it is less sensitive than the radioactive method.
8. Faint oligonucleotide synthesis byproduct bands may be visible below the 36-bp internal control when the radioactive method is used; however, these do not affect subsequent analysis.
9. When performing standard curves on extracts, we routinely carry out serial dilutions ranging from 0.1 µg to 2 µg; however, this may vary depending on whether the samples have very high or low telomerase activity. Alternatively, if only a limited number of cells are available, the cells can be counted and serial dilutions based on cell number can be used.
10. It is important to use good quality genomic DNA (OD 1.8–1.9) to avoid incomplete digestion and trapping in the well. If possible, spool out the DNA.
11. The amount of DNA required per lane will depend on the TRF lengths. Long TRF lengths can be visualized with less genomic DNA (1–2 µg) than short TRF lengths, because there are more binding sites for the probe when TRF lengths are long.
12. Alternatively, a nonradioactive method can be used such as the chemiluminescent system (Promega).
13. There are other methods available for analyzing telomere lengths, which require less starting material. These include Q-FISH (quantitative fluorescent *in situ* hybridization) ***(17)***, Flow-FISH ***(18)***, telomere PCR ***(19)***, and STELA (single telomere length analysis) ***(20)***.
14. Telomere dysfunction is not the sole mechanism to generate dicentric chromosomes and anaphase bridges.

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