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Transforming Growth Factor-Beta Protocols

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In Vitro Assays for Measuring TGF-β Growth Stimulation and Inhibition

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1. Introduction

Transforming growth factors (TGFs) were initially isolated from the conditioned medium of transformed cell lines through their ability to stimulate anchorage-dependent cells to form colonies in soft agar ***(1,2)***. The ability to proliferate in an anchorage-independent manner is still one of the best in vitro correlates with tumorigenicity. Subsequent studies demonstrated that the growth-promoting activity in the conditioned medium consisted of two unique peptides, TGF-α and TGF-β ***(3–5)***. Depending on the indicator cell line used, soft-agar colony growth could occur when TGF-α and TGF-β (i.e., NRK cells) or TGF-β alone (i.e., AKR-2B cells) were added to the serum-containing medium ***(6,7)***. This review will focus on TGF-β and cellular systems capable of responding in vitro to its growth modulatory activity independent of additional factors.

Transforming growth factor-β is a 25-kDa homodimeric protein representative of a family of molecules capable of regulating cell growth and differentiation ***(8–10)***. Three mammalian TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β 3, have been isolated ***(11)***. Although these molecules have similar and overlapping activity in the majority of in vitro assays, their role(s) in vivo appears to be quite distinct ***(12)***. This distinction becomes readily apparent when the phenotypes of TGF-β knockout mice are compared. For instance, whereas TGF-β1 null animals develop a multifocal inflammatory response and wasting following weaning, the lack of TGF-β2 or TGF-β3 results in a variety of developmental defects ***(13,14)***.

The cellular response to TGF-β is quite distinct, whereas mesenchymal cells are (in general) growth stimulated (both in vitro and in vivo), the majority of

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other cell types (i.e., epithelial, hematopoietic) are growth inhibited. It is unknown how a single growth factor, binding to the same set of receptors, can generate such divergent phenotypes as growth in soft agar, apoptosis, and/or growth arrest. Although studies on the growth-promoting activity of TGF-β have not recently generated as much interest as the growth-inhibitory response, a large body of literature exists documenting the importance of TGF-β in wound healing and various fibroproliferative disorders ***(15–18)***.

Although the approaches discussed in this chapter can be directly employed on any anchorage-dependent culture, they have primarily been utilized with mesenchymal cell cultures. Specifically, we will discuss methods for the following:

1. Thymidine incorporation
2. Autoradiography
3. Soft-agar colony formation
4. Morphological transformation

Although each of these assays can be readily modified to a variety of cell systems, this chapter will focus on two specific model systems: the AKR-2B cell line as a representative mesenchymal culture growth stimulated by TGF-β ***(19,20)***, and the Mv1Lu (CCL64) epithelial cell line, for which TGF-β acts as a late G1 phase growth inhibitor ***(21)***.

2. Materials

2.1. Cell Culture

1. Dulbecco's modified eagle medium (DMEM) (Life Technologies Inc., Gaithersburg, MD).
2. McCoy's 5A Medium (Life Technologies Inc.).
3. MCDB 402 (JRH Bioscience, Lenexa, KS).
4. Fetal bovine serum (Summit, Ft. Collins, CO).
5. Sea plaque agarose (FMC Bioproducts, Rockland, ME).
6. Transforming growth factor-beta (TGF-β): This can be obtained from a number of commercial sources. We have found all to be equally active.

2.2. DNA Synthesis

1. ^{3}H-Thymidine (64 Ci/mmol) (ICN, Costa Mesa, CA).
2. Methanol.
3. Emulsion (Kodak NTB2, Eastman Kodak, Rochester, NY).
4. Developer (D19) (Eastman Kodak, Rochester, NY).
5. Fixer: 75 g Na thiosulfate, 31.3 g K metabisulfite, water to 250 mL.
6. Hematoxylin or Giemsa (Fisher Scientific, Pittsburgh, PA).
7. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4.
8. Trichloroacetic acid (TCA): 10% (w/v) in water.

2.3. Soft-Agar Colony Growth

1. 1.6% sea plaque agarose is made in distilled water and autoclaved for 30 min. The liquefied agarose is then aliquoted (approx 60 mL) into sterile 125-mL glass bottles and stored at room temperature.
2. 2X serum-free DMEM is made according to the manufacturers suggestions using half the normal amount of water. The medium is sterilized through a 0.2-μm filter and stored at 4°C.
3. 35-mm sterile tissue culture dishes (warmed to 37°C).
4. Fetal bovine serum.

3. Methods

3.1. Thymidine Incorporation

This assay is based on the ability of TGF-β to modulate the incorporation of ^{3}H-thymidine in cultured cells. In general, the monolayer growth of most cell types is inhibited when TGF-β is simultaneously added to the serum-containing medium. Although conditions have been defined whereby TGF-β can stimulate mesenchymal cell growth, the response is usually weaker than that commonly observed with other mitogens ***(19)***.

A significant variable for all monolayer assays is the initial cellular seeding density. We find it is most convenient to report this based on the apparent usable growth area in the tissue culture dish/flask. The growth area reported by Cristofalo and Charpentier for various common tissue culture flasks and dishes is listed below ***(22)***:

T150 flask	150 cm^2
T75 flask	75 cm^2
T25 flask	25 cm^2
100-mm dish	64 cm^2
60-mm dish	22 cm^2
35-mm dish	9.6 cm^2
24-well dish	2.0 cm^2
96-well dish	0.32 cm^2 (approximately)

3.1.1. Epithelial Cells

3.1.1.1. Cycling Cultures

1. Mv1Lu cells (Mink Lung Epithelial Cells; CCL64) are plated at (1–2) × 10^4 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum (FBS). We routinely use 24-well dishes in a total volume of 1.0 mL.
2. Following 20–24 h at 37°C in a 5% CO_2 incubator, a 100X stock (10 μL) of TGF-β is directly added to duplicate wells for an additional 20–24 h. For most uses, the final TGF-β concentration ranges from 1.0 to 10.0 ng/mL (40–400 p*M*).
3. Add 10 μL of 100 μCi/mL ^{3}H-thymidine (64 Ci/mmol) for each 1.0 mL of medium and incubate at 37°C for 1–2 h. Final ^{3}H-thymidine concentration of 1.0 μCi/mL.

4. Remove (discard) labeled medium by aspiration and fix with 1.0 mL of 10% TCA per well for 10 min at room temperature.
5. Remove TCA (aspirate or dump out) and repeat TCA fixation (2X) described in **step 4**.
6. Aspirate TCA to dryness and solubilize in 300 µL (per 24 well) of 0.2 *N* NaOH containing 40 µg/mL sheared salmon sperm DNA.
7. Place on platform rocker and rock for 10–20 min at room temperature.
8. Take a 100-µL aliquot from each well of the 24-well plate, place in scintillation vial, and 5.0 mL scintillation fluid. A separate pipet tip should be used for each well (including duplicates).
9. Mix samples and count for 5 min. Shorter (i.e., 1 min) times can be used, however, if your counts are low, significant error can occur due to photoactivation.

3.1.1.2. Arrested/Restimulated Cultures

1. Mv1Lu cells are plated at 2×10^4 cells/cm^2 in DMEM supplemented with 10% FBS. We routinely use 24-well dishes in a total volume of 1.0 mL (4×10^4 cells/well).
2. Following 3 d growth, the medium is removed and the cultures rinsed 2X with 1.0 mL sterile PBS.
3. The second PBS rinse is removed and replaced with 1.0 mL DMEM containing 0.1% FBS for an additional 24 h incubation at 37°C.
4. Duplicate wells are pulsed with 1.0 µCi/mL ^{3}H-thymidine for 1–2 h at 37°C to determine the basal (quiescent) incorporation (*see* **Subheading 3.3.** for stopping the incorporation). For the remaining wells, the medium is removed and the cultures are restimulated at 37°C with fresh DMEM supplemented with 10% FBS, 10 ng/mL epidermal growth factor (EGF), ± [TGF-β]. If you wish to determine a particular cell cycle "window" where TGF-β acts ***(21,23,24)***, 10 µL of a 100X TGF-β stock can be directly added to the FBS/EGF-containing medium at the appropriate times.
5. Following 20–24 h stimulation, the cultures are pulsed with 1.0 µCi/mL ^{3}H-thymidine for 1–2 h at 37°C. To determine the minimum G1 transit time and/or rate of entry into S phase, cultures can be pulsed for 1–2 h at any time during the 24 h stimulation and the reaction stopped with ascorbic acid as described in **Subheading 3.3.**
6. Cultures are TCA fixed and processed as described in **Subheading 3.1.1.1, steps 4–9**.

3.1.2. Mesenchymal Cells

3.1.2.1. Cycling Cultures

1. We routinely use AKR-2B cells as a mesenchymal model. Similar studies can be performed on Balb/c-3T3, 10T1/2, NIH, and so forth murine fibroblasts with minimal changes (determined empirically).
2. All steps are performed as described in **Subheading 3.1.1.1.** with the exception that 5%-FBS supplemented McCoy's 5A medium is used. Although DMEM can be used, we have found that McCoy's 5A (Life Technologies) medium supports the continued passage of AKR-2B cells better.

3.1.2.2. Arrested/Restimulated Cultures

1. AKR-2B cells are plated at 2×10^4 cells/cm^2 in McCoy's 5A medium (Life Technologies) supplemented with 5% FBS. We routinely use 24-well dishes in a total volume of 1.0 mL (4×10^4 cells/well).
2. Following 2 d growth, the medium is removed and the cultures rinsed 2X with 1.0 mL sterile PBS.
3. The second PBS rinse is removed and replaced with 1.0 mL serum-free MCDB 402 for an additional 48 h incubation at 37°C. MCDB 402 is an outstanding medium for serum-free culture ***(25)***. Many cells show essentially no change in viability following 1–2 wk incubation in the absence of serum.
4. Duplicate wells are pulsed with 1.0 μCi/mL ^{3}H-thymidine for 1–2 h at 37°C to determine the basal (quiescent) incorporation (*see* **Subheading 3.3.** for stopping the incorporation). For the remaining wells, the medium is removed and cultures restimulated at 37°C with fresh McCoy's 5A medium (or DMEM) supplemented with the appropriate serum/growth factor "cocktail" ± [TGF-β].
5. Following 20–24 or 40–48 h stimulation, the cultures are pulsed with 1.0 μCi/mL ^{3}H-thymidine for 1–2 h at 37°C. The response of mesenchymal cells in monolayer to TGF-β has been controversial. There have been reports of normal growth stimulation, delayed stimulation presumably due to autocrine activity of an induced mitogen, as well as growth inhibition. To determine the minimum G1 transit time and/or rate of entry into S phase, cultures can be pulsed for 1–2 h at any time during the 24-to 48-h stimulation and the reaction stopped with ascorbic acid (*see* **Subheading 3.3.**).
6. Cultures are TCA fixed and processed as described in **Subheading 3.1.1.1., steps 4–9**.

3.2. Autoradiography

1. Cells are plated and/or arrested as described in **Subheading 3.1.1.1., 3.1.1.2., 3.1.2.1., or 3.1.2.2.**

2a. For cycling cultures, 5.0 μCi/mL ^{3}H-thymidine is added for 2–4 h at 37°C during the final 2–4 h prior to fixation. Remember to pulse cultures for a similar time prior to addition of TGF-β to obtain the 0-h control.

2b. Quiescent restimulated cultures can be similarly pulsed as described in **step 2a** at the end of the experiment or the label can be present continuously for the course of the study.

3. The medium is aspirated, the cells are washed one to two times with PBS, and the cultures fixed with two 20-min applications of 100% methanol (10% TCA can be used, but we find that methanol preserves the cellular structure slightly better). The PBS and methanol applications can be done by simply dumping the medium out and gently pouring.
4. Following the final methanol fixation, the plates are gently washed in water three to five times. A hand-held eye wash works well, or simply dunk the plates in a beaker of water. Again, the water is removed by pouring/shaking into the sink.
5. The excess water is removed and the plates are air-dried.

6a. Go to the dark room and add a *thin* film of emulsion to the entire well. We use Kodak NTB2 diluted equally (w/v) with water (*see* **Subheading 3.3.**).

6b. Adding emulsion is tricky. For microtiter and 24-well plates, a little (i.e., 50–500 μL) is added to each well to ensure complete coating and the excess removed by a hard shake. For larger plates, a few milliliters (i.e., 2–5 mL) are added, the plate is swirled to cover, and the excess is directly added to the next plate, where the process is repeated (a Pasteur pipet may be needed to obtain proper coating).

7. The cultures are placed in a light-tight container (a cookie tin or Tupperware container wrapped in foil works well) over a layer of Drierite (Fisher Scientific) for 2–4 d at room temperature (or 4°C).
8. Develop autoradiography in the darkroom. Many chemicals will work, but be careful if you buy a fixer that it is not too harsh. This will work:
   a. D19 Developer - 4 min; remove.
   b. Water wash (gently).
   c. Fixer - 2 min; remove.
      75 g Na thiosulfate, 31.3 g K metabisulfite, bring to 250 mL with water.
   d. Water wash (gently).
9. Counterstain with Giemsa or hematoxylin (Fisher Scientific) for approximately 15 min (determine empirically). Pour stain off, wash excess with water, and air-dry.
10. Count (or better yet, get someone else to count them for you) labeled/total nuclei in representative field(s) using a 10× to 20× objective.

3.3. Additional Comments

1. A common technical problem is how to utilize a single plate while stopping wells at distinct times (i.e., when determining the kinetics of G1 traverse and entry into S phase). Fixatives such as TCA are problematic because of the potential for fume carryover to adjacent wells. One easy method to overcome this is to use an organic acid such as ascorbic acid for fixation ***(26)***. A 1.0 *M* stock (in water) of the free acid (not the salt) is prepared and 300 μL is added for each 1.0 mL of culture medium. This will stop any incorporation and the plate can now be placed back into the incubator. At the end of the experiment, the entire plate can now be TCA fixed and processed appropriately.
2. Aliquots (10 mL) of the 1.0 *M* ascorbic acid are stored at –20°C. Once thawed, a sample can be maintained at room temperature for approx 1 wk (it will start to turn brownish).
3. The emulsion for autoradiography needs to be dissolved in a 50–55°C water bath. Once you get a stock diluted (i.e., 100 mL), it is convenient to aliquot the emulsion (i.e., 5–10 mL), wrap the tubes in foil, and store at 4°C. A tube(s) can then be used and any remaining discarded. Although the excess can be reused, this sometimes results in high-background problems.
4. Autoradiography with microtiter plates is difficult. An additional way to process those wells is (following fixation) to score the back of the well, use an appropriate size punch and hammer to knock the well out, and glue (use clear glue) the well-scored side down on to a microscope slide. Two rows of six wells can be

placed on a slide. The slides can then be dipped in emulsion, exposed, and developed as discussed in **Subheading 3.2.** Although initially more difficult, this method is preferred.

3.4. Colony Formation in Soft Agar

Transforming growth factor-β was initially identified by its ability to stimulate anchorage-dependent mesenchymal cells to grow in an anchorage-independent manner. The ability of anchorage-dependent cells to form colonies in soft agar is one of the best in vitro correlates with tumorigenicity. Although some cell lines (i.e., AKR-2B) only require the addition of TGF-β to the serum-supplemented medium ***(6)***, other lines (i.e., NRK) also need exogenous EGF (or TGF-α) plus TGF-β for optimal growth in soft agar ***(7)***. Finally, whereas the majority of studies presently focus on TGF-β's growth inhibitory actions, the in vivo growth-promoting role that TGF-β contributes during wound healing or in the pathogenesis of fibrotic disease(s) should not be underestimated ***(15–18)***.

3.4.1. Bottom Plugs

1. Bottom plugs consist of 1X DMEM supplemented with 10% FBS and 0.8% agarose. You need 1.0 mL for each 35-mm plate. Example: If 20 plates are required, combine 10 mL 1.6% agarose, 2.0 mL FBS, and 8.0 mL 2X DMEM. First, combine the serum and DMEM and set in a 37°C water bath to warm; second, microwave the agarose to liquefy; third, when the glass bottle is cool to your skin, mix with the media and serum and pipet 1.0 mL into the required number of 35-mm plates.
2. One milliliter does not flow easily over the plate bottom, you must tilt the plate while pipetting to ensure complete covering. These plates may be prepared 1 d in advance. After solidifying at room temperature, store at 37°C in a 5% CO_2 incubator.

3.4.2. Top Plugs

1. Top plugs consist of 1X DMEM supplemented with 10% FBS, 0.4% agarose, cells, ± TGF-β or other test reagents. The cell concentration can range from 5.0×10^3 to 2.0×10^4 cells/mL. If the cell concentration is too high ($>2.0 \times 10^4$ cells/mL), false positives can be obtained as a result of cell aggregation. We routinely use AKR-2B cells at 1.0×10^4 cells/mL (addition of cells discussed in **steps 3** and **4**).
2. For 35-mm plates, you need 1.0 mL/plate. Each sample is done in triplicate (total volume 4.0 mL) using a 17 × 100-mm or 15-mL conical tube.
3. Each tube will now receive 0.4 mL FBS and 2.0 mL 2X DMEM. Add 4.0×10^4 cells ± TGF-β (final concentration of 3–10 ng/mL) or any other test reagent(s) in a final volume of 0.6 mL 1X DMEM. Mix and place in a 37°C water bath. Be sure to have plates that do not receive TGF-β to determine spontaneous colony formation.
4. Microwave the 1.6% agarose to liquefy and cool until the bottle is not uncomfortable to check. This is the most critical part of the assay; you need to have agarose

warm enough so the top plugs do not solidify too soon, yet cool enough so you do not fry your cells.

5. Using a 5-mL pipet, pipet 1.0 mL of agarose into one tube and mix by pipetting up and down. Quickly pipet 3.0 mL, dispense 1.0 mL/plate, and tilt the plate to ensure complete covering. Do not add the agarose to a number of tubes prior to plating. This will likely result in the mixture prematurely solidifying (this can be avoided by placing the bottom plugs in a 37°C for 15–30 min prior to addition).
6. Let plates solidify at room temperature and then place at 37°C in a 5% CO_2 incubator for 1–2 wk.

3.4.3. Analysis

1. Quantitation is most easily performed using a computerized image analysis system where a defined size can be determined to represent significant colony growth. We have previously used an Omnicon Image Analyzer (BioLogics) with a threshold of 50 μm for AKR-2B cells. Other investigators ***(27)*** have utilized EagleSight analysis software (Stratagene, La Jolla, CA) following staining for 20 h at 37°C in a 1.0 μg/mL solution (in water) of iodonitrotetrazolium violet.
2. Because the above systems are quite expensive, an alternative method is to use a microscope with an eyepiece grid. The entire plate is analyzed and cell clusters of greater than 10 cells are counted as positive.
3. It is also possible to employ a qualitative analysis of the data by simply photographing representative fields on a 10× bright field.

3.5. Morphological Transformation

Cytoskeletal alterations were one of the earliest cellular findings associated with viral transformation ***(28,29)***. It was subsequently found that TGF-β modulated the expression of various cytoskeletal and extracellular matrix genes ***(30–32)***. Coincident with these effects on gene expression, TGF-β induces a morphologic change in mesenchymal cultures similar to that observed during the growth of transformed cell lines ***(33,34)***. The following assay was designed to optimize that phenotype in AKR-2B cells as a model of cytoskeletal rearrangement.

1. AKR-2B cells are plated in 60-mm culture dishes at a density of 1.36×10^4 cells/cm^2 in 4.0 mL (7.5×10^4 cells/mL) of McCoy's 5A medium supplemented with 5% FBS.
2. Incubate at 37°C for 2–4 d until confluence.
3. Wash 2X with 4.0 mL sterile PBS.
4. Remove the PBS and add 4.0 mL serum-free MCDB 402.
5. Incubate at 37°C for 2 d.
6. Remove the medium and replace with 2.0 mL serum-free MCDB 402 ± any test reagent (i.e., TGF-β at 10 ng/mL). Place back at 37°C.
7. Twenty-four hours later, directly add fresh TGF-β (10–100 μL) to a final concentration of 10 ng/mL.

8. Continue incubation at 37°C for an additional 24 h.
9. Remove the medium, wash 1X with PBS, add 2.0 mL PBS and photograph at 20X phase contrast.

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2

Measurement of Active TGF-β Generated by Cultured Cells

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1. Introduction

1.1. TGF-β Latency and Activation

The transforming growth factors-β (TGF-βs) constitute a family of potent regulators of cellular differentiation, proliferation, migration, and protein expression ***(1,2)***. Three isoforms of TGF-β have been described in mammals: TGF-β1, 2, and 3 ***(3–5)***. Most cell lines and tissues secrete TGF-β as a large latent complex formed by three components: TGF-β, LAP (latency-associated protein), and LTBP (latent TGF-β binding proteins). TGF-β is noncovalently associated to its prodomain LAP ***(6–8)***, and LAP is disulfide-bonded to LTBP ***(9)***. Four LTBPs (LTBP-1, 2, 3, and 4) have been described ***(10–14)***. Mature TGF-β must be released from the complex to bind to its high-affinity receptor and elicit its biological functions ***(15)***. This process, called TGF-β activation, appears to be a critical step in the control of TGF-β activity ***(16)***. An additional regulatory step involved in the activation process is the LTBP-mediated incorporation of latent TGF-β into the extracellular matrix ***(2)***. Activation of latent TGF-β has been described in various cell systems ***(17–19)***. However, the molecular mechanisms involved in extracellular TGF-β activation are not fully understood. It also remains to be elucidated whether latent TGF-β incorporation into the extracellular matrix regulates TGF-β activation in a positive or negative manner ***(2)***.

1.2. Detection of Active TGF-β

The availability of sensitive, specific, and quantitative assays for the detection of mature TGF-β is of fundamental importance in studying TGF-β

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activation. The purpose of this chapter is to describe some of the assays used in our laboratory to measure active TGF-β in cell systems. These assays are as follows:

1. Wound assay for bovine aortic endothelial cell migration.
2. Cellular plasminogen activator assay for TGF-β.
3. Mink lung-cell growth-inhibition assay.
4. Mink lung epithelial cells luciferase assay.

In all these assays, the active TGF-β generated by the test cells induces a known and measurable biological response in the reporter cells such as inhibition of endothelial cell migration ***(20,21)***, inhibition of epithelial cell proliferation ***(22)***, decreased plasminogen activator activity ***(23)***, and increased production of plasminogen activator inhibitor-1 ***(24)***. All of these assays can be used to measure active TGF-β released by the test cells into their medium.

Only a few primary cells and established cell lines secrete significant amounts of active TGF-β into their culture medium when properly treated. Some examples are treatment of keratinocytes with retinoids ***(25)*** or vitamin D analogs ***(26)***, treatment of cancer cells or normal fibroblasts with antiestrogens ***(27,28)***, and treatment of MG-63 osteosarcoma cells with corticosteroids ***(29,30)***. Otherwise, little, if any, soluble active TGF-β is generated by most cultured cells. The absence of detectable levels of active TGF-β in the medium of TGF-β-producing cells is a common situation. However, the lack of active TGF-β in a cell culture supernatant does not necessarily mean lack of TGF-β activation. This may be because of two reasons. First, in some cases, TGF-β activation occurs at the cell surface ***(17,31,32)***, generating a high local concentration of active TGF-β. Second, active TGF-β is cleared from solution by binding to cell-surface receptor and/or to the extracellular matrix. As a result, only a small fraction may be released into the medium and therefore diluted to undetectable levels. High local concentration of active TGF-β can be detected by reporter cells cocultured with the activating cells ***(17,33)***. A useful TGF-β assay must be both sensitive and specific. Neutralizing antibodies to TGF-β should be included to verify that there are no other factors present that may affect the assay. Addition of isoform-specific neutralizing antibodies and use of the appropriate standard curves will allow quantification of specific TGF-β isoforms. When analyzing the effect of a treatment on TGF-β activation, one must determine if increased active TGF-β is the result of increased activation of latent TGF-β or increased production of total (active plus latent) TGF-β without any change in the latent versus active TGF-β ratio. In most TGF-β assays, the amount of total TGF-β released into the culture medium can be measured upon activation of the latent fraction by either acidification ***(31)*** or heat treatment ***(34)***.

2. Materials

2.1. General

1. Minimum essential medium (αMEM), store at 4°C.
2. Dulbecco's modified Eagle medium (DMEM), store at 4°C.
3. Fetal calf serum (FCS), store at –20°C, keep at 4°C after thawing.
4. Bovine serum albumin (BSA), store at 4°C.
5. Penicillin–streptomycin–L-glutamine (PSG) stock (100×): 20 g/L strepto mycin, 50×10^6 U/L penicillin G, 29.2 g/L L-glutamine. Filter sterilize and store aliquots at –20°C. Keep at 4°C after thawing.
6. Phosphate-buffered saline (PBS) pH~7.3: 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4. Filter sterilize and store at 4°C.
7. Trypsin solution pH 7.2: 0.25% trypsin, 1 m*M* EDTA. Filter-sterilize and store aliquots at –20°C. Keep at 4°C after thawing.
8. Recombinamt TGF-β (rTGF-β) stock solution: 5 m*M* HCl, 0.1% BSA, 2 μg/mL TGF-β. Store at 4°C.
9. Neutralizing anti-TGF-β antibodies and nonimmune IgG. Store aliquots at –30°C. Keep at 4°C after thawing.
10. Control medium (serum-free medium): To avoid effects of serum factors, most experiments are conducted in the absence of serum. Serum-free medium contains αMEM or DMEM, depending on the cell type used in each assay, 0.1% BSA and 1× PSG. Filter sterilize and store at 4°C.
11. Test cells conditioned medium (*see* **Notes 1–4**): (a) Plate the cells at subconfluence in regular growth medium and let them attach at 37°C for 2–4 h; (b) wash twice with PBS, (c) add serum-free medium and incubate at 37°C for 24 h; (d) collect the medium and centrifuge to remove cell debris. The conditioned medium is ready to be tested for the presence and levels of total and active TGF-β.
12. Acid-or heat-activated conditioned medium (*see* **Notes 5** and **6**). Acidification: (a) Acidify the conditioned medium to pH 2 with 1 *M* HCl; (b) incubate 1 h at room temperature; (c) neutralize with 1*N* NaOH. Use immediately. Heat treatment: (a) incubate the conditioned medium for 10' at 80°C; (b) let the medium cool down to 37°C. Use immediately.

2.2. Wound Assay for BAE Cell Migration

1. Bovine aortic endothelial (BAE) cells.
2. Gelatin-coated dishes: (a) Prepare a 1.5% solution of gelatin in dH_2O; (b) dissolve and sterilize the solution by autoclaving; (c) cover the bottom surface of the dishes with the sterile solution; (d) incubate at 37°C for 15'; (e) wash twice with PBS. Use immediately.
3. Rigid razor blade (*see* **Note 7**).
4. Absolute methanol.
5. 1,1'-dioctodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetyl LDL (DiI-acetyl-LDL).

6. 3% Formaldehyde in PBS: Formaldehyde is usually obtained as a 37% solution in H_2O. Dilute the stock solution 1:12.3 in PBS. Formaldehyde vapors are toxic; prepare the solution in a chemical hood.
7. Light microscope with ocular grid.

2.3. Mink Lung-Cell Growth-Inhibition Assay

1. CCL-64 cells (American Type Culture Collection, Rockville, MD).
2. ^{3}H-Thymidine (^{3}H-TdR), 40-60 Ci/mmol.
3. ^{125}I-Deoxyuridine (^{125}I-UdR), 5 Ci/mg.
4. 3:1 (v/v) Methanol–acetic acid.
5. 80% methanol.
6. 0.5% trypsin.
7. 1% sodium dodecyl sulfate (SDS).
8. Liquid scintillation counter.
9. 1*N* NaOH.
10. Gamma counter.

2.4. Cellular Plasminogin Activator Assay

1. Bovine aortic endothelial (BAE) cells.
2. Gelatin-coated dishes: (a) Prepare a 1.5% solution of gelatin in dH_2O; (b) dissolve and sterilize the solution by autoclaving; (c) cover the bottom surface of the dishes with the sterile solution; (d) incubate at 37°C for 15'; (e) wash twice with PBS. Use immediately.
3. Lysis buffer: 0.1 *M* Tris–HCl, pH 8.1, 0.5% Triton X-100.
4. Bovine fibrinogen.
5. 0.1X PBS: 13,7 m*M* NaCl, 0.27 m*M* KCl, 0.43 m*M* Na_2HPO_4, 0.14 m*M* KH_2PO_4.
6. ^{125}I-Fibrinogen, prepared by the iodine chloride method ***(35)***.
7. 2.5% FCS in αMEM, prepare freshly.
8. Assay buffer: 0.1 *M* Tris–HCl, pH 8.1, 250 μg/mL BSA, 8 μg/mL plasminogen. Prepare freshly.
9. Urokinase stock: 0.1 *M* Tris–HCl, pH 8.1, 0.1% BSA, 1000 U/mL urokinase. Store 10-μL aliquots at –20°C. Just before use, diluted with 5 mL of Tris–HCl, 0.1 *M*, pH 8.1, 0.1% BSA. Keep the dilutions on ice. Urokinase activity is not stable to repeated freezing and thawing.
10. Plasminogen. Purification of plasminogen is carried out at 4°C using a 100-mL lysine–Sepharose column per 500 mL of serum:
    a. Equilibrate the column with PBS.
    b. Load the serum.
    c. Wash with at least three column volumes of 0.3 *M* potassium phosphate, pH 7.4, 2 m*M* EDTA; wash the column until the optical density (OD)λ_{280} returns to the basal value.
    d. Elute the plasminogen with 0.2 *M* ε-aminocaproic acid in 0.1 *M* potassium phosphate, pH 7.4; collect 5-mL fraction and read the ODλ_{280} to follow the elution profile (*see* **Note 8**).

e. Pool the eluted proteins and dialyze against PBS to remove the ε-amino-caproic acid.
f. Measure the $OD\lambda_{280}$ (OD of 1.7 units = 1.0 mg/mL of plasminogen), aliquot, and store at –20°C.

11. Gamma counter.

2.5. MLEC Luciferase Assay

1. Mink lung epithelial cells (MLEC) permanently transfected with the expression construct p800neoLUC ***(36)***.
2. Geneticin stock solution (Invitrogen, Carlsbad, CA): 80 mg/mL in PBS. Fillter-sterilize and store at –20°C.
3. Lysis buffer (Analytical Luminescence, San Diego, CA). Dilute 1:3 with dH_2O the 3X stock solution. Prepare freshly.
4. Assay buffer. Prepare freshly from the following stock solutions: 5X luciferin buffer [1 *M* tricine, 5.35 m*M* $(MgCO_3)_4Mg(OH)_2$, 13.35 m*M* $MgSO_4$, 0.5 m*M* EDTA, 166.5 m*M* DTT]; 50X ATP (37.5 m*M*); 20X luciferin (16 m*M*). Keep luciferin in the dark. (Lucifrerin is rapidly oxidized by exposure to light.) Store stock aliquots at –30°C.
5. Luminometer.

3. Methods

3.1. Wound Assay for BAE Cell Migration

This assay is based on the ability of TGF-β to inhibit cell migration in "wounded" monolayer cultures of BAE cells ***(20,21)***. The number of cells that migrate across the original edge of the wound is inversely proportional to the concentration of TGF-β present in the conditioned medium.

3.1.1. Cell Culture

1. Grow BAE cells on gelatin-coated dishes in αMEM containing 10% FCS and 1X PSG.
2. Use cells at early passages (not after passages 15–20).

3.1.2. Wound Assay

Portion of a confluent culture of BAE cells is removed by mechanical abrasion using a rigid razor blade ***(37)***.

1. Sterilize the razor blade in the pilot light of a Bunsen burner and let it cool down.
2. Use a surgical hemostat to manipulate the razor blade. Press the razor blade down onto the plate to cut the cell monolayer and to lightly mark the original edge of the wound by scoring the plastic surface (*see* **Notes 7** and **9–11**).
3. Gently move the blade to one side to remove part of the cell monolayer.
4. Wash twice with PBS to remove loose cells.
5. According to the experimental design, add the following:
   a. Control medium to determine the basal level of cell migration.

b. Control medium containing increasing amounts of rTGF-β to generate a standard curve; this assay can be used to detect concentrations of TGF-β as low as 0.4 p*M* ***(32)***.
c. Conditioned medium from the experimental cultures to measure active TGF-β.
d. Acid-or heat-activated conditioned medium to measure total (active plus latent) TGF-β.
e. Conditioned medium with neutralizing anti-TGF-β antibodies or nonimmune IgG to test the specificity of the migration inhibition.

6. Incubate at 37°C for 16–20 h.
7. Remove the medium and wash once with PBS.
8. Fix the cells with absolute methanol for 10–15' at room temperature.
9. Count the number of cells that have migrated more than 125 μm from the wound edge in seven successive 125-μm increments. The cells present in the first 125 μm segment are not included in the calculation in order to exclude those cells which moved across the origin before TGF-β had an effect ***(38,39)***. Cells are counted at 40X magnification using a light microscope with an ocular grid.
10. Data are presented as percent of migration observed in the control wound. For each experimental condition, the number of migrating cells is counted in four to six random fields from each of two replicate dishes and the mean value is used to calculate the percent of migration inhibition.

3.1.3. Coculture Assay

1. Immediately after wounding, the second cell type is suspended in serum-free medium and inoculated into the culture dish (*see* **Note 12**).
2. Incubate at 37°C for 16–20 h.
3. Count the migrating BAE cells as in the standard wound assay (*see* **Note 13**).

3.2. Mink Lung-Cell Growth-Inhibition Assay

CCL-64 mink lung epithelial cells have been shown to be extremely sensitive to growth inhibition by TGF-β ***(40)***. A very sensitive and specific assay for TGF-β has been described by Danielpour and colleagues ***(22)***. They have shown that CCL-64 cells plated in DMEM containing 0.2% FCS are half-maximally growth inhibited by about 0.5 p*M* of TGF-β after 22 h of treatment. Because of this sensitivity, conditioned media can be assayed without concentration. Because other growth factors such as insulin, EGF, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) have been shown not to stimulate or inhibit CCL-64 cell proliferation, this assay is relatively specific for TGF-β ***(22)***.

3.2.1. Cell Culture

1. Grow CCL-64 cells in the high-glucose formulation of DMEM supplemented with 10% FCS and 1X PSG.
2. Pass the cells at a seed density of 5×10^5 cells/75 cm^2 T-flask at 3 d intervals.

3.2.2. Growth-Inhibition Assay

CCL-64 cells in logarithmic growth phase are used to initiate the growth inhibition assay.

1. Trypsinize and suspend the cells in 10 mL of DMEM 10% FCS
2. Centrifuge the cells at 500g for 5'.
3. Wash the pellet once with 10 mL of DMEM containing: 0.2% FCS and 1X PSG.
4. Resuspend the cells in the same medium.
5. Count and dilute the cells to a final concentration of 10^6 cells/mL.
6. Seed 0.5 mL/well of cell suspension in 24-well plates.
7. Let the cells attach at 37°C for 2 h.
8. Remove the medium and add the following according to the experimental design:
   a. Control medium to determine the basal level of proliferation.
   b. Control medium containing various concentrations of rTGF-β to generate a standard curve; this assay can be used to measure TGF-β in the range 0.08–2.4 p*M* ***(41)***.
   c. Conditioned medium from the experimental culture to measure active TGF-β.
   d. Acid- or heat-activated conditioned medium to measure total (latent plus active) TGF-β.
   e. Conditioned medium with neutralizing anti-TGF-β antibodies or nonimmune IgG to test the specificity of the growth inhibitory response.
9. Incubate at 37°C for 22 h.
10. Remove the medium and pulse the cells with 0.25 mCi (40–60 Ci/mmol) of ^{3}H-TdR or 0.25 mCi (5 Ci/mg) of ^{125}I-UdR diluted in DMEM, 0.2% FCS, 1X PSG for 2 h at 37°C.
11. Remove the radioactive medium.
12. Fix the cells with 1 mL of methanol–acetic acid (3:1 v/v) for at least 1 h at room temperature.
13. Wash the wells twice with 1 mL of 80% methanol.
14. If ^{3}H-TdR is used, incubate with 250 μL of 0.5% trypsin for 30' at 37°C; solubilize the radioactivity with 250 μL of 1% SDS; measure the radioactivity by liquid scintillation counting.
15. If ^{125}I-UdR is used: lyse the cells with 1 mL of 1*N* NaOH for 30' at room temperature; ^{125}I-UdR is counted in a γ-counter.

3.3. Cellular PA Assay for TGF-β

This assay is based on the observation that TGF-β suppresses plasminogen activator (PA) activity of endothelial cells ***(23)***. The inhibitory effect of TGF-β is predominantly the result of the increased synthesis of plasminogen activator inhibitor-1 (PAI-1) ***(42)***.

Plasminogen activator activity in cell extracts or conditioned media can be measured using the ^{125}I-fibrin assay ***(43)***. Samples are tested in the presence of a known amount of plasminogen in ^{125}I-fibrin-coated plates. The PA present in

the test samples converts plasminogen into plasmin, and plasmin degrades fibrin. The amount of ^{125}I-fibrin degradation products released into the supernatant correlates with the levels of PA activity present in the sample. PA activity can be quantitated using a standard curve generated with purified urokinase plasminogen activator (uPA).

3.3.1. Cell Culture

Bovine aortic endothelial cells are grown as described in **Subheading 3.1.1.**

3.3.2. PA Assay

1. Grow BAE cells to confluence in 96-well plates in complete growth medium.
2. Remove the medium and wash the cells with PBS.
3. Add the following in duplicate:
   a. Control medium to determine the basal level of PA activity
   b. Control medium containing increasing concentration of rTGF-β to generate a standard curve; this assay can be used to measure TGF-β in the 0.08–2.4-p*M* range ***(41)***.
   c. Conditioned medium from the experimental medium to measure active TGF-β.
   d. Acid-or heat-activated conditioned medium to measure total (latent plus active) TGF-β.
   e. Conditioned medium with neutralizing anti-TGF-β antibodies or nonimmune IgG to test the specificity of the PA inhibitory response.
4. Incubate the cells at 37°C for 12 h.
5. Remove the medium and wash twice with ice-cold PBS.
6. Lyse with 50 μL/well of lysis buffer.
7. Determine protein concentration.
8. Measure PA levels in the cell extracts using the ^{125}I-fibrin plate assay (*see* **Note 14**).

3.3.3. ^{125}I-Fibrin Plate Assay

3.3.3.1. Preparation of ^{125}I-fibrin plates ***(43)***

1. Dilute bovine fibrinogen in warm (37°C) 0.1X PBS. Do not mix or vortex. Fibrinogen is diluted such that a volume can be spread over the bottom of the well to give a concentration of 10 μg/cm^2. If using 24-well plates, add 250 μL/well of 120 μg/mL fibrinogen solution.
2. Add ^{125}I-fibrinogen to bring the solution to approximately 160,000 counts per minute (cpm)/mL (40,000 cpm/well).
3. Aliquot 250 μL to each well, making sure that the entire bottom surface is covered.
4. Dry the open plates overnight under the hood.
5. Add 250 μL/well of medium containing 2.5% FCS. Fibrinogen is cleaved to fibrin by the action of thrombin present in serum.
6. Incubate at 37°C for 3 h.
7. Remove medium, wash twice with dH_2O, and store dry plates at 4°C.

3.3.3.2. ^{125}I-Fibrin Assay ***(44)***

Each sample is assayed in duplicate after dilution in assay buffer. Leave two wells with assay buffer only to give the background counts released by buffer alone. Add 500 μL of trypsin to each of the two wells. Trypsin will remove all the counts from the bottom of the wells, giving the total counts releasable.

1. Prepare 1 mL aliquots of assay buffer.
2. Add 1– 5 μg of total cell extract protein to the assay buffer aliquots.
3. Add increasing amounts of urokinase (2–20 mU) to a separate set of assay buffer aliquotes.
4. Add 500 μL/well of each aliquot to the ^{125}I-fibrin-coated wells.
5. Incubate at 37°C for 1–2 h.
6. After 1 and 2 h, take 100 μL from each well and count the amount of ^{125}I-fibrin degradation products with a γ-counter (*see* **Note 15**).
7. Use the urokinase standard curve to quantitate the PA activity in the BAE cell extracts (mU/μg). The data can also be presented as percentage of control, where 100% represents the PA activity of BAE cells incubated in serum-free medium.
8. Use the TGF-β standard curve to determine the TGF-β levels in the original experimental samples (pg/mL).

3.4. MLEC Luciferase Assay

This quantitative bioassay is based on the ability of TGF-β to upregulate PAI-1 ***(24)***. TGF-β activity is determined using MLEC permanently transfected with the expression construct p800neoLUC containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene ***(36)***. The specificity and sensitivity of the assay are the result of using a truncated PAI-1 promoter which retains the two regions responsible for maximal response to TGF-β ***(45)***.

3.4.1. Cell Culture

Mink lung epithelial cells are grown in DMEM containing 10% FCS, 1X PSG, and 250 μg/mL Geneticin (Invitrogen).

*3.4.2. Standard Luciferase Assay **(36)***

1. Detach MLEC with trypsin and suspend them at 5 × 10^5 cells/mL in complete growth medium.
2. Plate 50 μL/well (2.5 × 10^4 cells) in a 96-well plate (*see* **Note 16**).
3. Let the cells attach for 3–4 h.
4. According to the experimental design, replace the medium with 50 μL of each of the following:
   a. Control medium to determine the basal levels of TGF-β produced by the transfected MLEC.
   b. Control medium containing increasing concentrations of rTGF-β to generate a standard curve; this assay can be used to measure TGF-β in the 0.2–30 p*M* range ***(36)***.

c. Conditioned medium from the experimental culture to measure active TGF-β.
d. Acid- or heat-activated conditioned medium to measure total (latent plus active) TGF-β.
e. Conditioned medium with neutralizing anti-TGF-β antibodies or nonimmune IgG to test the specificity of the PAI-1–lucifarease induction.

5. Incubate the MLEC for 16–20 h at 37°C (*see* **Note 17**).
6. Wash the cells two times with PBS and aspirate all the PBS after the second wash.
7. Luciferase activity can be measured by various assays ***(46)***. In all protocols, cells transfected with luciferase expression plasmids are lysed to release the reporter protein luciferase. ATP and luciferin are added to the lysate in a luminometer. The enzyme catalyzes the ATP-dependent oxidation of the substrate, which emits light. Below, we describe the protocol used in our laboratory using a ML3000 Microtiter Plate Luminometer (Dynatech Labs. Inc., Chantilly, VA). Lyse the cells with 35 μL of 1X cell lysis buffer (Analytical Luminescence, San Diego, CA) for 20' at room temperature.
8. Transfer 30 μL of the cell-extract to a Microlight1 96-well plate (Dynatech Labs. Inc., Chantilly, VA).
9. 2" after injection of 110 μL of freshly prepared luciferin buffer containing 800 μ*M* luciferin and 750 μ*M* ATP, emitted light is measured for 3" (*see* **Note 18**).
10. The luciferase activity is recorded as relative light units (RLU). RLU values are converted to TGF-β activity (pg/mL) using the TGF-β standard curve.

3.4.3. Coculture Assay ***(17,33)***

1. Detach MLEC and test cells with trypsin and suspend them at 5×10^5 cells/mL in complete growth medium.
2. Plate 50 μL/well (2.5×10^4 cells) of MLEC in a 96-well plate.
3. Add 50 μL/well (2.5×10^4 cells) of test cells (*see* **Notes 19–21**).
4. Add neutralizing anti-TGF-β antibodies to one set of wells to test the specificity of the PAI-1–lucifarease induction (*see* **Note 22**).
5. Incubate the coculture for 16–20 h at 37°C.
6. Wash the cells two times with PBS and aspirate all the PBS after the second wash.
7. Lyse the cells and measure TGF-β activity as described in the standard luciferase assay.
8. The luciferase activity is recorded as relative light units (RLU). RLU values are converted to TGF-β activity (pg/mL) using the TGF-β standard curve obtained with the MLEC. The TGF-β activity in the coculture is compared with the TGF-β activity of the MLEC alone. TGF-β activity induced by different test cells can also be compared.

4. Notes

1. The amount of cells and the incubation time used to produce the conditioned medium may vary according to the experimental necessities and may need to be optimized.
2. Depending on the amount of active TGF-β produced by cells, the conditioned medium may need to be concentrated or diluted with fresh control medium in

order to fall within the optimal range of the assay. Note that concentration of the samples can result in losses of mature TGF-β ***(32)*** or activation of latent TGF-β.

3. When comparing several samples, one must normalize the conditioned medium to either the cell number or cell-extract protein concentration in the culture used to prepare the media.
4. The medium should be used immediately or kept at 4°C for short-term storage. Repeated freezing and thawing may result in activation of latent TGF-β.
5. The amount of TGF-β present in the conditioned medium after acidification or heating may be high. Serial dilutions of samples in fresh serum-free medium should be used in order for TGF-β concentrations to fall within the optimal range of the assay.
6. Although easier to perform, heat treatment may result in protein precipitation.
7. The razor blade must be a rigid one because the flexible type bends when pressure is applied and produces uneven wounds.
8. If using FCS, pass the eluate a second time through the column.
9. A blade should not be used for more then 20 wounds because a much-used blade has gaps in the cutting edge and leaves lines of cells attached to the plate.
10. Take care not to make the initial score in the plastic too deep, because cells will not be able to migrate across.
11. Multiple wounds can be made in the same plate.
12. The appropriate ratio between BAE cells and test cells has to be determined experimentally.
13. Bovine aortic epithelial cells can often be distinguished from other cell types by shape, size, and nuclear morphology. Otherwise, the BAE monolyer can be labeled before wounding with DiI-acetyl-LDL:
    a. Incubate the cells with 10 mg/mL of DiI-acetyl-LDL in regular growth medium for 4 h at 37°C.
    b. Wound the monolayer and wash three times with PBS.
    c. Add the second cell type suspended in serum-free medium.
    d. Incubate at 37°C for 16–20 h.
    e. Wash three times with PBS.
    f. Fix with 3% formaldehyde in PBS for 15' at room temperature.
    g. Visualize labeled BAE cells by fluorescence microscopy using standard rhodamine excitation.
14. If not analyzed immediately, the sample can be stored at –20°C. Repeated freezing and thawing may result in loss of PA activity.
15. If after 2 h the counts are still low, the incubation can be continued. The reaction is usually stopped when <20% of the total radioactivity is released from the plates.
16. Test triplicates of each sample for accurate statistical manipulation.
17. Keep assay times less than 20 h in order to avoid complications as a result of the effect of TGF-β on the MLEC proliferation.
18. The delay time and measuring time of light detection may need to be optimized ***(46)***.
19. Test triplicates of each sample for accurate statistical manipulation.
20. The optimal ratio between MLEC and test cells has to be determined experimentally.

21. Test cells may need to be plated first (24 h, 48 h in advance).
22. Various test cells may nonspecifically suppress or induce basal lucuferase expression by the MLEC.

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3

Iodination of TGF-β, TGF-β–Receptor Crosslinking, and Immunoprecipitation of TGF–β-Receptor Complexes

David Danielpour

1. Introduction

1.1. Iodination of TGF-β

Radioiodinated transforming growth factors (TGF-βs) are critical reagents for functional studies on TGF-β ligand–receptor binding ***(1–5)***, TGF-β–binding protein interactions ***(6–9)***, ligand uptake, degradation and clearance ***(1,10,11)***, titering of TGF-β neutralizing antibodies ***(9,10)***, and for quantitation of TGF-βs by radioimmuno and radioreceptor assays ***(12,13)***. Several methods have been developed for the iodination of TGF-βs, namely chloramine T, lactoperoxidase, and Bolton–Hunter ***(1,3,4,14)***. Although the latter method is gentler and believed to yield radiolabeled TGF-βs with greater biological activity than the first two, it is much more expensive, labor intensive and yields a poorly labeled product. Chloramine T is currently the most common method for iodination of TGF-βs, most likely because of its great efficiency of labeling. However, iodination with chloramine T can cause oxidative degradation of TGF-βs with excess treatment. For this reason, successful iodination of TGF-βs requires strict adherence to the amount of chloramine T used and the duration of treatment. Frolik et al. ***(1)*** have shown that three consecutive short treatments with low levels of chloramine T gives the best labeling with little oxidation of TGF-β. With the chloramine T method outlined later, all three isoforms of TGF-β can be iodinated with near-equal efficiency.

In this chapter, a modification the above chloramine T method for radioiodination of TGF-β ***(1)*** will be covered in detail. The principles of this method

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are briefly summarized here. Chloramine T rapidly decomposes in water to generate hypochlorous acid. Hypochlorous acid is believed to convert ^{125}I- to a cationic form (^{125}I+), which reacts with ionized tyrosine residues of proteins ***(15)***. At the end of the reaction, *N*-acetyl-tyrosine is added to quench unreacted ^{125}I+ species, and a large excess of nonradioactive iodide is added to lower the specific radioactivity of any residual carryover free iodide following fractionation. Acid–urea (8 *M*) is then added to the reaction tube to extract all TGF-β that bind to the tube under the neutral pH conditions of the reaction, and free ^{125}I, *N*-acetyl-tyrosine-[^{125}I], and other impurities are removed from ^{125}I-TGF-β by exclusion through a Sephadex-G25 gel filtration column. Gel filtration is run under acidic conditions with carrier BSA to reduce loss of TGF-β to the column, as TGF-β is very sticky at neutral pH.

1.2. Crosslinking of TGF-β to Cell-Surface Receptors

The presence of three classical TGF-β receptors (types I, II, and III) and a whole host of other cell-surface TGF-β binding proteins makes the classical Scatchard analysis of total specific binding of ^{125}I-TGF-β to cells an uninformative exercise. TGF-β binds to receptors or binding proteins on cells to form high-affinity noncovalent complexes that readily dissociate in the presence of ionic detergents such as sodium dodecyl sulfate (SDS). Thus, analysis of ^{125}I-TGF-β–receptor interactions under the denaturing conditions of SDS–polyacrylamide gel electrophoresis (SDS-PAGE) requires that the formed complexes be covalently linked with bifunctional crosslinkers. Disuccinimidyl suberate (DSS) is the most widely used agent for crosslinking TGF-β to receptors and other cell-surface binding proteins. DSS is a water-insoluble homobifunctional agent that crosslinks to free amino groups of proteins.

The general overview of the procedure ***(4,5,7,16)*** for crosslinking of ^{125}I-TGF-β to receptors/binding proteins is outlined as follows. ^{125}I-TGF-β is first allowed to bind to receptors on cells in the absence or presence of 100-fold molar excess of cold ligand. Unbound ligand is then washed away, and the ^{125}I-TGF-β bound to cell-surface proteins is then crosslinked with DSS. Free crosslinker is washed off, proteins are extracted from the cell membrane, and complexes are analyzed by SDS-PAGE and autoradiography.

1.3. Immunoprecipitation of ^{125}I-TGF-β–Labeled Receptors

The migration pattern of ^{125}I-TGF-β crosslinked species through SDS–polyacrylamide gels is not sufficient to confirm the presence of TGF-β receptors (TβRI, TβRII, TβRIII), not only because each of these receptors varies in the extent of glycosylation but also because of the potential confusion by TGF-β binding proteins that migrate at similar molecular weights (MWs) ***(7,8)***. TβRI, TβRII, and TβRIII migrate at about 53 kDa, 73–80 kDa, and 120–280 kDa,

respectively, depending on degree of glycosylation. When crosslinked to ^{125}I-TGF-β and then reduced, the receptors migrate at a MW 12.5 kDa larger. One straightforward way of confirming the status of the crosslinked species is by immunoprecipitation with specific antibodies to each of the TGF-β receptors. However, it is important to note that because these receptors form multimeric complexes with each other in the presence of ligand ***(5)***, they are expected to be all coimmunoprecipitated with antibodies against each receptor. Boiling the membrane lysates (in ristocetin-induced platelet adhesion [RIPA] buffer) for 2 min before immunoprecipitation can break receptor–receptor interactions and prevent coimmunoprecipitation.

2. Materials

2.1. Iodination of TGF-β

N-Acetyl tyrosine, chloramine T, potassium phosphase, sodium phosphate, bovine serum albumin (crystalline), ultrapure urea, and potassium iodide were obtained from Sigma (St. Louis, MO). Na^{125}I (cat. no. IMS.30) was obtained from Amersham (Arlington Heights, IL), PD 10 columns were obtained from Pharmacia (Piscataway, NJ), and carrier-free TGF-βs were obtained from R&D Systems, Inc. (Minneapolis, MN).

Prepare the following in advance of iodination.

1. PPB: 1.5 *M* potassium phosphate, pH 7.4 (store at room temperature [RT)].
2. SPB: 50 m*M* sodium phosphate, pH 7.4 (store at RT).
3. Storage solution: 4 m*M*, 1% crystalline bovine serum albumin (BSA) (store at 4°C).
4. Column buffer: 4 m*M* HCl, 75 m*M* NaCl, 0.1% crystalline BSA (store at 4°C).
5. PD 10 column: Equilibrate a PD 10 disposable column in column buffer and store overnight at 4°C.
6. TGF-β: 5 μg of carrier free BSA in 10–15 μL of either 4 m*M* HCl or high-performance liquid chromatographic (HPLC) solvent (30% CH_3CN, 0.1% trifluoroacetic acid [TFA]). This must be kept in a Sigma-coated or siliconized tube (store at –80°C).
7. Sigma-coated (siliconized) 1.5-mL microfuge tubes.

Prepare the following immediately prior to iodination:

1. 8 *M* urea, pH 3.2. Dissolve 2.4 g of ultrapure urea in 2 mL of 1 *M* acetic acid, add 1.3 mL of glacial acetic acid, and adjust the final volume to 5 mL with water.
2. 60 m*M* (or 10 mg/mL) KI in SPB.
3. 50 m*M* (11 mg/mL) *N*-acetyl tyrosine in PPB.
4. 50 μg/mL of chloramine T in SPB. Weigh out 2–10 mg of chloramine T in advance. Right before use, add the volume of SPB to give 5 mg/mL chloramine T. Dilute 10 μL of the resulting solution with 990 μL of PPB to yield the working concentration of 50 μg/mL.
5. Twelve numbered 12 × 100-mm tubes containing 0.5 mL of storage buffer.

2.2. Crosslinking of TGF-β to Cell-Surface Receptors

Disuccinimidyl suberate was purchased from Pierce (Rockford, IL), bicarbonate-free MEM from Gibco-BRL (Gaithersburg, MD), BSA V, dimethyl sulfoxide (DMSO), sucrose, EDTA, phenylmethyl sulfhonyl flouride (PMSF), pepstatin A, and leupeptin from Sigma (St. Louis, MO).

Prepare the following solutions in advance:

1. Binding buffer: bicarbonate-free MEM, 1 mg/mL BSA fraction V, 25 m*M* HEPES, pH 7.4. (Make fresh each time.)
2. Wash buffer 1: Binding buffer without BSA. (Make fresh each time.)
3. Wash buffer 2: 250 m*M* sucrose, 10 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA. (Store at 4°C.)
4. Solubilization buffer: 1% Triton X-100, 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA. (Store at 4°C.) Add 0.1 m*M* PMSF and 1 μg/mL pepstatin A and 1 μg/mL leupeptin before use.
5. DSS, prepare fresh each time. Make stock of 20 m*M* or 7.36 mg/mL in DMSO.

2.3. Immunoprecipitations of Labeled Receptors

RIPA buffer: 150 m*M* NaCl, 10 m*M* sodium phosphate (pH 7.0), 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate. Antibodies for immunoprecipitation of TGF-β RI (V-22, cat. no. sc-398) can be obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and that of TGF-β RII (cat. no. AF-241) and RIII (cat. no. AF-242-PB) can be obtained from R&D Systems, Inc. (Minneapolis, MN).

3. Methods

3.1. Iodination of TGF-β

Owing to the great volatility of $Na^{125}I$, all of the following steps should be conducted in a fume hood certified for radioiodination. It is an important safety issue that all pipet tips have filter plugs to reduce the chance of contamination.

1. To the tube containing 5 μg TGF-β, add 10 μL PPB, followed by 0.6 mCi of $Na^{125}I$ (8 μL), briefly mix, microfuge for 5 s, and immediately proceed to the next step.
2. Start the iodination reaction at room temperature as follows (microfuge for 5 s after each mixing step to keep sample at the bottom of tube):
   a. Add 4 μL of 50 μg/mL chloramine T, mix, and allow 2 min for reaction.
   b. Add 4 μL of 50 μg/mL chloramine T, mix, and allow 1.5 min for reaction.
   c. Add 4 μL of 50 μg/mL chloramine T, mix, and allow 1 min for reaction.
3. Stop the reaction at room temperature as follows:
   a. Add 20 μL of 50 m*M* *N*-acetyl tyrosine, mix, microfuge, and wait 2 min.
   b. Add 200 μL of 60 m*M* KI and 200 μL of 8 *M* urea (pH 3.2), mix, and microfuge.

4. Transfer 1 μL of this reaction mixture to a 1.5-mL tube containing 0.5 mL of storage buffer. This will later be precipitated by treatment with trichloroacetic acid (TCA) to determine the percent radiolabel incorporation into TGF-β.
5. Transfer the remaining reaction mixture to an equilibrated PD 10 column. Rinse the reaction tube with 200 μL of the KI solution and transfer to the column.
6. Elute column with column buffer, collecting ^{125}I-TGF-β in 12 × 100-mm tubes containing 0.5 mL of storage buffer. Collect 16 drops/tube in the first three tubes and 8 drops/tube in the next nine tubes.
7. Monitor cpms of column fractions to locate peak fractions (usually tubes 5–8). Pool peak tubes, dilute to 20 μCi/mL in storage buffer, aliquot in siliconized tubes, and store at –80°C.
8. Determine the cpms of tube in **step 4** containing 0.5 mL of storage buffer and 1 μL of reaction mix. Then, mix in 0.5 mL of 20% TCA to this tube, set on ice for 10 min, and microfuge for 10 min at top speed. Determine the cpms in 500 μL of the resulting supernatant to calculate the percentage precipitable counts per minute (%TPC). Calculate the specific activity of ^{125}I-TGF-β from the amount of ^{125}I added to the reaction mix, the %TPC, and the amount of TGF-β used for iodination, using the formula:

$$\text{Specific activity of } ^{125}\text{I-TGF-}\beta = \frac{\%\text{TPC} \times \mu\text{Ci used}}{\text{pmole TGF-}\beta\text{ used}}$$

The total picomoles of TGF-β recovered from the column can then be calculated using:

$$\text{pmoles recovered} = \frac{\mu\text{Ci of TGF-}\beta\text{ recovered from column}}{\text{Specific activity of } ^{125}\text{I-TGF-}\beta}$$

9. Determine the precipitability of the purified ^{125}I-TGF-β by precipitation with TCA as done in **step 8**. This is important for ensuring the absence of contaminating free ^{125}I (*see* **Note 1**).

3.2. Radioreceptor Crosslinking Procedure

1. Typically cells are cultured in six-well dishes until subconfluent. They are washed twice (4 mL/wash with cold binding buffer) and replaced with 1 mL of the above cold binding buffer containing 100 p*M* ^{125}I-TGF-β1 either without or with a 100-fold molar excess of unlabeled TGF-β1. Plates are then placed on a rocking shaker (10 cycles/min) at 4°C for 2 h.
2. Place dishes on ice, and wash cells twice with 2 mL of cold wash buffer 1.
3. Prepare 300 μ*M* of DSS in cold wash buffer 1 just before use. Aspirate wash buffer from wells and replace with 1 mL of the 300 μ*M* DSS solution. Preparation of this solution is critical for successful crosslinking. This should be done by directly adding 20 m*M* DSS in DMSO to a tube of the cold wash buffer and immediately mixing in the DSS by gently inverting the sealed tube several times.

Not mixing the solution immediately upon DSS addition leads to precipitation of DSS. Vigorously shaking or vortexing will cause DSS to fall out of solution, even if it has initially dissolved.

4. Place plates on a rocking platform for 15 min at 4°C.
5. Wash cells twice with 2 mL/well of ice-cold wash buffer 2. At this point, receptors could be extracted directly in the wells, or cells can be scraped off dishes transferred to microfuge tubes and extracted there.

Extraction in wells:

6. Place dish on an incline on ice for 1–2 min to allow excess buffer to collect at the base of the well and then aspirate. Add 200 μL of ice-cold solubilization buffer and place on a rocking platform at 4°C for 40 min.
7. Place plate at a 45° angle for 2 min to allow the lysate to collect at the base of the well. Transfer the supernatant to a microfuge tube, microfuge at 12,000*g* for 5 min, and transfer supernatant to a fresh microfuge tube. Proceed to **step 10**.

Extraction in tubes:

8. Scrape cells off wells with a cell scraper in the presence of 0.7 mL of wash buffer 2 containing 1 m*M* PMSF and transfer to microfuge tube. Collect remaining cells by rinsing wells with 0.7 mL of wash buffer 2.
9. Microfuge cells at 4000*g* for 2 min, resuspend the cell pellet in 80 μL of solubilization buffer, and extract for 40 min at 4°C on a shaker. Clarify sample by centrifugation at 12,000*g* for 5 min.
10. Add 15 μL of 4X SDS loading buffer containing 20% 2-mercaptoethanol to 45 μL of membrane lysate containing TGF-β crosslinked receptors, heat at 99°C for 5 min, and electrophorese proteins through 12% polyacrylamide Tris–glycine gels with SDS/Tris/glycine running buffer.
11. Fix gel by gentle shaking with 50% methanol–10% acetic acid for 1 h at room temperature, wash three times for 5 min each time with water, equilibrate for 20 min with 70% ethanol–1.5% glycerol with gentle shaking. Gel can then be placed between two layers of cellophane and air-dried in several hours at room temperature. A frame for drying minigels can be obtained from Novex (San Diego, CA).
12. Exposure times of autoradiography range anywhere from 1 d to 2 wk, depending on the level of receptor expression (*see* **Note 2**).

3.3. Immunoprecipitation of ^{125}I-TGF-β-labeled Receptors

1. To 200 μL clarified lysate of ^{125}I-TGF-β crosslinked cells add 35 μL of 100 mg/mL BSA and 35 μL of 10X RIPA buffer, mix well, and then add 2.5–5 μg of antibody against TβRI, TβRII, TβRIII, or control nonimmune IgG. Bring total volume up to 350 μL with water, mix well, and then incubate for 2 h at 4°C.
2. To each of the above tubes add 30 μL of 50% slurry of washed Protein A–Sepharose equilibrated in 1X RIPA buffer and 10 mg/mL BSA for 1 h.
3. Keep resin suspended for 2 h at 4°C by inverting tubes on a rotatory mixer (i.e., labquake shaker).

4. Wash resin 5 times with 1 mL of RIPA buffer by rapid (10,000*g* × 10 s) centrifugation.
5. Remove all but 15 μL of the supernatant from the last wash, add 30 μL of 2X SDS-PAGE sample loading buffer containing 10% 2-mercaptoethanol, boil for 5 min, and load up to 40 μL onto a 12% or a 4–20% gradient polyacrylamide Tris–glycine gel.
6. Following electrophoresis, fix gel with 50% methanol–10% acetic acid by gentle shaking for 1 h at room temperature, wash three times for 5 min each time with water, and equilibrate for 20 min with 70% ethanol–1.5% glycerol by gentle shaking. Gels can then be placed between two layers of cellophane and air-dried within several hours at room temperature. Frames and cellophane for air-drying minigels can be obtained from Novex (San Diego, CA).
7. Exposure times to autoradiographic film range anywhere from 6 h to 4 d, depending on the level of receptor expression (*see* **Note 3**).

4. Notes

1. For successful iodination of TGF-β it is important that $Na^{125}I$ be relatively fresh (< 2 wk old), as the radioactive decay by-products inhibit radiolabeling and also oxidize TGF-βs. We have also noticed variations in the suppliers of $Na^{125}I$, and recommend that provided by Amersham as the best source.

   Normally, iodination of TGF-β to a specific activity between 2.4 and 2.7 μCi/ pmol is considered ideal. Higher specific activities lead to higher nonspecific binding of the ligand with faster rates of radiolytic decay. Once prepared under optimal conditions, the radiolabeled material can be used for 1.5 mo it stored at –80°C. We recommend running the iodinated product on a gel right after preparation and 1 mo after storage to check for the presence of intact protein. For a more rapid check, determine the percentage of the purified ^{125}I-TGF-β that can be precipitated with TCA. This can be done similar to that described in **step 8**. If less than 5 μg of TGF-β is to be labeled, the volume of all solutions should be scaled down proportionately, with concentrations of solutions and timing of reactions kept constant. We recommend that no less than 1.2 μg TGF-β be iodinated at a time because of poor recovery of the iodinated material. To help increase recovery when labeling 1–2 μg of TGF-β, a smaller column (2 mL) of Sephadex G25 should be used, made from a 3-mL syringe. The concentration of TGF-β added to the iodination reaction should not be under 0.3 mg/mL. More dilute stocks of TGF-β can be concentrated with a Speed Vac, but extreme care should be used to avoid drying the sample, as TGF-β will be lost to the tube.
2. One critical aspect of radioreceptor crosslinking studies is the choice and proper use of the crosslinker. We have shown that DSS effectively crosslinks TGF-β to membrane-bound receptors and binding proteins. The water-soluble crosslinker, bis{sulfosuccinimidyl}suberate (BS3), which has been used for TGF-β receptor crosslinking in a few studies, is far less effective a crosslinker of TGF-βs to cell-surface receptors. Because DSS is very unstable and water insoluble, it must be stored and used properly for optimum crosslinking efficiency. DSS is extremely hydroscopic and water reactive. The stock bottle of DSS needs to be opened only

at room temperature and briefly. We recommend keeping the stock bottle of DSS properly sealed and in a desiccator box at –20°C. We have found that DSS solubilized in DMSO rapidly loses reactivity even when stored in cryotubes in a liquid-nitrogen freezer. Thus, it is essential that DSS be made fresh for each crosslinking reaction. Also important to proper crosslinking is the provision that DSS be kept in solution through the course of crosslinking. This can be prevented by avoiding vigorous mixing or agitation of the solution containing this crosslinker. In case the crosslinker precipitates, it should be replaced with a freshly prepared batch. The efficiency of crosslinking can be readily checked after running the gel: It is the amount of crosslinked material relative to the amount of TGF-β-competable free ^{125}I-TGF-β. If one wishes to extend the time of crosslinking, a fresh crosslinker should be added, as most of the DSS hydrolyzes after 15 min.

It is important that the cells being crosslinked be kept at 4°C all the times before and during crosslinking. Allowing cells to approach room temperature even for a brief moment can lead to internalization of occupied receptors with a corresponding loss of receptor crosslinking efficiency.

The concentration of ^{125}I-TGF-β1 used for optimal receptor crosslinking should be between 25 p*M* and 100 p*M*. Because the 100 p*M* ligand will saturate most of the TGF-β receptor binding, a greater than 100-p*M* ligand may increase the background with little gain. Use of 25 p*M* or less ^{125}I-TGF-β1 may be preferable to 100 p*M*, as it will substantially reduce the binding of TβRIII relative to that of TβRI and TβRII, in the event that the TβRIII signal is massive relative to the signaling receptors.

3. Immunoprecipitation is particularly useful for enhancing weak TGF-β receptor signals against a high background of other binding proteins, or weak TGF-β binding protein signals against a high background of TGF-β receptors. Moreover, the numbers of cells used can be substantially scaled up for detection of receptors in low expressers. It is important to know that there may be substantial lot-to-lot variability in the quality of commercially available TGF-β receptor antibodies. Thus, each lot needs to be carefully monitored for the ability to immunoprecipitate the TGF-β receptor. The MvLu mink lung cell line (ATCC # CCL-64), which expresses relatively high levels of TβRI, TβRII and TβRIII, can be used as a suitable positive control for receptor crosslinking and immunoprecipitation assays.

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4

Detection of TGF-β in Body Fluids and Tissues

Andrew H. Limper

Introduction

Transforming growth factor-β1 (TGF-β1) is a 25-kDa homodimeric protein secreted by numerous cells including platelets, monocytes, macrophages, epithelial cells, and fibroblasts ***(1–3)***. Active TGF-β1 exerts multiple and divergent effects on mesenchymal and epithelial cell cycle regulation ***(1)***. In addition, TGF-β1 influences the expression of multiple genes, which act in concert to enhance the synthesis of extracellular matrix components with resulting fibrosis ***(1,2)***. *In vitro* studies have demonstrated that TGF-β1 regulates the genetic expression of extracellular matrix components, including fibronectin (FN), the α5β1 fibronectin receptor (FNR), collagen, and proteoglycans ***(4–7)***.

Numerous studies on animal and human tissues have implicated roles for TGF-β1 in disease states ranging from malignancy, liver cirrhosis, fibro–proliferative lung diseases, and fibrotic diseases of the kidney ***(8–14)***. For instance, immunostain analysis has demonstrated enhanced tissue localization of TGF-β1 in patients with tissue fibrosis related to pulmonary sarcoidosis ***(11)***. In addition, enhanced TGF-β1 gene expression has been observed in early macrophage-rich lesions in patients with idiopathic pulmonary fibrosis using *in situ* hybridization ***(10)***. Notably, TGF-β1 protein was further localized to regions of active extracellular matrix protein gene expression in fibrosing lung ***(10,11,13,14)***. In addition to being localized to tissues, TGF-β1 has also been described to be present in enhanced quantities in the peripheral circulation in certain disease states ***(15–17)***. For instance, significantly elevated circulating TGF-β was observed in patients with advanced breast cancer that subsequently developed pulmonary and hepatic fibrosis after autologous bone marrow transplantation ***(15)***. Such studies suggest that circulating TGF-β measurements may indicate the degree of fibrotic activity following tissue injury.

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Enhanced local expression of TGF-β1 has been described using immunohistochemistry applied to tissue biopsies obtained from patients with a variety of chronic fibrotic disorders induced by bleomycin, collagen-vascular diseases, hypersensitivity pneumonitis, sarcoidosis, and idiopathic pulmonary fibrosis ***(10-19)***. Such studies provide useful qualitative information on cellular sources of this growth factor during tissue injury, repair, and fibrosis. Although not rigorously quantitative, with appropriate controls and concurrent analysis of diseased and normal tissues, TGF-β1 immunostaining can also provide semiquantitative information about the relative amounts of TGF-β1 in tissues ***(11)***.

In a parallel fashion, *in situ* hybridization studies provide useful insights into the cellular sources within diseased and normal tissues, which exhibit enhanced gene expression for TGF-β1 ***(10)***. Again, the technique of *in situ* hybridization should be viewed as providing qualitative information about localization of gene expression. *In situ* hybridization may be coupled with Northern hybridization, ribonuclease protection assays, or quantitative polymerase chain reaction (PCR) techniques to obtain quantitation of TGF-β1 RNA within tissue samples.

2. Materials

2.1. Materials for Quantifying TGF-β1 in Body Fluids by ELISA

2.1.1. Peripheral Blood or Other Body Fluids

Transforming growth factor–β1 has been detected both in circulating plasma and serum samples ***(15,20)***. Plasma collected in EDTA tubes is preferred when possible. Although not extensively studied, TGF-β1 determination may also be performed on pleural or peritoneal fluid, or on samples derived from bone marrow also collected in the presence of EDTA.

2.1.2. Antibody Pairs

Sandwich (or capture) enzyme-linked immunosorbent assay (ELISA) methods require paired antibodies derived from different host species, both of which recognize the TGF-β1 target. Specific monoclonal antibodies such as mouse monoclonal IgG1 (Code 80-1835-03, Genzyme Diagnostics, Cambridge, MA) or Mouse IgG1 MAB240 (R and D Systems Minneapolis, MN) are useful for coating plates to capture the growth factor from body fluids. Subsequently, a polyclonal antibody such as rabbit pan-specific anti-TGF-β (AB-100-NA, R and D Systems) or chicken IgY anti-TGF-β1 (AF-101-NA; R and D Systems) can be utilized as the detecting reagent.

2.2. Materials for Detecting TGF-β1 by Immunohistochemistry

2.2.1. Tissue Preparation

Immune localization of TGF-β1 can be performed on either freshly frozen tissues embedded in TBS Tissue Freezing Medium (Fisher Scientific, Pitts-

burgh, PA) and sectioned with a cryostat (10 μm). Our laboratory has instead preferred to study formalin-fixed, paraffin-embedded tissue sections. These specimens provide consistent outstanding morphological preservation of cellular structure. In addition, paraffin-embedded tissues have the advantage of evaluating TGF-β1 tissue deposition in archived pathological specimens. The procedures described herein will be in reference to paraffin-embedded tissue samples.

1. Tissue biopsies are obtained from diseased and normal subjects and immediately fixed in 10%-phosphate-buffered formalin and embedded in paraffin blocks.
2. Serial 5-μm sections are rendered from the paraffin blocks using a standard microtome technique and mounted onto glass microscope slides (Superfrost Plus Slides; Fisher Scientific).

2.2.2. TGF-β1 Antibodies

Tissue localization of TGF-β1 can be evaluated using any of several robust commercially available and investigator-provided antibodies. For instance, we have found good tissue localization of TGF-β1 in formalin-fixed, paraffin-embedded human tissues using AF-101-NA anti-TGF-β1 polyclonal antibody raised in chicken (R and D Systems). In addition, we have evaluated a variety of disease states, using a polyclonal rabbit antibody generated against a synthetic 30 amino acid peptide identical to the amino-terminal sequence of mature TGF-β1 ***(13,21)***. This reagent, referred to as polyclonal antibody A 1/30, was developed and generously provided by Dr. L. Ellingsworth (Celtrix, Inc., Santa Clara, CA). Polyclonal antibody A 1/30 recognizes cells that are actively producing TGF-β1 ***(22–24)***. When selecting an antibody for immunostaining, polyclonal antibodies are preferred over monoclonal antibodies, as they are usually significantly more sensitive.

Phosphate-Buffered Saline (PBS); pH 7.4
- 137 m*M* NaCl
- 4.3 m*M* Na2HPO4
- 1.4 m*M* KH2PO4
- 2.7 m*M* KCl

2.3. Materials for Detecting TGF-β1 by* In Situ *Hybridization

2.3.1. Tissue Preparation

In situ hybridization procedures to localize TGF-β1 mRNA can also be performed on either freshly frozen tissues or on formalin-fixed, paraffin-embedded tissue sections. Again, paraffin embedded tissue contains superior morphological preservation of cellular structure. In addition, rapid fixation of tissues in formalin inactivates endogenous RNAses. Such an approach will

permit TGF-β1 tissue localization in banked pathological specimens. Indeed, we have successfully detected TGF-β1 mRNA signal in tissues stored for over 20 yr ***(10)***.

2.3.2. Hybridization Probes

The choice of probes is at the discretion of the investigator, which include oligonucleotide probes, cDNA probes, and cRNA (riboprobe) reagents. We have employed cRNA riboprobes generated from specific regions of human TGF-β1. Specifically, we utilized a pSP64 plasmid (Promega) containing clone lBC1 for human TGF-β1, obtained from Dr. R. Derynck (University of California San Francisco) ***(25)***. The probe was linearized with Kpn1 and anti-sense RNA prepared with the SP6 polymerase, generating a 325-bp (base pair) probe including nucleotides 950–1274 ***(10)***.

Salt Sodium Citrate (SSC) Solution (20X Stock–1 L)
- 175 g NaCl
- Na_3citrate · $2H_2O$
- Water to 1n L
- Adjust pH to 7.0
- Autoclave and store

Hybridization Solution for ^{35}S-labeled cRNA Probes (final concentrations in water)
- Formamide solution—50% (final concentration)
- 2X SSC solution
- 20 m*M* Tris-HCl, pH 8.0
- 500 mg/mL Torula yeast tRNA
- 1X Denhardt's
- 1 m*M* EDTA
- 10% dextran SO_4
- 100 m*M* dithiothreitol

Denhardt's Solution (100X)
- 10 g Ficoll
- 10 g Polyvinylpyrrolidine
- 10 g bovine serum albumin (BSA) (Fraction V)
- Filter-sterilize (0.2 μm) and store at –20°C

Transcription Buffer (5X Stock)
- 200 m*M*; Tris–HCl buffer, pH 7.5
- 30 m*M* $MgCl_2$
- 10 m*M* Spermidine
- 50 m*M* NaCl

Carbonate Buffer: 0.2 *M*, pH 10.2
80 m*M* $NaHCO_3$
120 m*M* Na_2CO_3
Store at –20°C

Triethanolamine Stock (0.5 *M*)
Triethanolamine free base (67 mL) is added to diethyl pyrocarbonate (DEPC) treated and autoclaved water (800 mL).
The pH is adjusted to 8.0 with concentrated hydrochloric acid, and the volume is increased to a total of 1000 mL.
The stock is stored refrigerated.
For use, the solution is diluted to 0.1 *M* and filter sterilized.

RNAse A Buffer
0.5 *M* NaCl
10 m*M* Tris–HCl buffer; pH 8.0
1 m*M* EDTA

Eosin Y Working Solution
Add 10 g of Eosin Y to 200 mL of water.
Stir to dissolve and add 800 mL of 95% ethanol.
Dilute 1:3 with 80% ethanol and filter prior to use.

Harris Hematoxylin. Solutions obtained commercially (Sigma, St. Louis, MO) and filtered just prior to use.

3. Methods

3.1. Methods for Quantifying TGF-β1 by ELISA

Transforming growth factor-β1 can be detected in body fluids by the application of sandwich (or capture) ELISA assays. ELISA assays detect total immunoreactive TGF-β1. ELISA assays may be performed in parallel with functional TGF-β1 assays such as the mink lung epithelial cell growth-inhibition assay (*see* Chapter 1 ***[20]***) to contrast total TGF-β1 and levels of TGF-β1 activity during disease states. At present, many of the reagents for human TGF-β1 ELISA assays are now commercially available in kit form (e.g., Predicta, TGF-β1 assay; Genzyme, Cambridge, MA). These assays have been used to derive TGF-β1 concentrations in the sera of healthy volunteers and patients with lung fibrosis ***(15,16)***. A general TGF-β1 ELISA follows.

1. Plastic ELISA plates (96-well) are coated with primary antibody–mouse IgG1 MAB240 (R and D Systems) (10 μg/mL) in 0.1 *M* $NaHCO_3$, pH 8.0 overnight at 4°C. The following day, the plates are washed four times with TBS-Tween (TBS-T; 50 m*M* Tris–HCl, 150 m*M* NaCl, 0.05% Tween-20, pH 7.4). The plates are

blocked with TBS containing 0.5% Tween-20 for 2 h at room temperature and subsequently washed with TBS-T four times.

2. Ten microliters of each plasma sample are added to siliconized tubes containing 450 μL of diluent (TBS). The diluted samples are treated with transient acidification in order to activate all latent TGF-β1, thus making it accessible for measurement in the ELISA. This is accomplished by adding 1*N* HCl (20 μL) and incubating at 4°C for 60 min. Subsequently, the samples are neutralized with 1*N* NaOH.
3. The activated samples and diluted standards (rhTGF-β1; 0–5 ng/mL) are added into the appropriate test wells containing immobilized mouse antihuman TGF-β1.
4. The plates are incubated at room temperature for 2 h and are subsequently washed four times with TBS-T.
5. Next, rabbit polyclonal anti-TGF-β antibody (1 μg/mL in TBS-T; AB-100-NA; R and D Systems) is added. The plate is incubated for 1 h and washed four times with TBS-T.
6. Subsequently, goat anti-rabbit polyclonal immunoglobulin horseradish peroxidase conjugate (1:4000 dilution in TBS-T; Southern Biotechnology Associates, Birmingham, AL) is added and incubated for 1 h at room temperature.
7. After washing four times with TBS-T, 40 mg of *o*-phenylene diamine substrate (Sigma) is dissolved in 100 mL of buffer (24.7 mL of 0.1 *M* citric acid, 25.3 mL of 0.2 *M* Na_2HO_4, 50 mL of water, and 40 μL of 30% H_2O_2) and added to the plates (100 μL/well).
8. The substrate is allowed to develop under visual inspection (approx10 min) and readings are obtained, without quenching, at optical density (O.D.) = 450 n*M*.
9. A standard curve is generated and used to derive TGF-β1 in the body fluid samples.

3.2. Methods for Detecting TGF-β1 by Immunohistochemistry

Transforming growth factor-β1 protein can be localized in tissue sections using any one of a number of methods, including immunofluorescence or immunohistochemistry ***(11,14)***. The method described subsequently localizes TGF-β1 by the avidin–biotin complex method on paraffin-embedded tissues. Many of the reagents required are also available in commercial kit form (Vectastain ABC, Vector Labs, Burlingame, CA). The following method has been successfully utilized to localize abnormal TGF-β1 protein deposition in fibrotic lung ***(11,21)***.

1. Paraffin sections (5 μm thick) are heated to 60°C and deparaffinized by soaking in three exchanges of xylene (30 min each).
2. The sections are subsequently rehydrated through a graded series of alcohol washes (100% ethanol, 100%, 95%, 70%, 50%, and 30% ethanol, deionized water, and deionized water again; 20 min for each exchange).
3. The deparaffinized tissue sections are digested with hyaluronidase (1 mg/mL in 0.1 *M* sodium acetate and 150 m *M* sodium chloride, pH 5.5) for 30 min at 37°C to expose antigenic epitopes.

4. After washing the sections three times for 10 min each in phosphate-buffered saline (PBS), the sections are incubated for 30 min in methanol containing 0.3% hydrogen peroxide to quench endogenous peroxidase activity.
5. Wash the sections three times for 10 min each in PBS, then incubate with 1.5% normal goat serum to reduce nonspecific binding of antibodies.
6. After washing again, the sections are next incubated with primary polyclonal antibody A 1/30 (10 μg/mL) for 4 h at room temperature.
7. The sections are rinsed, as described earlier, and subsequently incubated with biotinylated goat anti-rabbit antibody (2 μg/mL, Dako Corporation; Carpinteria, CA) for 30 min at room temperature.
8. Next, the sections are washed three times and treated with peroxidase-conjugated streptavidin (2 μg/mL, Dako) for 30 min at room temperature.
9. Following three 10 min washes in PBS, bound antibodies are demonstrated using 3-amino-9-ethylcarbazole substrate (AEC substrate, Dako) in the presence of 3% hydrogen peroxide for 15 min.
10. Following substrate development, the sections are rinsed and counterstained by soaking for 1 min in Smith's hematoxylin stain (Sigma).
11. The samples may be randomized, coded, examined by a blinded observer, and scored in a semiquantitative manner ***(11)***.

3.3. Methods for Localizing TGF-β1 RNA Expression Using *In Situ* Hybridization

Tissue localization of TGF-β1 message can be accomplished through several methods using cRNA or DNA probes. Similarly, probe detection can be accomplished through autoradiography or by biotin or digoxigenin detection. The following method details a robust cRNA-based *in situ* hybridization strategy which has been successfully employed to detect TGF-β1 mRNA in formalin-fixed, paraffin-embedded tissues sections. Because this is a mRNA detection technique, care must be exercised to prevent contamination by exogenous RNAses. Gloves and RNAse-free plasticware or oven-baked glassware must be used throughout the procedure.

3.3.1. Glass Slide and Cover-Slip Preparation ("Subbing")

For optimal retention of tissue sections, the slides should be pretreated as follows. All aqueous solutions should be made with diethyl pyrocarbonate DEPC-treated water, which has been subsequently autoclaved to render it free of RNAse.

1. Glass slides (Superfrost Plus, Fisher Scientific) are successively dipped in 10% HCl in 70% ethanol, distilled water, and then 95% ethanol.
2. Dry the slides in a 150°C dry oven for 5 min.
3. Dip slides in 2% 3-aminopropyltriethoxysilane (Sigma) in acetone for 10 s, then wash twice in acetone, and three times in water.

4. Dry the slides overnight at 42°C.
5. Siliconize cover slips by dipping in Sigmacote (Sigma) for 10 s, then dip twice in 95% ethanol. Wipe clean with a fresh Kimwipe, then autoclave.

3.3.2. Tissue Sectioning and Deparaffinization

Formalin-fixed, paraffin-embedded tissues from both normal controls and disease states should be studied in parallel.

1. Sections (5 µm thick) are cut using a standard microtome and are floated on a sterile waterbath at 42°C.
2. The sections are lifted onto the subbed slides and dried at 42°C for 2 h. They may then be stored dry at 4°C. A few slides should be lifted onto unsubbed slides and stained with standard Hematoxylin & Eosin to assess tissue preservation and morphology.
3. Slides are heated at 60°C for 2 h in Coplin jars placed in a water bath.
4. Deparaffinize the slides by three exchanges of xylene at 60°C for 30 min each.
5. Dehydrate the sections with two 5 min exchanges of absolute ethanol.
6. The sections are subsequently rehydrated through a series of graded ethanol solutions (95%, 95%, 70%, 50%, and 30% made with RNAse-free, DEPC-treated, autoclaved water). Each exchange should last about 1 min.

3.3.3. Tissue Pretreatments

The purpose of these pretreatments is to render the sections more penetrable by the probes during the subsequent hybridization steps.

1. Slides are rinsed in PBS for 5 min.
2. The sections are then digested with nuclease-free Proteinase-K (1 mg/mL) in PBS at 37°C for 30 min.
3. Place the sections in 0.1 *M* triethanolamine buffer for 5 min.
4. Treat the section in freshly prepared 0.25% acetic anhydride in 0.1 *M* triethanolamine buffer for 10 min.
5. The slides are washed in two 5-min exchanges of 2X SSC solution. Next, the sections are dehydrated through a series of graded ethanol solutions (PBS, 30% ethanol, 50%, 70%, 95%, 100%, 100% ethanol again, xylene, and xylene again, for 1–2 min for each exchange). If storage is necessary during preparation of the probe, the slides may be stored in 95% ethanol at 4°C for up to several days. Finally, the samples are air-dried just prior to hybridization.

3.3.4. Probe Generation and Labeling.

Several approaches are available for TGF-β1 probe generation and labeling. We have utilized cRNA riboprobe strategy, with probes being generated from the lBC1 clone for human TGF-β1 in pSP64 (Promega, Inc., Madison, WI), described by Derynck and colleagues ***(25)***. The probe can be linearized by restriction endonuclease digestion with *Kpn*I, yielding a template of 325 bp representing nucleotides 950–1274 of TGF-β1. This template can be used to

generate antisense RNA probes via the SP6 polymerase ***(10)***. A general method of obtaining ^{35}S-UTP-labeled cRNA riboprobes is provided. Many of the reagents for riboprobe labeling are available in kit form through commercial vendors (e.g., Riboprobe System II; Promega).

1. Dry 15 µL of ^{35}S-UTP (1250 Ci/mmol, concentration 5 m *M*) in a speed vacuum concentrator over 1 h.
2. Add the following reagents to the dried ^{35}S-UTP totaling 10 µL:

RNAse-free (DEPC-treated and autoclaved) water	3.1 µL
100 m *M* dithiothreitol	2.0 µL
Unlabeled nucleotide mixture	2.0 µL
The cold nucleotide mixture consists of 250 µL each of 10 m*M* ATP, 10 m*M* GTP, 10 m*M* citadine triphosphate (CTP) combined with 250 µL of DEPC-treated water.	
5X transcription buffer	2.0 µL
RNAse inhibitor (60 U/µL)	0.4 µL

3. The reaction mixture is next incubated at either 37°C for T3 or T7 polymerases or at 40°C for SP6 polymerase.
4. After the first hour, an additional 0.5 µL of polymerase is added to the reaction solution and the reaction is again incubated for an additional hour at the same temperature as previously.
5. Remove 1 µL of the solution and dilute 1:10 in DEPC water. Reserve 1 µL of the diluted reaction mixture for determining total counts and 1 µL of the diluted reaction mixture is trichloroacetic acid precipitated and counted. The remaining 8 µL is returned to the general reaction mixture.
6. The following reagents are added to the reaction mixture:

   Magnesium chloride (0.5 *M*; 1.4 µL)
   Yeast tRNA (10 mg/mL; 5 µL)
   RNAse inhibitor (0.5 µL)
   DNAse I (RNAse free) (1.5 µL)
   DEPC-treated water (73.6 µL)

7. The reaction is incubated for an additional 15 min at 37°C.
8. Next, a volume of a 1:1 phenol–chloroform mixture equaling the reaction mixture is added and shaken vigorously for 5 min.
9. The mixture is centrifuged at 500 *g* for 5 min.
10. Centrifuge the extraction mixture at room temperature under 5000 *g* for 5 min.
11. The aqueous phase is collected from each of the two supernatants and combined. This generally totals approximately 175 µL.
12. An equal volume of chloroform is added to the solution and the extraction mixture is shaken vigorously for several minutes.
13. The aqueous phase is collected and an equal volume of ammonium acetate solution is added (4 *M*, 175 µL), cold absolute ethanol (1050 µL) is also added, and

the solution is mixed well. The nucleic acid is allowed to precipitate from the solution in a dry ice/ethanol bath for 15 min and for an additional hour at –70°C.

14. The suspension is allowed to reequilibrate to room temperature and then centrifuged at approx 14,000 *g* for 10 min.
15. The supernatant is gentle removed from the pellet and the pellet is carefully washed with absolute ethanol.
16. The pellet is air-dried.
17. The recovered pellet is dissolved in 175 μL of DEPC-treated water.
18. Repeat the chloroform extraction and alcohol precipitation steps listed in steps **11–16** for a second time.
19. The final pellet is resuspended in 100 μL of DEPC-treated autoclaved water containing 20 m *M* dithiothreitol
20. Count a 1-μL aliquot of the final labeled probe. A trichloroacetic acid precipitation to determine percentage of label incorporation and hence the specific activity can be calculated at this point using standard methods. However, in general this has not been necessary, as we have used the total cpm (counts per minute) as a guide to determine probe concentrations in the next step of hybridization.

3.4.5. Hybridization and Washing

Following successful probe labeling, the next process will include hybridization to the prepared tissue sections and washing.

1. Hybridization solution is added directly to the air-dried sections to minimize dilution effects. Plan on using approximately 3 or 4 μL of hybridization solution for each square centimeter of cover-slip area. This equates to 20 μL for standard 22 × 22-mm cover slips, and about 30 μL for 24 × 30-mm cover slips.
2. Using the above probe-labeling protocol, the specific activity of the probes is generally in the range of 4–5 dpm/μg (disintegrations per minute per μg) of probe. Under these conditions, the probe is generally mixed in the hybridization solution aiming to use approximately 0.5–1.0 × 10^6 cpm per hybridization under a 22 × 22 mm-cover slip.
3. The hybridization solution containing the radiolabeled TGF-β1 probe is applied to the previously siliconized cover slip and this is gently laid over the section. The hybridization solution is allowed to slowly spread over the tissue section, dissipating all bubbles from the field in the process. The cover slip is then sealed in position using rubber cement (diluted 1:1 with petroleum ether) extruded from a syringe.
4. The slides are placed horizontally in a humid chamber and incubated overnight at 50–55°C, based on an anticipated cRNA probe length of 150–200 nucleotides. This represents a T*i* (temperature of incubation) of approximately T*m* –30°C.
5. After overnight hybridization, the rubber cement is stripped away and the coverslips are removed by soaking in 4X SSC solution containing freshly instilled β-mercaptoethanol (25 m *M*). Next, the slides are washed twice in 4X SSC solution for 15 min each at room temperature.

6. The slides are next washed for 5 min in 0.5X SSC solution containing freshly added β-mercaptoethanol (25 m*M*) at room temperature.
7. The sections are soaked for 20 min in 0.1X SSC containing 25 m*M* β-mercaptoethanol at 60°C.
8. The slides are equilibrated in RNAse A buffer at 37°C for 1 min. Next, nonhybridized probe is digested away from the sample using RNAse A (20 μg/mL) for 30 min at 37°C. The samples are then soaked for an additional 30 min in RNAse A buffer alone at 37°C.
9. Subsequently, the sections are washed in 2X SSC solution at room temperature for 15 min.
10. The slides are washed once in 0.1X SSC for 20 min at 60°C, and once in 0.1X SSC maintained at room temperature for 30 min.
11. The sections are dehydrated by 1 minute exchanges through a graded series of alcohol solutions as described earlier, and allowed to air-dry before proceeding to coat the slide with emulsion, as described subsequently.

3.4.6. Autoradiography

For best results, it is essential that the autoradiography procedure be performed in a light-tight darkroom, from which one can exit without breaching the darkness. The darkroom should be equipped with a safelight composed of a 15-w bulb and a No. 2 Wratten filter. You will also require a water bath maintained at 42°C, and small light tight slide boxes with tight-fitting lids. For light security, these are usually placed within a second light-tight outer box and stored in a refrigerated light-tight dessicator during the subsequent autoradiographic exposure period.

1. The NTB emulsion (Kodak., Rochester, NY) is melted over 30 min in the 42°C water bath and diluted 1:1 in 42°C distilled water. Mix the emulsion carefully by inversion and return to the water bath for 30 min to allow bubbles to dissipate.
2. Test the emulsion by dipping several blank slides and examining them under the safelight to be certain that the emulsion is free of any bubbles.
3. Next, the tissue slides are coated with emulsion by immersing them twice slowly at a constant rate to obtain an even coating of emulsion. (They should be submerged in emulsion twice for 2 or 3 s each time.)
4. The slides are drained for several seconds by holding them vertically and blotting the edge on a paper towel. The slides are then allowed to dry vertically using a large test-tube rack
5. Allow the slides to air-dry undisturbed for at least 30 min to allow the emulsion to be firmly affixed to the slide
6. Next, the rack of slides is placed in a humid chamber such as a plastic Tupperware container inlaid with dampened paper towel. The sections are maintained in the darkroom in this humid chamber for 45 min. This step promotes fading of latent images and helps reduce background development of the emulsion.

7. The sections are removed from the humid chamber and again allowed to air-dry for at least 45 min in the dark. Avoid vacuum or heat-drying, as this will introduce artifacts into the emulsion.
8. Transfer the air-dried slides to black, plastic, tight-fitting, light-tight slide boxes. Include a small bit of desiccant, such as calcium chloride wrapped in a tissue, into each box. Enclose in a second light-tight outer box, also containing desiccant, and place in a light-tight dessicator at 4°C for exposure.

3.4.7. Development

After appropriate autoradiographic exposure times (1–4 wk), the slides are removed from the dessicator and permitted to reequilibrate to room temperature. This avoids condensation of water on the slides, which may adversely effect the quality of the signal. All developing solutions need to be equilibrated to an identical temperature (approx15°C cool water bath) for optimal results.

1. Place the slide in D19 Developer solution (Kodak; diluted 1:2 in distilled water) for 3 min.
2. Stop the photographic development by placing the sections in 1% acetic acid (prepared in distilled water) for 1 min.
3. Fix the slides by a 5-min exchange in rapid-fix solution (Kodak; diluted 1:4 in distilled water).
4. The slides are finally washed in three 10-min exchanges of distilled water.

3.4.8. Counterstaining

Counterstaining is necessary for morphologic identification of the cells expressing TGF-β1 message and for detecting normal and diseased regions of tissue. Counterstaining with a light Hematoxylin and Eosin procedure provides good morphological differentiation.

1. The developed samples are rehydrated by soaking in distilled water containing 0.5% acetic acid for 30 sec.
2. Harris hematoxylin (Sigma) is freshly filtered and used to stain the sections over 5 min.
3. The sections are washed in gently running tap water for at least 2 min.
4. Next, the samples are destained by dipping twice in 70% ethanol containing 0.5% hydrochloric acid.
5. The sections are washed in running tap water for an additional 2 min.
6. The slides are placed in 95% ethanol containing 0.5% acetic acid for a 10 s period.
7. The sections are further counterstained in working Eosin Y solution for 30 s.
8. The slides are dehydrated by a 5-min exchange in absolute ethanol.
9. The sections are next soaked in fresh xylene for 5 min.
10. While wet with xylene, fresh clean cover slips are mounted onto the sections using a xylene-based adhesive (Permount, Fisher Scientific) and the slides are permitted to air-dry for 2 h.
11. Finally, the excess emulsion is removed from the *back* of the slide using a household kitchen cleaner (e.g., Formula 409 Cleaner).

4. Notes

4.1. Notes for Quantifying TGF-β1 in Body Fluids by ELISA

1. Transforming growth factor-β1 is extremely sticky and easily lost on plastic and glass surfaces. Accordingly, all pipet tips, microcentrifuge tubes, and other laboratory vessels should be siliconized with a good commercial reagent prior to use (Sigmacote, Sigma).
2. Either plasma or serum can be used. Plasma, however, is preferred, in that serum involves clotting and centrifugation of the sample, which may cause some additional release of TGF-β1 from contained platelets. Therefore, if serum is employed, concomitant normal controls must be performed identically in parallel. The clotting process used during the generation of serum may lead to underestimation of differences between disease states and normal controls.
3. Most protocols for capture ELISA require preactivation of the samples with transient acidification. This is necessary for optimal recognition of the growth factor by the capturing antibody. As such, these procedures detect all immunoreactive TGF-β1, including fractions that were both active and inactive prior to acidification. Samples may be studied in parallel using a functional assay, such as the mink lung epithelial cell growth-inhibition assay, described in Chapter 1.
4. Body fluid samples are best assayed in triplicate.

4.2. Notes for Detecting TGF-β1 by Immunohistochemistry

1. Warming the xylene to 60°C in a water bath improves the efficiency of paraffin removal from the tissue sections.
2. Minimal antibody and other reagents can be used by using a hydrophobic marking pen (PAP Pen; Research Products International, Mt. Prospect, IL) to delineate a small zone around the tissue section. The antibody solutions are held in this zone by surface tension when incubated flat in a humidified chamber.
3. To confirm the specificity of tissue staining, semiserial sections should be stained in an identical fashion, substituting nonimmune rabbit immunoglobulins for the primary antibodies at identical dilutions.
4. In situations where the expression of TGF-β1 protein is difficult to detect, the primary antibody incubation may be extended overnight at 4°C for enhanced sensitivity.
5. The substrate development times listed are approximate and may vary with the application. Substrate development should be directly observed. In the case of the AEC substrate, the tissue assumes a light to moderate reddish-brown color. For validity in grading the relative TGF-β1 tissue deposition, it is essential that diseased and nondiseased tissues and immune and nonimmune control incubations be developed in parallel using the same batch of substrate, for an identical length of time.
6. AEC and other substrates may be dissolved by alcohols, xylene, and other organic solvents. Therefore, the final washes must be performed with water-based solutions only.
7. Similarly, xylene based cover-slip mounting media may also eliminate the substrate signal. Therefore, an aqueous-based mounting media is preferred (Biomeda Crystal/Mount Mounting Media, Fisher Scientific).

8. As an alternative, counterstaining with Methylene Green (1% in PBS) gives better contrast between peroxidase signal. However, Methylene Green provides less structural details for morphologic identification of the cells stained in the tissue section.

4.3. Notes for Localizing TGF-β1 RNA Expression Using In Situ *Hybridization*

1. The removal of paraffin is enhanced both by warming the xylene, and by frequent agitation of the sections in the xylene solution.
2. In general, probes are generated as pairs of antisense (binding) and sense (control) strands. Hybridizations performed on parallel sections with these pairs will help to ascertain the level of probe-binding background.
3. Additional parallel control sections can be included in which the contained RNA is digested away with RNAseA prior to the hybridization. If desired, this should be performed immediately prior to the Protease K digestion step. To accomplish this, the section are washed briefly in RNAse A buffer and subsequently incubated with RNAse A (50 mg/μL) for one hour at 37°C. Subsequently, these sections are rinsed at least three times before the Protease K digestion. The operator must be scrupulous to avoid contamination of the other sample sections with the RNAse prior to hybridization.
4. It is helpful to set up parallel hybridization in triplicate. The first set should be developed after roughly 4–7 d of exposure. This gives two more development time-points. Most TGF-β1 signals are usually detectable after 2 or 3 wk. However, strong signal development may require up to 1 m to 6 wk of exposure time.
5. Loss of tissue for the glass slides is a common problem during *in situ* hybridization procedures. This is improved by proper "subbing" of the slides, as described earlier. In addition, certain commercially treated slides (e.g., Superfrost Plus Slides) seem to have less associated tissue loss. As an additional step, briefly baking the sections for 30 min at 60°C, after air-drying and just prior to the hybridization step, helps assure firm adherence of the tissue.
6. Do not use ammonia water to "blue" the Hematoxylin solution. It will remove the emulsion deposits on the slide, rendering them worthless.

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5

Analysis of TGF-β-Mediated Synthesis of Extracellular Matrix Components

Barbara A. Hocevar and Philip H. Howe

1. Introduction

Coordinated regulation of production and turnover of extracellular matrix (ECM) components is essential for normal tissue homeostasis. The composition of the extracellular matrix can influence cell growth, state of differentiation, and specific gene induction. The multifunctional cytokine transforming growth factor-β (TGF-β) exerts its effects on cell proliferation, differentiation, and migration in part through its modulation of extracellular matrix components. TGF-β promotes net matrix deposition by increasing the expression of specific ECM components such as fibronectin (FN) and collagen, upregulating the expression of inhibitors of ECM proteases, such as plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitors of matrix metalloproteinases (TIMPs), while simultaneously suppressing the synthesis of proteases, which degrade matrix components, such as interstitial collagenase (reviewed in **refs. *1*** and ***2***). TGF-β also induces the expression of cell surface receptors for the ECM, the integrins, which enhance cell–matrix interactions ***(1,2)***.

Fibronectin, the most widely distributed component of the ECM, exists as two forms; plasma FN, a soluble dimeric form secreted by hepatocytes into the bloodstream, and cellular FN, a dimeric, crosslinked form deposited to the ECM, which is synthesized by virtually every cell type ***(3,4)***. Fibronectins exhibit alternatively spliced forms which are expressed during different developmental stages and pathological conditions. TGF-β, which is released by degranulating platelets at a wound site, has been shown to potently induce FN expression, both at the mRNA and protein levels ***(5,6)***.

One of the most dramatic increases in gene transcription and protein expression following TGF-β treatment is seen with PAI-1. PAI-1 is a rapid and spe-

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cific inhibitor of tissue-type and urokinase-type plasminogen activators and functions as the primary inhibitor of plasminogen activation in vivo ***(7)***. A new role for PAI-1 in the regulation of cellular adhesion, however, has recently emerged with the finding that PAI-1 binds to vitronectin, thereby preventing its association with both the urokinase-type plasminogen activator (uPA) receptor and integrin receptors ***(8,9)***. This leads to a net inhibition of cellular migration, which is independent of the role of PAI-1 as a plasminogen activator inhibitor ***(8,9)***. TGF-β has been shown to modulate PAI-1 synthesis at both the transcriptional and posttranscriptional levels ***(10)***.

In contrast to the well-documented effects of TGFβ on matrix deposition, less is known about the role of TGF-β in matrix breakdown. Matrix breakdown, mediated by the matrix metalloproteinase (MMP) family of proteolytic enzymes, is an essential process that allows for cell migration during development as well as for cell migration that occurs during normal tissue repair ***(11)***. The MMP family of enzymes consists of collagenases, such as interstitial collagenase or MMP1, gelatinases, MMP2 and MMP9, stromelysins, MMP3, and the membrane-type MMPs. Many of the effects of TGF-β on MMP expression appear to be cell-type dependent such that MMP1 expression is induced by TGF-β in keratinocytes ***(12)***, whereas MMP1 and MMP3 are suppressed by TGF-β in fibroblasts ***(13)***. Treatment of fibroblasts, keratinocytes, and HT1080 cells with TGF-β has been shown to increase MMP2 expression ***(14)***, whereas MMP9 expression was shown to be induced by TGF-β in keratinocytes ***(15)***. Aberrant expression of the MMP family of enzymes is observed in many pathological conditions such as rheumatoid arthritis, atherosclerosis, and tumor invasion and metastasis. As a result of the proposed role of TGF-β in promoting or inhibiting these disease states, a further examination of the effects of TGF-β on the MMP family of enzymes seems warranted.

This chapter describes methods to analyze the effects of TGF-β on the regulation of fibronectin, PAI-1, and MMP synthesis. Emphasis will be placed on methods that detect net protein synthesis, which reflects not only increased TGF-β-stimulated gene transcription but also TGF-β-regulated message stabilization, utilizing common reagents widely available to many researchers.

2. Materials

2.1. Fibronectin Assay

1. Methionine-free media (Gibco-BRL, Gaithersburg, MD).
2. TGFβ2 (Genzyme, Boston, MA).
3. [^{35}S]-Methionine, EXPRESS Methionine/cysteine protein labeling mix (NEN, NEG072).
4. Anti-rat fibronectin polyclonal antibody or anti-human fibronectin monoclonal antibody, clone 3E1 (Gibco-BRL).

5. Protein A–Sepharose or Protein G–Sepharose (Gibco-BRL).
6. Wash buffer: 50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 1% NP-40.
7. 2X Laemmli sample buffer: 120 m*M* Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 200 m*M* DTT, 0.01% bromophenol blue.
8. PPO (Sigma, St. Louis, MO) solution: 20 g dissolved in 100 mL glacial acetic acid.

2.2. PAI-1 Assay

1. Methionine-free media (Gibco-BRL).
2. TGFβ2 (Genzyme).
3. [^{35}S]-methionine, EXPRESS Methionine/cysteine protein labeling mix (NEN® Life Science Products, Boston, MA, NEG072).
4. 1X PBS (Sigma)
5. Extraction buffer: 10 m*M* Tris-HCl, pH 8.0, 0.5% deoxycholate, 1 m*M* phenylmethylsulfonyl fluoride (add just before using).
6. Wash buffer: 2 m*M* Tris, pH 8.0.
7. 2X Laemmli sample buffer: 120 m*M* Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 m*M* DTT, 0.01% bromophenol blue.
8. PPO (Sigma) solution: 20 g dissolved in 100 mL glacial acetic acid.

2.3. Gel Zymography

1. TGFβ2 (Genzyme).
2. 12-*O*-Tetradecanoylphorbol-13-acetate, TPA (Gibco-BRL).
3. Gelatin–agarose (Sigma).
4. NP-40 wash buffer: 50 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 1% NP-40.
5. Centricon 30 (Millipore Corp., Bedford, MA), optional.
6. 2X nonreducing Laemmli sample buffer: 120 m*M* Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% Bromophenol Blue.
7. Gelatin, type B from bovine skin (Sigma).
8. Triton X-100 buffer: 2.5% Triton X-100 (v/v).
9. Enzyme assay buffer: 50 m*M* Tris-HCl, pH 7.5, 200 m*M* NaCl, 20 mM $CaCl_2$.
10. Coomassie stain: Coomassie Brilliant Blue R-250 0.5% (w/v) in 45% methanol, 10% glacial acetic acid.
11. Destain: 45% methanol, 10% glacial acetic acid.

2.4. Quantitation of MMP Expression

2.4.1. Western Analysis

1. TGFβ2 (Genzyme).
2. 12-*O*-Tetradecanoylphorbol-13-acetate, TPA (Gibco-BRL).
3. Immobilon-P polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Bedford, MA).
4. Antibodies to human MMPs: anti-MMP1, anti-MMP2, anti-MMP9, supplied as 100 μg lyophilized, reconstituted with 1 mL sterile PBS (Oncogene Research Products, Cambridge, MA).

5. Rabbit anti-mouse IgG horseradish peroxidase (HRP) conjugate secondary antibody (Accurate, Chemical and Scientific, Westbury, NY).
6. Enhanced chemiluminescence reagent, Renaissance (NEN).

2.4.2. ELISA Analysis

1. Biotrak ELISA system for analysis of individual MMPs (Amersham, Arlington Heights, IL)
2. 1.0 *M* sulfuric acid.
3. Spectrophotomer plate reader capable of measuring 630 or 450 nm.

3. Methods

3.1. Analysis of TGF-β-Stimulated Fibronectin Synthesis

Fibronectin plays an important role during development and wound healing by promoting cell adhesion, migration, and cytoskeletal organization ***(3,4)***. Overproduction of matrix components including fibronectin is the main pathological finding in tissue fibrosis ***(16)***, whereas decreased fibronectin production is often observed following oncogenic transformation, leading to decreased adhesion and increased metastatic potential ***(3)***. The assay described here provides a simple method to assay for induction of fibronectin synthesis following TGF-β treatment. Further studies can then be conducted to demonstrate regulation at the mRNA or transcriptional level.

1. Plate 4×10^5 cells in a 60 mm plate in complete media (including serum).
2. The following day remove the media and replace with fresh complete media in the absence or presence of TGF-β (2 ng/mL) and continue incubation for 16–18 h (*see* **Note 1**).
3. Aspirate media and wash plates with 2 mL of methionine-free media. Replace with 1.5 mL of methionine-free media supplemented with or without TGF-β (2 ng/mL) and 50 μCi/mL of [^{35}S]-methionine. Continue the incubation for an additional 4–6 h.
4. Remove the media from each plate and place into 1.6-mL microfuge tubes. Add 5 μL of rat polyclonal anti-fibronectin antibody or 1 μL monoclonal anti-human fibronectin antibody to each tube and 40 μL of either Protein A– or Protein G–Sepharose for polyclonal or monoclonal antibodies, respectively. Rock the samples overnight at 4°C.
5. Spin the tubes at 14,000*g* for 20 s in a microfuge to pellet the Sepharose beads. Remove the media and wash the pellets three times with wash buffer. Resuspend the final pellet in 50 μL of 2X Laemmli sample buffer. Vortex the sample and heat for 3 min at 100°C. Vortex the samples again and spin down in the microfuge. Load the supernatant on 6% SDS-PAGE polyacrylamide gel electrophoresis gels and electrophorese at 180 V overnight.
6. Gels are fixed in 50% methanol and 10% glacial acetic acid for 30 min. The gel can then be dried and subjected to autoradiography or, alternatively, can be subjected to fluorography (**step 7**) to intensify the signal (*see* **Note 2**).

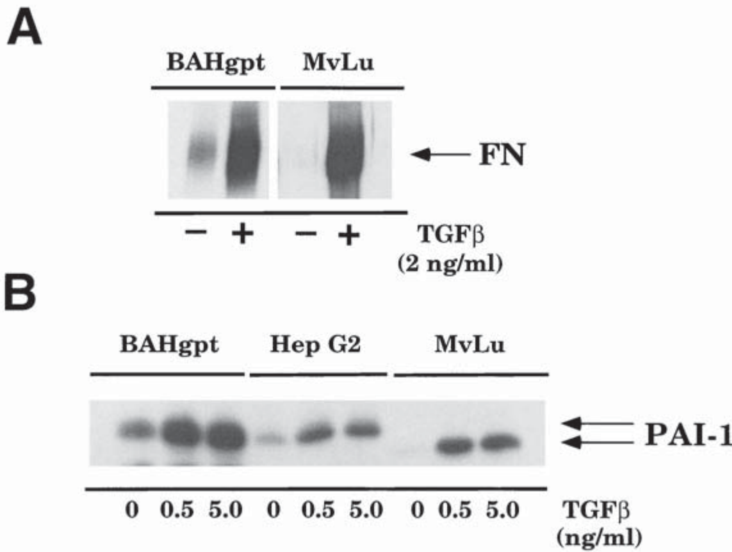

Fig. 1. Induction of FN and PAI-1 protein expression following TGFβ stimulation. **(A)** Fibronectin induction following TFG-β stimulation was assayed in BAHgpt and MvLu cells by immunoprecipitation of [^{35}S]-labeled fibronectin from the conditioned media using a polyclonal antibody to rat fibronectin. Immunocomplexes were resolved by electrophoresis in a 6% SDS-PAGE gel, subjected to fluorography and visualized by autoradiography. The position of the 240-kDa fibronectin polypeptide is shown by the arrow. **(B)** PAI-1 induction following stimulation with the indicated doses of TGF-β was assayed in BAH gpt, Hep G2, and MvLu cells. Extracellular matrices prepared from [^{35}S]-methionine-labeled cells were extracted in 2X Laemmli sample buffer and resolved on 8% SDS-PAGE gels, subjected to fluorography and visualized by autoradiography. The position of PAI-1 is indicated by the arrows, where PAI-1 is expressed as a 50-kDa protein in human cells and a 45-kDa protein in MvLu cells.

7. Following fixation, gels are placed in glacial acetic acid for 5 min. The acetic acid is removed and gels are placed in glacial acetic acid containing PPO for 45 min. Gels are washed with H_2O, dried, and subjected to autoradiography. An example of TGF-β-mediated FN induction is shown in **Fig. 1A**.

3.2. Analysis of TGF-β-Stimulated PAI-1 Synthesis

The PAI-1 gene is rapidly and potently induced following treatment with TGF-β *(10)*. Because regulation of PAI-1 occurs primarily at the level of transcription, constructs containing the PAI-1 promoter are commonly utilized in transient transfection assays to assess overall cellular TGF-β responsiveness. The assay described here, however, provides an easy assay to evaluate TGF-β-stimulated PAI-1 induction without relying on transient transfection and the availability of PAI-1 promoter constructs.

1. Plate 2×10^5 cells into each well of a six-well plate (or 35 mm dish) in 2 mL of complete media.
2. The following day, aspirate off the media, wash each well with 2 mL methionine-free media, and replace with 1 mL of methionine-free media. At this time, add TGF-β to the wells. Incubate for 2 h at 37°C.
3. After the 2 h, supplement the media with 50 μCi/mL of [^{35}S]-methionine. Incubate for 2 h at 37°C.
4. At this time, place the plates on ice. Perform the following washes, each with 2 mL/well of the various solutions: 1X with PBS, 3X with Extraction buffer (*see* **Note 3**), 2X with wash buffer, and 1X with PBS.
5. Add 100 μL of 2X Laemmli sample buffer to each well. Scrape each well using a rubber policeman into a microfuge tube. Heat tubes for 3 min at 100°C and load on 8% SDS/PAGE gels.
6. Gels are fixed in 50% methanol and 10% glacial acetic acid for 30 min. The gel can then be dried and subjected to autoradiography or, alternatively, can be subjected to fluorography (**step 7**) to intensify the signal (*see* **Note 2**).
7. Following fixation, gels are placed in glacial acetic acid for 5 min. The acetic acid is removed and gels are placed in glacial acetic acid containing PPO for 45 min. Gels are washed with H_2O, dried, and subjected to autoradiography. An example of TGF-β-mediated PAI-1 induction in various cell types is shown in **Fig. 1B**. PAI-1 is identified by its molecular weight of 45–50 kDa and is the major protein deposited to the ECM following TGF-β treatment.

3.3. Analysis of MMP Activity by Gel Zymography

Although the MMP family of enzymes plays a key role in normal development, aberrant expression and activation have been shown to contribute to various pathological states, including tumor invasion and metastasis. The activity of the MMP family of enzymes is tightly controlled at three levels: gene transcription, activation of latent enzyme, and inhibition by TIMPs. The expression and activation of MMP enzymes can be evaluated by the method of gelatin zymography, which takes advantage of the fact that many of the MMPs can utilize gelatin, or denatured collagen, as a substrate. Gelatin zymography can detect both the inactive or zymogen form as well as the activated form of the MMPs. This method of analysis is easy to perform, however, because the molecular weights of some MMPs overlap (such as MMP1 and MMP8); the presence of a particular MMP should also be confirmed by another method (i.e., Western analysis).

1. Seed cells to be tested in 100 mm dishes at a density of 2×10^7 cells/dish (approximately 70% confluent). After overnight incubation, remove media, wash plates with 5 mL serum-free media, and replace with 4 mL serum-free media with or without TGF-β (5 ng/mL). As a positive control, cells can also be treated with TPA at 40 ng/mL, as TPA has been shown to induce transcription and activation of the MMPs. Incubate cells at 37°C for 16–18 h.

2. The conditioned media is harvested and centrifuged at 500*g* for 5 min to remove cellular debris. The conditioned media can be used neat or concentrated using a Centricon 30 cartridge. Alternatively, for analysis of MMP2 or MMP9, media can be concentrated by incubation of the media with 50 μL of gelatin–agarose equilibrated in wash buffer, which is allowed to rock at 4°C for 1 h. The agarose beads are pelleted at 14,000*g* for 20 s and washed three times with 1 mL of wash buffer.
3. The gelatin–agarose beads that have been resuspended in 50 μL of 2X nonreducing Laemmli sample buffer or 50 μL of conditioned media mixed with 50 μL of 2X nonreducing Laemmli sample buffer are loaded without heating on an 8% SDS-PAGE gel containing 1 mg/mL gelatin (*see* **Note 4**).
4. Following electrophoresis, gels are washed with 200 mL of 2.5% Triton X-100 for 1 h to remove SDS, followed by three washes with H_2O for 5 min each to remove the Triton X-100. The gels are then incubated at 37°C for 18–24 h in enzyme buffer.
5. Gels are stained for 3 h with Coomassie Brilliant Blue and destained. The MMPs are visualized as clear bands where digestion of gelatin has occurred against a dark blue background. The various inactive zymogen MMPs are identified by their molecular weights (i.e., 92 kDa [MMP9] and 72 kDa [MMP2], whereas activated MMPs appear smaller (*see* **Note 5**). An example of gel zymography is shown in **Fig. 2A**.

3.4. Quantitation of MMP Expression

3.4.1. Immunological Detection of MMP Expression by Western Analysis

A semiquantitative analysis of expression of individual MMPs can be performed by standard Western analysis utilizing specific antibodies for each individual MMP. This approach is limited, however, by the availability of suitable antibodies, such that many commercial antibodies are species-specific and recognize only the inactive zymogen form of the MMPs.

1. Collect and harvest conditioned media from cells to be tested as described in **Subheading 3.3., steps 1** and **2**. Again, media can be concentrated, if desired, by Centricon cartridge or incubation with gelatin–agarose as described in **Subheading 3.3., step 2**.
2. The conditioned media (50 μL) or gelatin-agarose pellet is mixed with 50 μL 2X reducing Laemmli sample buffer and heated at 100°C for 3 min. Samples are resolved on 8% SDS-PAGE gels, transferred to Immobilon membrane (Millipore), and subjected to Western analysis using a 1:1000 dilution of the primary antibody. Specific MMPs are visualized by enhanced chemiluminescence (Renaissance). **Figure 2B** shows an example of MMP9 expression in the human fibrosarcoma HT1080-derived BAHgpt cell line ***(17)*** following treatment with TGF-β and TPA.

3.4.2. Quantitative Analysis of MMP Expression by ELISA

Matrix metalloproteinase expression can be accurately quantitated using an ELISA system which is commercially available (Biotrak, Amersham). The ELISA

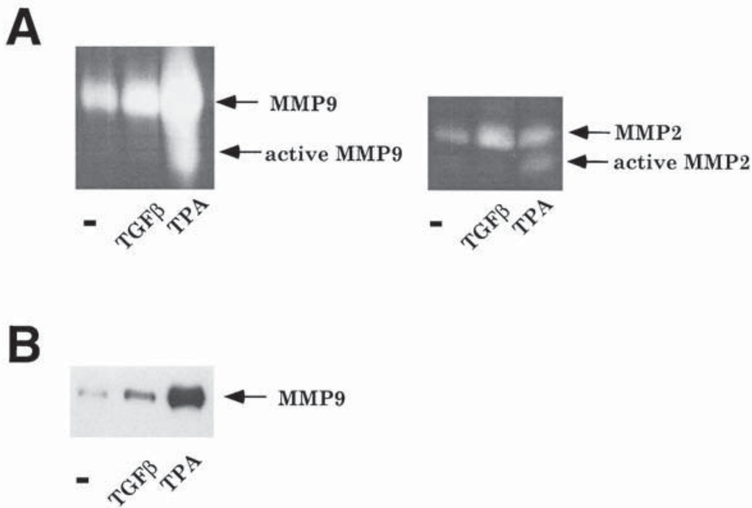

Fig. 2. Analysis of MMP expression in BAHgpt cells. **(A)** Conditioned media (1 mL) collected from BAHgpt cells untreated or stimulated with 5 ng-mL TGFβ or 40 ng/mL TPA was concentrated by incubation with 50-μL gelatin–agarose beads and resolved on a 8% SDS-PAGE gel containing 1 mg/mL gelatin. Following incubation of the gel in enzyme assay buffer, the gel was stained with Coomassie Blue and destained. A zone of clearing in the gel indicates the presence of the various MMPs which are identified by their molecular sizes: 92 kDa for MMP9 and 72 kDa for MMP2 zymogen forms. Note the presence of active MMP9 and MMP2 in the TPA-treated samples, which is indicated by the arrows. **(B)** conditioned media (1 mL) collected from BAHgpt cells untreated or stimulated with 5 ng/mL TGF-β or 40 ng/mL TPA was concentrated by incubation with 50-μL gelatin–agarose beads and was analyzed by denaturing gel electrophoresis on a 8% SDS-PAGE gel. Following transfer of the gel to an Immobilon membrane, MMP9 expression was detected by Western analysis utilizing a monoclonal anti-human MMP9 antibody (Oncogene Research Products), which only recognizes the inactive zymogen form of the enzyme at 92 kDa (indicated by the arrow).

system offers the advantage that it is highly specific and measures total MMP expression, which includes free as well as MMPs complexed to the TIMP proteins.

1. Plate 2×10^5 cells/well in a 24-well plate. Cells are incubated overnight in 1 mL complete media, including serum.
2. The following day, the media is removed, and the cells are washed with serum-free media and replaced with 300 μL serum-free media plus TGF-β (5 ng/mL). Again, TPA (40 ng/mL) treatment can be used as a positive control. After 16–18 h, the media is harvested and centrifuged at 500*g* for 5 min to remove cellular debris.
3. Conditioned media can be used straight, or if expression is very high, it can be diluted using diluent provided in the ELISA kit. The ELISA analysis is then per-

Table 1
Stimulation of MMP Expression in BAHgpt Cells by TGF-β and TPA

	MMP1 (ng/mL)	MMP2 (pg/mL)	MMP9 (ng/mL)
Untreated	12.50	BDL[a]	BDL[a]
TGF-β (5 ng/mL)	19.46	144.07	0.32
TPA (40 ng/mL)	58.63	92.66	39.15

[a]Below detection limits.

Conditioned media (100 μL) from 2×10^5 cells was analyzed by ELISA analysis (Biotrak, Amersham). In the case of TPA treatment, conditioned media was diluted 1:10 prior to analysis.

formed as specified by manufacturer's instructions. **Table 1** displays the MMP expression profile of a cell line derived from HT1080 cells, BAHgpt ***(17)***, following treatment with TGF-β and TPA.

A combination of the above methods may be necessary to determine both the expression and activities of the MMPs. Gel zymography can detect the activity of the MMPs; however, because MMPs bound to TIMPs are catalytically inactive, a spuriously low level of expression may be observed when high levels of TIMPs are present. Conversely, although the ELISA system detects both the free MMP and that bound to TIMPs, it does not provide an activity measurement.

4. Notes

1. Transforming growth factor-β2 is the isoform utilized in these experiments and was found to be equivalent to TGF-β1 in all experiments conducted.
2. A pilot experiment may need to be conducted to determine if fluorography needs to be used. If the expected level of FN or PAI-1 is low, fluorography is suggested to amplify the signal. At high levels of induction, however, fluorography produces signals that fall outside the linear range for autoradiography. Additionally, fluorography cannot be used if quantitation by phosphoimager analysis will be performed.
3. Following the extraction step, plates should be examined under the microscope to determine if any cellular debris remains. One should not be able to see cellular or nuclear outlines. If this is the case, then several additional washes with extraction buffer should be performed. This extra step ensures that only proteins deposited to the ECM are analyzed on the gel, not intracellular proteins.
4. It is important that the level of enzyme loaded onto the gel lies within the linear range for gel zymography. This is easily determined by loading different amounts or dilutions of the conditioned media.
5. Conditioned media can be pretreated with *p*-aminophenylmercuric acetate (APMA) to activate the zymogen form of the MMPs ***(18)***. In this way, the shift in molecular weight from inactive zymogen forms to activated forms of the MMPs is easily followed.

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6

Characterization of SMAD Phosphorylation and SMAD–Receptor Interaction

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1. Introduction

Signaling by transforming growth factor-β superfamily members is initiated when ligand binds to and induces formation of a heteromeric receptor complex composed of type I and type II serine/threonine kinase receptors (reviewed in **refs.** ***1–4***). Upon formation of this complex, receptor II phosphorylates receptor I in a highly conserved juxtamembrane region, known as the GS domain. Phosphorylated receptor I is then activated to phosphorylate its downstream targets, the members of the Smad family of proteins. These Smads are now known to be the critical intracellular mediators of TGF-β superfamily signaling (reviewed in **refs.** ***1–4***).

The Smad nomenclature is derived from a contraction of *sma* and *Mad* (*Mothers against dpp*), genes which encode the canonical family members identified in *Caenorhabditis elegans* and *Drosophila*, respectively ***(5,6)***. In addition to these and other invertebrate family members, 10 distinct Smads have been identified in vertebrates, Smad1–10. Sequence comparisons as well as functional analyses have led to the classification of Smads into three groups: the receptor-regulated Smads (R-Smads), the common Smad (Smad4), and the antagonistic Smads (reviewed in **refs.** ***1–4***). The activated type I receptor directly phosphorylates R-Smads on the last two serines within a conserved carboxy-terminal SSXS motif ***(7,8)***. These R-Smads function in specific pathways and, once phosphorylated, form heteromeric complexes with the common Smad, Smad4. The heteromeric complex then translocates to the nucleus, interacts with DNA binding proteins, and regulates the transcriptional activation of specific target genes. The antagonistic Smads appear to block positive signals, either through stably interacting with type I receptors or by preventing

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formation of R-Smad–Smad4 complexes. In this chapter, we will focus on methodological approaches to the analysis of R-Smad phosphorylation and association with its upstream kinase, the receptor complex.

1.1. Analysis of Smad Phosphorylation by [^{32}P]-Phosphate Labeling

Phosphorylation of R-Smads by receptor I is critical for the propagation of the intracellular portion of the signaling pathway. Prevention of phosphorylation by mutation of the C-terminal serine target sites prevents R-Smad association with Smad4, blocks its nuclear translocation, and abrogates TGF-β superfamily signaling ***(9)***. This phosphorylation is a highly specific event such that Smad2 and Smad3 are targets of TGF-β and activin type I receptors, whereas Smad1, 5, and 8 appear to be targets of the bone morphogenetic protein (BMP) receptors and of ALK1, a receptor for an unknown ligand. The specificity and importance of Smad phosphorylation was first established by [^{32}P]-phosphate labeling of transiently transfected cells. For this method, cells are transfected with epitope-tagged Smad proteins along with constitutively active type I receptors ***(10)*** to induce signaling, or with wild-type receptors as control ***(7,9,11)***. Cells are then labeled with [^{32}P]-phosphate and lysates are subjected to immunoprecipitation using antibodies that recognize the epitope-tagged Smads. [^{32}P]-Phosphate incorporation is then visualized by phosphorimaging or autoradiography. Immunoblotting of aliquots of total cell extracts is used to confirm equivalent Smad protein expression. Recently, highly specific antibodies that recognize Smads have become available; thus, the detection of Smad phosphorylation by ligand addition to cells endogenously expressing Smads and receptors is also possible.

1.2. Analysis of Smad2 Phosphorylation by Anti-phosphoserine Antibody

The detection of Smad phosphorylation using [^{32}P]-phosphate is highly sensitive but requires the use of very high levels of radioactivity (typically 1–5 mCi per experiment). Recently, antibodies that recognize phosphorylation on the last two serines of Smad2 have been developed. These antibodies are highly specific and extremely sensitive, permitting assessment of TGF-β-dependent phosphorylation of endogenously expressed Smad2 by immunoblotting using aliquots of total cell extracts. It is likely that antibodies specific for the phosphorylated forms of other Smad proteins will soon be developed. Here, we provide a description of the immunoblotting procedure used to detect endogenously phosphorylated Smad2.

1.3. In Vitro Kinase Assay

Examination of Smad phosphorylation using in vitro kinase assays has been difficult, primarily the result of the inability to produce active receptors in bac-

teria. To overcome this difficulty, we isolated activated receptor complexes from cells by immunopreciptation ***(7,9)***. This procedure can be used either with cells transiently transfected with receptors or with cells expressing endogenous receptor complexes. To measure phosphorylation in vitro, immunoprecipitated receptor complexes are incubated with bacterially expressed Smad fusion proteins in the presence of [γ-^{32}P]-ATP, and phosphate incorporation is visualized by autoradiography.

1.4. Receptor/Smad Interaction

Receptor-mediated phosphorylation of Smads involves a rapid and transient association of Smads with the receptor complex. Thus, in order to visualize receptor–Smad interactions, it is essential to stabilize this association ***(7,9)***. To accomplish this, we have utilized a kinase-deficient type I receptor that harbors a lysine to arginine mutation. This mutated type I receptor can be phosphorylated and activated by receptor II and can thus interact with Smads. However, because its kinase activity is abolished, the Smad substrate is "trapped" on the receptor. To visualize this interaction, receptors are affinity labeled by incubation of intact transiently transfected cells with [^{125}I]-ligand followed by crosslinking with disuccinimidyl suberate (DSS). Cell lysates are subjected to immunoprecipitation using antibodies against the appropriate Smad protein, and associated receptors are visualized by phosphorimaging or autoradiography. This method of radioactive labeling is extremely sensitive and allows analysis of Smad–receptor interactions not easily achievable by other methods.

2. Materials

2.1. Analysis of Smad Phosphorylation by [^{32}P]-Phosphate Labeling

2.1.1. Transfection of COS-1 Cells

1. Tissue culture medium: High-glucose Dulbecco's modified Eagle medium (HG-DMEM) (Gibco-BRL, Grand Island, NY) containing 10% fetal calf serum (FCS). Store at 4°C.
2. 10 mg/mL diethylaminoethyl-dextran (DEAE/Dex, Sigma D-9885; Sigma, St. Louis, MO) in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS–CaMg). Filter-sterilize using a 0.2-µm membrane and store at 4°C.
3. Nu-Serum™ (Becton Dickinson, Rutherford, NJ). Store aliquots at –20°C.
4. Chloroquine stock: 10 m*M* chloroquine diphosphate salt (Sigma C-6628) in ddH_2O. Filter-sterilize and store 5-mL aliquots at –20°C.
5. Transfection mix: 10% (v/v) NuSerum, 400 µg/mL DEAE/Dex, 0.1 m*M* chloroquine, prepared fresh in HG-DMEM.
6. 10 % (v/v) dimethylsulfoxide (DMSO) in PBS–CaMg. Filter-sterilize and store at 4°C.

7. 100X penicillin–streptomycin (Gibco-BRL 15140-122). Store at 4°C.
8. 500X Fungizone® (Gibco-BRL 15295-017). Resuspend in sterile ddH_2O and store at 4°C.

2.1.2. [^{32}P]-Orthophosphate Labeling

1. Radionuclide: [^{32}P]-orthophosphoric acid at a specific activity of approx 8000–9000 mCi/mmol and at a concentration of approx 10 mCi/mL. Order fresh as required (*see* **Note 1**).
2. Phosphate-free HG-DMEM. Store at 4°C (*see* **Note 2**).
3. Dialyzed FCS (Gibco-BRL, cat. no. 26300-061).

2.1.3. Lysis and Immunoprecipitation

1. 100X pepstatin: 1 mg/mL pepstatin A in DMSO. Store in 0.2 to1-mL aliquots at –20°C (*see* **Note 3**).
2. 100X phenylmethylsulfonyl fluoride (PMSF): 100 m*M* in 95% ethanol. Store at 4°C.
3. 100X protease inhibitor mix: 1 mg/mL antipain, 5 mg/mL aprotinin, 10 mg/mL benzamidine hydrochloride, 1 m*M* ethylenediaminotetraacetic acid (EDTA), 1 mg/mL leupeptin, in 10 m*M* Tris-HCl (pH 7.4). Store in 0.2-to 1-mL aliquots at –20°C.
4. 20X sodium fluoride (NaF): 1 *M*. Store at 4°C.
5. 100X sodium orthovanadate (Na_3O_4V): 0.1 *M*. Store at 4°C.
6. 10X sodium pyrophosphate ($Na_4O_7P_2$): 0.1 *M* prepared in 10 m*M* Tris–HCl (pH 7.4). Readjust pH to 7.4. Store at 4°C (*see* **Note 4**).
7. 100X trypsin inhibitor: 10 mg/mL in 1 m*M* EDTA and 10 m*M* Tris–HCl (pH 7.4). Store in 0.2- to 1-mL aliquots at –20°C.
8. 10X Tris/sodium/EDTA buffer (TNE): 0.5 *M* Tris–HCl (pH 7.4), 1.5 *M* NaCl, and 10 m*M* EDTA (pH 8.0). Store at 4°C.
9. 10% (v/v) Triton X-100 in ddH_2O. Store at 4°C.
10. 0.1% TNTE : 1X TNE containing 0.1% of Triton X-100.
11. 1000X protease-free ribonuclease A (RNase A, Sigma R-5500): 10 mg/mL stock in 10 m*M* Tris–HCl (pH 7.5), 15 m*M* NaCl. Prepare by heating to 100°C for 15 min, allow to cool to room temperature, aliquot, and store at –20°C.
12. Lysis buffer: 1X TNE solution containing 0.5% Triton X-100, pepstatin, phenylmethyl sulfonyl flouride (PMSF), protease inhibitor, sodium fluoride, sodium orthovanadate, sodium pyrophosphate, trypsin inhibitor, and RNase A to final concentrations of 1X in an appropriate lysis volume (*see* **Note 5**).
13. 8X protein sample loading buffer: 0.4 *M* Tris–HCl (pH 6.8), 40% v/v glycerol, 8% SDS, 0.4 *M* DL-dithiothreitol (DTT), 0.5% Bromophenol Blue.
14. Coomassie stain: 25% (v/v) 2-propanol, 7% acetic acid (v/v), and 0.05% Coomassie Blue (w/v).
15. Destain: 5% 2-propanol (v/v) and 7% acetic acid (v/v).
16. Monoclonal antibodies: Anti-FLAG M2 (Sigma F3165), anti-MYC 9E10 (Invitrogen R950-25; Invitrogen, San Diego, CA), anti-HA 12CA5 (Roche 1583816; Roche, Basel, Switzerland).

17. Polyclonal antisera: Anti-Smad1, anti-Smad2 and anti-Smad4 (Upstate Biotechnology, Lake Placid, NY).
18. Protein A bead slurry: Protein A Sepharose® CL-4B (Amersham Pharmacia Biotech, Piscataway, NJ,17-0780) (*see* **Note 6**).
19. Protein G bead slurry: Protein G Sepharose® 4 Fast Flow (Amersham Pharmacia Biotech, Piscataway, NJ, cat. no. 17-0618) (*see* **Note 7**).
20. SDS-radioimmunoprecipitation assay (RIPA) buffer: 150 m*M* NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 m*M* Tris–HCl, pH 8.0.

2.1.4. Western Blotting of Proteins

1. SDS–polyacrylamide gels: 8 × 10 cm, 0.75 mm thick, 8% acrylamide.
2. 10X transfer buffer: 0.25 *M* Trizma® Base (Sigma, cat. no. T-1503) and 1.92 *M* glycine dissolved in ddH_2O.
3. 1X transfer buffer: Mix 10X transfer buffer, methanol, and ddH_2O in a ratio of 1:2:7. Chill at 4°C.
4. 0.45 μm nitrocellulose membrane and 3MM Whatman filter paper.
5. Ponceau S solution (Sigma P7170).
6. 10X TBS: Tris-buffered saline containing 0.2 *M* Tris–HCl, 1.36 *M* NaCl, pH 7.6.
7. 1X TBST: 1X TBS containing 0.1% Tween-20.
8. Enhanced chemiluminescence (ECL) kit.
9. Secondary antibodies: Goat anti-mouse–horseradish peroxidase (HRP) and goat anti–rabbit–HRP secondary antibodies (Amersham Pharmacia Biotech).

2.2. Analysis of Smad2 Phosphorylation by Anti-phosphoserine Antibody

1. Anti-P-Smad2 antibody: Anti-phosphoserine-Smad2 (Ser465/467). Available from Upstate Biotechnology, cat. no. 06-829.
2. Anti-P-ser blocking solution: TBST containing 2 % BSA, prepare fresh.
3. Anti-P-ser wash solution: TBST containing 0.2 % BSA, prepare fresh.

2.3. In Vitro Kinase Assay

2.3.1. Preparation of Bacterial Fusion Protein

1. Luria Bertani (LB) medium: 10 g/L Bacto®-tryptone (Becton Dickinson, Sparks, MD) 5 g/L yeast extract, 0.17 *M* NaCl, pH to 7.0 with 5*N* NaOH. Sterilize by autoclaving.
2. 100X ampicillin: 10 mg/mL in ddH_2O. Store at 4°C.
3. Isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma I-6758) stock: 0.8 *M* in ddH_2O. Filter-sterilize and store 1-mL aliquots at –20°C.
4. Glutathione-Sepharose 4B (Pharmacia 17-0756-01) slurry. To prepare, wash three times with PBS–CaMg, once with 0.1% TNTE and resuspend as a 50% slurry in 0.1% TNTE.
5. Glutathione-*S*-transferase (GST) elution buffer: 10 m*M* reduced glutathione in 50 m*M* Tris–HCl (pH 8.0).

6. Dialysis membrane: Spectra-por (Spectrum Medical Industries Supplier, Carson, CA), 3-kDa pore size.
7. Dialysis buffer: 1 m*M* EDTA, 0.1 m*M* DTT in 50 m*M* Tris–HCl.
8. Concentration chamber: Centricon-3 concentrator (Amicon), 3-kDa pore size.

2.3.2. Isolation of Receptor Complexes

1. Mink lung epithelial cells (Mv1Lu; also known as CCL-64). Available from ATCC.
2. Tissue culture medium: Minimal essential medium (MEM) including 10% FBS supplemented with 1X MEM Non-essential amino acids (NEAA, Gibco-BRL 06881).
3. Transforming growth factor-β1 (R & D Systems, Minneapolis, MN).
4. Antibody: C16 (Santa Cruz) anti-TβRII antibody.
5. 10X kinase assay buffer: 50 m*M* Tris–HCl (pH 7.4), 10 m*M* $MgCl_2$, 1 m*M* $CaCl_2$.

2.3.3. Kinase Assay

1. Radionuclide: [γ–^{32}P]-adenosine 5'-triphosphate, 6000 Ci/mmol.
2. Adenosine triphosphate (ATP, Sigma A-9187).

2.4. Receptor/Smad Interaction

1. [^{125}I]-Iodinated TGF-β superfamily ligand (*see* **Note 8**).
2. 10X Krebs Ringer HEPES (KRH) buffer: 128 m*M* NaCl, 5 m*M* KCl, 5 m*M* $MgSO_4$, 1.3 m*M* $CaCl_2 \cdot 2H_2O$ and 50 m*M* HEPES (pH 7.4).
3. KRH/0.5% BSA: 1X KRH containing 0.5% w/v BSA, prepare fresh.
4. Disuccinimidyl suberate (DSS; Pierce Chemical, Rockford, IL, 21555ZZ): 10 mg/mL in DMSO. Prepare immediately before use.
5. Sucrose–Tris–EDTA (STE): 0.25 *M* sucrose, 10 m*M* Tris–HCl (pH 7.4), and 1 m*M* EDTA. Store at 4°C.
6. Modified lysis buffer: Prepare as in **Subheading 2.1.3., step 12**, but omit RNase A and phosphatase inhibitors and include 10% glycerol.

3. Methods

3.1. Analysis of Smad Phosphorylation by [^{32}P]-Phosphate Labeling

3.1.1. Transfection of COS-1 Cells

1. Maintain COS-1 cells in HG-DMEM containing 10% FCS at 37°C in a CO_2 incubator. One day prior to transfection, plate cells from an actively growing culture into 100-mm tissue culture dishes such that they will be approximately 80% confluent on the next day (*see* **Notes 9** and **10**).
2. Just prior to use, prepare 4 mL of transfection mix for each 100-mm dish. Add DNA to the mix to a final concentration of 2 μg/mL (*see* **Note 11**).
3. Wash cells once in serum-free HG-DMEM and add DNA–transfection mix.
4. Incubate cells 3–3.5 h at 37°C in a CO_2 incubator.
5. Remove the DNA–transfection mix and add 5 mL of 10% dimethyl sulfoxide (DMSO) per 100-mm dish for exactly 2 min at room temperature.

6. Remove the DMSO solution and wash cells once in 10 mL of HG-DMEM containing 10% FCS.
7. Incubate cells overnight in 10 mL of HG-DMEM containing 10% FCS, 1X penicillin–streptomycin, and 1X fungizone, at 37°C in a CO_2 incubator.
8. The next day, concentrate cells by trypsinization. Typically, 90% of cells are replated into a 60-mm dish for [^{32}P]-phosphate labeling and 10% of cells are replated into 1 well of a 12-well dish for determination of total protein levels.
9. Approximately 3–4 h after replating, after cells have attached, wash cells once in fresh media to remove dead cells and debris.
10. Incubate overnight at 37°C in a CO_2 incubator.

3.1.2. [^{32}P]-Orthophosphate Labeling

1. The next day, wash the 60-mm dishes twice in phosphate-free HG-DMEM.
2. Incubate in phosphate-free HG-DMEM for 15 min at 37°C in a CO_2 incubator.
3. Using appropriate radioactive safety measures (*see* **Note 1**), prepare phosphate-free HG-DMEM containing 0.2% dialyzed FCS and [^{32}P]-orthophosphate at 1 mCi/mL (*see* **Note 12**).
4. Add radioactive medium to cell layers and incubate 1–2 h at 37°C in a CO_2 incubator.
5. Following incubation with [^{32}P]-orthophosphate, place cells on ice and gently wash twice in ice-cold PBS–CaMg (*see* **Note 13**).

3.1.3. Lysis and Immunoprecipitation

1. Perform all subsequent manipulations at 4°C. Wash cells in ice-cold PBS–CaMg and remove all excess liquid.
2. Lyse the cells by shaking at 4°C for 15–30 min in 500 μL of lysis buffer per 60-mm dish.
3. Transfer cell lysates into 1.5-mL microcentrifuge tubes and remove cellular debris by microcentrifugation at 15,000*g* for 10 min at 4°C.
4. For determination of total protein, wash cells in 12-well dishes with ice-cold PBS–CaMg and lyse in 100 μL lysis buffer. Clear lysates by microcentrifugation and add 8X protein sample loading buffer to the supernatant to achieve a final concentration of 1X. Heat samples to 95°C for 2 min and then store at –20°C prior to electrophoresis. Proceed to immunoblotting, **Subheading 3.1.4.**
5. Subject cleared supernatants from 60-mm dishes to immunoprecipitation by adding the appropriate antibody and incubating at 4°C with rocking for 1–1.5 h (*see* **Note 14**).
6. Microcentrifuge lysates to remove any particulate matter that forms during the incubation.
7. Transfer cleared supernatants to fresh tubes containing 50 μL of Protein A Sepharose® or Protein G Sepharose®. Incubate at 4°C rocking for 1 h (*see* **Note 15**).
8. Wash Sepharose beads once in 0.1% TNTE, twice in RIPA, and twice again in 0.1% TNTE buffer. Use 1 mL of buffer per wash and mix by gently inverting tubes. Pellet beads between washes using a 10-s pulse on a benchtop microcentrifuge.
9. After the final wash, carefully remove all liquid and resuspend the washed beads in 30 μL of 0.1% TNTE containing 1X protein sample loading buffer.

10. Heat samples for 2 min at 95°C. Samples can be stored at –20°C and are reheated and microcentrifuged prior to loading.
11. Load samples onto an 8% SDS-PAGE gel. Avoid loading beads into well.
12. Perform electrophoresis at 130 V until sample has entered the separating gel and then at 200 V until Bromophenol Blue has reached the bottom of the gel.
13. Incubate gel in Coomassie stain for 15 min at room temperature with shaking.
14. Incubate gel in destain solution changing the solution every 10 min until background staining is removed and antibody bands are visualized.
15. Place gel on 3MM filter paper and dry under vacuum for 1–1.5 h at 80°C.
16. To visualize [^{32}P]-phosphate-labeled proteins, subject gel to phosphorimaging or to autoradiography by exposing to Kodak AR film at –70°C using intensifying screens (*see* **Note 16**).

3.1.4. Western Blotting of Proteins

1. Using gloves, cut one nitrocellulose membrane and two pieces of 3MM filter paper that match the dimensions of the gel.
2. Pre-wet nitrocellulose in ddH_2O for 10 min and then soak the nitrocellulose and 3MM papers in 1X transfer buffer.
3. Equilibrate acrylamide gel in 1X transfer buffer for 10 min.
4. Prepare a sandwich containing layers of 3MM paper, gel, nitrocellulose, and another sheet of 3MM in that order. Avoid trapping air bubbles between the layers.
5. Mark orientation and place sandwich into a transfer apparatus with the gel toward the negative electrode.
6. Transfer for approx 60 min according to transfer–apparatus manufacturer's instructions.
7. To confirm efficient transfer, stain nitrocellulose membrane with Ponceau S solution for approx 5 min. Rinse with ddH_2O until background staining is removed and proteins are visualized.
8. Perform subsequent steps at room temperature with shaking. Block nitrocellulose membrane in TBST containing 5% skim milk powder for 1 h.
9. Wash blot three times for 5 min each in TBST.
10. Incubate nitrocellulose for 1 h with an appropriate amount of primary antibody in 0.1% TBST. To detect FLAG-tagged proteins, use 1 μg/mL of anti-FLAG M2.
11. Wash blot three times for 5 min each in TBST.
12. Incubate nitrocellulose in appropriate secondary antibody (e.g., 1 : 10,000 dilution of Amersham goat-anti-mouse-HRP) for 45 min.
13. Wash blot three times for 5 min each in TBST.
14. Perform enhanced chemiluminescence as per manufacturer's instructions.

3.2. Analysis of Smad2 Phosphorylation by Anti-phosphoserine Antibody

1. Prepare cell lysates from transfected or nontransfected cells as described in **Subheading 3.1.3.**

2. Resolve proteins in cell lysates by SDS-PAGE and transfer to nitrocellulose as in **Subheading 3.1.4.**
3. Perform subsequent steps at room temperature with shaking. Incubate blot three times for 30 min in anti-P-ser blocking solution.
4. Add anti-P-Smad2 antibody diluted 1 : 500 in blocking solution and incubate blot for 1 h.
5. Wash three times for 5 min in anti-P-ser wash solution.
6. Dilute goat anti-rabbit-HRP 1 : 10 000 in anti-P-ser wash solution.
7. Incubate 45 min and then wash five times for 5 min each in anti-P-ser wash solution.
8. Proceed with enhanced chemiluminescence as per manufacturer's instructions.

3.3. In Vitro Kinase Assay

3.3.1. Preparation of Bacterial Fusion Protein

1. Prepare GST–Smad protein by diluting an overnight bacterial culture expressing GST–Smad 1 : 10 in 5 mL of LB containing 0.1 mg/mL ampicillin and grow 1 h at 37°C with shaking.
2. To induce protein expression, add isopropyl-beta-D-thiogalactopyranoside (IPTG) to 0.1 m*M* for 4 h.
3. Pellet bacteria by centrifugation for 10 min at 5000*g*.
4. Discard supernatant and resuspend in 1 mL ice-cold 0.5% TNTE.
5. Sonicate on ice three times for 15 s each at a medium setting. Avoid excess frothing.
6. Collect supernatants by microcentrifugation for 5 min at 4°C.
7. Incubate for 30 min at room temperature in 50 mL of 50% glutathione– Sepharose slurry with rocking.
8. Collect beads by microcentrifugation for 5 min at 1000*g*.
9. Wash pellet four times with 250 μL of cold 0.1% TNTE.
10. Gently resuspend pellet in 50 μL of GST elution buffer.
11. Collect supernatant after microcentrifugation for 5 min at 1000*g*.
12. Repeat elution twice and pool supernatants.
13. Dialyze bacterial proteins overnight at 4°C in 3-kDa cutoff dialysis membrane against large a volume of dialysis buffer.
14. Concentrate dialyzed proteins to approx 0.2 μg/μL with Centricon-3 concentrator. Store concentrated protein at 4°C until use.
15. Estimate protein concentration of bacterial proteins by comparison to protein standards by SDS-PAGE and Coomassie Blue staining.

3.3.2. Isolation of Receptor Complexes

1. To isolate TGF-β–receptor complexes, treat three 100-mm dishes of Mv1Lu cells with or without 0.5 n*M* TGF-β1 in MEM containing 0.2% FBS and NEAA, for 30 min at 37°C (*see* **Note 17**).
2. Wash cells with ice-cold PBS–CaMg.
3. Incubate for 15 min in 0.5 mL lysis buffer (as in **Subheading 2.1.3., item 12**) at 4°C with shaking and pool the lysates from different dishes.
4. Microcentrifuge lysate for 10 min at 4°C. Transfer supernatant to a new tube.

5. Incubate for 15 min with C16 (5 µL per dish) antibody at 4°C with rocking.
6. Microcentrifuge and transfer contents to a fresh tube containing 50 µL of Protein A–Sepharose bead slurry.
7. Incubate for 45 min at 4°C with rocking.
8. Wash three times with 0.1% TNTE and twice with 1X kinase assay buffer. Use 1 mL of buffer per wash and mix by gently inverting tubes. Pellet beads between washes using a 10-s pulse. After the last wash carefully draw off all of the supernatant.

3.3.3. Kinase Assay

1. Immediately resuspend receptor complexes isolated from the three 100-mm dishes in kinase reaction mix, made by combining 20–40 µCi of [γ-^{32}P]-ATP, 2 µg GST–Smad protein in 1X kinase assay buffer containing 1 µ*M* ATP in a final volume of 30 µL.
2. Incubate for 30 min at room temperature.
3. Add 8X protein sample buffer to a final concentration of 1X and resolve sample on 7.5% SDS-PAGE. Perform autoradiography as outlined previously.

3.4. Receptor–Smad Interaction

1. Transfect 100-mm COS-1 cells as in **Subheading 3.1.1.** and replate into 60-mm dishes (*see* **Note 18**).
2. On the second day after transfection, dilute iodinated ligand in HG-DMEM containing 0.2% FCS. For [^{125}I]-TGF-β we use a concentration of 50–100 p*M*.
3. Wash cells three times in HG-DMEM containing 0.2% FCS and incubate 15 min at 37°C in a CO_2 incubator.
4. Aspirate media from cells, add 1.5 mL media containing iodinated ligand and incubate for 30 min at 37°C.
5. During this incubation, prepare KRH/0.5% BSA and KRH/DSS on ice. Prepare KRH/DSS immediately prior to use by adding 6 µL of DSS stock to 1 mL of ice-cold KRH and mix well.
6. Aspirate medium from cells and gently wash three times with KRH/0.5% BSA and twice with KRH (no BSA).
7. Add 2 mL KRH/DSS per dish and incubate cells for 15–30 min at 4°C with shaking.
8. Perform subsequent steps on ice. Aspirate KRH/DSS and wash twice with ice-cold STE (*see* **Note 19**).
9. Lyse cells (as in **Subheading 3.1.3.**) in 1 mL of modified lysis buffer (*see* **Subheading 2.4., item 6**).
10. Add 1/8 volume of 8X protein sample buffer to 10% of cleared lysate and use to confirm equivalent expression of receptors and Smads.
11. On remainder of sample, isolate Smad-associated receptor complexes by immunoprecipitation with anti-Smad antibodies or anti-epitope antibodies for epitope tagged Smads as described in **Subheading 3.1.3.**
12. Quantitate receptors coimmunoprecipitating with Smads by counting immunoprecipitation tubes in a γ-counter.
13. After γ counting, resolve the immunoprecipitates by SDS-PAGE using 7% gels and visualize Smad-associated receptor complexes by autoradiography (*see* **Note 20**).

4. Notes

1. Using [^{32}P]-orthophosphate at high activity levels poses significant risk. Work according to radiation safety protocols. Monitor work area extensively using a Geiger counter.
2. If phosphate-free HG-DMEM is not readily available, phosphate-free MEM can be substituted because the labeling period is short.
3. The protease and phosphatase inhibitors are potentially hazardous. Read all safety precautions on materials data sheets and proceed accordingly.
4. Sodium fluoride, sodium orthovanadate, and sodium pyrophosphate act as phosphatase inhibitors. They are stable at 4°C for a few months.
5. Prepare just prior to use and keep on ice. Thaw inhibitor stocks at room temperature (or 37°C for pepstatin) and maintain on ice (or room temperature for pepstatin). Replace inhibitors to –20°C or 4°C immediately after use. Prepare lysis buffer no more than 30 min before use. RNase is not required in lysis buffers for non-phosphate-labeled cells.
6. Transfer beads into an equal volume of 0.1% TNTE containing 5% bovine serum albumin (BSA, Sigma A-7030), incubate 1 h at 4°C with rocking. Wash beads three times in 10 volumes of 0.1% TNTE, at 300*g* for 10 min to recover bead pellet. Store at 4°C in 0.1% TNTE at a 1:4 ratio of beads to TNTE.
7. Prepare Protein G–Sepharose slurry as in **Note 6**; however, omit BSA.
8. See Chapter 3 in this volume for preparation of iodinated ligands.
9. Typically a confluent dish of cells is split 1 : 3 and is then grown overnight.
10. As an alternative to transiently transfected COS-1 cells, phosphorylation of endogenously expressed Smads can also be examined. For Smad2 in Mv1Lu cells, we [^{32}P]-phosphate label 1 well of a 12-well dish of actively growing cells and stimulate the cells with 0.5 n*M* TGF-β, added 15–30 min prior to lysis. Cell layers are lysed and immunoprecipitated as described for COS-1 cells.
11. We typically use 0.25 μg/mL of R-Smads and 0.125 μg/mL of wild-type or constitutively activated receptors. Add empty vector as required to maintain a constant DNA concentration in all samples.
12. Minimize radioactivity used by preparing only enough radioactive medium required to just cover cells. Prepare 1.5 mL per 60-mm dish.
13. If cell layer is dense, wash carefully to avoid lifting sheets of cells. Do not use vacuum aspirator to remove radioactive solution as decontamination of tubing is difficult.
14. For epitope-tagged proteins, add monoclonal antibodies to a final concentration of approximately 1 μg/mL. For immunoprecipitation of endogenously expressed Smad1, Smad2, or Smad4, use concentrations of antibody as recommended by the manufacturer.
15. When measuring beads, cut pipet tip with a sharp razor blade to widen the opening so that beads do not block tips.
16. Exposure time varies from 1 h to 2 d depending on efficiency of immunoprecipitation and the level of phosphorylation.
17. A similar approach to isolate endogenous receptor complexes for other TGF-β superfamily members can be carried out using the appropriate cell lines. Alterna-

tively, receptor complexes can be isolated from COS-1 cells that have been transfected with epitope-tagged receptors. Follow immunoprecipitation up to and including **step 8** of **Subheading 3.1.3.** Continue with **step 5** of **Subheading 3.3.2.** and use appropriate anti-epitope antibodies.

18. We typically use wild-type type II receptors with kinase-deficient type I receptors to "trap" the Smad substrate on the receptor. Cotransfection of kinase-deficient type II receptor with wild-type type I receptor prevents activation of receptor I and thereby serves as a control. Alternatively, R-Smads harboring serine to alanine mutations at the target phosphorylation sites can be used with wild type receptors.
19. STE serves to inactivate free DSS.
20. To optimize the autoradiographic signal, use [^{125}I]-sensitive Kodak MS film and intensifying screens designed for [^{125}I] enhancement. Exposure time varies from 3 d to more than 1 wk depending on efficiency of crosslinking and immunoprecipitation.

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7

Promoter Analysis of TGF-β Responsive Genes by Transient Transfection and Deletional/Mutational Analysis

Ge Jin and Philip H. Howe

1. Introduction

The tranforming growth factor β (TGF-β) family of cytokines participates in regulating cell growth, morphogenesis, and differentiation by modulating expression of a variety of genes ***(1–4)***. These gene products broadly cover proteins involved in deposition of an extracellular matrix (ECM), such as fibronectin and its receptor, collagen and collagenase ***(5–9)***, the proto-oncogene products c-Jun and JunB ***(10–12)***, the cell-cycle regulators p15 and p21/Waf/Cip ***(13–15)***, and proteins involved in apoptotic cell death, such as clusterin ***(16)***.

Several TGF-β responsive *cis*-regulatory regions, which contain multiple protein binding sites (*cis*-elements) for transcriptional regulation, have been revealed mostly by transient transfection approaches. It has been shown that multiple activator protein-1 (AP-1) sites are important for TGF-β induction of plasminogen activator inhibitor type 1 (PAI-1) expression ***(17)***. Recently, the "CAGAbox" ***(18)*** and TSR elements ***(19)*** in PAI-1 promoter have been demonstrated to be *cis*-elements for interaction with activated Smad proteins upon TGF-β stimulation. Sp1 sites have also been shown to participate in the regulation of human α2(I) collagen ***(20)*** and p21/Waf1/Cip1 gene expression by TGF-β ***(21)***. Other *cis*-regulatory elements involved in TGF-β regulation include nuclear factor 1 (NF-1) ***(22)*** and TGF-β inhibitory elements (TIEs) ***(23)***. Our previous results have shown that the expression of clusterin, an 80-kDa glycoprotein, is upregulated by TGF-β ***(16)***. We have performed promoter studies by using deletional/mutational analysis and transient transfection assays to reveal that a consensus AP-1 site, located 72 bp (base pairs) upstream of tran-

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scription start site, is necessary and sufficient for TGF-β induction in a variety of cell lines ***(16)***. Using the same types of approaches, three AP-1 sites in the murine laminin α3A promoter have been shown to differentially contribute to induction of gene expression by TGF-β ***(24)***.

Transient transfection assays are an important tool for the study of genetic regulation and protein function within eukaryotic cells and tissues. It introduces nucleic acids into cells by nonviral methods, such as DEAE–dextran and liposomal techniques. This process is distinct from "infection," which is a viral method of nucleic acid introduction into cells. To study whether a promoter region contains a specific *cis*-regulatory element and how nuclear proteins interact with this region in response to a specific stimuli, generally the 5' promoter region of a specific gene, wide type and mutated, is joined with the gene encoding for firefly luciferase or bacterial chloramphinicol acetyltranferase (CAT). This fusion cDNA serves as a reporter gene for transfection into cells. Following transfection and activation by stimuli, cellular lysates are prepared and either luciferase or CAT activity is measured. Because the expression of luciferase or CAT is controlled by the promoter under study, luciferase or CAT activity is reflective of the transcriptional activation of the 5' regulatory promoter element.

In this chapter, we introduce, step by step, the methods of transient transfection of reporter genes into cells, measurement of luciferase and β-galactosidase activity, preparation of transfection reagents, and deletion/site-directed mutation of DNA.

2. Materials

2.1. Reagents and Materials

1. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethnaolamine (DOPE) from Avanti Polar Lipids (Alabaster, AL), cat. no. 85072.
2. 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) from Avanti Polar Lipids, cat. no. 890890.
3. LipofectAMINE Plus Reagent from Gibco-BRL (Grand Island, NY), cat. no. 10964-013.
4. Luciferase assay system with reporter lysis buffer from Promega (Madison, WI), cat.no. E4030.
5. FuGENE 6 transfection reagent from Boehringer Mannheim (Indianapolis, IN), cat. no. 1814443.
6. *o*-Nitropheny β-D-galactopyranoside (ONGP) from Sigma (St. Louis, MO), cat. no. N-1127.
7. Opaque white plates (Falcon), 96-well from Fisher Scientific (Pittsburgh, PA), cat. no. 08-771-26.
8. Agarose gel DNA extraction kit from Boehringer Mannheim, cat. no. 1696505.
9. *Pfu* DNA polymerase from Strategene (La Jolla, CA), cat. no. 600154.
10. Klenow DNA polymerase from Promega, cat. no. M2205.

11. T4 DNA ligase from Promega, cat. no. M1801.
12. Exonuclease III from Promega, cat. no. M1811.
13. MAX Efficiency DH5α competent cells: Gibco-BRL, cat. no. 18258-012.
14. T4 polynucleotide kinase from Gibco-BRL, cat. no. 18004-010.
15. SPIN-X centrifuge tube filters from Costar (Cambridge,MA), cat. no. 8160.
16. S1 nuclease from Promega, cat. no. M5761.
17. Luciferase assay reagent: reconstitute luciferase substrate (Promega) with 10 mL of luciferase buffer (Promega), aliquot, and store at –80°C.
18. TE (pH 8.0) saturated phenol: Add the same volume of TE buffer (10 m*M* Tris–HCl, pH 8.0, 1 m*M* EDTA) to molecular-biology-grade phenol (Sigma), shake vigorously, discard aqueous (upper) phase when two phases are clearly separated, and repeat this step once. Add the same volume of TE buffer and stir for several hours or overnight, discard the aqueous phase, and add enough TE buffer to cover the surface of phenol. Store at 4°C.
19. Phenol : chloroform : isoamylalcohol: Mix 250 mL of TE-saturated phenol, 240 mL of chloroform, and 10 mL of isoamylalcohol by shaking vigorously; add TE buffer to the surface of the reagent. Store at 4°C.

2.2. Buffers

1. 2X β-gal assay buffer: 120 m*M* Na_2HPO_4, 80 m*M* NaH_2PO_4, 2 m*M* $MgCl_2$, 100 m*M* β-mercaptoethanol, 1.33 mg/mL *o*-nitropheny β-D-galactopyranoside (ONGP, Sigma).
2. Reporter lysis buffer: Add 120 mL distilled H_2O to 30 mL of 5X reporter lysis buffer (Promega); mix well. Store at 4°C up to several months.
3. PBS (phosphate buffered saline, pH 7.4): 0.2 g/L KCl, 8.0 g/L NaCl, 0.2 g/L KH_2PO_4, 1.15 g/L Na_2HPO_4.
4. 1 *M* sodium carbonate.
5. 3 *M* sodium acetate (100 mL): 40.8 g sodium acetate–H_2O, adjust pH to 5.3 with glacial acetic acid, and add H_2O to 100 mL.
6. 10X Exo III buffer: 660 m*M* Tris–HCl (pH 8.0), 6.6 m*M* $MgCl_2$.
7. dNTP mix: 0.125 m*M* each of dATP, dCTP, dGTP, and dTTP in H_2O.
8. Klenow mix: Add 5 units of Klenow DNA polymerase to 30 μL of Klenow buffer (20 m*M* Tris–HCl, pH 8.0, 100 m*M* $MgCl_2$).
9. Ligase mix: 490 μL H_2O, 5 μL of 10X ligase buffer (500 m*M* Tris–HCl, pH 7.6, 100 m*M* $MgCl_2$, 10 m*M* ATP), 5 μL of 100 m*M* DTT, 5 units of T4 DNA ligase.
10. S1 nuclease mix: 172 μL H_2O, 27 μL of 7.4X S1 buffer (0.3 *M* potassium acetate, 2.25 *M* NaCl, 16.9 m*M* $ZnSO_4$, 45% glycerol), 70 units of S1 nuclease.
11. S1 stop buffer: 0.3 *M* Tris-base, 0.05 *M* EDTA.
12. Bacterial growth medium (SOC) medium: 5 g Bacto-tryptone, 2.5 g Bacto-yeast extract, 2.5 g NaCl, 10 m*M* $MgSO_4$, 10 m*M* $MgCl_2$, adjust pH to 7.0, add H_2O to 500 mL, autoclave, add 25 mL of sterile 40% glucose.
13. 10X phosphorylation buffer: 500 m*M* Tris–HCl, pH 7.6, 100 m*M* $MgCl_2$, 100 m*M* KCl, 100 m*M* DTT.
14. DNA elution buffer: 10 m*M* Tris–HCl, pH 8.0.

3. Methods

3.1. Transient Transfection Based on Artificial Liposomal Technique

Development of synthetic cationic lipids makes artificial liposomes a preferred carrier for the delivery of nucleic acids into cells. Artificial liposomes can be used to transfect cells with DNA of all sizes ***(25,26)***, RNA ***(27)***, and protein ***(28)*** with relatively high transfection efficiencies. A cationic lipid (such as DOTAP) with an overall net positive charge at physiological pH is mixed with a neutral lipid (such as DOPE) to form the liposomes. The cationic portion associates with the negatively charged DNA, resulting in compaction of the DNA in a DNA–liposome complex. Following endocytosis, the complexes appear in the endosomes, then in the nucleus, where the expression of imported DNA is controlled by cellular transcription machinery (**Fig. 1**).

3.1.1. Transient Transfection with DOTAP : DOPE Transfection Reagent

We have modified the protocol from Campbell ***(29)*** to prepare the liposomal-based transfection reagent in our lab. We have found that the DOTAP : DOPE transfection reagent is very efficient and suitable for use in transfecting nucleic acids into a variety of cell lines, such as mink lung epithelial cells (Mv1Lu, CCL64), HeLa, COS7, HT1080, HepG2 and 10T1/2 cells (unpublished data). The cells transfected with reporter genes using this protocol present very low cellular toxicity and high capability of TGF-β responsiveness.

1. The day before transfection, trypsinize and seed cells in six-well plates at 2×10^5 cell/well. Incubate the cells overnight in Dulbecco's modified Eagle medium (DMEM, Gibco-BRL, Grand Island, NY) supplemented with 10% serum (newborn calf serum or fetal bovine serum, depending on cell lines) at 37°C, 5% CO_2, without antibiotics (*see* **Note 1**).
2. Prepare the DNA–liposome complex: Add 30 µL of DOTAP : DOPE transfection reagent (*see* **Subheading 3.3.** for preparation; *see also* **Note 2**) to 200 µL of serum-free DMEM, add 5–10 µL of DNA solution containing 1 µg of reporter DNA of interest, and add 0.5 µg of pSV40 β-galactosidase plasmid (*see* **Note 2**) to the diluted transfection reagent; mix gently.
3. Incubate the DNA–liposome mix at room temperature for 30 min. Meanwhile, rinse the cells with 2 mL of 37°C warmed serum-free DMEM once, incubate the cells at 37°C in 5% CO_2 while waiting for formation of DNA–liposome complexes.
4. Add 800 µL of serum-free DMEM to the DNA–liposome complexes, mix well.
5. Remove serum-free medium from the cells, add 1 mL of the DNA–liposome complexes to the cells. Incubate the cells at 37°C, 5%CO_2 for 5 – 8 h.
6. Following incubation, aspirate the medium containing DNA–liposome complexes, add 2 mL of DMEM supplemented with 10% newborn calf serum. Add TGF-β to appropriate concentration.

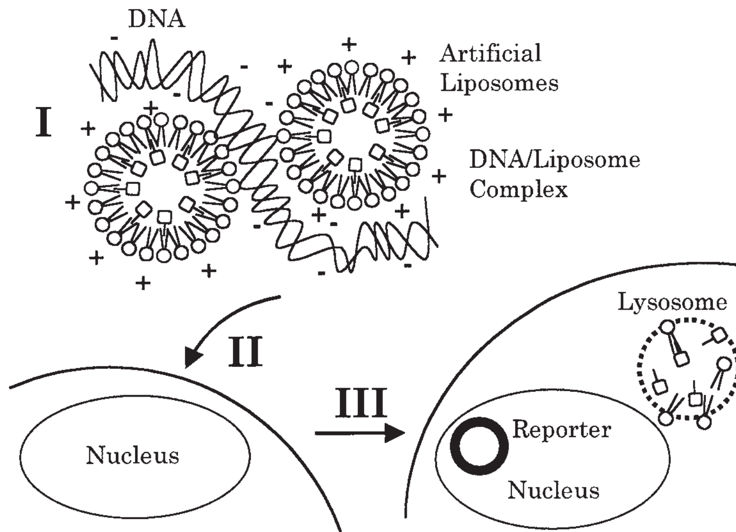

Fig. 1. Schematic representation of transient transfection assays with liposomal-based reagent. The transfection process can be depicted as three steps. The liposomes carry positively charged surfaces form compact complexes with negatively charged plasmid DNA **(I)**. The cells take up the DNA–liposome complexes through endocytosis **(II)**. In the cells, the DNA–liposome complexes appear first in the endosomes, then the DNA molecules present in the nucleus while the lipids are degraded presumably by lysosomes **(III)**.

7. Incubate the cells at 37°C in 5% CO_2 for 16 – 24 h, discard the medium, and wash the cells with phosphate-buffered serum (PBS) two times. Add 400 μL of reporter lysis buffer (Promega) to the cells, incubate at room temperature for 30 min to 1 h with rocking.
8. Scrap cells into microcentrifuge tubes. Pipet the lysate with a 1-mL tip to ensure that the enzyme protein is released from cells. Centrifuge at high speed (18,000*g*) for 1 min at room temperature. Transfer the supernatant to fresh tubes. The cell lysate is ready for luciferase and β-galactosidase measurements.

*3.1.2. Transient Transfection with LipofectAMINE PLUS Reagent (see **Note 3**)*

1. The day before transfection, plate 2 × 10^5 cells in six-well plates in 2 mL of DMEM containing 10% serum.
2. Add 1 μg of reporter DNA and 0.5 μg of pSV40 β-galactosidase DNA to 100 μL of serum-free medium, such as DMEM. Add 6 μL of PLUS reagent, mix gently. Incubate at room temperature for 15 min.
3. In another tube, mix 4 μL of LipofectAMINE reagent with 800 μL of serum-free medium. Add the diluted LipofectAMINE reagent to the DNA–PLUS complex; mix gently. Incubate the mix at room temperature for 15 min.

4. While the DNA–Plus–LipofectAMINE complexes are forming, rinse the cells with serum-free medium once and incubate the cells in 1 mL of the medium.
5. Add 1 mL of DNA–PLUS–LipofectAMINE complexes to each well; mix gently. Incubate cells in transfection medium at 37°C, in 5% CO_2 for 3 h.
6. After 3 h incubation, add 1 mL of complete DMEM to the wells. Add TGF-β to the appropriate concentration. Incubate the cells for 16 – 24 h at 37°C in 5% CO_2.
7. Wash cells and prepare cell lysates as described in **steps 7** and **8** in **Subheading 3.1.1.**

3.2. Transient Transfection Based on Nonliposomal Reagent

Transfection reagents based on nonliposomal formulation, such as lipopolyamines ***(30)*** and dendrimers ***(31)***, have been proven to be effective in transfecting mammalian cells with minimal cytotoxicity. FuGENE 6 transfection reagent (Boehringer) provides high levels of transfection with minimal cell toxicity for many eukaryotic cell lines. The transfection can be performed in the presence or absence of serum with minimal optimization.

1. The day before transfection, plate 2×10^5 cells in six-well plates in 2 mL of DMEM containing 10% serum.
2. For preparation of transfection, add 100 μL of serum-free medium to a microcentrifuge tube, add 5 μL of FuGENE 6 directly into the medium. Incubate at room temperature for 5 min.
3. Dilute 1 μg of reporter DNA and 0.5 μg of SV40 β-galactosidase DNA with serum-free medium to a final volume of 5 μL. Dropwise, add diluted FuGENE 6 reagent to the DNA solution. Mix by tapping the tube gently. Incubate at room temperature for 15 min.
4. Dropwise, add the DNA/FuGENE 6 complexes into the medium on the cells, swirl the plates to ensure even dispersal. There is no need to remove the medium and wash the cells prior to transfection.
5. Incubate the cells at 37°C in 5% CO_2 for 3 h. Add TGF-β to the desired concentration and incubate the cells for 16 – 24 h. Extract the cell lysates for luciferase and β-galactosidase assays as described in **Subheading 3.1.1., steps 7** and **8**.

3.3. Measurement of Luciferase and β-Galactosidase Activity

The promoter reporter DNA (such as 3TPLux) containing the luciferase coding region provides the basis for the quantitative analysis of promoter transactivation. The luciferase activity reflects the strength of the promoter, which controls luciferase expression and its responsiveness to TGF-β stimulation. The assay measuring luciferase activity is rapid, sensitive, and quantitative, and the enzyme is a monomeric protein that does not require posttranslational modification for its activity. Because transfection efficiency can be affected by many factors, such as cell status, transfection reagent, and cell types, it is important to cotransfect the cells with another reporter vector of which the expression is not affected by TGF-β treatment. The transcription of pSV40

β-galactosidase reporter (Promega) is driven by an SV40 promoter and the enzyme can be constitutively expressed in transfected cells. TGF-β treatment does not alter the expression of this reporter in the transfected cells. It can therefore be cotransfected with the reporter construct of interest and used as an internal control to normalize luciferase activity (*see* **Note 3**).

3.3.1. Measurement of Luciferase Activity

1. For measurement of luciferase activity with 96-well plates in a luminometer (Dynatech, Chantilly, VA; ML-2250): Add 10 μL of cell lysate from **Subheading 3.1.1., step 8** to a 96-well plate (opaque white plate, 96-well). To reduce variations in luminometer reading when working with multiple samples, limit each measurement to 12 wells and add the samples along the wells marked alphabetically.
2. With a pipet helper, rapidly add 50 μL of luciferase assay reagent to each well containing cell lysate. Mix lysate and the luciferase assay reagent gently by hand-rotating the plate on a flat surface for several times. To measure background levels, read either a H_2O blank or lysate from nontransfected cells.
3. Put the plate into sample shutter of the luminometer, close the door, and start to read the relative light unit (RLU). The data will be printed out on the data sheet.
4. Sometimes, the samples will produce too much light, which would damage the photomultiplier tube, and the RLU data will be recorded as "Over." If this is the case, either the sample should be diluted or the "Gain" parameter has to be set at "HI" or "MED" to reduce photomutiplier tube sensitivity.
5. For measurement of luciferase activity with a scintillation counter: Luciferase activity can also be measured by a scintillation counter, such as Tri-Carb 2100TR or Tri-Carb 4000 series liquid scintillation analyzer from Packard Instruments (Downers Grove, IL). Before measurement, make sure the coincidence circuit of the scintillation counter is turned off and the machine is in manual mode. Open all channels of the scintillation counter.
6. Immediately before measurement, add 20 μL of cell lysate into a microcentrifuge tube. Mix the lysate with 100 μL of luciferase assay reagent at room temperature.
7. Place the tube in a scintillation vial (either clear or translucent vials are acceptable), and put the vial onto the countersample rocker. Read the samples one at a time.
8. If the samples produce too much light output, the photomultiplier tube will be saturated and give out no reading or identical readings from sample to sample. It is necessary to make significant dilution of the sample (1 – 10 or 100 times dilution).

3.3.2. Measurement of β-Galactosidase Activity

1. Mix 250 μL of cell lysate with 250 μL of 2X β-gal assay buffer in a microcentrifuge tube. Use lysate from nontransfected cells as a blank to measure background enzyme activity.
2. Incubate the mixture in a 37°C H_2O bath for 30 min or until a faint yellow color appears. If the yellow color development is still not obvious, incubate the mixture at 37°C overnight.

3. Add 500 µL of 1 *M* sodium carbonate to stop the reaction. Mix by vortexing.
4. Read A_{420} with a spectrophotometer for β-galactosidase activity.

3.4. Preparation of DOTAP : DOPE Liposomes

DOTAP : DOPE is a liposomal formulation of the cationic lipid 1,2-dioleoyl-3-trimethyammonium-propane (DOTAP) and neutral lipid 1,2-dioleoyl-an-glycero-3-phosphoehtanolamine (DOPE) presenting at a ratio of 1 : 1 (w/w). The reagent is prepared in our lab and has been used for transfection of DNA into a wide variety of cell types. We have found that this transfection reagent is very efficient for use in transfection with very low cellular toxicity. Furthermore, the reagent is quite inexpensive. Preparation of the reagent is easy and quick. The reagent has little variation in transfection efficiency from batch to batch, and it is stable at 4°C for at least 6 mo.

1. Dissolve 10 mg of DOTAP and 10 mg of DOPE in 1 mL of absolute ethanol (*see* **Note 4**).
2. Pipet 50 µL of the lipids with a white or yellow tip (200 µL). Rapidly inject the lipid mix into a 17×10-mm round-bottomed tube containing 1 mL of sterile H_2O while vortex mixing (with Fisher's vortexer, set 4).
3. Store the transfection reagent at 4°C up to 6 mo. Concentration of the lipid is 1 mg/mL.
4. Transfect the cells as described in **Subheading 3.1.1.**

3.5. Deletion and Mutagenesis of Promoter Region for Promoter Analysis

The regulatory region of TGF-β responsive genes contains *cis*-regulatory elements that provide binding sites for activated transcription factors to regulate transcription. The activity of a TGF-β responsive *cis*-regulatory region can generally be pinpointed to a segment of DNA that ranges from about 100 to 200 bp long. To identify the transcription factors, it is critical to determine the exact nucleotide sequences important for the function of the regulatory elements upon TGF-β stimulation. For this purpose, a series of deletions of the promoter region can be fused to a reporter construct (luciferase), so that the effect of each deletion on transcription can be tested, and the responsiveness of these deletions to TGF-β can also be easily determined. Because numerous *cis*-elements have been identified during the decades and their sequences have been deposited in data banks, we suggest that, when possible, the regulatory region be analyzed by computer programs, such as GeneWorks, MacVector, or GCG, and through database on the World Wide Web (*see* **Note 5**).

3.5.1. Generation of Deletions of Promoter Region with Exonuclease III

Unidirectional deletions of inserted promoters in a reporter vector can be made using a protocol developed by Henikoff ***(32)***. The insert DNA is specifically digested by restriction enzymes which produce a 5' protruding or blunt

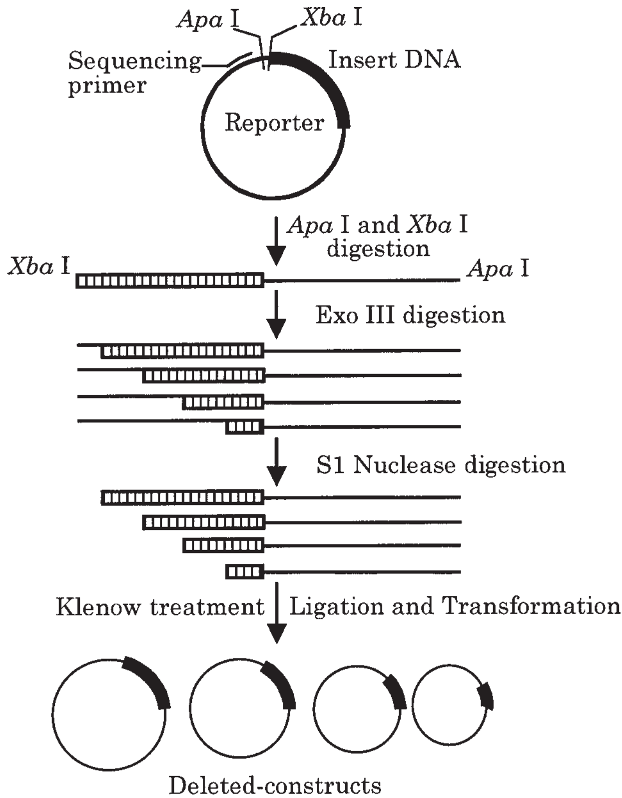

Fig. 2. Schematic diagram of unidirectional deletion assay with Exonuclease III. The insert DNA is first digested by two unique restriction enzymes which provide a 5' protruding (or blunt) end (i.e., *Xba*I) adjacent to the deletion start site of the DNA, and a 3' overhang end (i.e., *Apa*I) which is resistant to Exo III digestion. Exo III uniformly digests the insert DNA from the 5' protruding end and results in only single-stranded DNA. S1 nuclease treatment removes the single-stranded DNA resulting in deleted DNA fragments for ligation and transformation.

end, as well as an adjacent 3' overhang end in the DNA. Digestion of the DNA with Exonuclease III (Exo III) will uniformly generate 5' deletions at the 5' protruding or blund end. Following ligation and transformation, the deleted reporters can be transfected into cells and their responsiveness to TGF–β can be determined (**Fig. 2**). To generate deletions with this protocol, the promoter DNA is first subcloned into the multiple cloning site of either pGL2 or pGEM (Promega) vector series, so that at least two unique restriction sites are located between the ends of the insert DNA and the sequencing primer binding site (GLprimer1 for pGL2 and SP6 or T7 for pGEM). For this purpose, it is suggested that the insert DNA would be inserted between *Nhe* I (or *Xho*I) and

*Hind*III in the pGL2 basic vector (Promega, cat. no. E1641), *Xba* I and *Bam*H I in the pGEM-7Zf(+) vector (Promega, cat. no. P2251) (*see* **Note 6**).

1. Mix 40 μL of DNA (20 μg) with 5 μL each of restriction enzymes, 15 μL of 10X restriction reaction buffer, and 85 μL H_2O. Incubate at 37°C for 3 h.
2. Check the digestion by electrophoresis of 5 μL digestion mix on a 1% agarose minigel.
3. Add 100 μL of H_2O, 250 μL of TE-saturated phenol : chloroform : isoamyl alcohol (25 : 24 : 1), vortex for 30 s and centrifuge at 12,000*g* for 3 min.
4. Transfer the upper phase to a fresh tube. Add one volume of chloroform : isoamyl alcohol (24 : 1), vortex, and centrifuge as above.
5. Transfer the upper phase to a fresh tube. Add 25 μL of 3 *M* sodium acetate and 600 μL of ice-cold 100% ethanol; mix well. Centrifuge at 12,000*g* for 5 min.
6. Carefully remove the supernatant and wash the pellet with 1 mL of 70% ethanol. Air dry the pellet.
7. Dissolve DNA in 20 μL of sterile H_2O. Mix 10 μL of the DNA solution with 6 μL of 10X Exo III buffer and 44 μL H_2O; leave at room temperature. Meanwhile, for each DNA deletion series, add 7.5 μL of S1 mix to each of 20 or 30 microcentrifuge tubes (or use a 96-well plate); leave on ice.
8. Add 300 units of Exo III to the DNA samples, mix rapidly. Incubate at room temperature for 30 s.
9. Remove 2.5 μL sample to an S1 tube on ice at 30 s intervals. Pipet briefly to mix.
10. After all the samples have been taken, move the tubes to room temperature for 30 min. Add 1 μL of S1 stop buffer to each tube. Heat at 70°C for 10 min to inactivate the S1 nuclease. Centrifuge briefly to collect the liquid.
11. Remove 3 μL of samples from each time point, run the DNA on 1% agarose gel to determine the extent of digestion.
12. Add 40 μL H_2O, 15 μL of 7.5 *M* ammonium acetate, and 120 μL of 100% ethanol; incubate at –20°C overnight.
13. Centrifuge at 12,000*g* for 10 min at 4°C. Remove supernatant and wash the pellet with 0.5 mL of 70% ethanol. Centrifuge briefly, drain the tube, and air-dry the pellet. Resuspend the pellet in 9 μL of H_2O.
14. Add 1 μL of Klenow mix to samples from each time point, incubate at 30°C for 3 min, then add 1 μL of the dNTP mix. Incubate for an additional 5 min at 37°C. Heat the samples at 65°C for 10 min to inactivate the enzyme.
15. Transfer the samples to room temperature. Add 40 μL of ligase mix to each sample; mix and incubate at room temperature overnight.
16. Thaw 50 μL of DH5α super competent cells (Gibco-BRL) on ice; add 10 μL of ligation product into 50 μL of the competent cells. Incubate on ice for 30 min.
17. Heat to 42°C in a H_2O bath for 45 s, then place on ice for 3 min. Add 400 μL of 37°C prewarmed SOC medium. Shake at 37°C for 1 h at 250 rpm.
18. Plate 200 μL of the cells on a 100-mm LB/agarose plate containing 100 μg/mL ampicillin. Incubate overnight at 37°C.
19. Screen 10–20 recombinants from each time point to select deletions with appropriate sizes for further analysis. This can be easily done by colony polymerase chain reaction (PCR) ***(33)***.

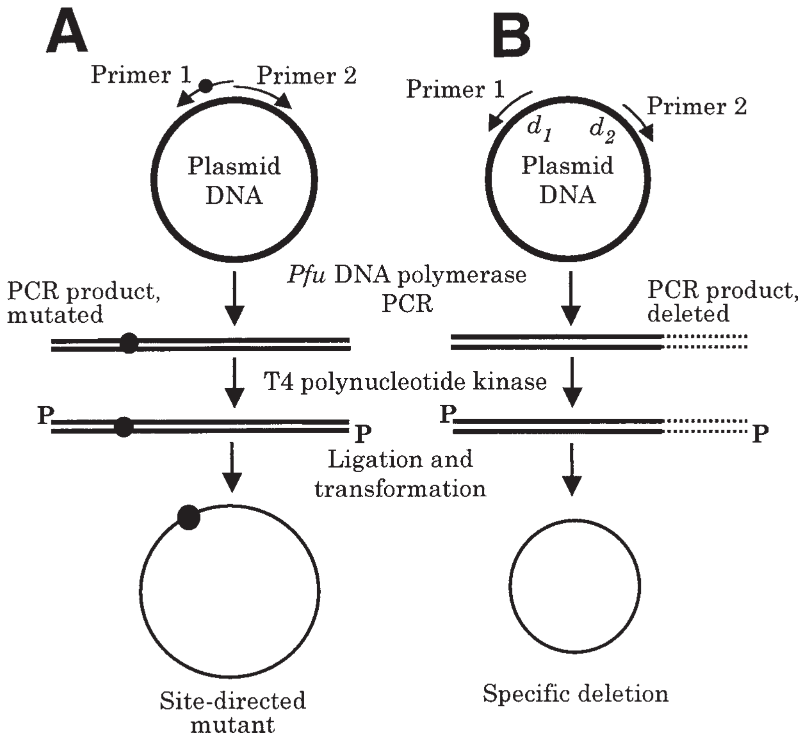

Fig. 3. Schematic diagram of site-directed mutagenesis and specific deletion assays. **(A)** Site-directed mutation. Two directly adjacent but nonoverlapping primers are used for PCR reaction. One primer should contain the site that is to be mutated. **(B)** Generation of specific deletions. After PCR amplification the prominent PCR product excludes the deleted region and contains the blunt ends for ligation.

3.5.2. Generation of Site-Directed Mutation of Promoter Region

With deletional analysis, the TGF-β responsive region within the promoter can be determined. This region generally contains several binding sites for transcription factors and can be further dissected by mutagenic analysis into specific small DNA segments, which can only be activated by TGF-β stimulation and for specific transcription factor binding. The protocol presented here is based on PCR techniques using *Pfu* DNA polymerase, which is a proofreading DNA polymerase and provides PCR product with blunt ends for intramolecular ligation. This protocol can be easily adopted for site-specific deletions of DNA without modification (**Fig. 3**). We have successfully generated specific deletions and site-directed mutations by using the protocol described here (*see* **Note 7**).

1. For insertion of mutations, two directly adjacent but nonoverlapping primers need to be synthesized. One of the primers should contain the site that has

been mutated. The primers can be dissolved in H_2O at the concentration of 20 μ*M* (20 pmol/μL).

2. Prepare a PCR reaction mixture on ice in a thin-wall PCR tube as follows:

H_2O	75 μL
10X native or cloned *Pfu* buffer	10 μL
5 m*M* dNTP	4 μL
Template DNA (10 ng/μL)	5 μL
Primer #1 (20 μ*M*)	2 μL
Primer #2 (20 μ*M*)	2 μL
Native or cloned *Pfu*	2 μL

The final volume for each reaction is 100 mL.

3. Overlay each reaction with a drop of mineral oil (Molecular Biology Reagent, Sigma, cat. no. M5940).
4. Perform PCR using the cycling conditions as provided. These conditions have been optimized for a plasmid of 5–7 kbp long in our lab. The PCR was performed using a thermal cycler manufactured by Ericomp Inc. (San Diego, CA).

No. of cycles	Temp (°C)	Duration
1	98	45 s
30	95	45 s
	55[a]	45 s
	72	14 min[b]
1	72	10 min

[a]The annealing temperature can be determined by primer T_m-5°C, however, it can be lowered further to obtain optimal results.

[b]The extension time is critical for yield of PCR product using *Pfu* polymerase. *Pfu*-based PCR amplification requires a minimun extension time of 2 min/kb of amplified template. From our experience, 14 min will produce enough PCR product from a 6.3-kbp template.

5. Check that the PCR is successful by electrophoresis of 5-μL sample on a 0.7% agarose gel.
6. Purify the specific PCR product by electrophoresis of whole PCR reaction on a 0.7% agarose gel. Cut out the DNA band of interest with a sharp scalpel and move the gel slice into a microcentrifuge tube. Try to leave as much as agarose gel possible.
7. Add 600 μL of the agarose solubilization buffer (Agarose Gel DNA Extraction Kit, Boehringer). Resuspend the silica matrix and add 20 μL of the silica matrix to the sample.
8. Incubate the mixture at 60°C for 15 min and vortex every 3 min. Make sure the gel is completely dissolved in the mixture.
9. Centrifuge for 30 s at high speed. Discard the supernatant. Resuspend the matrix containing the DNA with 500 μL of binding buffer on a vortexer. Centrifuge and discard the supernatant.

10. Wash the pellet with 500 μL of washing buffer and vortex. Centrifuge and discard the supernatant. Repeat this step once.
11. Remove all the liquid with a pipet. Air-dry at room temperature for 15 min. The matrix color turns bright white when dry.
12. Add 20 μL of DNA elution buffer; incubate at 55°C for 10 min, vortexing every 2–3 min. Transfer the suspended matrix to a SpinX column; spin at 6000*g* in a microcentrifuge at room temperature. Add 10 μL of elution buffer onto the pellet in the column; spin again. Repeat this process once.
13. Take 10 μL of the eluted DNA, mix with 2 μL of 10X phosphorylation buffer, 2 μL of 10 m*M* ATP, and 15 units of T4 Polynucleotide Kinase (Gibco or Promega). Add H_2O to 20 μL. Incubate at 37°C for 20 min.
14. Heat the phosphorylation mixture at 65°C for 10 min to inactivate the polynucleotide kinase. Centrifuge briefly to collect the liquid down to the bottom of the tube.
15. Add 1 μL of ligase to the phosphorylation mixture; mix thoroughly. Incubate at room temperature overnight.
16. Transform E.coli with 5 μL of ligation mixture as described in **Subheading 3.5.1., steps 16–18**.
17. Perform plasmid miniprep from 5–10 colonies to obtain enough DNA for sequencing.

4. Notes

1. For transient transfection with artificial liposomes, the transfection efficiency is usually lower if the transfection is carried out in medium-containing serum. If the cells cannot tolerate serum-free medium, the cells have to be tested with different transfection reagents in transfection solution containing serum. FuGENE 6 is a choice for cells that cannot grow in serum-free medium, as the transfection reagent provides even higher transfection efficiency in the presence of serum than that in serum-free medium. Transient transfection efficiency is also partly determined by the amount of liposomes used in the DNA–liposome complexes. We have found that the transfection of MvLu1; CCL64 cells requires a higher concentration of liposome reagent than the transfection of HT1080 cells.
2. We divide the luciferase reading (RLU) by the β-galactosidase to normalize for transfection efficiency. The transcription activity is calculated as "induction fold" by comparing normalized luciferase activity of TGF-β-treated cells and untreated control. In each experiment, duplicate transfections are performed. A Dual-Luciferase Reporter Assay System (Promega, cat. no. E1910) is available for the purpose of normalizing transfection efficiency. This assay system uses firefly luciferase as a reporter of the promoter construct, whereas SV40 or CMV promoters drive expression of *Renilla* luciferase used as an internal control vector. The luciferase assay with these two luciferases can be simply performed in one tube, because the firefly and *Renilla* luciferases are of distinct evolutionary origins and substrate requirements. This assay system combines the speed, sensitivity, and convenience of two luciferase reporter enzymes into single-tube, dual-reporter format.
3. Many commercial transfection reagents based on artificial liposomes are available. If one encounters difficulty in transient transfection, such as low transfec-

tion efficiency or high cellular toxicity, different transfection reagents need to be tested to obtain optimistic transfection. The transfection reagent can be prepared in one's lab (*see* **Subheading 3.4.**) or purchased from different sources. The vendors that provide transfection reagents: Tfx-50 Reagent (Promega, cat. no. E1811); Transfectam Reagent (Promega, cat. no. E1231); LipoTAXI Transfection Reagent (Stratagene, cat. no. 204110); DOSPER Liposomal Transfection Reagent (Boehringer, cat. no. 1811169); pFx-1–pFx-8 transfection reagent (Invitrogen, cat. no. T930-1).

4. If the lipids are difficult to dissolve in 100% ethanol at room temperature, the ethanol solution can be warmed up to 37°C for 5 – 10 min in a H_2O bath with the tube tightly capped. The lipid solution can be kept in the H_2O bath during preparation of the liposomes to prevent the lipids from separating from the ethanol.
5. The promoter region of a known gene can be analyzed by aligning the sequence of the promoter of interest with the sequences deposited in the transcription factor databases. Listed are the searchable World Wide Web sites; these sites also contain the links to different databases in molecular biology.

   http://www.embl-heidelberg.de/srs5: This is a network browser for databanks in molecular biology. http://transfac.gbf.de/TRANSFAC/: A transcription factor database. http://www.bionet.nsc.ru/trrd/: The Transcription Regulatory Regions Database (TRRD) is designed for the accumulation of experimental data on extended regulatory regions of eukaryotic genes and provides information regarding the modular structure of the transcription regulatory region.
6. Restriction enzymes that produce 3' overhang-end and resistant to Exo III digestion: *Aat*II; *Apa*I; *Ban*II; *Bgl*I; *Bst* XI; *Kpn*I; *Nsi*I; *Pst*I; *Sac*I. However, *Pvu*I- and *Sac* II- generated 3' overhang ends are not protected from Exo III digestion. Restriction enzymes that produce 5' protruding or blunt ends: *BamH*I; *Cla*I; *EcoR*I; *EcoR*V; *Hind*III; *Nco*I; *Nde*I; *Not*I; *Sal*I; *Sma*I; *Xba*I; *Xho*I.
7. Some transcription factors and their cognate consensus binding sites are listed below. The site-specific mutants are also listed and the mutated-sites are underlined.

Transcription factor	Consensus binding site motif	Mutant
AP1	TGAGTCA	TGAGTAG or TGAGGCA
Sp1	GGGCGGG	GTTCGGG
CREB	TGACGTCA	TGTGGTCA
NF-1	TTGGATTGAAGCCAAT	TTGGATTGAATAAAAT
NFkB	GGGACTTTCC	GCGACTTTCC
Stat3	TTCTGGGAATT	TTCTGGGCCGT
Myc-Max	CACGTG	CACGGA
E2F1	TTCGCGC	TTCGATC
Ets	GAGGAA	GAGAGA

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8

Regulation of AP-1 Activity by TGF-β

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1. Introduction

The activator protein-1 (AP-1) transcriptional complex is composed of DNA binding proteins belonging to the Jun and Fos proto-oncogene families, which play an important role in cell proliferation and transformation (reviewed in **ref. *1***). AP-1 activity is required to stimulate many genes that are induced following growth factor and cytokine treatment, and also is required for transformation mediated by oncogenes such as *ras* ***(2)***. Members of the Jun family can homodimerize or heterodimerize with each other or Fos family members, which cannot bind DNA on their own. The dimerization, which is mediated by a leucine zipper motif, allows the complex to bind to a consensus DNA sequence 5'-TGA G/C TCA-3', termed an AP-1 site. This same sequence was found in several 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive genes and, hence, is also designated a TRE or TPA-responsive element. Heterodimers can form between c-Jun and ATF-2, a member of the CREB (CRE binding) protein family, allowing the complex to bind to CRE (cAMP regulatory element) sites, which differ from an AP-1 site by one nucleotide ***(3,4)***.

The Jun family is composed of three members, c-Jun, JunB, and Jun D; the Fos family consists of c-Fos, FosB, fra1, and fra2. Even though the AP-1 family members share homology in their DNA binding domains and bind to the same sequence, different AP-1 partners display different gene-transactivating properties. c-Jun homodimers and heterodimers between c-Jun and c-Fos are potent transactivators of AP-1 sites, whereas dimers containing JunB have been shown to repress AP-1-containing promoters ***(5)***. JunB however, has been shown to strongly transactivate a cAMP-responsive promoter ***(5)*** and also promoters containing multiple AP-1 sites ***(1)***.

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Regulation of AP-1 activity is achieved both at the level of gene transcription and by posttranslational modification of existing AP-1 complexes by phosphorylation ***(6)***. Much of this regulation is mediated by the the mitogen-activated protein kinase (MAPK) family. This family consists of the extracellular-signal-regulated kinase (ERK) pathway ***(7)*** and two stress-activated pathways, the c-Jun N-terminal kinase (JNK) pathway and the p38 pathway ***(8,9)***. Transcription of the *c-fos* gene involves cooperation between the serum response factor (SRF) which is constitutively bound to the serum response element (SRE), and a ternary complex factor (TCF), such as Elk-1. Phosphorylation of the TCF increases the transcription of *c-fos*, and this phosphorylation has been shown to correlate temporally with the activation and repression of the *c-fos* gene ***(10)***. All three members of the MAPK family, the ERK, JNK, and p38 pathways, have been shown to participate in the phosphorylation of Elk-1 ***(11–13)*** which leads to increased *c-fos* gene induction. Whereas increased c-Fos protein plays a role in repression of its own promoter, c-Jun actually plays a positive role in its gene regulation. The *c-jun* promoter contains a TRE site which is recognized by a c-Jun/ATF-2 heterodimer ***(14)***. Phosphorylation of both c-Jun by JNK kinases ***(15,16)*** and ATF-2, which can be phosphorylated by both JNK and p38 kinases ***(17,18)***, leads to increased *c-jun* transcription. The promoter of the *junB* gene has recently been shown to contain several Smad binding elements (SBEs), which, when occupied by the transforming growth factor-β (TGF-β)-activated transcription factors Smad3 and Smad4, mediates upregulation of *junB* in response to TGF-β ***(19)***. c-Fos phosphorylation is mediated by Fos kinase, which has recently been identified as pp90rsk2 in PC12 cells ***(20)***. In all of these cases, phosphorylation leads to increased transcriptional activity.

Many promoters of TGF-β-regulated genes contain either AP-1 sites, such as the PAI-1 ***(21)***, TIMP-1 ***(22)***, TGF-β1 ***(23)***, c-Jun ***(24)***, α2(I) collagen ***(25)***, and apoJ/clusterin promoters ***(26)***, or CRE sites occupied by c-Jun/ATF-2 heterodimers, such as the FN ***(27)***, c-Jun ***(14)***, and TGF-β2 ***(28)*** promoters, suggesting that control of AP-1 activity is one mechanism by which TGF-β mediates gene regulation. TGF-β has been shown to influence AP-1 activity by triggering gene induction of the *c-jun*, *junB*, and *c-fos* genes in a variety of cell types. Additionally, TGF-β has been shown to activate the MAP kinase cascade leading to increased phosphorylation of c-Jun, ATF-2, and Elk-1 ***(29)***. This chapter focuses on methods to study the regulation of AP-1 activity by TGF-β, which includes a study of gene induction by Northern analysis, as well as analysis of AP-1 transcriptional activity mediated by TGF-β.

2. Materials

2.1. Northern Blot Analysis

1. TGF-β2 (Genzyme, Boston, MA) (*see* **Note 1**).
2. Kits for preparation of poly-A+ RNA {Poly(A) Pure Kit [Ambion, Austin, TX] or Oligotex mRNA kit [QIAGEN), Valencia, CA]}.

3. 10X MOPS buffer: 0.4 *M* MOPS (3-[*N*-morpholino]propanesulfonic acid), 100 m*M* sodium acetate, 10 m*M* EDTA.
4. RNA loading buffer: 170 µL 10X MOPS, 830 µL formamide, 290 µL formaldehyde (37%), 100 µL glycerol, 30 µL dye mix (1.5% Bromophenol Blue and xylene cyanol FF in dH_2O).
5. 1.2% MOPS/formaldehyde–agarose gel: 4.8 g agarose and 288 mL dH_2O are mixed and microwaved until agarose is melted, the solution is cooled to 60°C, followed by addition of 40 mL 10X MOPS and 72 mL formaldehyde (37%).
6. 20X SSC: 175.3 g NaCl and 88.2 g sodium citrate is dissolved in 800 mL dH_2O, pH is adjusted to 7.0 with 10*N* NaOH, and brought up to a volume of 1 L with dH_2O.
7. NytranPlus nylon membrane (Schleicher and Schuell, Dassell, Germany).
8. Hybridization buffer: 15 mL formamide, 3 mL 50X Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 7.5 mL 20X SSC, 1.5 mL 10% sodium dodecyl sulfate (SDS), 2.25 mL dH_2O, 750 µL of 10 mg/mL denatured salmon sperm DNA (Boehringer Mannheim, Mannheim, Germany).
9. 10% SDS: dissolve 100 g electrophoresis-grade SDS in 900 mL dH_2O, adjust pH to 7.2 with HCl, and adjust volume to 1 L with dH_2O.
10. α-[^{32}P]-dCTP, 3000 Ci/mmol (NEG/013H, NEN® Life Science Products, Boston, MA).
11. Rediprime random labeling system (Amersham, Arlington Heights, IL).
12. cDNA probes for analyis of *c-jun*, *junB* and *c-fos* messages: a 1.1 kb *Eco*RI fragment of human *c-jun* ***(2)***, a 1.5 kb *Eco*RI fragment of mouse *junB* ***(30)***, a 1.2 kb *Eco*RI/*Sal*I fragment of mouse *c-fos* ***(31)***, a 700-bp *Bam*HI/*Hind*III fragment of rat cyclophilin ***(32)***.

2.2. AP-1 Luciferase Reporter Assay

1. pAP1-Luc plasmid (Stratagene, La Jolla, CA).
2. pRL-SV40 vector (Promega, Madison, WI).
3. FuGENE 6 transfection reagent (Boehringer Mannheim).
4. TGF-β2 (Genzyme).
5. Dual-Luciferase Reporter Assay System (Promega): This kit contains both lysis and reaction buffers necessary for luciferase measurements.
6. 1X PBS (phosphate-buffered saline).
7. Microtiter plates (Dynatech, Chantilly, VA) and luminometer (ML2250, Dynatech).

2.3. GAL4-Fusion trans-Reporting Assay

1. PathDetect *Trans*-Reporting System (Stratagene): pFR-Luc plasmid containing five copies of the GAL4 UAS linked to a firefly luciferase reporter, pFA-cJun, pFA-ATF-2, and pFA-Elk1 plasmids containing the GAL4 DNA binding domain fused to each respective transactivating gene, and pFC-dbd plasmid containing only the GAL4 DNA binding domain as a negative control.
2. pRL-SV40 vector (Promega).
3. FuGENE 6 transfection reagent (Boehringer Mannheim).
4. TGF-β2 (Genzyme).

5. Dual-Luciferase Reporter Assay System (Promega): This kit contains both lysis and reaction buffers necessary for luciferase measurements.
6. 1X PBS.
7. Microtiter plates (Dynatech) and luminometer (ML2250, Dynatech).

3. Methods

3.1. TGF-β-Stimulated Induction of AP-1 mRNA

Transforming growth factor-β has previously been shown to induce the *c-jun*, *junB*, and *c-fos* genes in various cell types, regardless of whether these cells are growth inhibited or growth stimulated by TGF-β. In A549 lung adenocarcinoma cells, which are growth-inhibited by TGF-β, TGF-β causes a rapid and transient increase of *c-fos* mRNA, while the stimulation of both *c-jun* and *junB* mRNA reaches a peak at 4 h which remains elevated through 8–24 h of treatment ***(33)***. By contrast, whereas both human and mouse AKR-2B fibroblasts demonstrate a potent induction of *junB* mRNA, *c-jun* is not induced following TGF-β treatment ***(33,34)***. TGF-β treatment of AKR-2B fibroblasts results in *c-fos* induction indirectly by first mediating the expression of *c-sis* ***(35)***. TGF-β treatment of keratinocytes induces a *c-jun* message, which leads to upregulation of collagenase expression, whereas in fibroblasts, collagenase expression is repressed following TGF-β treatment as a result of strong induction of *junB* ***(34)***. The expression of individual AP-1 genes following TGF-β stimulation therefore can help to delineate how induction of other TGF-β-stimulated genes occurs. Because induction of the various AP-1 family members following TGF-β treatment appears to be cell-type-specific, it is helpful to first characterize induction of AP-1 gene expression following TGF-β treatment by Northern analysis.

1. Poly-A+RNA is prepared from cells untreated or treated with 5 ng/ml TGF-β for various times using a commercially available kit [Poly(A) Pure Kit, Ambion]. Alternatively, poly-A+RNA can be prepared from total RNA (isolated using TRIzol Reagent, Gibco-BRL) using Oligotex mRNA kits (Qiagen) (*see* **Note 2**).
2. Mix 5 μg of poly-A+RNA per time-point with five volumes of RNA loading buffer and incubate at 55°C for 15 min. Transfer the samples to ice.
3. Load samples on a prepared 1.2% MOPS/formaldehyde–agarose gel and electrophorese in 1X MOPS buffer. Gels are run at 100–140 V until the marker dyes are sufficiently separated (about 4 h). The time required to run the gel will depend on the size and thickness of the gel.
4. The gel is soaked in 3X SSC for 20 min to remove formaldehyde. Wet the membrane to be used for transfer in dH_2O, followed by equilibration in 20X SSC. Transfer is performed by capillary blotting using 20X SSC according to Maniatis et al. ***(36)*** or, alternatively, a vacuum apparatus (PosiBlot 30-30 Pressure Blotter, Stratagene) can be used for transfer.
5. Following transfer, immobilize the RNA onto the filter by ultraviolet (UV) crosslinking (Stratalinker UV Crosslinker, Stratagene).

6. The nylon filter is prehybridized in 10–20 mL of prewarmed hybridization buffer at 42°C for at least 1 h. At this time, the cDNA probe fragment is labeled by random incorporation of α-[^{32}P]-dCTP using the Rediprime kit (Amersham). The labeled probe is denatured for 10 min at 100°C and placed on ice.
7. Remove prehybridization buffer from the nylon membrane and replace with 5–10 mL of fresh hybridization buffer containing denatured probe ([1–5] × 10^6 cpm/mL [counts per minute]). Incubate in a shaking water bath at 42°C overnight (18–20 h).
8. The following washes are performed each with a volume of 150–250 mL: two washes in 6X SSC/0.2% SDS for 15 min at room temperature; two washes in 1X SSC/0.2% SDS for 15 min at 37°C. The filter should be monitored for the intensity of radioactive signal during washes. If the background signal is still high, a more stringent wash can be performed in 0.1X SSC/0.2% for 15–30 min at 55°C. The filter is then blotted to remove excess liquid, placed in Saran wrap, and subjected to autoradiography (*see* **Note 3**).
9. The probe can be removed by placing the membrane in boiling dH_2O for 5 min or can be stripped in 55% formamide, 2X SSC, 1% SDS at 65°C for 1 h. Sequential reprobing of the membrane can now be performed starting at **step 6**.

An example of Northern analysis for TGF-β-stimulated induction of *c-jun*, *junB*, and *c-fos* gene expression in the HT1080-derived cell line BAHgpt ***(37)*** is shown in **Fig. 1**. In this cell line, both *c-jun* and *junB* are potently induced by TGF-β, reaching a peak at 2 h. The *c-fos* message is constitutively expressed in this cell line, presumably the result of the presence of an activated N-ras allele, and thus does not show any induction in response to TGF-β. The cyclophilin (1B15) message is utilized here as a control for equal RNA loading, as TGF-β treatment does not alter its expression.

3.2. TGF-β-Stimulated Induction of AP-1 Activity

Traditionally, AP-1 binding activity measured by electrophoretic mobility shift assay (EMSA) has been utilized to demonstrate the binding and presumed activity of different transcription factors bound to an AP-1 promoter. This binding does not measure transcriptional activity however, as many promoters are constitutively occupied in vivo. Because the AP-1 members are subject to post-translational modifications such as phosphorylation, which alter their transcriptional activity without altering their DNA binding, it is essential to monitor AP-1 activity using an AP-1-containing reporter construct. This section describes an assay to monitor AP-1 transcriptional activity utilizing a commercially available luciferase reporter construct (pAP1-Luc, Stratagene) which contains seven tandem AP-1 repeats upstream of a TATA box that controls transcription of the luciferase reporter. This construct can indicate whether AP-1 activity is stimulated in cells by various treatments i.e., TGF-β stimulation, and can be used in cotransfection assays with exogenous genes to assess their contribution

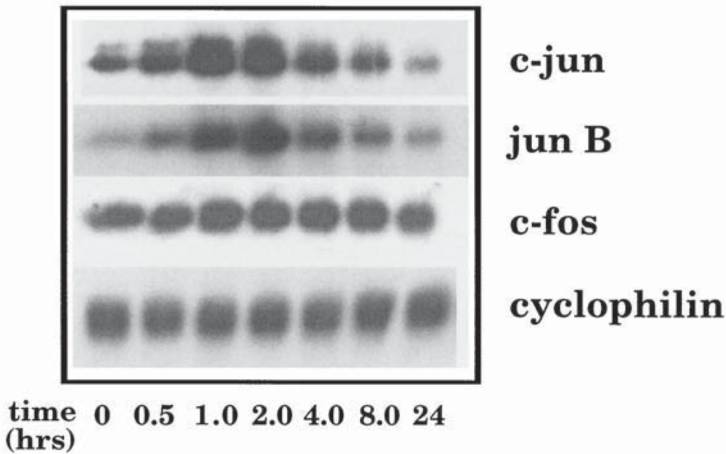

Fig. 1. Northern analysis of TGF-β-mediated induction of AP-1 family members in BAHgpt cells. Poly A + RNA (5 μg) isolated from BAHgpt cells treated with 5 ng/mL TGF-β for the times indicated was fractionated on a 1.2% MOPS/formaldehyde–agarose gel and transferred to nylon membrane. The membrane was sequentially hybridized with α-[^{32}P]-dCTP random-labeled probes for c-*jun*, *junB*, c-*fos*, and cyclophilin mRNA, with the previous probe stripped from the membrane prior to hybridization with the next probe. mRNA was visualized by autoradiography. Message sizes for the AP-1 family genes are as follows: c-*jun* mRNA appears as two transcripts at 2.6 and 3.4 kb, *junB* mRNA at 2.0 kb, and c-*fos* mRNA at 2.2 kb.

to regulation of AP-1 activity. The demonstration of the importance and activity of an AP-1 sequence in the regulation of a gene, however, requires the use of that specific gene promoter, as has been described ***(26)***.

1. Plate cells to be tested at the density of 2×10^5 cells/well in a six-well plate in serum-containing media.
2. The following day, cells are transfected with 0.5 μg pAP1-Luc (Stratagene) and 0.2 μg pRL-SV40 (Promega) per well utilizing 3 μL FuGENE 6 transfection reagent (Boehringer Mannheim) according to manufacturer's instructions (*see* **Note 4**). The pRL-SV40 plasmid, which expresses *Renilla* luciferase from a constitutive promoter, is included as an internal control to equalize for transfection efficiency.
3. The transfection is allowed to proceed overnight (18–24 h). At this time, the transfection media can be removed and replaced with fresh serum-containing media if cell toxicity is observed. Cells are now treated with 5 ng/mL TGF-β for an additional 20–24 h.
4. Cells are washed twice with 2 mL of 1X PBS/well. Cell lysates are then prepared and luciferase activity is measured utilizing the Promega Dual-Luciferase Reporter Assay System according to manufacturer's protocols. Luciferase

measurements are determined using 20 μL of cell extract in a 96-well plate on a ML2250 Microtiter Plate Luminometer (Dynatech).

5. To normalize for transfection efficiency between wells (or between different cell types), the luciferase measurement of the pAP1-Luc plasmid is divided by the luciferase measurement of the internal standard pRL-SV40 plasmid. This ratio can then be used to directly compare activities between treatments and various cell types.

Figure 2A illustrates the stimulation of AP-1 transcriptional activity following TGF-β treatment in two cell types: the HT1080-derived BAHgpt cell line and the mink lung epithelial MvLu cell line.

3.3. TGF-β-Mediated Transactivation of GAL4-Transcription Factor Fusion Plasmids

The activation of specific domains of transcription factors leading to increased transcriptional activity can be monitored using a reporting system that is based on the yeast transcription factor GAL4. The GAL4 DNA-binding domain binds and activates transcription from the GAL4 upstream activating sequence (UAS). Because the GAL4 protein and the GAL4 UAS are not expressed in mammalian cells, this system has been used to study transactivation of mammalian transcription factors. In this system, a plasmid bearing the transactivation domain of a specific transcription factor fused to the DNA-binding domain of GAL4 is cotransfected into mammalian cells along with a reporter plasmid bearing the GAL4 UAS linked to a reporter gene (**Fig. 3**). If the transactivation domain of the transcription factor becomes phosphorylated following a specific treatment, the reporter gene is now expressed and can be measured. This system is commercially available (PathDetect, Stratagene) and can be used to study the activation of c-Jun, c-Fos, ATF-2, and Elk-1 transcriptional activity. Additionally, this method can be used to study the potential transcriptional transactivation of newly identified factors and has been utilized to study the transcriptional activity of the TGF-β-stimulated Smad family of transcription factors ***(38,39)***.

1. Plate cells to be tested at the density of 2×10^5 cells/well in a six-well plate in serum-containing media.
2. The following day, cells are transfected with 1.0 μg pFR-Luc (reporter containing the GAL4 UAS), 0.2 μg pRL-SV40 (Promega), and 0.25 μg of the specific transactivator fusion plasmid per well utilizing 3 μL FuGENE 6 transfection reagent (Boehringer Mannheim) according to manufacturer's instructions (*see* **Notes 4** and **5**). The pRL-SV40 plasmid, which expresses *Renilla* luciferase from a constitutive promoter, is included as an internal control to equalize for transfection efficiency.
3. The transfection is allowed to proceed overnight (18–24 h). At this time, the transfection media can be removed and replaced with fresh serum-containing

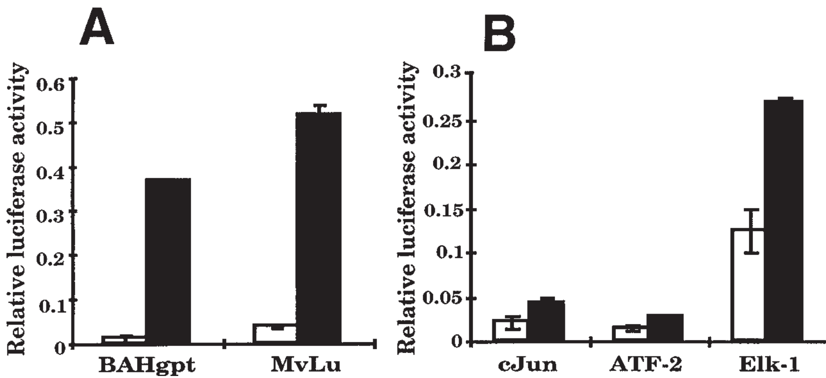

Fig. 2. Stimulation of AP-1 activity following TGF-β treatment. **(A)** BAHgpt and MvLu cells were transiently cotransfected with 0.5 μg of pAP1-Luc and 0.2 μg pRL-SV40 plasmids using 3 μL FuGene 6 (Boehringer Mannheim). After 18 h, cells were incubated in the absence (open bars) or presence of TGF-β (5 ng/mL) (closed bars) for an additional 24 h. Cells were lysed and luciferase activity determined using the Promega Dual-Luciferase Reporter Assay according to manufacturer's instructions. Luciferase activity is expressed as a ratio of specific luciferase activity divided by the luciferase activity of the internal standard. Shown is the mean ± SD of duplicates from a representative experiment. **(B)** BAHgpt were transiently cotransfected with μg pFR-Luc, 0.2 μg pRL-SV40, and 0.25 μg of either pFA-cJun, pFA-ATF-2, or pFA-Elk-1 (PathDetect Trans-Reporting System, Stratagene) utilizing 3 μL per well FuGene 6 transfection reagent (Boehringer Mannheim). After 18 h, cells were incubated in the absence (open bars) or presence of TGF-β (5 ng/mL) (closed bars) for an additional 24 h. Cells were lysed and luciferase activity determined using the Promega Dual-Luciferase Reporter Assay according to manufacturer's instructions. Luciferase activity is expressed as a ratio of specific luciferase activity divided by the luciferase activity of the internal standard. Shown is the mean ± SD of duplicates from a representative experiment.

media if cell toxicity is observed. Cells are now treated with 5 ng/mL TGF-β for an additional 20–24 h.

4. Cells are washed twice with 2 mL of 1X PBS/well. Cell lysates are then prepared and luciferase activity is measured utilizing the Promega Dual-Luciferase Reporter Assay System according to manufacturer's protocols. Luciferase measurements are determined using 20 μL of cell extract in a 96-well plate on a ML2250 Microtiter Plate Luminometer (Dynatech).
5. To normalize for transfection efficiency between wells (or between different cell types), the luciferase measurement of the pFR-Luc plasmid is divided by the luciferase measurement of the internal standard pRL-SV40 plasmid. This ratio can then be used to directly compare activities between treatments and various cell types. **Figure 2B** illustrates the stimulation of c-Jun, ATF-2, and Elk-1 transcriptional activity following TGF-β treatment in the HT1080-derived BAHgpt cell line.

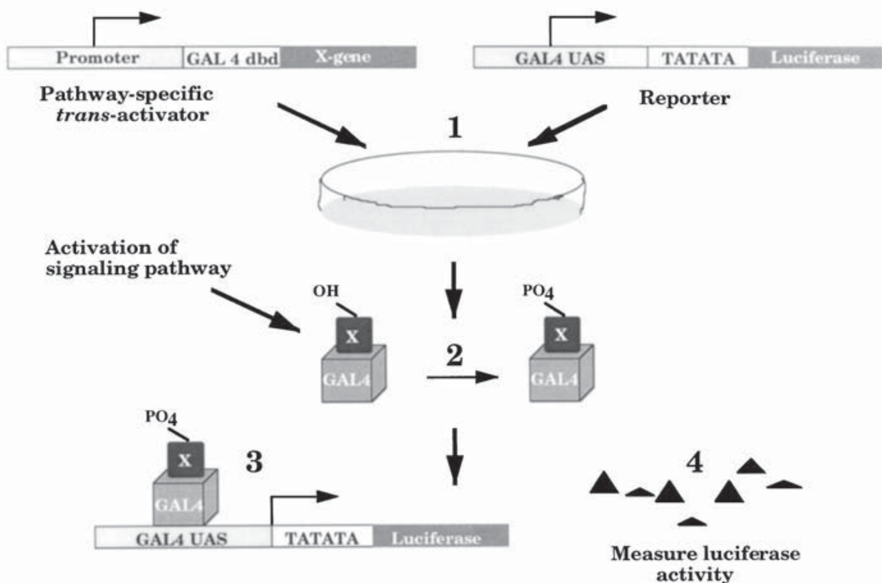

Fig. 3. The GAL4 fusion *trans*-activating system. **(1)** A fusion plasmid consisting of the transactivating domain of a specific transcription factor fused to the GAL4 DNA-binding domain is cotransfected with a luciferase reporter plasmid containing a promoter consisting of the GAL4 UAS fused to the firefly luciferase gene into mammalian cells. **(2)** The fusion transactivator protein is expressed in mammalian cells and becomes phosphorylated as a result of stimulation of a signaling pathway following cell treatment or by the expression of exogenously transfected genes. **(3)** The phosphorylated transactivator fusion protein binds to the GAL4 UAS present in the luciferase reporter plasmid and activates transcription of the luciferase gene. **(4)** Luciferase activity is measured following cell lysis.

4. Notes

1. Transforming growth factor–β2 is the isoform utilized in these experiments and was found to be equivalent to TGF-β1 in all experiments conducted.
2. It is suggested that poly-A+ rather than total RNA be used for Northern analysis because the expression of AP-1 family members is generally very low. Additionally, it becomes difficult to visualize induction of genes whose message sizes are close to the size of either 28S or 18S ribosomal RNA, which has a higher abundance in total RNA preparations than the AP-1 family genes.
3. The membrane should not be allowed to dry but should remain slightly damp. In the case that autoradiography demonstrates a high-background signal, the filter can be subjected to additional washes. Additionally, probe removal is easier and more complete if the membrane has been kept slightly damp.
4. The choice of transfection reagents is determined by the efficiency of transfection, which must be determined for each individual cell line. In our hands,

FuGENE 6 transfection reagent (Boehringer Mannheim) has proven to transfect a wide variety of different cell types.

5. The amount of specific transcription factor fusion plasmid to be transfected that yields the highest induction following cell treatment should be determined for each cell type and each construct. It is suggested that a series of transfections be conducted utilizing a range of fusion plasmid from 50–1000 ng/well, keeping the reporter plasmid at a concentration of 1.0 μg/well.

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9

Cdk Pathway: Cyclin-Dependent Kinases and Cyclin-Dependent Kinase Inhibitors

Diana M. Gitig and Andrew Koff

1. Introduction

In elucidating a novel cell-cycle pathway or mode of regulation, it will first be necessary to establish that the cell is arrested in a particular stage of the cell cycle following arrest-inducing conditions. This is achieved by staining the DNA in a population of cells with propidium iodide and then determining the cell-cycle profile of that population by fluorescence-activated cell sorter (FACS) or by measuring ^{3}H–thymidine incorporation into DNA as it is being replicated.

Second, measure the amount of cyclin-dependent kinase (cdk) kinase activity in treated and untreated cells. This can be accomplished in a G1 cell by assessing the phosphorylation of histone H1 by cdk2 and of GST-Rb by cdk4 and cdk6. These results can be correlated with the phosphorylation and expression of the various cdk substrates (i.e., Rb) as determined by immunoblotting.

If kinase activity differs from control levels, it is important to determine why. To do this, first develop the steady-state expression profile of the cyclins, cdks, and cyclin-dependent kinase inhibitors (ckis) by immunoblotting. Next, investigate the formation of cyclin–cdk complexes by performing coimmunoprecipitations. If you detect an absence of kinase activity, it will be interesting to determine whether this is due to cki binding, posttranslational modifications, aberrant localization of one of the cyclins or cdks, or other events. Finally, it may be relevant to determine whether cyclin–cdk complexes form at all. Reconstitution experiments are indispensible in answering these types of questions (*see* **Fig. 1**).

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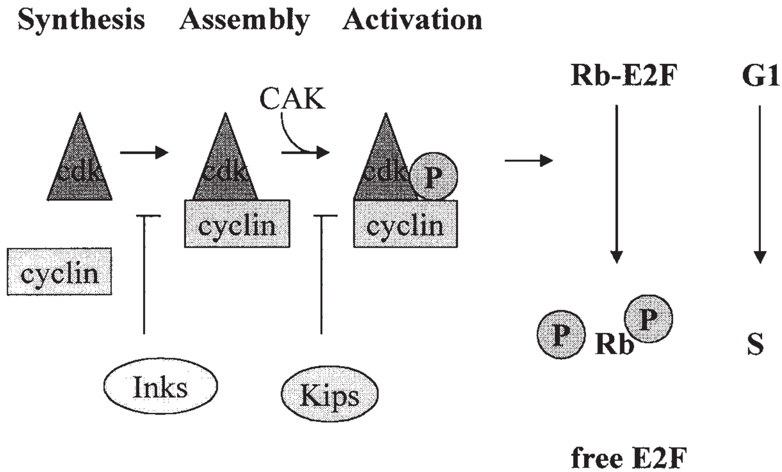

Fig. 1. Activation pathway of cyclin-dependent kinases.

Ultimately, these questions may give you an idea to the mechanism of cell-cycle arrest and a source of ideas for future directions, such as in vitro and cellular localization studies. However, if all the results indicate that the cell should not be in an arrested state, yet it clearly is in arrest, you may be on your way to elucidating a novel mechanism.

The paradigm in which this series of experiments was developed and successfully used to discover p27Kip1 is laid out in **refs. *1–4***. Some other references in which this path has been successfully followed include **refs. *5–9***.

2. Materials

1. PI stain: 10 mg propidium iodide, 100 μL Triton X-100, 3.7 mg EDTA, from Sigma (St. Louis, MO) cat. no. E5134. Add phosphate-buffered saline (PBS) to 100 mL.

   Note: Propidium iodide is a known mutagen and suspected carcinogen, so be careful with it and dispose of it properly. This solution should be stored at 4°C in the dark.
2. RNAse solution (200 units/mL): 10 mg RNAse, 5 mL PBS. Combine in a 15-mL tube. Heat to 75°C for 20 min, then cool to room temperature (RT). Store in 1 mL aliquots at –20°C for up to 6 mo; do not freeze–thaw.
3. Solution I: 10 m*M* Tris–HCl, pH 7.4, 30 m*M* NaCl, 20 m*M* $MgCl_2$.
4. Solution II: Solution I + 1% NP40.
5. NP40–RIPA: 50 m*M* Tris–HCl pH 7.5, 250 m*M* NaCl, 0.5% NP40, 5 m*M* EDTA pH 8.0, 1 m*M* phenylmethylsulphonyl fluoride (PMSF),* 50 m*M* NaF,* 3 m*M*

*These protease inhibitors should be added fresh. However, after the first wash, the buffer without them may be used.

Na_3VO_4,*10 μg/mL leupeptin,* 10 μg/mL soybean trypsin inhibitor,* 10 μg/mL aprotinin,* 10 m*M* β-glycerophosphate.*

6. 10X H1 kinase buffer: 200 m*M* Tris–HCl pH 7.4, 75 m*M* $MgCl_2$, 10 m*M* dithrothrectol (DTT) (add just before use).
7. Tween lysis buffer (approx 5 mL is needed/immunoprecipitation [IP]): 50 m*M* HEPES–KOH pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA pH 8.0, 2.5 m*M* EGTA, 1 m*M* DTT, 0.1% Tween-20, 10% glycerol, 10 m*M* β-glycerophosphate, 1 m*M* NaF,* 0.1 m*M* Na_3VO_4, 0.2 m*M* PMSF,* 10 μg/mL aprotinin,* 10 μg/mL leupeptin.*
8. 5X Rb kinase buffer: 250 m*M* HEPES–KOH pH 7.5, 50 m*M* $MgCl_2$, 5 m*M* DTT, 12.5 m*M* EGTA, 50 m*M* β-glycerophosphate.
9. TNE: 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 1 m*M* EDTA pH 8.0, 5 m*M* DTT, 1 m*M* PMSF.
10. HKM: 20 m*M* HEPES–KOH pH 7.5, 5 m*M* KCl, 0.5 m*M* $MgCl_2$. Autoclave and store in 10 mL aliquots at –20°C.
11. Transfer Buffer A: 100 mL 0.4 *M* ε-amino-*n*-caproic acid 0.25 *M* Tris base, 200 mL isopropanol, 700 mL H_2O.
12. Transfer Buffer B: 20 mL 1.25 *M* Tris base, 200 mL isopropanol, 800 mL H_2O.
13. Transfer Buffer C: 200 mL 1.25 *M* Tris base, 200 mL isopropanol, 600 mL H_2O.
14. TNT (standard = 0.05% or 1X): 150 m*M* NaCl, 25 m*M* Tris–HCl, pH 7.5, 0.05% Tween-20.
15. TMD: 20 m*M* Tris–HCl, pH 7.5, 7.5 m*M* $MgCl_2$, 1 m*M* DTT.
16. ATP regenerating system: 3 m*M* ATP from a 0.3 *M* stock in H_2O, 0.04 mg creatine phosphokinase from a 2-mg/mL stock in 50% glycerol, 0.04 *M* phosphocreatine from a 0.4 *M* stock in H_2O. All working stocks should be stored at –20°C; long-term storage is at –80°C.

3. Methods

3.1. Propidium Iodide Staining

Propidium iodide (PI) stains nucleic acids, and thus after RNAse treatment is used to determine the DNA content of a given cell. This allows you to determine the percentage of cells in each phase of the cell cycle in a population. G1 cells have a 2n DNA content, G2/M cells have a 4n DNA content, and S phase cells have an intermediate amount. Apoptotic cells, which degrade their DNA, have a content less than 2N; and cells in which M has been uncoupled from S and cells exhibiting genomic instability for other reasons may have DNA contents exceeding 4N. The cells in the different phases of the cycle are determined by flow cytometry on a fluorescence activated cell sorter. Propidium iodide can be used on either whole cells or nuclei. The following protocol is for staining of whole cells from suspensions and was adapted from the *Becton Dickinson Sourcebook*, section 1.11 ***(9a)***.

1. Chill PBS, methanol, and samples on ice.
2. Prepare single-cell suspensions of samples in ice-cold PBS. To accomplish this, look at the cells under the microscope after trypsinization to make sure there are no clumps.

3. Gently mix each suspension and count in a hemacytometer or Coulter counter. Adjust the concentration to (1–2) × 10^6 cells/mL with ice-cold PBS.
4. Transfer 1 mL of each sample to a labeled 12 × 75-mm test tube.
5. To fix the cells, add cold methanol or ethanol dropwise from a Pasteur pipet while gently mixing the cells on a vortex on setting 4. Add 2 mL of methanol to 1 mL of the cell suspension.
6. Incubate the tubes on ice for at least 30 min. Samples will remain stable for up to 1 wk at 4°C in this methanol solution.
7. Centrifuge at 300*g* for 5 min. Aspirate the supernatant with a Pasteur pipet.
8. Add 500λ of PI stain solution to each tube and vortex gently to resuspend. Add 500λ of thawed RNAse solution (final concentration = 100 units/mL) and vortex to mix.
9. Incubate at RT for 30 min in the dark.
10. Filter samples through 35-mm nylon mesh.
11. Keep samples at 4°C in the dark until ready to analyze; analyze on a flow cytometer within 1 h after staining. Propidium iodide fluoresces primarily at wavelengths above 610 nm.

3.2. Alternative Protocol: Nusse Nuclear Preparation for Cell-Cycle Analysis (DNA Content)

Nuclear staining has the advantage of producing “sharper” results (i.e., lower coefficient of variation on the G1 peak) than whole-cell staining.

1. Trypsinize and harvest cells. Spin down.
2. Wash cells in PBS and spin down. Aspirate dry.
3. Resuspend 5 × 10^5 – 1 × 10^6 cells in 250λ in solution I, pipetting up and down gently.
4. Incubate on ice for 15 min.
5. Add an equal volume of solution II, pipetting up and down gently. Now you have nuclei.
6. Add 5λ 10 mg/mL RNase A.
7. This can be stored at 4°C for 2–3 d before analysis. Transfer to FACS tubes and add 500λ PI solution.

3.3. ^{3}H-Tdr Incorporation Assay

Tritiated thymidine is incorporated into DNA that is being replicated. Thus, it is often used in combination with PI staining to ascertain what percentage of a cell population is in S phase or to confirm that cells are growth arrested. Unlike PI staining, however, tritiated thymidine incorporation can be measured entering a time-course, indicating that certain cells (treated, mutant, etc.) are now traversing the S phase faster or slower than controls. The following protocol has been optimized for exponentially growing mouse embryonic fibroblasts. For caveats, *see* **Note 1**.

1. Seed 1.5 × 10^5 cells/well in 3 ml in a six-well TC plate; let grow overnight. Note that (3–5) × 10^5 cells is the optimal number for ^{3}H-Tdr labeling; the number of cells seeded per well should be adjusted according to cell doubling time.
2. Prepare 0.5% ^{3}H-Tdr labeled media.

3. Aspirate media and add 1 mL labeled media to each well or dish. Incubate at 37°C for 2h.
4. Aspirate media and wash 3X with PBS.
5. Add 700λ 1X trypsin (stock = 10X, Gibco-BRL [Grand Island, NY] cat. no. 15400-054) to each well. Count the cells so the amount of ^{3}H-Tdr incorporated per cell can be calculated later.
6. Add 800λ cold 10% TCA to harvested cells; vortex and let tubes sit on ice for 30 min. TCA (trichloroacetic acid) preferentially precipitates DNA and RNA polymers over free nucleotides.
7. Spin at 4°C for 15 min.
8. Wash with 1 mL cold 10% TCA and spin at 4°C for 15 min.
9. Resuspend pellet in 200λ 2 *M* NaOH.
10. Incubate in a 65°C water bath for 10–20 min.
11. Add to scintillation vial containing 3 mL scintillation fluid; vortex well and let sit at RT for 30 min.
12. Count in a scintillation counter.

3.4. H1 Kinase Assays

As the cyclin–cdks that propel the cell through the cell cycle are highly regulated enzymes, it is often essential to measure their activity (or lack thereof). Histone H1, one of the proteins important in maintaining the structure of chromatin, is the substrate most commonly used to measure cdk2 activity in vitro, although it may not be a substrate for these kinases in vivo. H1 kinase assays can be preceded by an anti-cyclin A IP or anti-cyclin E IP to measure the amount of activity associated with those cyclins, or an anti-cdk2 IP to measure total cdk2 activity. Because relative kinase activities are often being compared (i.e., in differently treated cells), the linearity of this assay is important and must be determined empirically by titrating the extract against a constant amount of antibody. The linear range is the range in which the kinase activity increases proportionally to the amount of extract used.

1. Lyse cells in NP40–RIPA (Radio Immunoprecipitation Assay).
2. Immunoprecipitate (*see* **Subheading 3.9.**).
3. After two washes of the protein A–Sepharose pellet with NP40–RIPA, wash four times with 900λ H1 kinase buffer.
4. For each reaction, mix the following:
   5λ 10X H1 kinase buffer
   5λ 0.3 mM ATP–lithium salt pH 7.0 (BMB [Roche Molecular Biochemicals, Indianapolis cat. no. 1140965; stock is 100 m*M*)
   0.5λ histone H1 (BMB cat. no. 223549; 4 mg/mL in H_2O; store at –20°C.)
   1λ γ–^{32}P-ATP (3000 Ci/mmol)
   38.5λ dH_2O
   Mix well and let sit on ice.

   In our experience, BMB produces the best available and most reproducible batches of histone H1.

5. Pellet the protein A–Sepharose from the final H1 kinase wash in a microfuge at 4°C for 3–5 s. Aspirate the supernatent.
6. Add 45λ of the above kinase assay reaction mix.
7. Incubate at 37°C for 30 min. To keep the protein A–Sepharose in suspension, flick the tubes at 7, 15, and 22 min into the incubation.
8. Microfuge 4°C for 30 s.
9. Add 15λ 4X sample buffer, boil 5 min, microfuge and load 20λ of the supernatent onto 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Run the gel for five minutes after the dye front runs off. Histone H1 is approximately 34 kDa and runs as a doublet.
10. Stain the gel with Coomassie for 20 min and then destain overnight. This serves a dual purpose; it both fixes the gel to eliminate background and demonstrates that equal amounts of substrate were loaded in each lane. Then, dry and expose to X-ray film at –80°C with an intensifying screen for desired length of time and/or expose the gel to a phosphorimager screen if the signal is to be quantitated.

3.5. Rb Kinase Assays

It is difficult to detect cdk4,6 activity in most cells. The substrate of choice is Rb. Rb and its family members are important substrates of cyclin D/cdk4,6 in vivo. In these kinase assays, the substrate is generally either purified GST-Rb or an Rb peptide available from Santa Cruz Biotechnology, Santa Cruz, CA; (cat. no. sc4112). This peptide lacks the pocket domain of Rb, so it is not the ideal substrate for measuring cdk6 activity. If the information being sought is the phosphorylation state of Rb, and not the level of cdk4/6 activity, this should be ascertained by Western blot.

1. Lyse the cells in Tween–lysis buffer.
2. Immunoprecipitate as usual, but use Tween–lysis buffer rather than NP40–RIPA.
3. After incubation with protein A–Sepharose, wash the pellet 4X in Tween–lysis buffer and 2X in 1X kinase buffer.
4. Before aspirating the sup of the final wash prepare the reaction mix:
   For each reaction:
   6λ 5X kinase buffer
   5μg GST-Rb or 0.5λ Rb peptide
   5λ 0.3 m*M* ATP-lithium salt pH7.0 (BMB cat. no. 1140965; stock is 100 m*M*)
   1λ γ^{32}P-ATP
   dH_2O to 30λ
5. Aspirate the sup of the final wash completely with a gel loading tip. Resuspend the pellet in 30λ of the ice-cold reaction mix.
6. Incubate at 30°C for 30 min, mixing every 10 min.
7. Microfuge and add 10λ 4X SDS–sample buffer.
8. Boil for 5 min, microfuge, and run 20λ on 10% SDS-PAGE.
10. Stain the gel with Coomasie for 20 min and then destain overnight; then, dry and expose to X-ray film at –80°C with an intensifying screen for desired length of time and/or expose the gel to a phosphorimager screen if the signal is to be quantitated.

3.6. Purification of GST-Rb

The trick to good GST-Rb is concentration. The more concentrated it is, the better it stores. *See* **Note 2**.

1. Inoculate a single colony of pGEX-Rb in BL21-DE3 cells in a 1-L flask containing 200 mL LB/Amp. Shake at 37°C overnight.
2. Inoculate 50 mL of this overnight culture into each of four 2 L flasks containing 450 mL LB/Amp and shake at 37°C until absorbance as measured by (OD_{600}) of each culture is 0.5–0.65 (2–4 h).
3. Remove 1 mL of the uninduced culture to run on your gel later. Add IPTG to 0.2 m*M* (add 1 mL of a freshly made 100 m*M* stock to each 500 mL culture) and shake at 37°C for 3 h.
4. Spin 4000*g* at 4°C (in a Sorvall H6000A rotor [Kendro Laboratory Products, Newtown, CT] or equivalent) for 15 min and resuspend the pellet in 40 mL cold PBS. Transfer to 50-mL Falcon tubes.
5. Spin at 1924*g* in tabletop centrifuge (Sorvall RT6000D; rotor H1000B) at 4°C for 15 min. Discard the supernatent and store the pellet at –80°C overnight.
6. Pre-equilibrate 1.5 mL glutathione–agarose slurry (Sigma cat. no. G9761) in TNE + 0.3% Tween in a 50-mL Falcon tube by rocking at 4°C overnight.
7. Rususpend the frozen pellet in 15 mL TNE on ice.
8. Add 10 mg lysozyme (200λ of a 50-mg/mL stock) to resuspended pellet and let sit on ice for 30 min; vortex occasionally.
9. Add 150λ of a 2-mg/mL stock of DNaseI and of 1 *M* $MgCl_2$. Let sit on ice for 20 min; viscosity should decrease.
10. Add 465λ of a 10% stock of Tween-20 to yield a final concentration of 0.3%. Transfer the suspension to a 200-mL conical tube that has had its top cut off. You can cut these tubes by heating a metal spatula in a Bunsen burner and slicing through the plastic. Using a large tube enhances the efficiency of the sonication by increasing the surface area.
11. Sonicate using an internal probe for 45–60 s, then let rest on ice for an equal amount of time. Repeat this 3X. As cells are lysed, the solution should darken in color.
12. Transfer the lysed cells to 50-mL centrifuge tubes and spin at 15,000*g* at 4°C for 20 min in a Sorvall SS-34 rotor.
13. Save the supernatent and dilute it to 30 mL with TNE + 0.3% Tween.
14. Add this 30 mL of lysate to the 1.5 mL of pre-equilibrated glutathione–agarose beads in a 50-mL Falcon tube and rotate at 4°C for 3–5 h.
15. Spin down the beads (11,000 rpm at 4°C for 3–4 min in the tabletop centrifuge) and resuspend them in 10 mL TNE + 0.3% Tween. Pipet this suspension into a Bio-Rad (Hercules, CA) poly-prep column (cat. no. 731-1550). Let the beads settle in the cold room for about 30 min.
16. Let the buffer drain and wash the column with 25–30 mL TNE + 0.3% Tween. Next, wash with 25–30 mL 1X kinase buffer. Again, this is all done in the cold room (4°C).
17. Elute with 1X kinase buffer + 0.1% Tween and 20 m*M* glutathione. Adjust the pH of the elution buffer to 7.5–8.0 with 3 *M* KOH.

18. Use 0.7–1 mL of elution buffer to "elute" the void volume of wash buffer, and then start collecting 2-mL fractions. Keep the eluted fractions on ice. Immediately quantitate against bovine serum albumin (BSA) standards using the Bradford protein assay. The more concentrated the prep is, the longer it will remain stable at –80°C. Aliquot the most concentrated fraction into smaller volumes before storing at –80°C.
19. Use 5 μg of GST-Rb as the substrate per kinase assay.

3.7. Preparation of Cell Extracts in "HKM"

The lysis method and lysis buffer used to make cell extracts depend both on the type of cells and the purpose of the extracts. The following protocol lyses whole cells in a hypotonic lysis buffer, and the resulting extracts can be stored at –80°C for years. This protocol was designed to prepare extracts for immunoblots and reconstitution experiments, in which samples are normalized by mass. In contrast, samples for immunoprecipitations and kinase assays are generally normalized by cell number in our lab. In cell-cycle research, this may be an important distinction, as cells in G2 have approximately twice as much mass as cells in G1. Thus, it is not ideal to use HKM extracts for immunoprecipitations or kinase assays.

1. If cells are adherent, trypsinize them. Collect the cells in ice-cold complete media (5 mL per 150-mm plate).
2. Collect by centrifugation at 1000*g* at 4°C for 5 min.
3. Resuspend cell pellet in 10 mL cold PBS and transfer to a 15-mL Falcon tube.
4. Collect by centrifugation at 1000*g* at 4°C for 5 min. Wash 2 more times with PBS.
5. Resuspend cells in 1 mL cold PBS and transfer to an Eppendorf tube with a 1-mL pipetman. Cut off the end of the blue tip to make a larger hole so the cells do not get sheared.
6. Collect the cells in a microfuge at 4°C.
7. Aspirate the supernatant and approximate the volume of the cell pellet by comparing it by eye to a known volume in another Eppendorf tube. Be conservative; it is preferable to underestimate the volume of lysis buffer and end up with more concentrated lysates. Ideally, lysates should be between 10 and 25 mg/mL.
8. Add 1.2 volumes of HKM buffer.
9. Adjust to 2 m*M* PMSF (based on the volume of HKM buffer) from a 0.1-*M* stock prepared in EtOH.
10. Adjust to 0.5 m*M* DTT (based on the volume of HKM buffer) from a 0.2-*M* stock that has been stored at –20°C.
11. If the cells were trypsinized, add 1 μg/mL of soybean trypsin inhibitor from a 10-mg/mL stock.
12. Vortex quickly to resuspend the cell pellet; wrap the top of the tube in parafilm so that nothing spills.

13. Sonicate at 20–30% power using a cuphorn sonicator/550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) filled with ice H_2O using the following Guidelines:

1–200 μL total volume	2.0 min
2–300 μL	2.5 min
3–400 μL	3.0 min
4–700 μL	4.0 min
>700 μL divide in half	

Sonication is performed in 1-min intervals with cooling on ice for 30 s in between.
14. Pellet the cellular debris at 13,000*g* at 4°C for 10 min.
15. Transfer the supernatent to an ultracentrifuge tube, measure the volume of the supernatant, and adjust to 0.1 *M* NaCl.
16. Clarify the extract by ultracentrifugation at 100,000*g* at 4°C for 10 min.
17. Determine the protein concentration by Bradford assay, aliquot the supernatent, and store at –80°C.

3.8. Western Blotting

Western blotting, or immunoblotting, is a quick and reliable means of determining the steady-state levels of proteins expressed in a cell. ECL, the detection method most commonly used in immunoblotting, is nonquantitative; however, it can yield relative measurements. For example, a given protein may be present in control cells but absent in treated cells. It is essential to remember that it is the steady-state level of protein that is being determined. Thus, if a decrease in protein level is observed, the blot cannot tell you if the decrease is the result of increased degradation or decreased transcription or translation. The mechanism behind the observed decrease must be ascertained by a different assay. For cell-cycle analysis, the accumulation of cyclins, cdks, ckis (Inks and Cip/Kips), Rb family members (to see their phosphorylation state), and cdc25 should be determined. For practical hints, *see* **Notes 3** and **4**.

1. Run protein gel according to *Current Protocols in Molecular Biology*, Chapter 9 ***(10)***. Samples and markers should be in 4X sample buffer, heated to 100°C for 3 min and then microfuged for 3 min before loaded onto gel.
2. Cut Whatman 3MM paper to the size of the resolving gel. For a minigel consisting of 4.5 mL resolving gel solution and using 1-mm spacers, this is 8.5 × 5.5 cm. You need 15 papers per gel.
3. Cut a piece of immobilon membrane (Millipore [Bedford, MA] cat. no. PIPVH00010) to this same size. Make sure to wear gloves, and even so, do not touch the membrane even with gloved fingers — use flat-tip forceps.
4. With a blue or black ballpoint pen, mark the lower right corner of the membrane with the antibody with which you are blotting, your initials, or anything else to help you identify it.
5. Place five large weigh boats (or tip boxes) on your bench and partially fill with methanol, H_2O, transfer buffer A, transfer buffer B, and transfer buffer C.
6. Using flat-tip forceps, place the membrane in methanol for a few seconds to wet the hydrophobic surface. Then, submerge completely in water for at least 5 min.

7. Submerge the wet membrane in transfer buffer B for at least 5 min.
8. Place six sheets of Whatman paper in transfer buffer A, three sheets of Whatman paper in transfer buffer B, and six sheets of Whatman paper in transfer buffer C.
9. Stack as follows on flat Saran wrap, avoiding air bubbles between layers:
   Six sheets of buffer A
   Gel
   Membrane with the marked side facing gel (the marking will now be in the lower left corner)
   Three sheets of buffer B
   Six sheets of buffer C

   The best way to do this is to wet your bench and then put Saran wrap down so it sticks and makes a flat surface. When removing the papers from the buffers, slightly squeeze them with gloved fingers to remove excess liquid. After placing papers down, flatten by running gloved fingers over them and then rolling the wide end of a Pasteur pipet over them to squeeze out any air bubbles.
10. Invert the stack onto the semidry blotter. The membrane is now below the gel, as proteins will transfer toward the (+) from the gel onto the membrane.
11. Close the blotter and connect the leads. Transfer at constant current, 0.8 mA/cm^2, for 1.5 h. For a minigel with the above dimensions, this is 34 mA.
12. Remove the membrane and place into 0.05% TNT; agitate gently.
13. The gel may be stained with Coomassie or discarded, as desired. Wipe down both sides of the semidry blotter with a wet paper towel. It is normal for some black color to come off on the paper towel, but do not try to remove all of it and wipe the blotter "clean." Place an open paper towel inside the blotter before closing it up.
14. Decant the 0.05% TNT from the membrane and replace with 50–150 mL of 5% nonfat milk in 0.05% TNT. Agitate 45 min to overnight at RT. If leaving this overnight, add 0.02% NaN_3 from a 10% stock to the milk solution to prevent bacterial growth.
15. Decant and wash membrane 2X 15 min in 0.05% TNT.
16. Transfer membrane to a Falcon 1012 dish containing 15 mL of primary antibody probe solution; incubate, agitating at RT for 1 h to overnight. The optimal conditions (dilution, percentage of Tween-20 in TNT) for each antibody must be determined empirically. The primary antibody solution can be stored at 4°C and reused several times if 0.02% azide is added. In our experience, the following conditions have worked:

cyclin A sc-596	1 : 1000 TNT	cdk2 sc-163	1 : 1000 TNT
cyclin E sc-247	1 : 1000 0.5% TNT	cdk4 sc-260	1 : 1000 1% TNT
cyclin D1 CC11 (Calbiochem, La Jolla, CA)	1 : 1000 TNT	cdk6 sc-177	1 : 1000 TNT
cyclin D2 sc-452	1 : 500 0.5% TNT	p21 sc-6246	1 : 1000 TNT
cyclin D3 sc-453	1 : 500 0.5% TNT	p27 sc-528	1 : 1000 TNT
Rb 14001A (Pharmingen, San Diego, CA)	1 : 1000 0.1% milk in TNT		

   All antibodies are from Santa Cruz Biotechnology unless otherwise indicated.

17. Rinse the membrane and then wash it 3X 20 min with the percentage of TNT used for the primary antibody incubation.
18. Transfer membrane to a Falcon 1012 dish containing 15 mL of the appropriate secondary antibody probe solution diluted at 1:10,000 in 0.5% TNT.
19. Incubate on rocker at RT for 30 min to 1 h.
20. Wash 3X 15 min in 0.5% TNT.
21. Discard secondary antibody probe solution. For ECL, it is best to put two pieces of Saran wrap on your bench: one for adding ECL and the other for wrapping the membrane for film exposure.
22. Immediately before use, thoroughly mix equal amounts of ECL reagents 1 and 2; 2 mL of each per blot.
23. Remove membrane from wash buffer and shake off excess liquid, but do not dry. Place it marked side up on one of the pieces of saran. Completely cover the blot with 4 mL ECL solution and let sit 1 min.
24. Remove membrane from ECL and shake off excess liquid, but do not dry. Place it marked side down on the other piece of Saran wrap completely and expose to film. ECL stays good for about 1 h, so multiple exposures can be taken; and although many bands show up in 10–30 s, others do not come up for 10–20 min. Note that ECL's intensity diminishes with time.

3.9. Immunoprecipitation

There are two main reasons to immunoprecipitate (or IP) a protein: first, to isolate it away from other components of a mixture, such as a cell extract or chromatography fraction; and second, to see what, if anything, is bound to the protein. This latter technique is often referred to as a co-IP and is particularly relevant in cell-cycle analysis, where one wants to ascertain whether cyclin–cdk complexes are forming at the proper time and in the proper abundance and if they have inhibitors or anything else bound to them. Although the strength and specificity of the antibody–antigen interaction ensures that immunoprecipitation is a sensitive means of isolating proteins, the IP does not provide any way to detect the protein(s) that are immunoprecipitated; another technique must be employed for this detection. Traditionally, cell extracts were metabolically labeled with ^{35}S-methionine or ^{32}P before the IP, so the immunoprecipitated protein(s) could be detected by autoradiography. Other ways of visualizing the immunoprecipitated protein(s) include functional enzyme assays and Western blotting. In a co-IP, one binding partner (i.e., the cyclin) may be immunoprecipitated and the other (i.e., the cdk) may be immunoblotted to examine the complex formation.

An important thing to take into consideration before beginning an IP is the reason you are doing the experiment. Do you want to measure kinase activity or the strength of an inhibitor? If you are looking for complexes, are they strong or weak complexes? It is essential to know these things before starting the IP,

as the type of information you are seeking will determine many parameters of the experiment: the lysis buffer and method used to make the extracts; the amount of starting material and antibody used; and the detection method that will subsequently be employed.

Another reason to do an IP is to deplete an extract of a particular protein. In a depletion experiment, the pellet containing the immune complex is discarded and the remaining supernatant is retained. Depletions are done in order to study the effect the absence of a protein may have on a cellular process, or to confirm a protein's function by demonstrating that the loss of the protein results in the loss of that function. Generally, two to three sequential IPs are used to "completely" deplete a protein from an extract, for example, when the antibody is in excess, each round should deplete approximately 90% of the protein present. Of course, it must be remembered that anything bound to the protein of interest will be depleted as well.

1. Generally, 100λ volume is used. A very rough guideline is 5×10^6 to 5×10^7 cells, or 200–1000 μg extract, per IP. A commonly used buffer is NP40–RIPA.
2. Add 10 μg of the desired antibody, mix, and let sit on ice for 1 h.This corresponds to 10λ of most Santa Cruz antibodies. Again, the optimal amount of antibody must be determined empirically, depending on the amount of starting material and the purpose of the experiment. The antibody should be in excess of the antigen. To ensure that this is the case, titrate the antibody against a constant amount of antigen (i.e., extract) and the extract against a constant amount of antibody; when the signal no longer increases, the antibody is in excess.
3. Add 100λ protein A–Sepharose slurry equilibrated 4X in the lysis buffer used; rotate at 4°C for 45 min. When purchased, protein A–Sepharose is in ethanol to prevent bacterial growth. To equilibrate it, transfer 400 μL of the slurry to an Eppendorf tube and allow it to settle naturally. Aspirate the supernatant and replace it with 1 mL of lysis buffer. Make sure the beads are completely resuspended and again allow them to settle naturally. Repeat this process three times. This can be done during the hour when you are IPing. Never vortex this slurry, as this will break the beads into smaller particles called fines that will interfere with your experiment. For the same reason, try to pipet it as little as possible; if it needs to be resuspended, invert the tube by hand. Some antibodies bind to protein G better than to protein A. To determine which you need, refer to **ref. *13*** (Table 15.2, p. 168).
4. Microfuge at 4°C for 3 s. Aspirate the supernatant and wash the beads with 1 mL of lysis buffer. Make sure the beads are completely resuspended. Microfuge again at 4°C for 3 s, and wash twice more.
5. If proceeding on to a Western blot or autoradiography, wash once with Tris, $MgCl_2$, and DTT, then resuspend the beads in 20λ 4X sample buffer, boil 5 min, and load onto SDS-PAGE. If proceeding on to a kinase assay, *see* **Subheadings 3.4.** or **3.5.**

3.10. Reconstitution Assays

p27Kip1 was identified using this technique as described in **ref.** ***2***. It can be used to measure the ability of a particular extract—a cell type, or phase of the cell cycle, or drug-treated cell, or fraction of an extract—to activate, inhibit, degrade, assemble, or stabilize an exogenously added protein or proteins. Once the activity is identified in an extract, it can then be isolated, purified, and cloned. Conversely, the exogenously added protein can "rescue" an activity impaired in the extract by permitting its occurrence, thereby determining what is blocking that event in the extract. *See* **Note 5**.

1. Prepare whole-cell extracts in hypotonic lysis buffer. Alternatively, cytoplasmic extracts prepared by Dounce homogenization can be used.
2. To 50 μg of extract, add the desired combination of cyclins, cdks, inhibitors, and/or other proteins in the form of Sf 9 cell lysates, purified proteins, or rabbit reticulocyte lysates. Alternatively, different extracts (i.e., in different phases of the cell cycle) can be mixed in different ratios to determine which is dominant.
3. Incubate at 37°C for 30 min with ATP and an ATP–regenerating system.
4. Adjust conditions to NP40–RIPA.
5. Proceed onto immunoprecipitation, kinase assay, and/or Western blot, as desired.

4. Notes

1. A major caveat of ^{3}H-Tdr incorporation is that it cannot distinguish between a change in the *rate* of cells traversing the S phase and a change in the *number* of cells traversing the S phase. Thus, it is often combined with either PI staining and FACS or with BrdU staining. Like tritiated thymidine, BrdU (bromodeoxyuridine) is incorporated into DNA that is being replicated. However, it can be used to label individual cells, rather than a population, and thus it can give a sense of the number of cells in a population that are in the S phase. Follow manufacturer's instructions for BrdU labeling; we have achieved good results with Boehringer BrdU.
2. Most problems with purified GST-Rb are due to low concentrations. Although it is not clear why, dilute GST-Rb does not fare well during long-term storage. The protein should be at least 1.5 mg/mL to stay stable for up to six months at –80°C.
3. The key thing about a Western blot is optimizing the signal-to-noise ratio. This can be accomplished by modifying a number of steps: blocking, washing, and both primary and secondary antibody incubations. To enhance a weak signal, you can increase the amount of primary antibody and/or use the primary antibody in the blocking solution (after the membrane has been blocked). You can alter the stringency by altering the percentage of Tween-20 or the concentration of NaCl in the primary antibody solution, or by incubating the primary antibody in blocking buffer. Additionally, although milk is standard, you can also try other blocking agents like 3% cold-fish (telostatin) gelatin or BSA. When possible, it is advisable to run a positive control. The ideal is the expected amount of a recombinant form of protein being blotted for; in this way, the identity of your band is

confirmed, and if the film is entirely blank, it probably indicates that either the primary or secondary antibody is bad.

4. As there are two potentially overnight steps, Western blots are flexible and can be planned around your schedule. To get a quick result, a blot can be finished in 1 d; if the gel is loaded first thing in the morning, the blocking step is 45 min to 2 h and the primary antibody incubation is only for 1–2 h. Alternatively, both the block in milk and the primary antibody can go overnight. Normally, though, when only one of these steps is going overnight, it is best to plan which will be based on the strength of the anticipated signal and background levels as discussed earlier.
5. If the extract is limiting, an alternative method to reconstitution assays is to IP straight out of the extract and add the exogenous proteins to the washed immune complex.

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10

Activation of the Mitogen-Activated Protein Kinase Pathway by Transforming Growth Factor-β

Jianbo Yue and Kathleen M. Mulder

1. Introduction

The mitogen-activated protein kinase (MAPK) superfamily includes three major subfamilies: extracellular signal-regulated kinase (Erks), c-Jun-N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), and p38 kinases ***(1–3)***. Activation of MAPK requires the phosphorylation of both tyrosine (Y) and threonine (T) residues in a conserved motif: TEY for Erks, TPY for SAPKs, and TGY for p38 ***(1–3)***. These MAPKs, in turn, activate downstream substrates by phosphorylating a minimum consensus target sequence of Ser/Thr-Pro ***(1,2)***.

In our laboratory we have shown that transforming growth factor-β (TGF-β) can activate the cytoplasmic cell signaling proteins Ras ***(4)***, Erks1 and 2 ***(5,6)***, and SAPK/JNK ***(7–9)***. TGF-β resulted in a rapid activation of these signaling components within 3–10 min of treatment with TGF-β in several epithelial cell types and tumor cells ***(4–9)***. The rapid activation of these proteins indicates that their activation is a direct effect of TGF-β. Moreover, Ras is required for TGF-β-mediated Erk and JNK activation in intestinal epithelial cells (IECs) (***9***; unpublished data). We have also demonstrated that the MAP and Erk kinase 1 (MEK1) inhibitor PD98059 can block the ability of TGF-β to activate Erks1 and 2 (***8***; unpublished data). Further, we have demonstrated that there is not only a tight correlation between TGF-β–mediated MAPK activation and growth inhibition ***(9)***, but also a requirement for Ras activation in order for TGF-β to upregulate the Cdk inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ ***(10)***. Collectively, the data from our lab ***(4–10)*** and other labs ***(11–14)*** have indicated that MAPK subfamily members play an important role in the signaling of TGF-β responses. However, the mechanism of TGF-β regulation of these MAPK members is still not clear.

Here, we describe a method that has been successfully used in our laboratory to detect TGF-β-mediated Erks1 and 2 and SAPK/JNK activation ***(5–8)***.

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We have found that the in vitro kinase assay is the most sensitive method for detecting the TGF-β-activated MAPKs. By using the proper specific antibody, the activated MAPKs are immunoprecipitated, the kinase immunocomplexes are collected, and the complexes are mixed with the appropriate kinase substrate to perform an in vitro kinase assay. After the reaction is terminated, the reaction mixture is analyzed by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE), and the gel is directly exposed to film. The phosphorylated substrate is detectable by autoradiography, thereby demonstrating an increase in MAPK activity by TGF-β.

The method to be described is divided into three sections. We will introduce how to treat the cells with TGF-β first. Next, we will discuss how to perform the in vitro Erk assays. Finally, we will introduce how to perform the in vitro SAPK/JNK kinase assay.

2. Materials

1. Anti-Erk1 antibody (SC-93; Santa Cruz Biotechnology, Santa Cruz, CA).
2. Anti-Erk2 antibody (SC-154, Santa Cruz).
3. Anti-JNK1 antibody (SC-474, Santa Cruz).
4. Protein A–agarose (15918-014, Life Technologies, Bethesda, MD).
5. c-Jun (79) (SC-4113, Santa Cruz).
6. Myelin-Basic Protein (MBP) (M1891, Sigma)
7. ERK kinase cell lysis buffer: 1% Triton X-100, 10 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 30 m*M* Na pyrophosphate, 50 m*M* NaF, 1 m*M* phenylmethyl sulphonyl flouride (PMSF), 1 m*M* Na orthovanadate, 0.1% aprotinin, 5 m*M* benzamidine, 1 m*M* ethylene glycol tetraacetic acid (EGTA), and 100 n*M* okadaic acid.
8. TBS wash buffer: 25 m*M* Tris–HCl pH 7.6, 130 m*M* NaCl, 3 m*M* KCl, 1m*M* Na orthovanadate, 5 m*M* benzamidine.
9. ERK reaction buffer: 30 m*M* HEPES, 10 m*M* $MgCl_2$, 1 m*M* dithiothreitol (DTT), 20 μ*M* ATP, 5 m*M* benzamidine, 4 mg/mL MBP, 1 μCi/μL γ-^{32}P-ATP (3000 Ci/mmol; DuPont NEN, Boston, MA).
10. SAPK/JNK cell lysis buffer: 20 m*M* HEPES pH 7.4, 2 m*M* EGTA, 50 m*M* β-glycerophosphate, 1 m*M* DTT, 1 m*M* Na_3VO_4, 1% Triton X-100, 10% glycerol, 2 μ*M* Leupeptin, 0.1% Aprotinin, 400 μ*M* PMSF.
11. LiCl wash buffer: 500 m*M* LiCl, 100 m*M* Tris–HCl pH 7.6, 0.1% Triton X-100, and 1 m*M* DTT.
12. SAPK/JNK reaction buffer: 20 m*M* morpholinepropanesulfonic acid (MOPS), 2 m*M* EGTA, 10 m*M* $MgCl_2$, 1 m*M* DTT, 0.1% Triton X-100, 1 m*M* $NaVO_4$, 15 μ*M* ATP, 0.1 μg/μL c-JUN, 1 μCi/μL γ-^{32}P-ATP (3000 Ci/mmol; DuPont NEN).

3. Methods

3.1. Cell Plating and TGF-β Treatment

1. Plate cells at a cell density that will permit them to reach approx 50% confluence on the following day (*see* **Note 1**).

2. The next day, wash cells twice with phosphate-buffered saline (PBS), and change cell culture medium to serum-free conditions (*see* **Note 2**).
3. Treat cells with TGF-β (10 ng/mL) for the indicated times, such as 5 min, 10 min, 30 min, 1 h, and so forth (*see* **Note 3**).
4. Rinse cells twice with PBS (ice-cold), add 250 μL of lysis buffer (for T-75 flask), and scrape cells (*see* **Note 4**).
   For ERK assay, use Erk cell lysis buffer.
   For JNK assay, use SAPK/JNK cell lysis buffer.
5. Cell lysates are passed through a 21-gauge needle several times to disperse any large aggregates. Insoluble material is then removed by centrifuging the cell lysates (12,000*g*) for 20 min at 4°C.
6. Protein concentrations of cell lysates are determined by the bicinchonic acid (BCA) protein assay (N. 23223 and N. 23224; Pierce, Rockford, IL).

3.2. ERK In Vitro Kinase Assay

1. Add equal amounts of protein for each sample (100 μg/sample) into each microcentrifuge tube.
2. Add 5 μg of anti-Erk1 antibody (50 μL) to each tube (see **Note 5**). We also normally add 5 μg of normal rabbit IgG to one extra tube containing an equal amount of protein of one sample as a nonspecific control.
3. Incubate tubes for 1–2 h at 4°C in a rotator.
4. After 1–2 h, add 30 μL of protein A–agarose to each tube. Rotate at 4°C for another 30 min to 1 h.
5. After 30–60 min, spin tubes in microcentrifuge (3500*g*) for 5 min at 4°C, then place on ice.
6. Aspirate the supernatant and wash the beads in each tube with 200 μL of ice-cold Erk lysis buffer by gentle mixing. Spin in microcentrifuge (3500*g*) for 2 min at 4°C, and aspirate the supernatant. Repeat for a second wash.
7. Add 1 mL of ice-cold TBS wash buffer, gently mix, spin in microcentrifuge (3500*g*) for 2 min at 4°C, and aspirate the supernatant. Repeat for a second wash.
8. In a hood designed for radioactive work, in 30-s intervals, add 30 μL of ERK reaction buffer (containing 1 μCi/μL γ ^{32}P-ATP) to each tube. Gently mix the buffer with the agarose beads. Put the tube at 30°C for 30 min. During this period, tap the tube every 5 min to mix the buffer with beads.
9. After the 30-min reaction, add 30 μL of 2X SDS sample buffer to each tube in 30-s intervals and place on ice.
10. Boil the samples for 5 min.
11. Centrifuge the tubes for 10 min to separate the supernatant and beads.
12. Carefully pipet out 55 μL sample buffer from each tube, and try not to touch or disturb the beads. Load samples on a 15% SDS-PAGE (MBP is a 17-kDa protein). Remember samples are radioactive; use a shield when loading gel.
13. Run gel at 25 mA constant current for approx 1 h until the front dye passes through the stacking gel.

14. Run gel at 35 mA constant current for approx 3 h until dye front reaches the bottom of the resolving gel.
15. After running the gel, remove gel from the apparatus, place on a piece of filter paper, and cover the gel with Saran Wrap.
16. Place the gel in a film cassette and take several short exposures of wet gel, such as 2 min, 5 min, 10 min, 30 min (*see* **Note 6**).
17. After exposing the gel, set up a transfer apparatus to transfer proteins from the gel to the membrane (using radioactive precautions).
18. After transfer, perform immunoblot analysis by using the anti-Erk antibody (SC-93-G) (*see* **Note 8**) as the primary antibody (1 μg/mL) as a control to determine the amount of Erk protein in each sample.

3.3. JNK In Vitro Kinase Assay

1. Add equal amounts of protein of each sample (100 μg/ sample) to each microcentrifuge tube.
2. Add 5 μg of anti-JNK1 (*see* **Note 5**) to each tube, rotate at 4°C for 1–2 h.
3. After 1–2 h rotation, add 30 μL protein A–agarose to each tube and rotate for another 30–60 min .
4. Centrifuge for 5 min, aspirate supernatant, and wash the beads three times with JNK cell lysis buffer.
5. Wash the beads three times with LiCl wash buffer.
6. Wash the beads three times with JNK assay reaction buffer (without c-Jun and γ-^{32}P-ATP).
7. Add 30 μL of JNK assay reaction buffer (with c-Jun and 1 μCi/μL γ-^{32}P-ATP) to each tube, and gently mix the buffer with the beads.
8. Incubate the reaction for 30 min at 30°C. Every five min, tap the tube to mix.
9. Add 30 μL 2X SDS loading buffer and boil for 5 min.
10. Run on a 10% SDS-PAGE gel.
11. Expose the gel to X-ray film; the c-Jun (79) migrates as a 37-kDa protein (*see* **Note 7**).
12. After exposing the gel as for Erk assay, transfer the gel to a membrane and perform immunoblot analysis with anti-JNK1 (SC-474-G) (*see* **Note 8**) as the primary antibody to determine the amount of JNK protein in each sample.

4. Notes

1. The cell density at the time of TGF-β treatment is important for the outcome of the experiments. We recommend that the cells be in log phase growth (usually about 60–80% confluency) at the time they are treated with TGF-β. Also, we always set up a parallel experiment to check the cellular responsiveness to TGF-β by a ^{3}H-thymidine incorporation assay ***(3)***.
2. Serum in the cell culture medium interferes with TGF-β effects on cells. We recommend that you change the culture medium to serum-free conditions prior to TGF-β treatment. For many cell types, a 13-h incubation in serum-free medium does not produce a quiescent state ***(15)***. If this time period does induce quiescence, a shorter time period must be used, because the cells should be in exponential phase

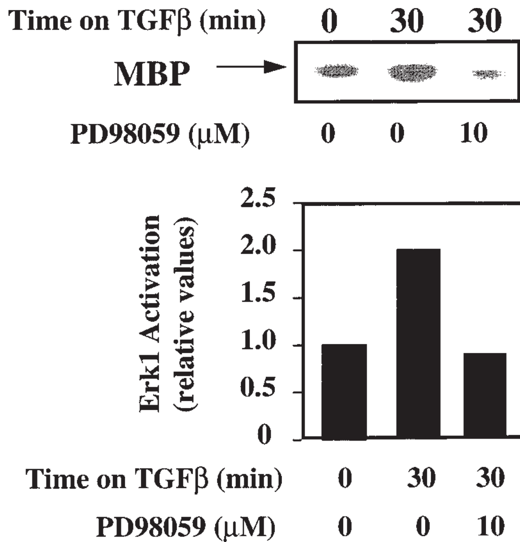

Fig. 1. Activation of Erk1 by TGFβ in IEC 4-1 cells. Exponentially proliferating cultures of IEC 4-1 cells were treated with or without TGFβ_3 (10 ng/mL) for 30 min in the presence or absence of 10 μ*M* of the MEK1 inhibitor PD98059. The in vitro Erk kinase assay was performed as described in the text.

growth when treated with TGF-β. In the protocol described here, no growth stimulators will be added with the TGF-β to promote cells re-entering from quiescence. In addition, if the serum-free incubation causes extensive cell death, cells may need to be incubated in serum-free medium for a shorter time period. A 15–30-min incubation after two PBS washes can be used, which did not interfere with the effects of TGF-β on Erks1 and 2 in IECs (unpublished data).

3. We found that TGF-β activates SAPK/JNK transiently in some cell types. It activates JNK within 5–10 min in IEC 4-1 cells, followed by a return to basal levels by 1 h. Thus, kinetic analyses are recommended for each cell type to determine the optimal TGF-b treatment time.
4. One key aspect to this assay is that the cell lysis buffer must be made up fresh each time the cells are lysed. We recommend that the stock solutions be prepared for each reagent and that the final lysis buffer is prepared by adding the appropriate volumes of each stock solution to arrive at the correct final concentrations.
5. A key to the success of the in vitro kinase assay is the quality of the antibodies used. After trying different commercially available SAPK/JNK and Erks1 and 2 antibodies, we found that anti-Erk1 (SC-93), anti-Erk2 (SC-154), and anti-JNK1 (SC-474) from Santa Cruz were the most specific. We also found that the phospho-specific antibodies did not work well for TGF-β-mediated Erk activation in our systems.
6. MBP migrates as a 17-kDa protein. After 30 min of TGF-β treatment, MBP phosphorylation by Erk1 was increased by twofold above basal levels in IEC 4-1 cells (**Fig. 1**).

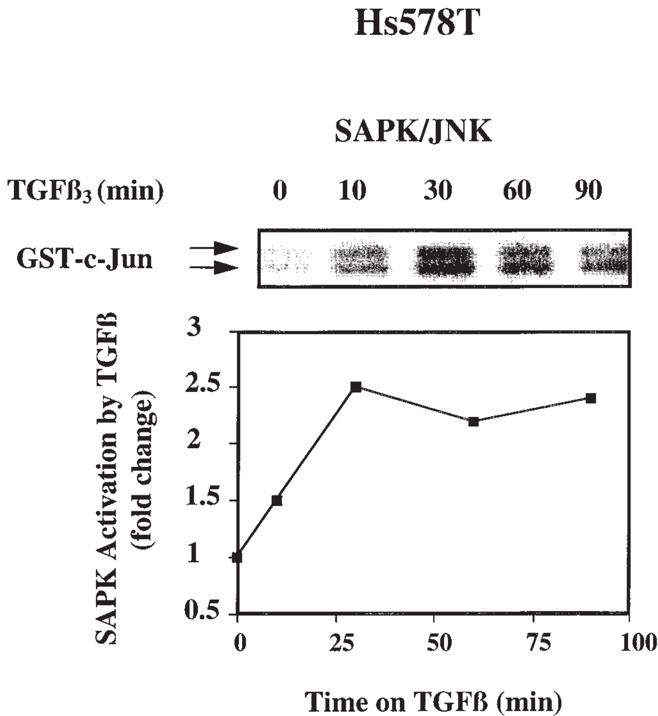

Fig. 2. Activation of SAPK/JNK by TGF-β in Hs578T human breast cancer cells. Hs578T cells were treated with or without TGFβ_3 (10 ng/mL) for the indicated times. The in vitro SAPK kinase assay was performed as described in the text.

Moreover, the MEK1 inhibitor PD98059, at a concentration of 10 μ*M*, blocked the ability of TGF-β to activate Erk1 in these cells. Also, there is no MBP phosphorylation in the nonspecific lanes (normal rabbit IgG immunocomplex lane) for the exposure times we routinely use.

7. The c-Jun (79) peptide migrates at the 37-kDa position. After 30 min of TGF-β treatment, c-Jun phosphorylation by JNK1 was increased by 2.6-fold above basal levels in the Hs578T human breast cancer cells (**Fig. 2**). Also, there is no c-Jun phosphorylation in the nonspecific lanes (normal rabbit IgG immunocomplex).
8. If the antibody used in the immunoprecipitation is rabbit antiserum, it is best to use either goat or antiserum obtained from another species as the immunoblotting antibody. The SAPKs or Erks migrate very close to the IgG heavy chain.

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11

Detection of TGF-β Type II Receptor Hot-Spot Mutations: The BAT-RII Assay

Lois L. Myeroff and Sanford Markowitz

1. Introduction

The transforming growth factor-β (TGF-β) pathway is important in the growth control of many different cell types, particularly epithelial cells, and is often disrupted in the development of cancer ***(1,2)***. The most common mutation in the TGF-β pathway detected thus far is mutation of the type II receptor (RII) in colon cancers, particularly in tumors with defective mismatch repair or microsatellite instability (MSI) ***(3–5)***. These RII mutations occur in a repetitive region of coding sequence, a stretch of 10 adenines, and occur in greater than 90% of colon tumors with MSI ***(5)***. Frameshift mutations, with insertion or deletion of one or two adenines, result in premature termination of the receptor protein, with loss of the membrane anchor and intracellular kinase domain. This RII mutation also occurs in gastric and endometrial tumors ***(4)*** and may be a hot spot in other tumor types with microsatellite instability as well.

Our laboratory has modified our originally published method for detection of this mutation, known as the BAT-RII assay ***(4,5)***. This assay is designed only to detect frameshift mutations in the hot-spot 10 bp adenine repeat in RII (bases 709–713; Genbank accession number M85079). The name BAT refers to the adenine repeat — "**B**ig **A** **T**ract." The forward primer is end-labeled with γ^{32}P-ATP or γ^{33}P-ATP, and then used in a polymerase chain reaction (PCR) with unlabeled reverse primer and genomic DNA template. Deletion and/or insertion mutations are detected as a shift in size of the 73-bp band by 1 or 2 base pairs on a sequencing-type polyacrylamide gel.

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2. Materials

1. TA10-F1 primer (5'-CTTTATTCTGGAAGATGCTGC-3'); diluted to 300 ng/μL in H_2O.
2. TA10-R1 primer (5'-GAAGAAAGTCTCACCAGG-3'); diluted to 300 ng/μL in H_2O.
3. Polynucleotide kinase (PNK) (10 U/μL) and 10X PNK buffer, supplied with enzyme (Roche Molecular Biochemicals, Indianapolis, IN).
4. γ–^{32}P-ATP (10 μCi/μL) or γ^{33}P-ATP (10 μCi/μL) (*see* **Note 1**).
5. Genomic DNA from test samples and controls (*see* **Note 2**).
6. DNAse- and RNAse-free H_2O for PCR.
7. 1.25 m*M* dNTPs for PCR (Perkin Elmer, Norwalk,CT).
8. *Pwo* enzyme (5 U/μL) and 10X buffer, supplied with enzyme (Roche Molecular Biochemicals, Indianapolis, IN).
9. Sephadex G-50 (Pharmacia, St. Albans, UK) in Tris-EDTA (TE).
10. 1cc syringe.
11. Glass wool.
12. LongRanger gel solution (FMC BioProducts, Rockland, ME) or other sequencing gel polyacrylamide solution.
13. Urea.
14. 10X Tris-borate-EDTA (TBE) electrophoresis buffer (Gibco-BRL, Grand Island, NY).
15. Ammonium persulfate (APS) (Sigma, St. Louis, MO) 10% in H_2O.
16. N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma).
17. Formamide (Fisher Scientific, Pittsburgh, PA).
18. Xylene Cyanol (Sigma).
19. Bromophenol Blue (Sigma).

3. Methods

3.1. End-Label Primer

1. The labeling reaction consists of the following:
   7 μL TA10-F1 primer (300 ng/μL)
   7.5 μL 10X PNK buffer
   54 μL H_2O
   6 μL γ–^{32}P-ATP (10 μCi/μL) or γ-^{33}P-ATP (10 μCi/μL) (*see* **Note 1**)
   0.5 μL PNK enzyme
   75 μL total volume
   We mix primer, water, buffer, and PNK together in a microfuge tube in a PCR setup area, and then move quickly to a PCR radioactive area where the isotope is added to the tube, mixed, and then taken to a 37°C preheated thermocycler, heat block, or water bath.
2. Incubate at 37°C for 30 min; then heat at 70°C 5 min to inactivate the enzyme.
3. Remove the unincorporated isotope with a size-separation column purification. Removal of unincorporated nucleotides means the amount of radioactivity in the electrophoresis bottom buffer after electrophoresis is within the limits on sewer radioactivity disposal for our institution (< 7 μCi). This step is optional, but helpful.

a. To prepare an inexpensive, do-it-yourself column, remove the plunger from a 1-mL syringe.
b. Insert a small plug of glass wool into the bottom, and pack down with the plunger. Pipet in 1 mL of G-50. Place syringe in a 15-mL disposable plastic tube, and spin 1 min at 250*g* in a tabletop centrifuge. Add more G-50 and spin until the bed volume reaches 1 mL. Remove all liquid from the tube and replace the syringe column in the tube.
c. Add 25 μL H_2O to the inactivated reaction and load onto the column. Spin 1 min at 250*g*, then wash column with 150 μL H_2O, respin, and collect the effluent.
d. If column purification is not used, add 175 μL H_2O to the inactivated reaction to create labeled primer solution of 8 ng/μL. Labeled primer can be stored at –20°C for about 1 mo for ^{32}P and 2–3 mo for ^{33}P.

3.2. BAT-RII PCR Reaction

1. In 0.5-mL or 0.2-mL PCR tubes, aliquot approximately 50–100 ng of genomic DNA for each sample to be tested (*see* **Notes 2** and **3**) and add H_2O for a total volume of 7.95 μL in each tube.
2. Mix a reaction cocktail for $n + 2$ reactions (n = number of samples). Multiply the following volumes by $n + 2$ to determine volume of each item in cocktail:
   0.75 μL TA10-R1 primer, unlabeled
   2.4 μL 1.25 m*M* dNTPs
   1.5 μL 10X *Pwo* buffer
3. Add $(n + 2) \times 0.15$ μL *Pwo* polymerase to the cocktail, mix briefly, and spin down in microfuge quickly. Then, add $(n + 2) \times 2.25$ μL of end-labeled TA10-F1 to cocktail. Aliquot 7.05 μL of cocktail to each sample tube, then layer on 15 μL mineral oil. See **Note 4** for more details of PCR reaction setup procedure.
4. Run a "touchdown" PCR program: 95°C for 5 min; then three cycles of 95°C for 1 min, 62°C for 30 s, 72°C for 30 s, and decrease annealing temperature 2°C per cycle until 56°C annealing is reached and continue cycling for total of 30 cycles; 70°C for 10 min final extension, 4°C hold. This "touchdown" program is more stringent than a typical, one-temperature annealing program and decreases artifact bands.

3.3. Electrophoresis

1. While the PCR reaction is running, prepare a 6% LongRanger/7M urea/1X TBE sequencing gel with shark-stooth combs. Allow the gel to cure for 45 min. Prerun gel in 1X TBE at 75 W constant power for about 20 min.
2. When PCR cycles are complete, add 15 μL of sequencing stop solution (95% formamide, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol) to each 15-μL PCR reaction under oil, heat at 85°C for 5 min, then place on ice.
3. After cleaning all urea out of the wells with a syringe and 1X TBE, load 4–6 μL of each reaction into wells. Remember to load the gel asymmetrically so you can orient it later. Run the gel at 75 W constant power (52°C) until the Bromophenol Blue dye is about 1 in. from bottom.
4. Dry gel; expose to film or phosphoimager screen.

3.4. Interpretation of Results

Expect the predominant radioactive PCR band to be at 73 bp. Use of a normal control will demonstrate the wild-type size band, as well as a bit of artifact band at the –1 (72 bp) position. Use of a control mutant sample with a mixture of wild type and –1 mutant alleles (such as RL-952, *see* **Note 2**) is particularly useful. In such wild-type/-1 heterozygous samples, the 73- and 72-bp bands should be of equal intensity. Homozygous –1 mutant samples may have a faint band at the 73-bp position, but a very strong band at the 72-bp position. Tumors with +1 and/or –2 mutations are rare, but when such bands are seen, they are invariably authentic.

4. Notes

1. Either ^{32}P or ^{33}P can be used for primer labeling. ^{32}P has greater signal strength, which helps detection for small amounts of DNA, but ^{33}P gives sharper bands that can make interpretation easier, especially for wt/-1 heterozygous samples.
2. Normal human genomic DNA should be included in each experiment as a control. Genomic DNA from cell lines with known BAT-RII mutations should also be used as standards. RL-952 (wt/-1 heterozygote) and HCT-116 (-1 homozygous) are examples of tumor cell lines with defined BAT-RII mutations, and are available from ATCC.
3. The amount of genomic DNA used can vary from 10 to 300 ng, but the most consistent results are achieved with about 50 ng. If the DNA has been extracted from paraffin-embedded fixed tissue, more than 50 ng may be necessary.
4. Use of the proofreading thermostable polymerase *Pwo* lessens the errors made by *Taq* polymerase reading through the repetitive A_{10} tract, as well as the variable addition of nontemplated adenines to the PCR product ends. *Pwo* thus reduces the appearance of the artifact shadow band at the –1 (72 bp) position. However, *Pwo* polymerase has intrinsic exonuclease activity, which will chew up the primers in the absence of genomic DNA, so we set up the reaction in this order:
    a. Thaw the labeled TA10-F1 primer in PCR radioactive working area.
    b. Aliquot the sample DNA and H_2O into tubes on the PCR bench and set aside.
    c. Make up the cocktail with cold TA10-R1 primer, 10X buffer and dNTPs, set aside.
    d. Transfer sample tubes to radioactive PCR working area, open lids. Preheat PCR block to 95°C.
5. Add *Pwo* enzyme to cocktail, mix, spin down, and go immediately to the radioactive area. Add labeled TA10-F1 primer to the cocktail,pipet up and down several times to mix, and start transferring into DNA tubes. Move as quickly as you can safely, with no delays.
6. Layer on oil, close tubes, and immediately transfer tubes to preheated 95°C PCR block; run program. We use an oil overlay even with a heated lid PCR block because of the small volumes.

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12

Mutation Detection in the TGF-β Receptors and *smad* Genes: RT-PCR and Sequencing

Lois L. Myeroff, Hongmei He, Stephen P. Fink, and Sanford Markowitz

1. Introduction

The transforming growth factor-β (TGF-β) pathway is important in the growth control of many different cell types, particularly epithelial cells, and is often disrupted in the development of several different types of cancer. Many tumor cell lines are resistant to the growth suppressive effects of TGF-β ***(1–3)***. In some tumors, this resistance the result of mutations in the TGF-β receptors or the *smad* family of downstream TGF-β signaling proteins ***(4–8)***. The purpose of this chapter is to give this laboratory's current protocols for mutation detection in the TGF-β receptors and the *smad* genes.

The most common lesion in the TGF-β pathway detected thus far is mutation of the type II receptor (RII) in colon cancers ***(3,4)***. A protocol for detection of mutations in the hot-spot BAT-RII tract, found in tumors with defective mismatch repair, is detailed in Chapter 11. Missense mutations in the type II receptor, found throughout the coding sequence but most commonly in the kinase domain, have been detected in MSI+ and MSI- colon carcinomas, head and neck cancers, and cutaneous T-cell lymphomas ***(3,9–12)***. Although some laboratories screen for RII mutations with polymerase chain reaction/single-stranded conformation polymorphism (PCR/SSCP) or chemical mismatch assays ***(12–14)***, these screening assays can miss mutations, so we prefer to sequence RT-PCR products. Unfortunately, the RII cDNA can be difficult to amplify full length with traditional two-step reverse transcriptase – polymerase chain reaction (RT-PCR). We have determined that the Titan one-step RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, IN) is the most consistent method to amplify the full-length RII coding region. This easy-to-use kit utilizes a mixture of reverse transcriptase, *Pwo* polymerase, and *Taq* polymerase for high fidelity and processivity. However, if the RII message

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level is low or if the Titan kit is unavailable, it may be preferable to amplify the coding region in two overlapping pieces (referred to as "5'-half" and "3'-half") with a two-step reverse transcription and PCR method.

Mutations in the type I TGF-β receptor (RI) appear to be rare, but examples in the literature are beginning to appear ***(14,15)***. Although mutations in RI may be infrequent, we believe it is necessary to rule out RI mutation as the cause of TGF-β resistance in a cell line under study. Because of the very GC-rich 5' end of the RI gene, it can be a difficult cDNA to amplify and sequence. We have tested many different methods, and the protocol detailed in this chapter is the most consistent. We amplify the cDNA in two pieces, a 1600-bp "full-length" RI PCR product for sequencing the coding region from about base 240 to the stop codon at base 1586, and a 582-bp 5' end RI fragment to sequence the start site and GC-rich 5' end cleanly.

Mutations of the *smad* genes have been detected in pancreatic, colon and other cancers ***(7,8,16–18)***. Mutations in the *smad4* gene have been identified in several different tumor types, including pancreas ***(7)***, colon ***(16)***, and gastric tumors ***(19)***. However, mutations in the *smad2* and *smad3* genes have been relatively rare, at least with the tumor types and methods used thus far ***(18,20)***. We use a two-step method of reverse transcription followed by full-length PCR and direct sequencing to be sure that we are not missing any potential mutations.

For RII, RI, and the *smad* genes, the primer numbers correspond to the numbering used in the Genbank entries (accession numbers listed below). The numbers refer to the base that the primer starts, and "s" and "as" refer to sense and antisense, respectively.

2. Materials

2.1. Titan RT-PCR Amplification of Full-Length RII

1. Titan RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, IN).
2. RNAse-and DNAse-free H_2O.
3. 1.25 m*M* deoxynucleoside triphosphates (dNTPs) for PCR (Perkin-Elmer, Norwalk, CT).
4. Forward (RII-297s) and reverse (RII-2077as) amplification primers, diluted to 4 μ*M* in H_2O (*see* **Table 1**).
5. Total RNA (1 μg per reaction) from tissues or cell lines of interest.

2.2. Two-Step RT-PCR

1. RNAse-and DNAse-free H_2O.
2. 1.25 m*M* dNTPs for PCR (Perkin-Elmer).
3. Random hexamers, 2 mg/mL.
4. AMV reverse transcriptase and 5X buffer (Roche Molecular Biochemicals, Indianapolis, IN).
5. *Taq* polymerase and 10X buffer (5 U/μL; Roche Molecular Biochemicals.

6. Forward and reverse amplification primers, diluted to 4 μ*M* in H_2O (*see* **Table 1**).
7. Total RNA (5 μg per cDNA reaction) from tissues or cell lines of interest.

2.3. Amplification of RI

1. Advantage–GC genomic polymerase mix, 5X GC genomic PCR buffer, 5X GC-melt buffer, and 25 m*M* $Mg(OAc)_2$, supplied with enzyme (Clontech, Palo Alto, CA).
2. Advantage–GC cDNA polymerase mix, 5X GC cDNA PCR buffer and 5X GC-melt buffer, supplied with enzyme (Clontech).

2.4. PCR Product Purification

1. Qiaquick gel extraction kit (Qiagen, Chatsworth, CA).
2. Qiaquick PCR purification kit (Qiagen)

2.5. Primers for Sequencing, Diluted to 4 μM in H_2O

(*See* **Table 1**.)

3. Methods

3.1. One-Step RT-PCR for Full-Length RII with Titan Kit

1. For each reaction, aliquot 1 μL each of primers RII-297s and RII-2077as into PCR tubes, and add 1 μg total RNA in up to 1 μl (*see* **Note 4**).
2. For *n* samples (including an H_2O control), multiply the following volumes by $n + 1$ and make a cocktail (*see* **Note 5**):
   - 8 μL dNTPs (1.25 m*M*)
   - 2.5 μL DTT
   - 10 μL 5X Titan buffer
   - 25.5 μL H_2O
   - 1 μL Titan enzyme mix
3. Aliquot 47 μL of cocktail into each RNA sample tube, pipet several times to mix, and transfer to 50°C preheated PCR block. Run a program: 50°C 30 min, 95°C 2 min; then cycle 95°C for 45 s, 58°C for 1 min at 68°C, 2 min for 36 cycles, 68°C 5 min final extension, 4°C hold.
4. Proceed to **Subheading 3.3.**

3.2. Two-Step Reverse Transcription and PCR Amplification

3.2.1. Reverse Transcription of cDNA

One reaction will make enough cDNA for five subsequent PCR reactions (*see* **Note 4**).

1. For each cDNA sample desired, mix the following:
   - 8.5 μL total volume for 5 mg total RNA and H_2O
   - 0.5 μL random hexamers (2 mg/mL)
   - 10 μL 1.25 mM dNTPs
   - 5 μL 5X AMV RT buffer
   - 1 μL AMV RT (Roche Molecular Biochemicals; 25 U/μL)
2. Incubate at 42°C for 1.5 h. Store cDNAs at –20°C for up to several months.

Table 1
Primers for PCR Amplification and Sequencing

PCR product	Accession #	Expected size	Forward primer	Reverse primer	Sequencing primers
RII full length	M85079	1780 bp	RII-297s	RII-2077as	See **Notes 1** and **2**
			(5'-CGCTGGGGGCTCGGTCTATG-3')	(5'-GCAGCCTCTTTGGACATGC-3')	RII-297s (5'-CGCTGGGGGCTCGGTCTAT-3')
					RII-425s (5'-GTCGGTTAATAACGACATGATAG-3')
					RII-719s (5'-GCCTGGTGAGACTTTCTT-3')
					RII-926s (5'-GAGTTCAACCTGGGAAACC-3')
					RII-1028s (5'-TGCCAACAACATCAACCACAA-3')
					RII-1323s (5'-AAGGGCAACCTACAGGAGTACC-3')
					RII-1562s (5'-TGTGGATGACCTGGCTAA-3')
					RII-463as (5'-CCGTTGTTGTCAGTGACTATC-3')
					RII-708as (5'-CTTTCATAATGCACTTTGGA-3')
					RII-1160as (5'- CCACTGTCTCAAACTGCTCT-3')
					RII-2073as (5'- CCTCTTTGGACATGCCCAGCC-3')
RII 5' half	M85079	863 bp	RII-297s	RII-1160as	See RII primers above
			(5'-CGCTGGGGGCTCGGTCTATG-3')	(5'-CCACTGTCTCAAACTGCTCT-3')	
RII 3' half	M85079	1045 bp	RII-1028s	RII-2073as	See RII primers above
			(5'-TGCCAACAACATCAACCACA-3')	(5'-CCTCTTTGGACATGCCCAGCC-3')	
RI "full length"	L11695	1600 bp	RI-75s	RI-1675as	See **Note 3**
			(5'-CCATGGAGGCGGCGGTCGCTGCTCCGCG-3')	(5'-CTCAGTGAGGTAGAACAACTGACCTCCC-3')	RI-228s (5'-ATGGGCTCTGCTTTGTCTC-3')
					RI-459s (5'-CTGTCATTGCTGGACCAG-3')
					RI-764s (5'-GCTGTTAAGATATTCTCCT-3')
					RI-828as (5'-GTTTGATAAATCTCTGCCTC-3')
					RI-1669as (5'-GAGGTAGAACAACTGACCT-3')
RI 5'-end	L11695	582 bp	RI-5s	RI-586as	
			(5'-AGGCGAGGTTTGCTGGGG-3')	(5'-AAAAGGGCGATCTAATGAAGGG-3')	RI-13s (5'-TTTGCTGGGGTGAGGCAGCG-3')
					RI-278as (5'-TGTGTATAACTTTGTCTGTGGTCTC-3')

					smad2-1343s (5'-GAATGAGTTTTGTGAAAGGG-3')
					smad2-180as (5'-TCCTCCAGACCCACCAGCTGACTTC-3')
					smad2-1530as (5'-TTTCCATGGGACTTGATTGG-3')
smad3	U68019	1308 bp	smad3-56s (5'-CCTCCCCAGCCATGTCGTC-3')	smad3-1363as (5'-TCCCCTACCATACTTGATGTC-3')	smad3-56s (5'-CCTCCCCAGCCATGTCGTC-3')
					smad3-718s (5'-TTGGACCTGCAGCCAGTTACC-3')
					smad3-902as (5'-TTCCTGTTGACATTGGAGAG-3')
					smad3-187as (5'-TCTTGACCAGGCTCTTGACCG-3')
					smad3-1363as (5'-TCCCCTACCATACTTGATGTC-3')
smad4	U44378	1725 bp	smad4-129s (5'-ATGGACAATATGTCTATTACGAATAC-3')	smad4-1854as (5'-TATTTGTCCTAACATAAAACATCAGG-3')	smad4-420s (5'-CATGTGATCTATGCCCGTC-3')
					smad4-745s (5'-GTTAGCCCCATCTGAGTC-3')
					smad4-1004s (5'-CATCTTCAGCACCACCCG-3')
					smad4-1211s (5'-CCTTCTGGAGGAGATCGC-3')
					smad4-1388s (5'-GACAGAGAAGCTGGGCGT-3')
					smad4-1618s (5'-TGTTGATGACCTTCGTCG-3')
					smad4-524as(5'-GGTGATACACACTCGTTCGTAG-3')
					smad4-741as (5'-GTGCTGGTAGCATTAGACTC-3')
					smad4-1148as (5'-GGGCAGCTTGAAGGAACC-3')
					smad4-1446as (5'-TCGATGACACTGACGCAA-3')
					smad4-1750as (5'-CTGCAATCGGCATGGTAT-3')

3.2.2. PCR Amplification of RII, smad2, smad3, or smad4

1. Aliquot 5 µL of cDNA from **Subheading 3.2.1.** into PCR tubes (*see* **Note 5**).
2. For *n* reactions, make up a cocktail of *n* + 1 times the following volumes:
   1 µL forward primer (*see* **Table 1**)
   1 µL reverse primer (*see* **Table 1**)
   5 µL 1.25 m*M* dNTPs
   5 µL 10X PCR buffer
   32.8 µL H_2O
   0.2 µL *Taq* (5 U/µL)
3. Aliquot 45 µL of cocktail into each PCR tube, pipet briefly to mix.
4. Run the following PCR program for each gene:
   RII: 95°C, 5 min; [95°C 45 s, 61°C 1 min, 72°C 2 min] 35 cycles, 72°C 5 min final extension, 4°C hold
   smad2: 95°C, 5 min; [95°C 45 s, 56°C 1 min, 72°C 3 min] 38 cycles, 72°C 5 min final extension, 4°C hold
   smad3: 95°C, 5 min; [94°C 30 s, 56°C 30 s, 72°C 2 min] 35 cycles, 72°C 5 min final extension, 4°C hold
   smad 4: 95°C, 5 min; [95°C 30 s, 58°C 1 min 30 s, 72°C 1 min 30 s] 35 cycles, 72°C 7 min final extension, 4°C hold
5. Proceed to **Subheading 3.3.**

3.2.3. PCR Amplification of RI

1. Aliquot 5 µL cDNA from **Subheading 3.2.1.** into PCR tubes.
2. For *n* reactions, multiply the following volumes by *n* + 1 to make a cocktail.
   For full-length RI PCR:
   1 µL forward primer RI-75s (5 µ*M*)
   1 µL reverse primer RI-1675 as (5 µ*M*)
   5 µL 1.25 m*M* dNTPs
   10 µL 5X GC genomic PCR reaction buffer
   10 µL GC-melt
   2.2 µL 25 m*M* $Mg(OAc)_2$
   1 µL Advantage–GC genomic polymerase mix
   14.8 µL H_2O
   For the 5'-end RI PCR:
   1 µL forward primer RI-5s (5 µ*M*)
   1 µL reverse primer RI-586as (5 µ*M*)
   5 µL 1.25 mM dNTPs
   10 µL 5X GC cDNA PCR reaction buffer
   10 µL GC-melt
   1 µL Advantage–GC cDNA polymerase mix
   17 µL H_2O
   Aliquot 45 µL of cocktail into each sample tube, mix briefly, and close tubes.
3. Run PCR cycles:
   For full length: 5 min, 95°C; [95°C 30 s, 64°C 1 min, 70°C, 3 min] 38 cycles, 70°C, 5 min final extension, 4°C hold. For 5'-end PCR: 5 min, 95°C;

[95°C 30 s, 60°C 1 min, 70°C, 3 min] 38 cycles, 70°C, 5 min final extension, 4°C hold.
4. Proceed to **Subheading 3.3.**

3.3. PCR Product Purification

1. Run 3–5 µL of each sample on 0.8% agarose gel to check amplification of the expected band.
2. If only one band is visible, use Qiaquick PCR purification kit to remove primers and nucleotides. Follow the manufacturer's directions for preparation for direct sequencing and elute DNA with 50 µL H_2O.
3. If more than one band or a smear is visible, use the Qiaquick gel extraction kit exactly according to the manufacturer's instructions. Elute DNA with 50 µL H_2O.
4. After Qiaquick purification, estimate the concentration of DNA either by electrophoresis next to standards of known concentration, or by spotting 1 µL on an ethidium bromide–agarose plate next to standards of known concentration.

3.4. Direct Sequencing

We recommend sequencing RT-PCR products directly, without cloning (*see* **Note 6**). Many institutions have a facility for automated sequencing, and there are also small companies that provide sequencing service at low cost. We have had success with automated sequencing with the ABI system 377, used exactly according to the directions provided by the manufacturer. We estimate the concentration of RT-PCR product as earlier, and then prepare a premixed reaction containing 10–20 ng of RT-PCR product for each 200 bp of length, 2 µL of 4 m*M* primer, and H_2O to 24 µL. Some facilities may have different requirements for premixed reactions. If automated sequencing is not available, another good option is manual cycle sequencing with one of the many kits available commercially.

4. Notes

1. Sequencing through the RII A_{10} tract can be difficult. We sequence away from it on either side with RII-708as and RII-719s, and sequence toward it as well with RII-425s and RII-1160as.
2. At base 429 in RII, the junction between exons 1 and 2, there may be an in-frame insertion of 75 bp in the cDNA ***(21,22)***. This alternatively spliced RII receptor has function indistinguishable from the predominant RII species and is present at lower levels. However, its presence in the RT-PCR product often causes the sequence after that point to be an unreadable mixture of two sequences. For this reason, we have included the RII-425s primer to sequence only the predominant RII cDNA.
3. In the 5' end of the RI gene, there is a nine amino acid alanine repeat. However, the number of alanines is polymorphic in the population ***(15)*** and heterozygous individuals create a sequencing problem. For this reason, we use 5'-end partial PCR to amplify and sequence the GC-rich 5' end and the region of GCG alanine

repeats. It is necessary to sequence the 5'-end PCR product from both ends into this region in order to get a readable sequence.

4. One RT-PCR reaction usually gives enough product to sequence with only three or four primers, so we usually amplify multiple reactions from the same template to generate enough product for complete sequencing.
5. Because the Titan enzyme mix contains *Pwo* polymerase, complete work quickly after mixing in the enzyme to minimize the exonuclease effect on the primers.
6. If you wish to clone an RT-PCR product, we prefer the TOPO-TA cloning kit from Invitrogen (San Diego, CA). It is best to purify and clone as soon as possible after PCR reaction because the one-base overhangs generated by the *Taq* polymerase degrade with time.

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13

Analysis of TGF-β-Inducible Apoptosis

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1. Introduction

Transforming growth factor-β (TGF-β) is an important regulator of cellular growth and immune homeostasis ***(1–3)***. TGFβ is a homodimeric 25-kDa protein which activates cellular signaling through the recruitment and transphosphorylation of specific heterodimeric cell-surface receptors ***(4)***. Although activation of TGF-β receptors initiates serine/threonine kinase activity, the subsequent signaling mechanisms involved in a wide array of diverse functional consequences is currently under investigation. TGF-β has been postulated to play important roles in tissue fibrosis and the regulation of the extracellular matrix ***(5)***, growth arrest of epithelial cells ***(6)***, regulation of the immune response, and induction of apoptotic cell death ***(7–11)***. The essential nature of this cytokine is exemplified by the lethal and nonoverlapping phenotypes of the three highly homologous mammalian TGF-β isoforms, each of which exhibit cell-specific expression ***(3,12)***. TGF-β_1 deficient mice exhibit extensive lymphocytic hyperproliferation and the production of autoimmune antibodies found in several human diseases ***(3,13–16)***.

The mouse immature B-cell line, WEHI 231, has been used as an in vitro model of B-cell tolerance ***(17)***, growth inhibition ***(6,7,9,11)***, and apoptosis ***(7–11,18–20)***. TGF-β has been shown to induce growth arrest at the G1/S phase border in WEHI 231 cells ***(6,7)***. Growth inhibition in WEHI 231 cells is controlled by several cell-cycle regulators that include the cyclins and the cyclin-dependent kinases ***(7,17,21,22)***. TGF-β has also been shown to induce apoptosis in WEHI 231 cells, as well as other cell types ***(23–31)***; however, the mechanisms regulating this process are not well understood and appear to be cell-specific. In vivo, lymphocyte apoptosis during development is essential in controlling the immune repertoire and preventing autoimmunity ***(17)***. The abil-

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ity of TGF-β to induce apoptosis in immature B-cells may be a major mechanism controlling lymphocyte growth and supports the essential nature of this growth regulator in the maintenance of normal immunological homeostasis ***(3,12,17)***. Immature B-lymphocytes provide a physiologically relevant in vitro model to study the mechanisms involved in TGF-β-induced apoptotic cell death.

Apoptosis is an active process of cell death characterized by a lack of an inflammatory response, crosslinking of the plasma membrane to seal the cellular contents, and formation of apoptotic bodies ***(32)***. The specificity of apoptotic signaling pathways appears to be mediated by the initiating stimulus. Apoptosis is mediated by specific cysteine proteases (caspases) that are unique in cleaving substrates specifically following aspartate residues ***(33,34)***. Cleavage of cellular substrates is commonly associated with cells undergoing apoptosis. αII-Spectrin (α-fodrin), is an actin-binding cytoskeletal protein postulated to play a vital role in the structural integrity of cells ***(35)***. Plasma membrane blebbing, an early apoptotic phenotype, has been proposed to result as a consequence of the loss or cleavage of intact αII-spectrin ***(36–40)***. We have recently demonstrated that αII-spectrin is cleaved into novel fragments coincident with the onset of TGF-β-induced apoptosis in WEHI 231 cells ***(8)***. Whether this cleavage is a direct or indirect consequence of the apoptotic process and whether cleavage of specific cellular substrates is essential for apoptotic cell death are a matter of active investigation. Recent advances have led to commercially available caspase inhibitors that irreversibly inhibit enzyme activation (**Table 1**). Specific, as well as broad, spectrum caspase inhibitors consist of monopeptide to tetrapeptide amino acids conjugated to a lipophilic, methyl ketone. These inhibitors can efficiently block DNA ladder formation, further caspase activation and substrate cleavage, and are dependent on specific caspase and apoptotic signaling mechanisms following activation. It is becoming evident, however, that the mechanism of activation via specific caspases as well as cleavage of particular substrates are cell-type-specific. The object of this chapter is to provide detailed methodologies for the analysis of apoptotic cell death.

2. Reagents and Buffers

1. Ac-YVAD-pNA (Calbiochem, [La Jolla, CA]; C7752).
2. Annexin V Apoptosis Detection Kit (R & D Systems, [Minneapolis, MN]; KNX50-20).
3. Biotin-VAD (Enzyme Systems Products Life Sciences [St. Petersburg, FL]; FK-014).
4. Chemiluminesence Reagent (New England Nuclear [Boston, MA]; NEL101).
5. Complete Protease Inhibitor Tablets (Boehringer Mannheim [Mannheim, Germany]; 1836170).
6. Formaldehyde, 16% solution (Electron Microscopy Sciences [Fort Washington, PA]; 15710).

7. GF-C Glassfibre Filters—24 mm (Whatman [Clifton, NJ]; 1822 024).
8. Goat Anti-Rabbit IgG-HRP (Transduction Labs [Lexington, KY]; R14745).
9. αII-Spectrin Monoclonal Antibody (Chemicon [El Segundo, CA]; MAb 1622).
10. Rabbit anti-mouse IgG–HRP (Accurate [Chemical and Scientific, Westbury, NY]; JZM035003).
11. Streptavidin–agarose (Gibco-BRL [Grand Island, NY]; 15942-014).
12. Streptavidin-HRP (Accurate Antibodies; SIL-SAH1).
13. Tacs 2 TdT–HRP Blue Label (TUNEL) kit (Trevigen [Gatherburg, MD]; 4811-30K).
14. TGFβ2, human recombinant (Genzyme [Boston, MA]; 302-B2-002).
15. TNT T7 Transcription/Translation System (Promega, [Madison, WI]; L1170).
16. Vacuum Manifold Filtration Unit (Millipore [Bedford, MA]; XX2702550).
17. Cleavage assay buffer: 20 m*M* HEPES, pH 7.4, 0.5% NP-40, 100 m*M* NaCl, 20 m*M* dithiothreitol (DTT).
18. CC buffer: 20 m*M* HEPES 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EDTA, and 1 m*M* DTT.
19. Fluorography solution: 20% polypropylene oxide (PPO) in glacial acetic acid.
20. High-salt buffer: 10 m*M* HEPES, pH 7.0, 0.5 *M* NaCl, 0.5% NP-40, 1 m*M* EDTA.
21. HL buffer: 10 m*M* Tris base, pH 8.0, 1 m*M* EDTA pH 8.0, 0.2% Triton X-100.
22. Molybdopterin guanine dinucleotide (MGD) buffer: 50 m*M* NaCl, 2 m*M* $MgCl_2$, 5 m*M* EDTA, 10 m*M* HEPES, pH 7.0, 1 m*M* DTT.
23. pNA assay buffer: 20 m*M* HEPES buffer, pH 7.4, 0.1 *M* NaCl, 0.05% Nonidet NP-40.
24. Western blocking buffer: 60 m*M* Tris base, 204 m*M* NaCl, 0.1% Tween-20, pH 7.3.
25. Western transfer buffer: 25 m*M* Tris base, 192 m*M* glycine, 20% methanol.
26. Whole-cell lysis buffer: 20 m*M* Tris base, pH 7.4, 1% Triton X-100, 10% glycerol.
27. ^{3}H-Methyl thymidine (Sp. Act. 74 Ci/mmol, ICN Biomedicals [Costa Mesa, CA]; 24060).

3. Methods

3.1. Growth Arrest and Cell Death

3.1.1. ^{3}H-Thymidine Assay for Suspension Cells

1. In a 24-well plate, add 5×10^4 cells in 1 mL in each well, in quadruplicate.
2. Treat cells with appropriate concentration of TGF-β for 20 h.
3. Add 1–2 μCi/well ^{3}H-methyl thymidine in 100 μL of serum-free media.
4. Incubate 4 h at 37°C in a tissue culture incubator.
5. Process for ^{3}H counts after filter assay.

3.1.1.1. Filter Assay

1. Apply GF-C glassfibre filters to a vacuum filtration unit.
2. Wet each filter with 1 mL of fresh, ice-cold 10% trichloroacetic acid (TCA) and vacuum.
3. Apply cell suspension and media from each well to individual filters and vacuum.
4. Wash 3X 5 mL each with 10% TCA and vacuum after each wash.
5. Remove filter from filtration unit, place in glass vial, add 4 mL of scintillation fluid, cap, and count.

3.1.2. Propidium Iodide Staining by FACS for Cell Cycle Anaylsis

Progression through the cell-cycle results in changes in the cellular DNA content that can be analyzed and quantitated by various fluorochromes after fluorescence-activated cell sorting (FACS). Propidium iodide (PI) is a DNA-binding fluorochrome that can also bind double-stranded RNA; thus, an RNAse digestion step is included. PI staining identifies cell populations in the G1, S, and G2/M phases of the cell cycle. A sub-G1 peak identifies the population of cells that are apoptotic.

3.1.2.1. Cell Fixation

1. Collect at least 1×10^6 cells by centrifugation at 300*g* for 5 min.
2. Wash 1X with ice-cold 1X phosphate-buffered saline (PBS).
3. Resuspend the cells in 300 μL of ice cold 1X PBS to get a single-cell suspension.
4. Set the vortex to shaking at speed "2" and shake the tube.
5. Add 700 μL of ice-cold 100% ethanol, dropwise.
6. Close the tube, vortex once to achieve a single-cell suspension.
7. Keep the tubes at 4°C overnight.

3.1.2.2. Staining the Fixed Cells

1. Centrifuge the cells at 300*g* for 3 min.
2. Wash with ice-cold 1X PBS twice.
3. Resuspend the cells in 100 μL of 1X PBS containing 100 μg/mL of RNase A.
4. Incubate at 37°C for 30 min.
5. Add 100 μL of 1X PBS containing 800 μg/mL of PI.
6. Incubate at 37°C for 30 min, in the dark.
7. Keep the cells on ice and analyze using a 536-nm excitation and 617-nm emission filters.

3.1.3. Determination of Cellular Viability via Trypan Blue

Cell survival, as well as cell number, are assayed by direct bright-field microscopic evaluation of the proportion of cells able to exclude the vital dye, Trypan Blue. Apoptotic cells undergo extensive intracellular protein crosslinking, thereby excluding Trypan Blue. In vivo, apoptotic cells are rapidly phagocytozed by macrophages or neighboring epithelial cells. In cell culture however, apoptotic cells exclude Trypan Blue for a limited time as a result of the failure of the cells to be removed, also known as secondary necrosis.

1. Treat cells with vehicle or TGF-β and collect by centrifugation at 300*g* for 5 min.
2. The media is aspirated and the cells are resuspended in a minimal volume.
3. 20 μL of cell suspension is removed and combined with 20 μL of 0.4% Trypan Blue.
4. Cells able to exclude the dye are clear and viable, whereas those that are blue are dead.
5. The number of cells are counted in duplicate and added. The number obtained is the number of cells multiplied by 1×10^4/mL.

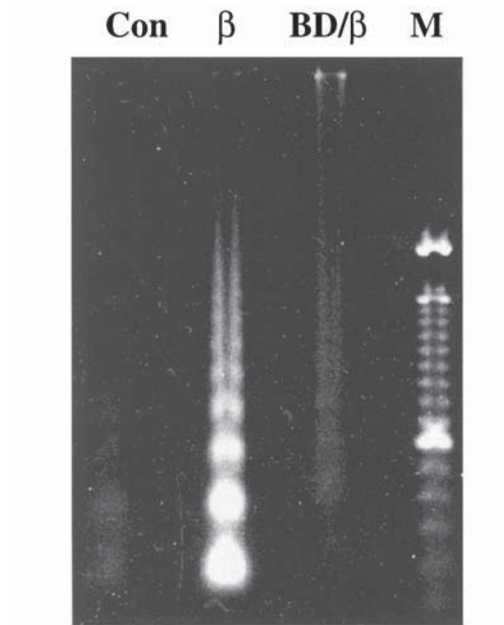

Fig. 1. TGF-β induces oligonucleosomal DNA laddering. 1 × 10^6 WEHI 231 cells were treated for 24 h with control (Con, 4 m*M* HCl / 0.5 mg/mL BSA), 5 ng/mL TGF-β (β), 5 ng/mL TGF-β following a 1-h preincubation with 100 μ*M* BD-fmk (BD/β); 100-bp DNA ladder (M).

3.2. Identification of Apoptotic Cell Death

3.2.1. DNA Fragmentation Assay

Identification of oligonucleosomal DNA laddering in repeating 180-bp units is a classic hallmark of apoptotic cell death (**Fig. 1**). This differs from necrotic cell death, which is represented by randomly cleaved DNA fragments and results in a smear upon DNA analysis. The apoptotic DNA fragmentation pattern represents the cleavage of DNA between nucleosomal linker units by a specific DNAse(s). These small DNA fragments are capable of escaping the nuclear membrane and are, therefore, enriched and can be isolated from the cytosol of cells undergoing apoptosis.

3.2.1.1. Isolation of Cytosolic Oligonucleosomal DNA

1. Spin media and cells (*see* **Note 1**), 300*g* for 5 min.
2. Carefully aspirate the supernatant.
3. Add 700 μL of HL buffer to the cells, resuspend, and transfer to an 1.5-mL Eppendorf tube.
4. Incubate 15 min at room temperature.

5. Add one volume of Tris-saturated phenol and shake to mix.
6. Spin for 2 min at 14,000*g* at room temperature.
7. Transfer supernatant to a new tube and add one volume of phenol:chloroform : IAA (25 : 24 : 1).
8. Spin for 2 min at 14,000*g* at room temperature (aqueous phase should be clear or repeat **step 7**).
9. Transfer the upper, aqueous layer to a new tube and add 1/10 volume of 5 *M* NaCl and an equal volume of isopropanol.
10. Precipitate at –20°C overnight.
11. Spin for 20 min at 4°C, 14,000*g*.
12. Wash the pellet with 1 mL of 70% ethanol
13. Air-dry pellet for approx 20 min.
14. Resuspend in 20 µL TE pH 8.0 and add 1 µL of 0.5 mg/mL DNAse-free RNase A (25 µg/mL final) at 37°C for 15 min.
15. Add 3 µL of 10X DNA dye.
16. Load dry on 1.2% agarose gel containing ethidium bromide and run in 1X TBE at 50 V (constant voltage) for 30 min, then cover with buffer and run at 18 V overnight.

3.2.2. Annexin V Staining

Translocation of phosphatidylserine (PS) from the inner plasma membrane to the outer leaflet is one of the earliest detectable events in the apoptotic process. Annexin V, a calcium-dependent phospholipid-binding protein, binds to exposed PS. Thus, a combination of PI and Annexin V–fluorescein isothiocyanate (FITC) staining can distinguish apoptotic cells (positive for Annexin V–FITC) from live cells (negative for both dyes) and dead cells (positive for both dyes).

3.2.2.1. Cell Fixation

1. 2×10^6 cells are washed 2X with ice-cold 1X PBS and resuspended in 200 µL of 1X Annexin V binding buffer.
2. 100 µL of the above cell suspension is transferred to a new tube and 1 µL of 50 µg/mL FITC-conjugated Annexin V and 10 µL of PI reagent are added, mixed gently, and incubated for 15 min at room temperature in the dark.
3. 400 µL of the 1X Annexin V binding buffer is added and analyzed within 1 h.

3.2.3. TUNEL Assay for Apoptotic Analysis of Suspension Cells

The TUNEL assay is used to identify cells with free 3' hydroxyl groups in the DNA that are present in cells undergoing apoptosis. This free 3' hydroxyl in DNA is presumably the result of cellular DNAse(s) that is activated during the apoptotic process and are responsible for generating a oligonucleosomal DNA ladder. Identification of these cells is detectable *in situ* by incorporating a biotinylated deoxynucleotide via terminal deoxytransferase (TdT) to the free 3' hydroxyl end. Subsequent exposure to streptavidin–horseradish peroxidase (HRP) allows for visual identification of these cells by exposure to the HRP

substrate, 3,3' diaminobenzidine. This procedure is highly sensitive and results in positively labeled cells that are brown with minimal background. Labeled cells can be quantitated to determine the percentage of cells undergoing apoptosis at any given time.

3.2.3.1. Preparation

1. For suspension cells, collect 1×10^5 cells in 1 mL of media.
2. Spin at 1000*g* in a microfuge for 5 min.
3. Carefully remove supernatant and resuspend in 10 µL of 1X PBS
4. Apply to a slide in a circle, let semi-air-dry (approx 10 min).
5. Fix for 20 min in 4% formaldehyde /1X PBS (50 µL).
6. Remove solution and place in 70% ethanol for 5 min.
7. Dehydrate the slides through graded ethanols; 70%, 95%, 100%, 1 min each and air-dry.

3.2.3.2. In advance (For Every 10 Slides)

1. Make 50 mL of 1X labeling buffer (5mL/45mL water).
2. Reserve 50 µL/sample in a separate tube of 1X labeling buffer.
3. Make 50 mL of 1X Stop buffer (5mL/45mL water).
4. Prepare 50 mL 2% H_2O_2 (3.3 mL of 30% peroxide stock in 46.7 mL H_2O).

3.2.3.3. Analysis

1. Rehydrate slides 1 min each through graded ethanols in 1X PBS; 100%, 90% (e.g., 90 mL absolute ethanol and 10 mL water), 75%, 50%, 25%, and, finally, 1X PBS.
2. Wash slides in 1X PBS for 10 min and carefully remove the solution.
3. Incubate each slide in 50 µL of Proteinase K solution.
4. Cover slip and incubate for 20 min at room temperature.
5. Remove endogenous peroxidase activity by incubating slides for 5 min in 2% H_2O_2 solution.
6. Wash slides 3X in 1X PBS and then immerse in 1X labeling buffer.

3.2.3.4. TdT Labeling (*n* Samples)

TdT dNTP mix	$n \times 0.5$ µL
50X divalent cation (Mn^{+2})	$n \times 0.5$ µL
TdT	$n \times 0.5$ µL
1X labeling buffer	$n \times 50.0$ µL

Incubate with 50 µL/sample and cover slip at 37°C for 1 h in a humid chamber.

3.2.3.5. Detection

1. Stop the reaction with 50 mL 1X Stop buffer.
2. Incubate 5 min at room temperature.
3. Quick dip in H_2O for less than 1 min.

4. Dilute streptavidin–HRP conjugate 1/500 in Blue Strep–HRP diluent.
5. Incubate 50 µL/sample of diluted Strep–HRP at room temperature for 15 min.
6. Wash 2X in H_2O,1.5 min each and remove water.
7. Add 25 µL of Blue label/sample for 0–7 min at room temperature; then quick dip slides 3–4X in H_2O.
8. Briefly counterstain with Red C, monitor carefully 30–60 s.
9. Dehydrate 3X in 100% ethanol; followed by Para-Xylene, and cover slip after a drop of Permount.
10. View under bright-field optics.

3.3. Determination of Caspase Activity

Caspases (cysteine aspartyl proteinases) are unique cysteine proteases that are intricately involved in apoptotic cell death. Caspases are present intracellularly as inactive proenzymes and activated by aspartate cleavage of proenzyme caspases or conformational changes associated with the recruitment of binding factors ***(1)***. Cleavage of procaspases initiates a signaling cascade and the formation of an active heterodimeric enzyme composed of larger and smaller subunits ***(2)***. Caspase activation leads to limited proteolysis of cellular substrates that culminate in apoptotic cell death ***(3)***. Proteolytic caspase cleavage is dependent on a tripeptide amino acid sequence that conforms to the caspase binding pocket and is immediately upstream and adjacent to the aspartate residue where cleavage occurs ***(2,3)***. The aspartate residue is absolutely required for proteolysis; however, the different adjacent tripeptide amino acids most likely determine caspase substrate specificity.

3.3.1. Spectrophotometric Caspase Activity Assay

Ac-YVAD-pNA is a para-nitroanilide reagent used for colorimetric analysis of caspase activity generated in vitro or in cell lysates via hydrolysis. YVAD is a tetrapeptide caspase inhibitor for interleukin-1β converting enzyme (ICE). Ac-DEVD-pNA is a similar reagent that is more specific for the caspase 3 family of proteases. These reagents are also commercially available as fluorogenic, AFC (7-amino-4-trifluoromethyl coumarin)-conjugated compounds.

In 90 µL of pNA assay buffer:

Add purified caspase preparations (approx 15 ng) or cellular lysates (10–200 µg) at the same time as 10–500 µ*M* Ac-YVAD-pNA.
Incubate at 37°C for 0–24 h.
Remove aliqouts at 30-min to 1-h intervals.
Analyze the reactions in a spectrophotometer at 405 nm (*see* **Note 2**).

3.3.2. In Vitro Caspase Activation Assay via Cytochrome C

Cytochrome C is often released from the mitochondria into the cytosol during apoptosis. Upon entering the cytosol, cytochrome C induces a dATP-dependent formation of a protein complex that results in the proteolytic activation of

procaspases and apoptotic destruction of nuclei. In vitro incubation with cytochrome C and dATP with untreated cellular lysates or purified procaspases results in caspase activation and initiation of the proteolytic cascade.

3.3.2.1. Prepare Cytosolic Extract from Untreated (unactivated) Cells

1. Spin the cells at 300*g* for 5 min and wash 1X in 1X PBS.
2. Resuspend in a small volume of CC buffer and put on ice for 20 min.
3. Pass through a 26-gauge needle 15X, or sonicate 15 s on ice.
4. Spin cells at 16,000*g* for 30 min at 4°C.
5. Transfer supernatant to a new tube, determine protein concentration, and aliquot. Store at –70°C.

3.3.2.2. Incubate

50 μL of 50 μg untreated whole-cell lysate (10–15 mg/mL) (*see* **Note 3**)
48 μL cleavage assay buffer
1 μL of 1 m*M* horse heart cytochrome C (10 μ*M* final)
1 mL of 100 m*M* dATP (1 m*M* final)

Incubate at 30°C for 1 h and add 35 μL of 4X Laemmli buffer. Run samples on an sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and analyze for cleavage (activation) of specific caspases or substrates via Western blot. Alternatively, DNA can be isolated and examined for the generation of an oligonucleosomal DNA ladder.

3.3.3. Preparation and Use of Caspase Inhibitors

Determination of caspase cleavage sites has allowed the design of specific tetrapeptide inhibitors that can prevent substrate cleavage as well as block the apoptosis process in response to cell death activators (**Figs. 1–3**). These inhibitors are lipophilic and cell permeable, and can be reversible (aldehyde conjugated) or irreversible (fluoro or chloro methylketone). General as well as specific caspase inhibitors are commercially available and are made up of monopeptide to tetrapeptide amino acids that bind to critical aspartyl residues that inactivate subsequent caspase activity and further apoptotic progression (**Table 1**).

1. All caspase inhibitors are prepared in 100% dimethyl sulfoxide (DMSO) (*see* **Note 4**) at a concentration of 10–100 μ*M*.
2. Caspase inhibitors are preincubated with cells for 1 h at 37°C before treatment with TGF-β.
3. The concentrations of caspase inhibitor vary; however, as a guideline, the broad-spectrum caspase inhibitor (BD) is effective from 25 to 100 μ*M*.
4. The more specific caspase inhibitor, ZVAD, is generally effective between 50 and 250 μ*M*.
5. Specific caspase inhibitors should be effective within the range of 5–50 μ*M* (*see* **Note 5**).

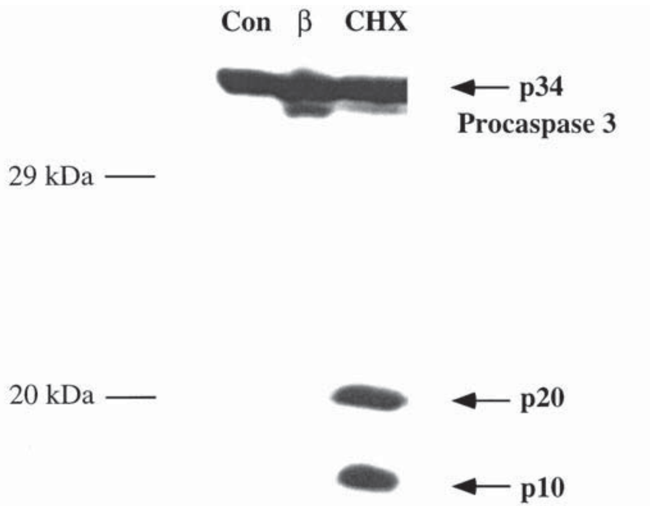

Fig. 2. Induction of capase 3 activity. WEHI 231 cells were treated for 24 h with control (Con, 4 m*M* HCl/0.5 mg/mL BSA) or 5 ng/mL TGF-β (β). Cells were also treated with 10 μg/mL cycloheximide (CHX) for 4 h. Cell lysates (50 μg) were analyzed by Western blotting with caspase 3 antibody.

6. The half-life of irreversible caspase inhibitors is 48–60 h in cell culture at 37°C, 95% O_2/5%CO_2.
7. Analysis of a delay in apoptotic cell death versus a true block can be assessed by sequential daily addition of caspase inhibitor to prevent loss of activity (*see* **Note 6**).

3.3.4. Specific Caspase Activation

Caspase activation is determined by the cleavage of procaspase (approx 30–40 kDa) into activated ~p20 and ~p10 subunits, depending on the protease (**Table 2**). Western blot analysis using specific caspase antibodies demonstrate this activation.

1. Separate 50–100 μg of whole-cell lysate by 10–12% SDS-PAGE.
2. Incubate the gel in Western blot transfer buffer for 15 min.
3. At the same time, cut Immobilon membrane to the same size as the gel, immerse in 100% methanol for 1–2 min, wash 3X with H_2O, and incubate in Western blot transfer buffer for 10 min.
4. Transfer proteins from the gel to Immobilon-P PVDF membrane (Millipore) 18 h at 100 mA.
5. Determine protein transfer empirically to Immobilon by staining with Ponceau S solution for 1 min followed by several H_2O washes.
6. The membrane is incubated for 1 h in Western blocking buffer.
7. The membrane is subsequently incubated with a 1/1000 to 1/5000 dilution (depending on titer) of primary caspase antibody (here rabbit anti-human caspase 3) in blocking buffer and incubated for 2 h at room temperature with rocking.

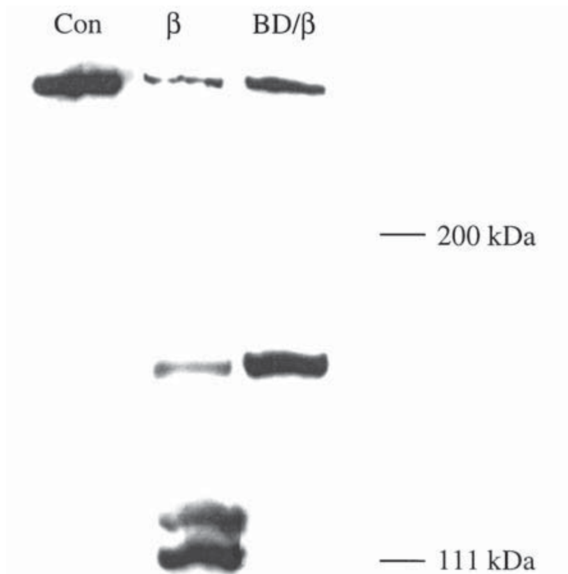

Fig. 3. Induction of αII-spectrin cleavage. WEHI 231 cells were treated for 24 h with control (Con, 4 m*M* HCl/0.5 mg/ml BSA) or 5ng/ml TGF-β (β). Cells were also pretreated for 1 h with 100 μ*M* BD-fmk followed by 24 h with 5 ng/mL TGF-β. Cell lysates (50μg) were analyzed by Western blotting with αII-spectrin monoclonal antibody.

8. The blot is washed 3X 10 min each with 1X PBS/0.05% Tween-20.
9. The membrane is incubated with a 1/2500 dilution of goat anti-rabbit–HRP for polyclonals for 1 h at room temperature.
10. The blot is washed 3X 10 min each with 1X PBS/0.05% Tween-20.
11. The blot is washed in H_2O, and incubated with a 1 : 1 solution of Renaissance Chemiluminescence Reagents (NEN) according to the manufacturer's directions for 1 min.
12. The blot is then briefly dried between filter paper, wrapped in Saran wrap, placed in a film cassette, and exposed to XAR-5 film (Kodak) in the dark from 5 s to 5 min.

3.3.5. Identification of Caspases Using Biotinylated Inhibitors

Caspases can be identified using labeled inhibitors. Biotinylated caspase inhibitors readily detect activation of cellular caspases for molecular-weight determination, activation, or microsequencing.

3.3.5.1. Western Blotting

1. Grow and treat 1×10^7 cells at the appropriate dose and time to obtain 50–60% apoptosis.
2. Collect cells by centrifugation, remove the supernatant, and resuspend the cell pellet in 1/1000 of the original media volume (10 μL for 10 mL of media).

Table 1
Specificity of Caspase Inhibitors

Inhibitor	Target caspase(s)
Z-YVAD	1, 4
Z-DEVD	3, 6, 7, 8, 10
Z-VAD 0	1, 3, 4
Z-IETD	8
Z-VEID	6
Z-DVAD	2
Z-VDVAD	2
Z-WEHD	1, 4, 5
Z-LEHD	9
BD	Broad-spectrum inhibitor
Z-AAD	Granzyme B[a]
Z-FA	Cathepsin[b]

Note: Caspase Inhibitors are available through Enzyme Systems Products. Z and B (Boc) denote the N-terminal blocking group followed by amino acids in one-letter code. All inhibitors are *O*-methylated to enhance cellular permeability and conjugated to a fluoromethyl ketone moiety to ensure irreversible binding.

[a]Z-AAD is an aspartyl cleaving serine protease inhibitor for granzyme B and is also used as a negative control.

[b]Z-FA is an inhibitor of nonaspartyl cleaving cysteine proteases (cathepsin) used as a negative control for caspases (1–4, 6–9, and 10).

3. Add 20 mL of 2X biotin–VAD in MGD. (Concentrations of 20 m*M*, 10 m*M*, 1 m*M*, 0.1 m*M*).
4. Freeze–thaw 3X in liquid nitrogen/37°C water bath.
5. Incubate at 37°C for 15 min, spin 2 min at 14,000*g* to remove cell debris.
6. Transfer the supernatant to new tube, add 13 µL of 4X SDS-PAGE buffer and run all of the sample on 10–12% gel and transfer to 0.1 µm PVDF.
7. Block for 1 h and incubate with streptavidin–HRP (1/1000) for 4 h. Develop by ECL.

3.3.5.2. Immunoprecipitation

Steps 1–5 are the same as Western blotting.

6. Transfer the supernatant to new tube and bring up the volume to 1.0 mL with MGD buffer and immunoprecipitate with 50–100 µL of streptavidin–agarose for 1 h at 4°C with rocking.
7. Centrifuge at 14,000*g* for 2 min and wash the agarose pellet 1X with MGD buffer.

Table 2
Caspase Nomenclature

Caspase	Alternative names
Caspase 1	IL 1-β-converting enzyme, ICE
Caspase 2	ICH-1, Nedd-2
Caspase 3	Cpp32, Yama, apopain
Caspase 4	TX, ICH-2,ICE_{rel}II
Caspase 5	TY, ICE_{rel}III
Caspase 6	Mch 2
Caspase 7	Mch 3, ICE-LAP 3, CMH-1
Caspase 8	MACH, FLICE I, Mch 5
Caspase 9	ICE-LAP 6, Mch 6
Caspase 8	Mch 4, FLICE 2
Caspase 11	
Caspase 12	
Caspase 13	ERICE
Caspase 14	mICE

8. Remove the supernatant and wash the agarose pellet 3X with 1.0 mL of high-salt buffer.
9. Wash the agarose pellet 1X with 1.0 mL of 10 m*M* HEPES pH 7.0.
10. Remove the supernatant and add 100 μL of 1X Laemmli buffer, heat for 5 min at 95°C and, separate on gel.

3.4. Analysis of Substrate Cleavage During Apoptosis

Activation of caspases results in aspartyl cleaving activity. In addition to the activation of other procaspases, this activity is responsible for the cleavage of numerous cellular substrates that may be critical in maintaining cellular survival (**Table 3**). As with caspase activation, substrate cleavage appears to be a cell-specific phenomenon. Cleavage of cellular proteins and prevention by caspase inhibitors can easily be determined by standard Western blotting.

3.4.1. Western Blotting for In Vivo Substrate Analysis

Analysis of cellular αII-spectrin as a caspase substrate (**Fig. 3**):

1. Separate 50–100 μg of whole-cell lysate by SDS-PAGE at 100–120 V constant voltage for 18 h.
2. Incubate the gel in Western Blot transfer buffer for 15 min.
3. At the same time, cut Immobilon membrane to the same size as the gel, immerse in 100% methanol for 1–2 min, wash 3X with H_2O, and incubate in Western transfer buffer for 10 min.
4. Transfer proteins from the gel to Immobilon-P PVDF membrane (Millipore) 18 h at 100 mA.

Table 3
Caspase Substrate Cleavage

Structural proteins	Signaling molecules
Actin	Ras-GAP
αII	Cbl/Cbl-b
βII Spectrin	Rho DA-GDI
Lamins A, B1, B2, C	Raf-1
NuMA	Akt-1
Kinectin	MEKK-1
Gelsolin	PKC8
Gas2	FAK
RNA/DNA regulation or repair	**Others**
DNA-PK c.s.	72-kDa SRP
DNA-RC 140	SREBP 1
PARP	SREBP 2
Heteroribonuclear proteins C1 and C2	Huntingtin
U_1 70-kDa SNP	Pro-IL $_1$β
Apoptotic regulators	**Cell cycle regulators**
Bcl-2	Rb
Bcl-xL	p21 WAF1/CIP1
Procaspases	MDM2
DFF45/ICAD	Pak2 (PAK1)

Note: Substrates reported to be cleaved after caspase activation. Caspase activation and substrate cleavage appear to be cell-type-specific. Therefore, identification of the caspases responsible for cleaving these proteins has been omitted and should be verified in each particular system.

5. Determine protein transfer empirically to Immobilon by staining with Ponceau S solution for 1 min, followed by several H_2O washes.
6. The membrane is incubated for 1 h in Western blocking buffer.
7. The membrane is subsequently incubated with a 1/1000–1/5000 dilution (depending on titer) of primary antibody (here αII spectrin monoclonal antibody) in blocking buffer and incubated for 2 h at room temperature with rocking.
8. The blot is washed 3X for 10 min each with 1X PBS/0.05% Tween-20.
9. The membrane is incubated with a 1/2000 dilution of rabbit α-mouse–HRP for monoclonals for 1 h at room temperature.
10. The blot is washed 3X 10 min each with 1X PBS/0.05% Tween-20.
11. The blot is washed in H_2O, and incubated with a 1 : 1 solution of Renaissance Chemiluminescence Reagents (NEN) according to the manufacturer's directions for 1 min.
12. The blot is then briefly dried between filter paper, wrapped in Saran wrap and placed in a film cassette.

13. The blot is exposed to XAR-5 film (Kodak) in the dark from 5 s to 5 min and developed. Several exposures may be necessary.

3.4.2. In Vitro Transcription and Translation (Promega)

In vitro transcription and translation systems (TNT) are useful molecular biology tools that can generate native protein from a cDNA plasmid. Therefore entire proteins or specific parts of an individual protein can be generated and used for further analysis. A further advantage is that these reactions can be radioactivley labeled to enhance the detection.

3.4.2.1. TNT Labeling Assay

25 μL of rabbit reticulocyte lysate (rapidly thaw and place on ice)
2 μL of TNT reaction buffer
1 μL of RNA polymerase (T7, T3, SP6)
1 μL of amino acid mixture minus methionine
2 μL of [^{35}S]-methionine (1000 Ci/mmol at 10 mCi/mL)
X mL of plasmid cDNA 1 mg

Bring total volume of reaction to 50 μL with the addition of H_2O, incubate at 30°C for 2 h, add 50 μL of 2X Laemmli buffer.

3.4.3. In Vitro Substrate Cleavage Assay

Although there are many uses of the TNT system, the proteins generated from this reaction are used to identify enzymatic cleavage (caspase) activity, as well as evaluate the cleavage sites present within the protein.

3.4.3.1. Preparation of Whole-Cell Lysates

1. Wash 5 × 10^6 cells 1X in 1X PBS.
2. Resuspend cells in whole-cell lysis buffer on ice for 10 min.
3. Sonicate for 15 s on ice.
4. Centrifuge at 14,000*g* at 4°C for 20 min (to clear cellular debris).
5. Transfer clarified supernatant to a new tube.
6. Determine protein concentration
7. Store at –20°C.

3.4.3.2. In Vitro αII-Spectrin Cleavage Assay

1. Mix untreated (control) or TGF-β–treated whole-cell lysates (0–30 μg) with αII-spectrin (3 μL of ^{35}S-methionine-labeled TNT reaction) (*see* **Note 7**). Equalize the volume by addition of the cleavage assay buffer.
2. Incubate for 1 h at 37°C.
3. Stop the reaction with 1X Laemmli buffer and boil for 5 min.
4. Run the samples on SDS-PAGE.
5. Fluorograph and autoradiograph.

3.4.3.3. Fluorography

Destain the gel for 30 min.
Incubate in 100% glacial acetic acid 5 min to dehydrate.
Add PPO solution for 45 min.
Wash extensively with H_2O, dry, and expose to film for 24–72 h at –70°C.

4. Notes

1. Cell number should be greater than 5×10^5 total cells to visualize by ethidium bromide.
2. Several dilutions should be used. A calibration curve should be generated using known amounts of the colorimetric compound (pNA).
3. Controls for cytochrome C assay include (1) omission of dATP, (2) omission of cytochrome C, and (3) inclusion of a caspase inhibitor in the reaction.
4. Although the maximum permissible concentrations of DMSO need to be independently tested for toxicity and most likely vary with cell type, for WEHI 231 cells the concentration of DMSO used for analysis should not exceed 0.25%.
5. The use of high concentrations of specific caspase inhibitors (> 50 μ*M*) to block apoptosis or substrate cleavage is usually indicative of nonspecificity.
6. A delay in apoptosis is easily recognizable as cell death will eventually occur in the presence of a caspase inhibitor over time, whereas a true block in the apoptotic pathway would be present if the cells do not die in the presence of the inhibitor.
7. When using caspase inhibitors in vitro, preincubate 5 min before beginning the reaction. Samples should include substrate only (background), sample+inhibitor+ substrate (negative control), and sample + substrate (enzymatic activity).

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