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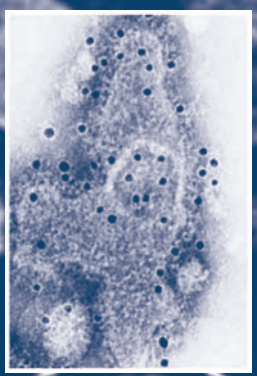
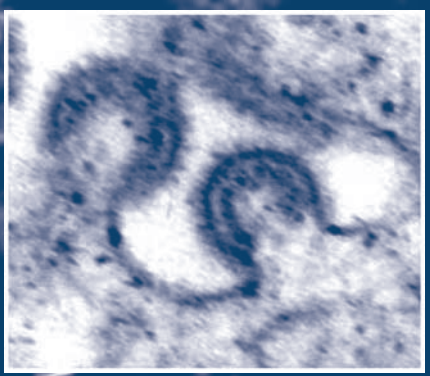
Glycovirology Protocols

Edited by

Richard J. Sugrue





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Cover Illustration. Background: Panel of Figure 5 from Chapter 5, "Functional Analysis of the *N*-Linked Glycans Within the Fusion Protein of Respiratory Syncytial Virus" by Ping Li, Helen W. McL. Rixon, Gaie Brown, and Richard J. Sugrue. Left inset: The morphology of mature respiratory syncytial virus particles. Virus particles were labeled with an antibody against the virus G (attachment) glycoprotein and visualized using electron microscopy. The location of the bound antibody was detected using a second antibody conjugated to 10-nm gold particles, which are visualized as black spots in the image. Right inset: Electron micrograph showing respiratory syncytial virus budding from the surface of infected Hep2 cells..

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Preface

The application of molecular biological techniques in the field of virology over the last 25 years has enhanced our understanding of how viruses interact with their hosts. Such studies have demonstrated that intimate interactions between virus and cell proteins occur during the virus replication cycle. Many of these interactions are mediated via posttranslational modifications, either of virus or host-cell proteins. The addition of carbohydrate molecules, also referred to as glycans, is one of the most important ways in which proteins are modified during virus infection. From the initial stages of cell attachment to the final stages of virus maturation, glycans are involved. Receptor specificity, which governs virus attachment to the host cell and is hence a major determinant of tissue tropism, is in many cases largely dependent on the structure of the glycan moieties present on the cell surface. Additionally, glycans mediate the interaction between many virus proteins and cellular chaperones during transport through the secretory pathyway, thus preventing the formation of misfolded proteins during virus maturation.

Given the increasing importance of glycosylation to the field of virology, it is pertinent and timely to produce a book that describes, and collates, some of the strategies that have been used to study the glycobiology of viruses. The focus of *Glycovirology Protocols* is restricted to glycoproteins, although it is acknowledged that other glycan-modified biomolecules, such as glycolipids, also play an important role during virus replication. The opening chapter provides an overview of glycosylation in relation to virus infection and the generic techniques that are used to analyze and characterize glycoproteins. However, many of these techniques cannot simply be taken "off the shelf," rather they must be modified to suit the specific virus system in question. It is for this reason that expert virologists have been asked to contribute chapters that describe the application of these techniques to their own specific areas of interest. *Glycovirology Protocols* is written for researchers with different levels of experience, from PhD students to senior scientists. It is intended that the information presented in this book will provide insight as to how the techniques of glycobiology can be applied in virology to answer questions that are of interest to the reader.

Richard J. Sugrue

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1

Viruses and Glycosylation

An Overview

Richard J. Sugrue

Summary

Although many virus proteins are glycosylated, the pattern of glycosylation that is exhibited can be highly variable, and it is largely dependent on how a specific virus protein is processed by the host cell during infection. However, irrespective of their glycosylation pattern, many virus glycoptoteins have been found to play essential roles during the virus replication cycle. Consequently, it is therefore becoming necessary to understand the effect that the attached glycans have on the function of different virus glycoproteins. As a first step towards understanding how glycans can influence the activity of a specific glycoprotein, we need to both establish the mechanism of glycosylation, and determine the nature of the attached glycans. This chapter provides an overview of some of the different ways in which viruses proteins are glycosylated, and highlights some of the generic techniques by which they can be examined.

Key Words: *N*-linked glycosylation; *O*-linked glycosylation; GPI-linked; lectins; virus glycoproteins.

1. Glycosylation and the Virus Life Cycle

Protein glycosylation is important at several stages of the virus replication cycle, from the initial stages of cell attachment, to the final stages of virus maturation. In general, virus proteins are glycosylated by the host cell machinery, which has circumvented the need for many viruses to encode enzymes for the glycosylation of their proteins. Therefore, the process by which virus proteins are glycosylated is very similar to the way that cellular proteins are glycosylated. Indeed, virus glycoproteins have been used as model systems with which to better understand the general mechanisms involved, and the consequences of, protein glycosylation in the cell.

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Many virus proteins are glycosylated, but the degrees to which they are glycosylated, and the structure of the attached glycans, varies. Although in most cases they are glycosylated at relatively few sites (e.g., the dengue virus E protein), some virus glycoproteins display very high levels of glycosylation. The HIV-1 glycoprotein gp160 is unusual in that it is heavily glycosylated by the addition of *N*-linked glycans, making a significant contribution to its apparent mass when analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). In this case, it is envisaged that high levels of glycosylation may have consequences in immune evasion by shielding the protein from the host's immune system. Although most virus glycoproteins are modified by *N*-linked glycosylation, some virus glycoproteins, for example the respiratory syncytial virus G protein, are extensively modified by the addition of *O*-linked glycans (discussed later).

In general, the effect of glycosylation on specific properties of virus glycoproteins is not well characterized. However, there is evidence that in some instances, the attached glycans may play a direct role in the biological activity of the virus protein in question. For example, the paramyxoviruses fusion (F) protein mediates the fusion of the cell membrane and virus envelope, and several studies have shown that glycosylation at specific sites in the F protein is required for its biological activity.

The other major role played by glycosylation during virus replication occurs during virus entry. At the initial stages of infection, the virus interacts with cell receptors, which are in many cases glycoproteins. The ability of a virus to attach to a target cell may be largely dependent on the structure of the attached glycans on the cell receptor. One of the best understood examples is the way that the influenza virus attaches to its cell receptor, sialic acid. Human influenza viruses use sialic acid that is terminated by α2,6 galactose, while avian viruses bind preferentially to silica acid, which is terminated by α2,3 galactose. These differences in glycan specificity reflect subtle differences in the structure of the haemagglutinin protein receptor binding pocket in avian and human viruses. Therefore, in the case of influenza virus, the structure of the glycan receptor is a major determinant of host range specificity.

Apart from influenza virus, several other viruses (e.g., sendai viruses) use sialic acid-containing oligosaccharides as a cell receptor. However, it should be noted that sialic acid is not the only glycan that can be used by viruses for cell attachment. For example, several viruses have been identified that use proteoglycans as a point of attachment to cells. The most commonly used proteoglycan is heparan sulphate (HS), which is used by several divergent viruses, a list that includes herpes simplex virus type 1, human cytomegalovirus and human respiratory syncytial virus.

2. Glycosylation of Virus Proteins

Virus proteins are usually glycosylated via one of three different mechanisms. These processes not only differ in cellular enzymes that are involved, but they also give rise to different types of glycan structure. The resulting glycans are referred to as being either *N*-linked, *O*-linked, or glycosylphosphatidyl inositol (GPI)-anchored, and these glycan structures will be briefly described below.

2.1. N*-Linked Glycosylation*

In the process of *N*-linked glycosylation, the glycan chains are added to the virus protein via an asparagine residue. *N*-linked glycosylation occurs at sites within the protein where the consensus amino acid sequence Asn-X-Ser/Thr is present. This is by far the most common way in which virus proteins are glycosylated, and the process by which they undergo *N*-linked glycosylation is similar to that which occurs on cellular glycoproteins.

A distinct series of steps occur during this process, which are mediated by specific enzyme activities (**Fig. 1**). This process starts by the transfer of a glycan chain from a lipid carrier (dolichyphosphate) to the polypeptide chain, as the latter is synthesised in the endoplasmic reticulum (ER). At this stage, the glycan chain exists as trimannosyl-chitobiose core ([*N*-acetyl glucosamine]$_2$ [mannose]$_3$) (**Fig. 2**) to which chains of mannose residues are attached. At the initial stage in this process, glucose residues are attached to the mannose chains by oligosaccharyl transferase activity in the ER. The glucose residues are subsequently removed by glucosidase 1 and 2. At this stage in the process, the glycan chains exist in a form that have a relatively high mannose content, and they are referred to as being high mannose or simple glycans. The mannose chains are selectively removed (trimmed) in the Golgi complex by mannosidase 1 and 2 activities, which leaves the trimannosyl-chitobiose core attached to the protein. This core structure is then further modified by the addition of different terminal monosaccharides, such as galactose, fucose and *N*-acetyl glucosamine. Fucose is commonly attached to the *N*-acetyl glucosamine that is involved in the linkage of the glycan chain to the protein. The overall effect of this is to convert the attached glycan chains into structures that exhibit a high degree of complexity, and they are referred to as complex glycans. In some instances, glycan chains can be produced that are intermediate between the high mannose and complex types, and these are referred to as hybrid glycans. Schematic diagrams showing the different N-linked glycan structures are shown (**Fig. 2**).

Although glycans may show a degree of variability in their component terminal monosaccharides, heterogeneity of complex glycans may also arise as a result of branching of the glycan chains (**Fig. 3**). This results in glycan structures that exhibit a distinct number of glycan chains arising from the trimannosyl-chitobiose core. Therefore, complex glycans can exist in a form that has either

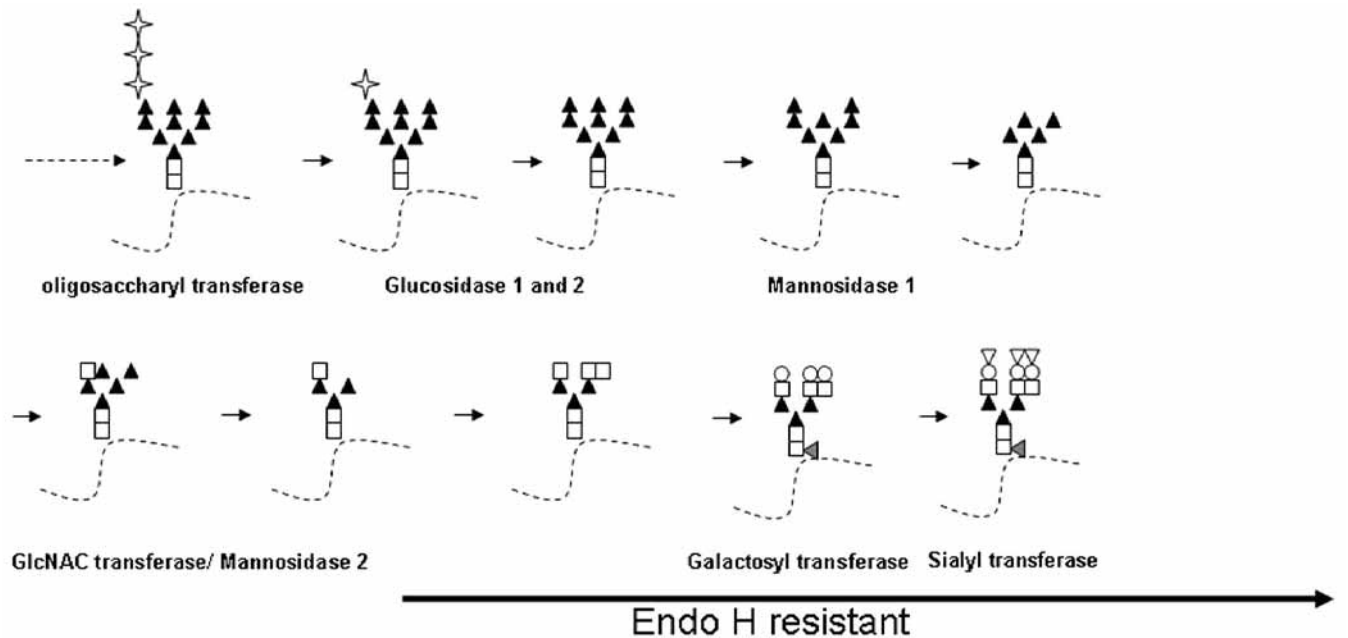

Fig. 1. Schematic diagram showing the process of *N*-linked glycosylation. Some of the various enzymatic activities that are associated with this biochemical pathway are shown. Also highlighted is the region of this pathway where *N*-linked glycans exhibit Endoglycosidase H (Endo H) resistance (open box, *N*-acetyl glucosamine; closed triangle, mannose; open star, glucose; open circle, galactose; inverted open triangle, sialic acid; grey triangle, fucose; dashed-line represents the polypeptide backbone).

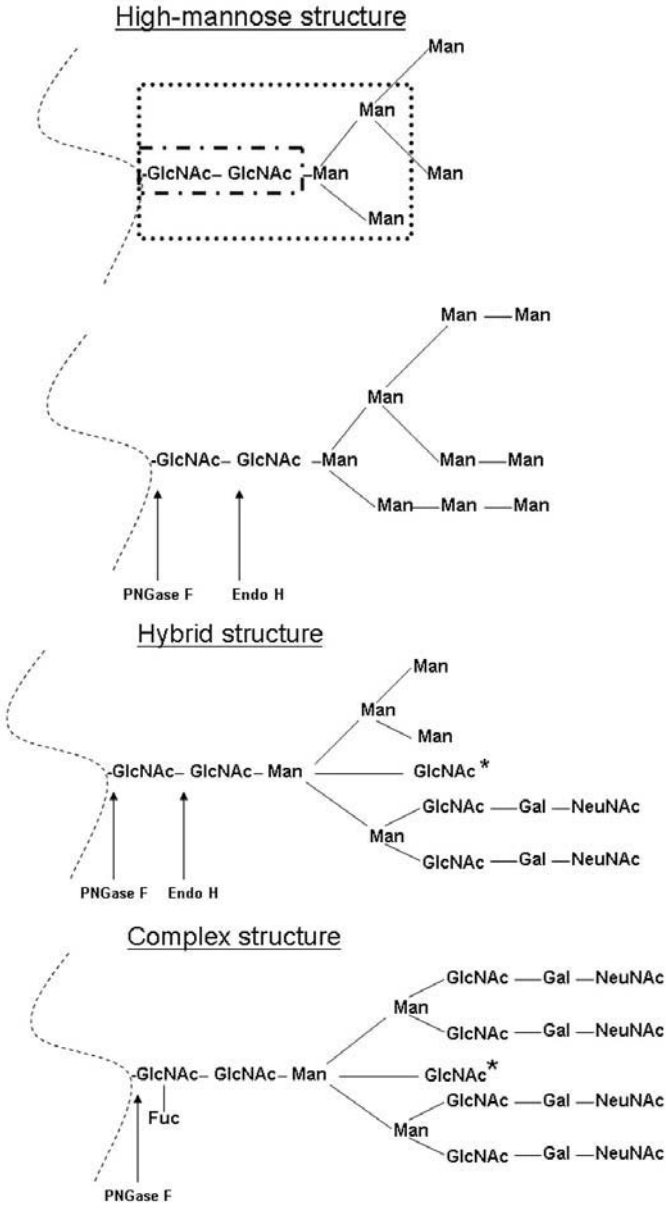

Fig. 2. A schematic diagram showing the general structures of high-mannose, hybrid and complex N-linked glycans. The chitobiose (_ . _ . _ .) and trimannosyl-chitobiose (……) core structures are high-lighted (GlcNAc, *N*-acetyl glucosamine; Man, mannose; Gal, galactose; NeuNAc, sialic acid; Fuc, fucose; dashed-line represents the polypeptide backbone. * Indicates a bisecting N-acetyl glucosamine. Also highlighted are the sites of action of PNGase F and Endo-H).

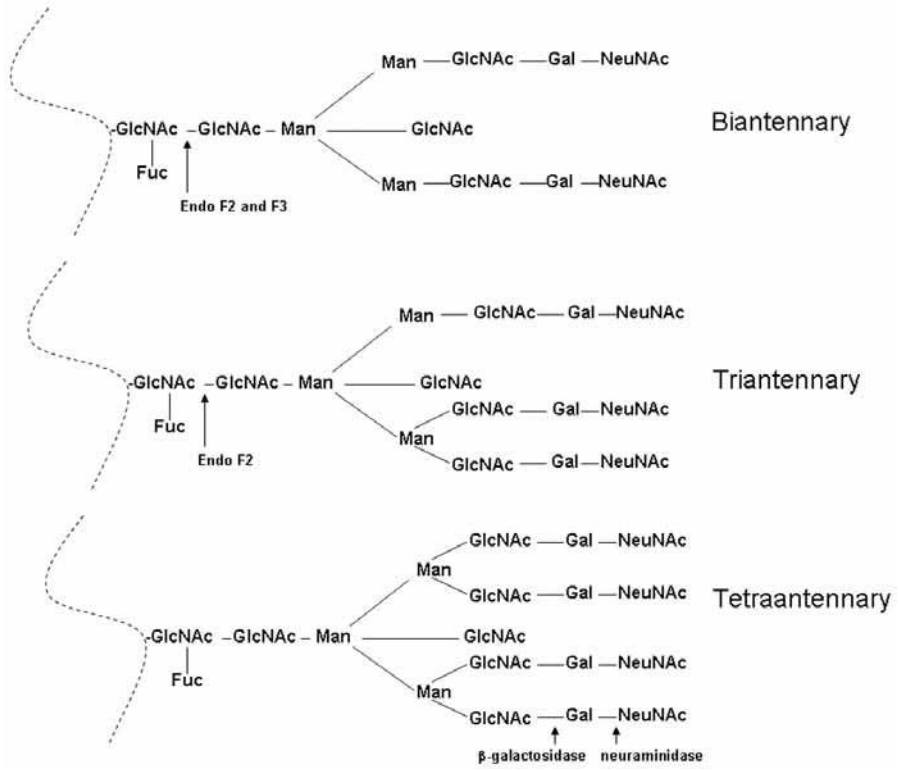

Fig. 3. Branching occurs within the mature *N*-linked glycan structure. Schematic representation showing glycans with either two (biantennary), three (triantennary), or four (tetraantennary) branches. (Key: GlcNAc, N-acetyl glucosamine; Man, mannose; Gal, galactose; NeuNAc, sialic acid; Fuc, fucose; dashed-line represents the polypeptide backbone). The sites of action of Endo F2, Endo F3, β-galactosidase and neuraminidase are highlighted.

two (bi-antennary), three (tri- antennary), or four (tetra-antennary) glycan chains attached to the core structure. In addition, in some instances an additional. *N*-acetyl glucosamine is linked to the branching mannose within the core structure (**Fig. 2**, highlighted by *), and this is referred to as a bisecting *N*-acetyl glucosamine residue.

The glycosylation process described above involves several distinct steps, which are mediated by specific enzymic activities. These different cell enzyme activities can be selectively inhibited by the use of various compounds. For example, castanospermine and deoxymannojirimycin are potent inhibitors of

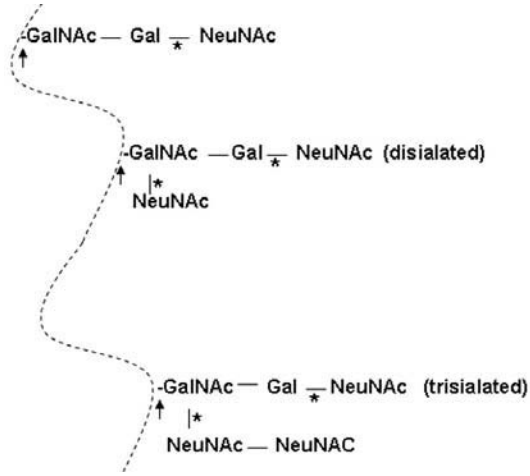

Fig 4. Schematic diagram showing the general structure of *O*-linked glycans. Also shown are chains terminating in two (disialated) and three (trisialted) sialic acid residues (GalNAc, *N*-acetyl galactosamine; Gal, galactose; NeuNAc, sialic acid; dashed-line represents the polypeptide backbone. The site of action of *O*-glycanase is also highlighted by the upward arrow).

α-glucosidase 1 and α-mannosidase 1 respectively. In the presence of these inhibitors, virus glycoproteins are expressed that contain modified *N*-linked glycans, which are structurally distinct from those that are present on the mature protein. Some of these inhibitors are currently being evaluated as potential antiviral compounds.

2.2. O-Linked Glycosylation

O-linked glycans are usually attached to the polypeptide chain through a serine or threonine residue. Unlike *N*-linked glycosylation, the addition of *O*-linked glycans does not require a consensus sequence for the transfer of the glycans to the protein. Although *N*-acetyl galactosamine is a common monosaccharide that is used for the initial attachment of the glycan to the protein, other monosaccharides can also be used, e.g., glucosamine and mannose. The *O*-linked glycans are then elongated by specific glycosyltransferase activities. The termination of the *O*-linked glycan chain is achieved by the addition of specific monosaccharides, such as *N*-acetyl glucosamine, *N*-acetyl galactosamine, and sialic acid (**Fig. 4**). *O*-linked glycosylation generally gives rise to high molecular mass glycoproteins when they are analyzed by SDS PAGE, which is due to the fact that they are usually present at several different sites on

the protein, giving rise to a high density of glycan chains. Although this type of modification is not as frequent as that of *N*-linked glycosylation, several different viruses have proteins that are extensively modified by the addition of *O*-linked glycans, e.g.. the respiratory syncytial virus G protein.

O-linked glycosylation is also involved in the formation of proteoglycans, a class of carbohydrate that is of special interest to the virologist. These carbohydrates consist of a core structure, to which one or more glycosaminoglycan (GAG) chains are attached. GAG chains are unbranched, high molecular weight polysaccharides, that consist of a backbone of repeating disaccharide units, consisting of an aminosugar and uronic acid. Heparan sulphate (HS) is an example of one of these structures, and several viruses are able to bind to HS during the initial stages of cell attachment. HS consists of repeating units of *N*-acetylglucosamine and glucuroic acid. The GAG chain is initiated by the addition of glucuronic acid to a tetrasaccharide linker. This structure is attached to a serine (that is immediately distal to a glycine residue) within the polypeptide backbone, a process that occurs via *O*-linked glycosylation. This unit is extended by the addition of the glucuroic acid and *N*-acetyl glucosamine to give long GAG chains, which are further modified to varying degrees by sulphation.

2.3. Glycosylphosphatidyl Inositol-Anchored Proteins

In some instances glycoproteins are tethered to a lipid membrane via a lipid linkage. These proteins are covalently linked at their C-terminus, via phosphoethanolamine, to a trimannosyl-non-acetyled glucosamine glycan, which in turn is attached to the membrane via a phosphatidylinositol group. This type of modification is often referred to as a glycosylphosphatidyl inositol (GPI) anchor. The complement regulatory factor CD55 is an example of a GPI-anchored protein, and this protein is the host cell receptor for some picornaviruses. Although GPI-anchored virus proteins are not as common as those modified by *N*- and *O*-linked glycosylation, there are some examples of virus proteins with GPI anchors. The dengue virus NS1 protein has recently been shown to be capable of being modified by the addition of a GPI anchor and this form of the protein is reported to have a role in cell signalling during dengue virus infection ***(1)***.

3. Generic Techniques That Are Used to Analyze Virus Glycoproteins

The following provides an overview of some of the different generic strategies that can be used to analyse virus glycoproteins.

3.1. Detection.

3.1.1. Radioactive

This is perhaps one of the oldest and most commonly used techniques to detect virus glycoproteins. In this procedure, virus-infected cells are incubated

in a tissue culture medium formulation that does not contain glucose. Glucose-free medium is commercially available from several suppliers (e.g., glucose-free DMEM from invitrogen). The proteins are metabolically radiolabelled by the inclusion of a radioactive monosaccharide in the glucose-free medium. The cells are then detergent extracted, and the radiolabeled proteins examined by SDS-PAGE. In general, the proteins must be immunoprecipitated prior to the SDS-PAGE analysis. However, in some cases, such as influenza virus infected cells, the virus glycoproteins can be analysed directly without the need for immunoprecipitation.

The list of available radiolabeled sugars from specialist companies, such as Amersham and Dupont, has now increased. These are usually available in several different specific activities, but in general, the use of high specific activity isotope is preferred. Examples of some of these isotopes that are available from Amersham include D-[6-^{3}H]Glucosamine hydrochloride (15-35Ci/mmol) and D-[2-^{3}H]Mannose (10–20 Ci/mmol).

3.1.2. Nonradioactive

Glycoproteins, as with many other proteins, can be detected using conventional proteins stains, such as Cooomassie and silver stain. However, a recent development has been the use of nonradioactive procedures to specifically detect glycoproteins in polyacrylamide gels following SDS-PAGE analysis. These reagents are usually based on a fluorescence stain which binds to glycans that are present on glycoproteins. Pro-Q-Emerald (molecular probes) and GlycoProfile™ III (Sigma) are two examples of fluorescence stains that allow the detection of glycoproteins. This type of procedure offers sensitivity of detection and obviates the costly disposal of radioisotoptes. In addition, these stains can be used in conjunction with other techniques, such as proteomics, because they are compatible with mass spectrometry. This allows glycoproteins to be identified in protein mixtures that are separated by two-dimensional (2D)-SDS PAGE, allowing the characterisation of the glycoprotein profile.

A variation of the previously mentioned techniques is called fluorophore-assisted carbohydrate electrophoresis (FACE). In this procedure, glycans are removed from a protein (e.g., enzymatic digestion) and labeled with a fluorescence tag (e.g., 8-Aminonapthalene 1,3,6-trisulfonic acid [ANTS]). The labeled glycans are then resolved on 20–40% polyacrylamide gels. However, the resolution of this procedure can be disappointing because of the pore size of the polyacrylamide gel and heating effects.

Products are also commercially available that will tag the carbohydrates on a glycoprotein, and thus allow their detection. A good example of this is a biotin-labeled hydrazide compound (Pierce chemical company). A glycopro-

tein can be immunoprecipitated, and transferred by Western blotting onto a polyvinylidine difluoride (PVDF) membrane. Treatment of membrane-bound glycoproteins with sodium metaperiodate causes oxidation of the carbohydrates within the glycan chain. Biotin hydrazine reacts with these oxidized carbohydrates, and the biotin becomes covalently linked to the glycoprotein. The presence of the biotin tag is detected using strepavidin conjugated to horse radish peroxidase. This strategy was recently used by Zimmer and colleagues to analyze the glycans on the RSV fusion protein ***(2)***.

3.2. Glycan Analysis

3.2.1. Glycosidases

The structure of a glycan that is attached to a protein determines its sensitivity to cleavage by specific glycosidases. These glycosidases were originally purified from different microorganisms, however, some of these enzymes have now been produced using recombinant expression systems, which has reduced their cost. Suppliers normally provide these enzymes together with their respective reaction buffers and additives. The list of glycosidases that are now available has increased dramatically, and the reader is invited to view the catalogues from major suppliers (e.g., Sigma and New England Biolabs) to see the availability of enzymes. The enzymes are available from suppliers in a variety of formulations. For example, PNGase F is supplied by NEB at 500,000 U/mL in 50 m*M* NaCl, 20 m*M* Tris-HCl, pH 7.5 either with or without glycerol.

Of those enzymes that are currently used to analyse virus glycoproteins, the glycosidases that process *N*-linked glycans are perhaps the most commonly used. The enzyme PNGase F removes the entire glycan chain from proteins that are modified by *N*-linked glycosylation. The sensitivity to treatment with PNGase F is often used as a default experimental method to determine if a particular protein is *N*-linked glycosylated. PNGase F is sensitive to the structural conformation of the protein, and therefore, protein denaturation may be required for efficient deglycosylation. The presence of small amounts of detergent, such as NP40, also increases the rate of cleavage. Typical reaction conditions during PNGase F treatment of a denatured protein is 1% NP40, 50 m*M* sodium phosphate, pH 7.5 at 37°C.

In contrast to PNGase F, most glycosidases cleave specific types of glycan structure, and they have been traditionally used to gain information about the structure of the attached glycans. Endoglycosidase H (EndoH) is a commonly used enzyme that cleaves between the two *N*-acetyl glucosamine residues of the chitobiose core, thus removing the latter from proteins that are modified by either high mannose or hybrid glycans. Complex glycans are resistant to EndoH treatment, therefore this enzyme can be used to determine the maturation sta-

tus of a glycan. In a similar manner to PNGase F, this enzyme works more efficiently following the denaturation of the protein to be analysed, and typical reaction conditions are 50 m*M* sodium acetate, pH 5.5 at 37°C.

Although PNGase F and EndoH are the most commonly used endoglycosidases that are used to examine virus glycoproteins, there are now several other endoglycosidases available. In particular, Endo F1, F2, and F3 (Sigma) are able to remove *N*-liked glycans from proteins under native conditions, by cleaving between the two *N*-aceytylglucosamine residues of the chitobiose glycan core. However, unlike EndoH, these enzymes exhibit an additional degree of specificity, which enables them to be used to provide extra structural information about the glycans in question. Whereas complex glycans are resistant to endo F1 treatment, complex glycans are removed by Endo F2 and F3 treatment. Endo F2 preferentially cleaves biantennary complex glycans, and its activity is not affected by the presence of core fucosylation. In contrast, endo F3 is able to remove biantennary complex glycans from glycoproteins, but it will cleave at a significantly reduced rate if core fucosylation is absent. Additionally, whereas Endo F2 has some activity against high mannose glycans, both Endo F2 and Endo F3 will not cleave hybrid glycan structures.

Glycosidases are available that will remove specific types of terminal sugars from *N*-linked glycans without removing the entire glycan. For example, enzymes such as *N*-acetyl-hexosaminidase, *N*-acetyl-galactosaminidase, and neuraminidase are able to remove terminal *N*-acetyl-galactosamine, *N*-acetyl glucosamine, and sialic acid, respectively, from the attached glycans. In addition, core fucosylation can be removed by α-fucosidase. These enzymes can be used to obtain information about the nature of the terminal sugars that are present on the glycans, and hence provide structural information about the attached glycans.

Although those enzymes that specifically recognize *N*-linked glycans have been discussed previously, enzymes are now available that allow the selective removal of *O*-linked glycans from proteins. An example of such an enzyme is Endo-a-*N*-acetylgalactosaminidase (*O*-glycosidase, Sigma), which hydrolyses the *N*-acetylgalactosamine linkage, thus liberating the core glycan from *O*-linked glycosylated proteins.

3.2.2. Lectins

Lectins are proteins that bind to carbohydrates. There are now recognized to be many different types of lectins, and they are classified according to their source. Different lectins exhibit a high degree of selectivity with respect to the structure of the carbohydrates that they bind to, which is determined by their carbohydrate recognition domain. For example, concanavalin A has a high affinity for high mannose glycans, whereas lentil lectin which has an affinity

Table 1
list of Some Common Lectins That are Available From Suppliers to Analyze Glycan Structure

Lectin/source	Specificity	Eluting carbohydrate
Concanavalin A	α-Man; α-Glc	Methyl-D-α-mannopyranoside
Dolichos biflorus	Terminal GalNAc	GalNAc
Lentil lectin	α-Man, fucosylated chitobiose core enhances binding	Methyl-D-α-glucopyranoside / Methyl-D-α-mannopyranoside
Lotus lectin	Terminal α-Fuc	L-Fuc
Peanut lectin	Galβ1-3GalNAc	Lac
PHA-E	Complex biantennary with outer galactose and bisecting GlcNAc	GalNAc
PHA-L	Complex triantennary and tetraantennary	GalNAc
Soybean	α- or β- GalNAc	GalNAc
Tomato	GlcNAcβ1-4 GlcNAc oligomers	
Wheat germ lectin	$(GlcNAc)_2$ Chitobiose core, NeuNAc	Chitotriose

for the fucosylated core region of biantennary and triantennary *N*-linked glycans. Others, such as Peanut agglutinin, have a high affinity for sugar structures that are found in *O*-linked glycans. As a result of their carbohydrate specificity, lectins have become valuable tools in the characterization of glycoproteins. A list of some common lectins that are available from suppliers to analyze glycan structure is provided (**Table 1**).

The format in which lectins are used to analyze glycan structure varies. In some cases, lectins have been conjugated to inert supports (e.g., Sepharose or Agaose), which allow the "pull down" of proteins containing specific types of glycan structure. The bound glycoproteins can subsequently be eluted using a competing carbohydrate, and the eluted protein identified using other means. In other cases, the lectins are conjugated to chemical tags (e.g., peroxidase or biotin). Proteins are first immunoprecipiated using specific antibodies, and then transferred by Western blotting onto membranes. The presence of specific glycan structures can then subsequently be detected by probing the membrane with the tagged lectin.

4. Conclusions

This chapter has provided an overview of the interaction between viruses and the process of glycosylation. However, it should be noted that this was not intended to be an extensive review of the ways in which virus proteins are glycosylated, beause it is clear that different viruses interact with the cellular glycosylation process in unique ways. It is therefore intended that this introduction will provide some general basic background information to the material that is covered in the following chapters. Furthermore, only the most common techniques used to study virus glycoproteins has been included in this introduction. However, it is likely that the techniques used to investigate the role of glycosylation in virology will expand in the future. For example, it is likely that postgenomic techniques will be increasingly applied to study the structure of glycans that are attached to virus glycoproteins and virus receptors, which will aid in the elucidation of the role that they play in virus infection.

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2

Interaction Between Respiratory Syncytial Virus and Glycosaminoglycans, Including Heparan Sulfate

Louay K. Hallak, Steven A. Kwilas, and Mark E. Peeples

Summary

Glycosaminoglycans (GAGs), including heparan sulfate (HS), are expressed on the surface of nearly all cells, linked to transmembrane proteins. These GAGs are sulfated to varying extents, lending a negative charge, and are used by a large number of viruses to initiate infection of immortalized cell lines. Here we describe the rationale and methods for analyzing GAG usage by one such virus, respiratory syncytial virus (RSV). The protocols presented allow the determination of which GAG(s) is employed by the virus, which GAG modification(s) is important, and whether the important GAG is on the cell or on the virus. We also discuss the finding that many viruses are selected for GAG usage during passage in culture and present a method for rapidly determining whether GAG usage is characteristic of a wild virus or is limited to laboratory-adapted virus.

Key Words: Glycosaminoglycans; heparan sulfate; heparin; chondroitin sulfate; proteoglycan; respiratory syncytial virus; RSV; GAG; paramyxovirus; sulfation.

1. Introduction

Glycosaminoglycans (GAGs) are linear, unbranched polymers of repeating disaccharide units produced by and associated with most mammalian cells and some bacterial cells *(1)*. The two sugars that compose the disaccharide are glucuronic acid or its epimer, iduronic acid, and an amino sugar, either glucosamine or galactosamine. Sulfates are added at various positions on the disaccharide chain, lending a negative charge to these molecules. Although the biochemistry of these GAGs is not the topic of this review, we refer the reader to reviews on GAG biosynthesis elsewhere *(2,3)*.

GAGs are found in intracellular vesicles and on the outer face of the plasma membrane, where they can act as virus receptors, as well as receptors for growth

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factors and other molecules. Many GAG–protein interactions are attributed to the affinity between the negative charges of GAGs and groups of positive charges on the protein. But evidence has accumulated that shows specific structural and conformational requirements for both the GAGs and the interacting protein to create physiologically relevant binding. Heparin binding to antithrombin was the first specific GAG–protein interaction to be recognized *(4)*. There have been more than 100 reported specific GAG–protein interactions. For example, fibroblast growth factor binds to heparin, heparan sulfate (HS), or dermatan sulfate (chondroitin sulfate type B) in the extracellular matrix and is thereby protected from degradation. The same types of GAGs are required for the activation of its receptor *(5,6)*. The binding of fibronectin to GAGs is required for cell adhesion to the extracellular matrix *(7)*.

Many bacteria *(8–13)* and viruses *(14–26)* have been shown to use GAGs, particularly HS, for attachment to, and entry of cultured, immortalized cells. In some of these viruses, such as herpes simplex virus type 1, the virus uses HS in vivo *(27)*. In other cases, particularly among RNA viruses, it is clear that the efficient use of HS is often an adaptation, selected by growth in culture *(28,29)*. For this reason, in addition to the laboratory strains, the examination of a virus for its GAG usage should include wild virus taken directly from patient samples. A reasonable and simple approach for testing virus from patient samples is included under **Subheading 3.5.1.**

We and others *(18,30–36)* have studied the dependence of respiratory syncytial virus (RSV) on GAGs for efficient attachment to and infection of cultured, immortalized cells. GAGs such as HS, chondroitin sulfate A, chondroitin sulfate B, chondroitin sulfate C, and hyaluronic acid have been tested for their role in RSV infection. Keratan sulfate is another GAG found on some specialized tissues such as cornea *(37–39)*, bone *(40)*, and epithelial cells *(41)*, including the human airway epithelium *(42)*. Although not found on the cell surface, heparin has been used as a model GAG in these studies. Heparin is more heavily sulfated than HS. It is also cheaper, and easier to derivatize.

Most cell surface GAGs are covalently linked to transmembrane "core" proteins via an *O*-glycosidic trisaccharide linkage to Ser (**Fig. 1**) within a signature sequence: Ser-Gly preceded by acidic amino acids, though all such sites are not modified. The various GAGs are distinguished both by the composition of their disaccharide subunits and by postsynthetic modifications. Some GAG chains occur in copolymer forms. For example, chondroitin sulfate A is often present on the same chain as chondroitin sulfate B and/or chondroitin sulfate C. Likewise, chondroitin sulfate B is present in copolymers with other chondroitin sulfates *(43,44)*. Whereas most of the GAGs are attached to the cell surface via core proteins, hyaluronic acid is secreted. Nevertheless, some hyaluronic acid is found on the cell surface via a noncovalent binding to its receptor, CD44.

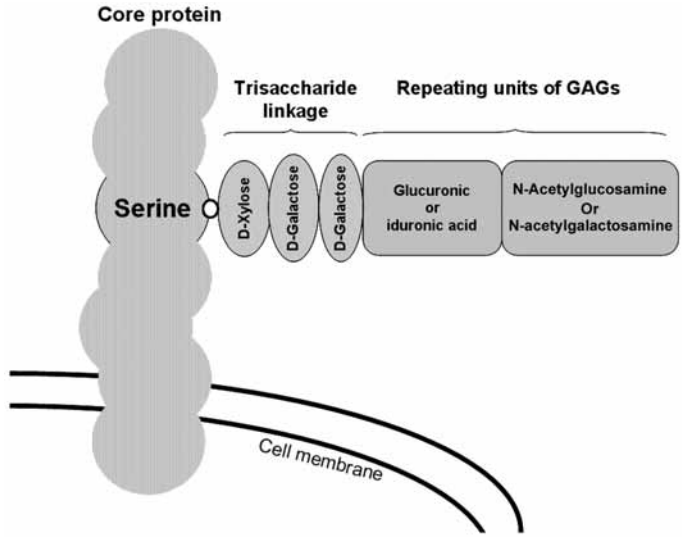

Fig. 1. Cell surface anchored proteoglycans are composed of a transmembrane core protein with one or more serine-linked glycosaminoglycan chains.

Biosynthesis of cell surface GAGs involves several steps:

1. Translation of the core protein and its translocation and anchoring in the ER.
2. Transfer of xylose from UDP-xylose to the hydroxyl group of serine followed by the addition of two galactose residues to complete the trihexose linkage region.
3. Sequential, repeated addition of hexuronic acid and aminosugars.
4. *N*-deacetylation and *N*-sulfation of the *N*-acetylglucosamine.
5. Epimerization of some of the glucuronic acid at the C5 position to generate iduronic acid in HS and chondroitin sulfate B.
6. Sulfation at positions C6, C3, or C2.

The number of cells in a culture that have been infected by a virus can be determined by staining cells with antibodies against one or more viral proteins (*see* **Note 1**). Alternatively, insertion of the green fluorescent protein (GFP) marker gene into the genome of a virus can serve as a useful tracer for virus infection. By constructing and using recombinant GFP-expressing RSV (rgRSV), we have been able to quantify the role of GAGs in mediating RSV infection of cultured cells *(33)*. We have examined cells in which particular GAGs have been removed by mutation or enzymatic digestion, and studied purified GAGs for their ability to block infection. We found that cell surface HS, and perhaps chondroitin sulfate B, are involved in infection of cultured target cells, and that binding activity correlates with the presence of certain structural sulfation patterns and the presence of iduronic acid in the GAG repeating disaccharides. As

anticipated, the major role that GAGs play in initiating infection is the attachment of virions to target cells (*see* **Note 2**).

2. Materials

2.1. Cell Lines, Viruses, and Media

1. rgRSV with GFP as an infection marker (available from the authors).
2. Chinese hamster ovary (CHO) cell lines K1 (American Type Culture Collecion [ATCC], CCL-61), pgsA-745 (ATCC, CRL-2242), pgsE-606 (ATCC, CRL-2246), pgsF-17, pgsB-761, and pgsD-677 (ATCC, CRL-2244).
3. HEp-2, a human epithelial tumor cell line (ATCC, CCL-23), is used for growing RSV stocks.
4. DMEM/F12 medium.
5. Sulfate-free medium such as Joklik-modified S-MEM medium.
6. Opti-MEM medium.
7. Fetal bovine serum (FBS).
8. Penicillin/streptomycin.
9. Glutamine.
10. Phosphate-buffered saline (PBS).
11. Cell washing/blocking buffer (PBS with Ca^{2+} and 5% FBS).
12. Trypsin/EDTA.
13. Dialyzed FBS.

2.2. Chemicals and Enzymes

1. Soluble GAGs from Sigma-Aldrich Co. (St Louis, MO): bovine lung heparin (H-9133), porcine intestinal mucosa HS (H-9902), bovine intestinal mucosa HS (H-5393), bovine kidney HS (H-7640), chondroitin sulfate A (C-9819), B (C-3788), and C (C-4384).
2. Basic fibroblast growth factor (bFGF) from Sigma-Aldrich Co. (F-0291).
3. 4% Paraformaldehyde solution (20 g paraformaldehyde, 28 m*M* KH_2PO_4 [1.90 g], 36 m*M* $NaHPO_4$ [2.48 g], water to 500 mL). Heat to 65°C with stirring until dissolved. Filter to remove undissolved particles.
4. Heparinase I and heparinase III (heparitinase) from Sigma-Aldrich Co. (H2519 and H8891, respectively).
5. Protamine sulfate from Sigma-Aldrich Co. (P-4020)
7. Chemically modified heparin: *N*-De-sulfated, fully *N*-sulfated, 6-*O*-De-sulfated, and 2-*O*-De-sulfated are available from Neoparin Inc. (Alameda, CA; GT6030, GT6041, GT6013, and GT6012, respectively). Heparin fragments for size assays are prepared commercially by Neoparin Inc., or similar services.
8. Sulfur-35 sulfate ($^{35}S[SO_4]$) is available from GE Healthcare Life Sciences, formerly Amersham Biosciences, (SJS1).
9. Sucrose to prepare ultracentrifugation density gradient.
10. Nonfat dry milk to make a 2% solution in PBS for blocking cells before antibody staining.
11. 0.02% Triton X-100 in PBS.

3. Methods

3.1. Establishing a Correlation Between Viral Infection and Cell Surface GAGs

3.1.1. Infection of GAG-Deficient CHO Cell Lines

The most informative first experiment to determine whether a virus uses GAGs to enter target cells is to compare its relative infection efficiency on GAG-expressing and GAG-deficient CHO cells. This assay is simple and can be analyzed by flow cytometry, as described in the following. If the amount of virus is limiting, as for clinical samples, as little as 1000 plaque-forming units (pfu) can be used, as described in **Note 3**.

A series of CHO cell lines, each deficient in one of the enzymes required for GAG synthesis, have been generated by chemical mutagenesis in the laboratory of Jeff Esko *(45–47)*. These cell lines can be obtained from the ATCC. The most severely GAG-deficient cell line, CHO-*pgsA-745,* lacks nearly all of its xylosyltransferase activity. Because this enzyme adds the first sugar to initiate GAG chain formation, the cell line is nearly devoid of HS and chondroitin sulfates. This mutant is used in our protocols to assess the usage of cell surface GAGs to initiate RSV infection.

Similar experiments can be performed using other CHO mutants such as pgsE-606 (lacks *N*-sulfotransferase), pgsF-17 (deficient in 2-*O*-sulfotransferase), pgsB-761 (lacks galactosyltransferase I required for HS and chondroitin sulfate expression), and pgsD-677 (does not express HS due to lack of *N*-acetylglucosaminyltransferase and glucuronosyltransferase required for HS polymerization). These additional mutants can be very helpful in identifying which of GAG modifications are important for virus binding, as discussed under **Subheading 3.2.3.**

3.1.1.1. Infection of CHO Cell Lines With rgRSV

1. Plate 1×10^5 cells of CHO K1 or CHO pgsA-745 in each well of a 12-well tissue culture dish, in 1 mL of DMEM/F12 medium supplemented with 10% FBS, glutamine, penicillin, and streptomycin. Incubate overnight at 37°C in a humidified 5% CO_2 incubator. (These conditions are used for incubation throughout this report.)
2. On the following day, inoculate cells with a multiplicity of infection (MOI) of 1, in a volume of 0.25 mL Opti-MEM without serum. Incubate plates at 37°C for 2 h with redistribution every 15 min. Redistribution of viral inoculum can be done manually or by using a mechanical tilting table at a low speed.
3. Remove unbound virus and rinse cell monolayers twice with PBS.
4. Feed cells with 2 mL of DMEM/F12 medium supplemented with 10% FBS and incubate the plates at 37°C for 36 h to allow the virus in infected cells to produce GFP.

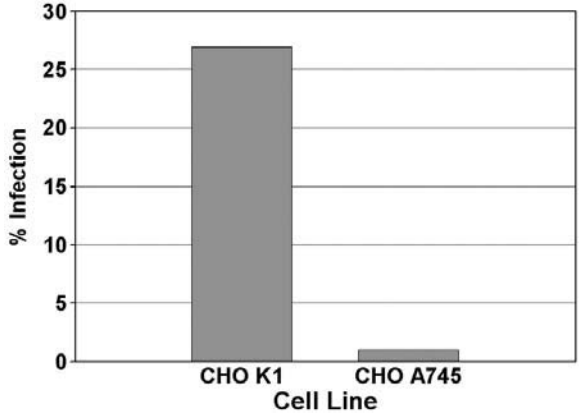

Fig. 2. The recombinant green fluorescent protein-expressing respiratory syncytial virus infects Chinese hamster ovary (CHO) K1 cells (express glycosaminoglycans [GAGs]) more than 20-fold more efficiently than it infects CHO PGSA-745 cells (severely deficient in GAG expression). Both sets of cells were inoculated with the same virus 24 h prior to analysis by flow cytometry.

5. Examine infected control CHO-K1 cells under a fluorescence microscope equipped with the appropriate filter for GFP emission. About 60% of the cells should fluoresce green. If the virus used does not express GFP, see **Note 1**.

3.1.1.2. Fixing Infected Cells for Flow Cytometric Analysis

1. Remove the medium and rinse cell monolayers with PBS. Add 1X trypsin-EDTA and allow sufficient time for cells to detach from plastic. Do not overtrypsinize.
2. Stop the trypsin and EDTA action by adding 5 mL cell wash buffer. This step is essential to minimize cell clumping.
3. Transfer the cell suspension into a labeled conical 15-mL tube and centrifuge at 1000*g* for 5 min.
4. Remove supernatant and resuspend cells by repeated pipetting in 0.3 mL cell washing buffer. Make certain cells are completely dispersed by testing a small sample under a microscope.
5. Add 0.3 mL of 4% paraformaldehyde solution. Incubate for 20 min at room temperature, then wash and resuspend cells in 300 µL PBS or an appropriate solution for FACS analysis such as FACSFlow™ sheath buffer (BD Biosciences, cat. no. 342003) (*see* **Note 4**).

3.1.1.3. Analyzing Infected Cells by Flow Cytometry

GFP produced by the virus can easily be used to quantify infected cells by flow cytometry (**Fig. 2**). However, the fixation step appears to lead to a slow

leakage of soluble GFP from cells, so flow cytometry analysis should be performed the same day that the cells are fixed. RSV infectivity is rapidly inactivated by paraformaldehyde solution. On the flow cytometer, run:

1. The negative control, uninfected but fixed CHO-K1 and CHO-pgsA-745 samples to gate cells as negative. Set the cursor between 1% and 3% to allow for basal autofluorescence. Note that fixed uninfected cells have lower mean channel fluorescence than unfixed cells; therefore, it is important that negative control cells be fixed at the same time and under the same conditions as the infected cells.
2. The positive control CHO-K1 cells to determine the percentage of infected cells. The percentage should roughly correspond to visual examination under an ultraviolet light microscope.
3. Run infected CHO-pgsA-745 to determine the percentage of infected cells. The ratio between the percentage of CHO-K1 and CHO pgsA-745 cells infected, which we have termed the "GAG Dependency Index," reflects the level of GAG usage, particularly HS and the chondroitin sulfates (*see* **Note 5**). Because CHO-pgsA-745 lacks these GAGs, the type of GAG involved in RSV infection cannot be determined with certainty from this experiment. Therefore, we have also used other cell lines such as CHO pgsD-677, which lacks only HS *(33)*.

3.1.2. Identification of the GAGs Involved in RSV Attachment: Removal of Cell Surface GAGs by Specific Enzymes

The human lung epithelial tumor cell line, HEp-2, is highly susceptible to RSV infection. In this protocol, we examined the effects of removal of GAG chains from the cell surface on viral infection of these cells. Commercially, there are several enzymes that can cleave HS, chondroitin sulfate, keratan sulfate, or cell-associated hyaluronic acid, but some of these enzymes have overlapping specificities and they should be used with caution. For example, sheep hyaluronidase type III randomly cleaves β-*N*-acetyl-hexamine-[1-4] glycosidic bonds in hyaluronic acid and chondroitin sulfates A, B, and C. Similarly, chondroitinase AC can cleave chondroitin sulfate A and C, but it can also remove chondroitin sulfate B if it is in a co-polymer with A or C. Chondroitin sulfate B lyase that cleaves chondroitin sulfate B polymers, and heparinase I and heparitinase that cleave HS are more specific.

1. Plate 1×10^5 HEp-2 cells in each well of a 12-well tissue culture plate.
2. Incubate cells overnight at 37°C with DMEM supplemented with 10% FBS. Cells should form approx 70% confluent monolayers.
3. Rinse cell monolayers with cell washing/blocking buffer. The FBS in this buffer blocks nonspecific enzymatic activities.
4. Prepare enzymes at 3.3 U/mL in cell washing/blocking buffer and add 250 μL to each well.
5. Include a mock-digested well, treated with the same amount of buffer but no enzyme.

6. Incubate cells at 37°C on a rocker platform, moving slowly to redistribute the enzyme. Alternatively, tip the plates in several directions every 15 min for 3 h.
7. Remove enzymes and rinse cells with cell washing/blocking buffer.
8. Inoculate each well with 0.25 mL inoculum from diluted rgRSV stock containing 4×10^5 pfu/mL in serum-free Opti-MEM (MOI = 1 in each well).
9. Incubate plates at 37°C for 2 h on a slow rocker, or manually shake the plates every 15 min.
10. Remove inoculum, rinse cells once with PBS and add 1 mL complete medium.
11. Incubate cells at 37°C for 18 to 24 h until GFP expression becomes apparent, but before syncytia start to form.
12. Harvest, fix, and analyze cells to determine the percentage infected, as outlined under **Subheadings 3.1.1.2.** and **3.1.1.3.**

Some cells may be removed from the substrate by the enzymatic treatment. For that reason, we have expressed the results of such experiments as a reduction in the percentage of cells infected, due to the removal of GAGs.

3.1.3. Identification of the Specific GAG Structures Used by RSV: Competition for RSV Infection by Purified, Soluble GAGs

If RSV binds to a specific GAG on the cell surface, it should also bind to the soluble form of that GAG. If a soluble GAG occupies the attachment sites on the virus, the virus will be unable to bind to target cells, thereby preventing infection. To determine which GAGs RSV binds, and the relative avidities, we have tested their abilities to block rgRSV infection (**Fig. 3**).

1. Prepare HEp-2 cells as in **Subheading 3.1.2., steps 1** and **2**.
2. Serially dilute heparin (1:2) in PBS beginning at 50 μg/mL and similarly dilute the other GAGs from 400 μg/mL. Include 0 μg/mL control wells.
3. Transfer 125 μL from each dilution to a fresh tube.
4. Add 125 μL inoculum from diluted rgRSV stock containing 8×10^5 pfu/mL in serum-free Opti-MEM (MOI = 1) to each tube and mix well. This dilution results in a final GAG concentration in the first tube of 25 μg/mL for heparin, and 200 μg/mL for the other GAGs.
5. Incubate virus/GAG mixtures 45 min at room temperature.
6. Remove the medium from cell monolayers and rinse the cells once with PBS.
7. Inoculate the HEp-2 cells with the virus/GAG mixtures and incubate at 37°C for 2 h.
8. Remove unbound virus, rinse cells twice with PBS, and add 1 mL complete medium. Incubate at 37°C for 16–20 h.
9. Examine and trypsinize cells, and proceed with fixation and flow cytometric analysis as outlined under **Subheadings 3.1.1.2.** and **3.1.1.3.**

3.2. Determining the Role of Sulfate in the rgRSV–GAG Interaction

The degree and pattern of sulfation in GAGs affects its interaction with rgRSV. The following experiments will test the importance of sulfate groups in rgRSV infection.

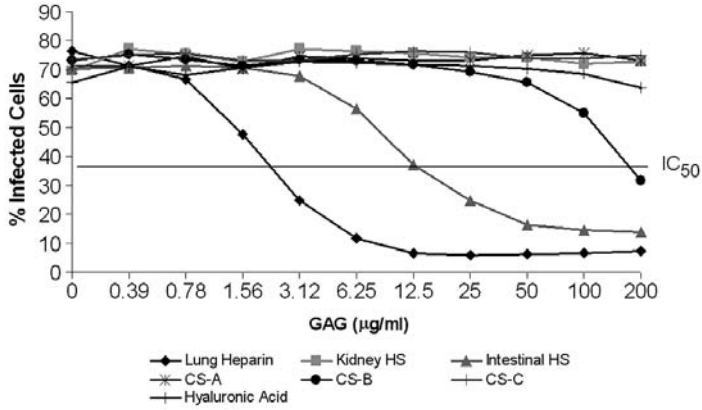

Fig. 3. Blocking experiment to determine the ability of various soluble glycosaminoglycans (GAGs) to inhibit recombinant green fluorescent protein-expressing respiratory syncytial virus (rgRSV) infection. Each GAG was serially diluted, the same amount of rgRSV added to each tube, and the mixture was added to HEp-2 cells. The cells were analyzed by flow cytometry 20 h later. The 50% inhibitory concentration can be determined from these data and used to compare the relative efficiency of inhibition as a measure of the avidity of these GAGs to bind rgRSV. These data were normalized and compiled from several experiments *(33)*.

3.2.1. Inhibition of rgRSV Interaction With Susceptible Cells by Dextran Sulfate

In contrast to GAGs, which are found on mammalian cells, dextran sulfate is a branched glucose polysaccharide produced by *Leuconostoc dextranicum* and *Leuconostoc mesenteroides* bacteria under certain conditions. It is isolated and chemically modified to contain a large number of sulfate groups that lend a strong negative charge to the compound. Dextran sulfate can be obtained commercially in various average molecular sizes. For neutralizing rgRSV, we use dextran sulfate of an average molecular weight of 5 kDa and 10 kDa and unsulfated dextran as a control with similar average molecular weight of 10 kDa.

1. Prepare HEp-2 cells as indicated in **Subheading 3.1.2., steps 1** and **2**.
2. Serially dilute dextran and the 5 and 10 kDa dextran sulfates in PBS 1:2, beginning with 50 μg/mL. Include a negative control without dextran.
3. Transfer 125 μL from each dilution to a fresh tube.
4. Add 125 μL inoculum from diluted rgRSV stock containing 8×10^5 pfu/mL in serum-free Opti-MEM to each tube and mix well. This results in a final dilution of 25 μg/mL and lower.
5. Incubate the mixtures 30 min at room temperature.
6. Remove medium from cell monolayers and rinse the cells once with PBS.

7. Inoculate the cells with the virus mixtures and incubate at 37°C for 2 h.
8. Remove unbound virus, rinse cells twice with PBS, and add 1 mL complete medium. Incubate at 37°C for 16–20 h.
9. Trypsinize cells and proceed with fixation and flow cytometry analysis as outlined under **Subheadings 3.1.1.2.** and **3.1.1.3.**

3.2.2. Blocking GAG Sulfation by Sodium Chlorate Treatment

Sodium chlorate is a general inhibitor of GAG sulfation that selectively inhibits the activity of ATP sulfate adenylyltransferase (ATP sulfurylase), the first enzyme in the sulfation pathway of cellular GAGs. However, we have observed that sodium chlorate slows cell growth, perhaps by affecting other cellular functions. Controls for this problem, particularly counting the cells after the 48-h treatment and adjusting the inoculum to maintain the same MOI as added to the control cells, should be included. In this protocol, all exogenous sources of sulfate must be removed by using medium and reagents that are sulfate-free.

1. Grow HEp-2 cells for 48 h in sulfate-free Joklik-modified S-MEM medium supplemented with 50 m*M* sodium chloride and 10% dialyzed FBS in a 75 cm^2 tissue culture flask.
2. Trypsinize chlorate-treated cells and centrifuge the cell suspension to remove trypsin.
3. Resuspend cells in the sodium-free medium with 50 m*M* sodium chlorate and plate them in a six-well tissue culture dish at 2×10^5 cells/well.
4. Incubate overnight at 37°C.
5. Remove medium and rinse cells with PBS.
6. Inoculate cells with 500 µL from diluted rgRSV inoculum in PBS containing 4×10^5 pfu/ml. Do not use Opti-MEM medium for inoculation because it contains sulfate in the form of $MgSO_4$.
7. Incubate cells 2 h at 37°C.
8. Remove unbound virus and cover the cells in each well with 2 mL sulfate-free medium supplemented with 10% dialyzed FBS.
9. Incubate cells for 24 h at 37°C.
10. Examine cells under a fluorescence microscope.
11. Remove medium, rinse and trypsinize cells, then proceed with fixation and flow cytometry analysis as outlined under **Subheadings 3.1.1.2.** and **3.1.1.3.**

3.2.3. Infection of CHO Cell Lines That Lack N- or O-Sulfation

The most common sulfation positions in heparin and HS are the *N* position of the amino sugar, the C6 of glucuronic acid, and the C2 of iduronic acid. *N*-sulfation is required for the epimerization of glucuronic acid to iduronic acid in HS. The physiological relevance of *N*-sulfation vs *O*-sulfation to the ability of rgRSV to infect cells can be tested on CHO cell lines that lack the ability to add either *N*- or *O*-sulfate groups to their HS chains. The cell line CHO pgsE-606 is

deficient in *N*-sulfotransferase activity and CHO pgsF-17 is deficient in 2-*O*-sulfotransferase activity. We found that *N*-sulfation was important whereas *O*-sulfation was not important for RSV infection *(32)*.

1. Plate 1×10^5 cells of CHO-K1, CHO pgsE606, and CHO pgsF-17 and follow the inoculation, washing and incubation steps as outlined under **Subheading 3.1.1.1.**
2. Remove medium, rinse and trypsinize cells, then proceed with fixation and flow cytometry analysis as outlined under **Subheadings 3.1.1.2.** and **3.1.1.3.**

3.2.4. Blocking rgRSV Interaction With Cellular GAGs by Protamine Sulfate

Protamine sulfate has basic charges that can efficiently neutralize heparin, HS, and chondroitin sulfate. If cell surface GAGs are involved in rgRSV binding, then treatment of cells with protamine sulfate should abrogate the ability of rgRSV to bind and therefore to infect. We found that protamine pretreatment of cells partially blocked rgRSV infection *(33)*.

1. Plate 2×10^5 HEp-2 cells in each well of a six-well plate in 2 mL Opti-MEM medium supplemented with 2% FBS. Incubate cells overnight to allow cell attachment.
2. Remove medium and rinse cells once with PBS.
3. Block cell surface GAGs by adding 1 mL protamine sulfate solution (200 μg/mL in PBS) to cells for 1 h at room temperature.
4. Remove unbound protamine sulfate and rinse cells once with PBS.
5. Add 0.5 mL rgRSV inoculum (4×10^5 pfu/mL). Incubate plates at 37°C for 2 h. Shake plates every 15 min.
6. Remove unbound virus and rinse cell monolayers twice with PBS. Add 2 mL complete Opti-MEM medium supplemented with 2% FBS and incubate cells 18–24 h at 37°C.
7. Remove medium, rinse and trypsinize cells, then proceed with fixation and flow cytometry analysis as outlined under **Subheadings 3.1.1.2.** and **3.1.1.3.**

3.3. Examining Specific Structural Requirements Within GAGs for Efficient RSV Interaction

3.3.1. The Use of Chemically Modified Heparin to Confirm N-Sulfation as a Requirement for Efficient Infection

Under **Subheading 3.2.3.**, rgRSV interaction with CHO cell lines that lack *N*- or *O*-sulfation was examined. In this section, we use a different approach to test the same rgRSV interaction with *N*- and *O*-sulfated GAGs. Chemically modified heparin with *N*-sulfate groups replaced by *N*-acetyl groups and heparin in which the 2-*O*-sulfate or 6-*O*-sulfate groups have been removed are commercially available. The neutralizing activity of these reagents can be compared to unmodified heparin. A reduced ability to neutralize rgRSV would indicate the importance of that modification for rgRSV infection. We found

that only heparin lacking the *N*-sulfation failed to block rgRSV infection *(32)*.

1. Make 1:2 serial dilutions in Eppendorf tubes of unmodified and chemically modified heparins from 100 μg/mL. Also, include a negative control with no heparin.
2. Transfer 125 μL of each diluted solution to a fresh 1.5-mL Eppendorf tube.
3. Add 125 μL of 8×10^5 pfu/mL rgRSV to each tube. The virus addition will result in an MOI =1 and reduce each heparin or modified heparin concentration to half of that in the first series of Eppendorf tubes.
4. Incubate the virus/modified heparin mixtures for 45 min at room temperature.
5. Transfer the mixture of each dilution onto a monolayer of HEp-2 cells prepared as in **Subheading 3.1.2., steps 1** and **2**.
6. Proceed as in **Subheading 3.1.3., steps 6–9**.

3.3.2. Determination of the Minimal Heparin Chain Size That Can Block rgRSV Infection

The length of heparin and HS chains vary from a few disaccharide units to several hundred disaccharide units. For rgRSV, the minimal heparin chain size that has a blocking effect on rgRSV is 10 saccharides *(32)*. But this length requires much higher concentration to achieve the same inhibitory effect of a longer heparin chain but at a lower concentration. Heparin chains smaller than 10 saccharides do not have any appreciable effect on rgRSV infection even at 200 μg/mL. This result suggests that some structural pattern, perhaps multivalency rather than the amount of sulfate is required for efficient rgRSV binding.

1. Make 1:2 serial dilutions of 2-, 4-, 6-, 8-, 10-, 14-, 16-, 18-mer, and full-length heparins from 400 μg/mL to 0 μg/mL.
2. Follow **Subheading 3.3.1., steps 2–6** above.

3.3.3. Using bFGF to Block the Iduronic Acid Subcomponent of HS and Chondroitin Sulfate on the Cell Surface

Three types of GAGs, heparin, HS, and chondroitin sulfate B, contain iduronic acid in their polymers and three other GAGs, chondroitin sulfate A, chondroitin sulfate C, and hyaluronic acid do not contain iduronic acid but contain its epimer glucuronic acid. We found that all GAGs that contain iduronic acid can neutralize rgRSV but GAGs that do not contain this component do not *(33)*. Iduronic acid in heparin and HS is required for bFGF binding. If rgRSV infection is inhibited when cells are pretreated with bFGF, it would indicate the importance of this component for rgRSV infection. We found that bFGF pretreatment of cells inhibited rgRSV infection *(33)*.

1. Plate 1×10^5 HEp-2 cells in each well of a 12-well plate in 1 mL Opti-MEM medium supplemented with 2% FBS.
2. Incubate cells overnight. Remove medium and rinse cells once with PBS.
3. Treat cell surface GAGs with 250 μL of 10 μg/mL bFGF in PBS.

4. Incubate at room temperature for 1 h.
5. Remove bFGF solution and rinse cells once with PBS.
6. Add 250 µL of a diluted rgRSV suspension (4×10^5 pfu/mL).
7. Remove unbound virus and add 1 mL complete Opti-MEM medium supplemented with 2% FBS and incubate cells 18–24 h at 37°C.
8. Remove medium, rinse and trypsinize cells then proceed with fixation and flow cytometry as outlined under **Subheadings 3.1.1.2.** and **3.1.1.3.**.

3.4. Determine Whether Sulfated GAGs are Associated With Virus Particles

The majority of cell sulfation occurs in cellular HS and chondroitin sulfates. This test will determine whether viral particles contain any sulfated GAGs associated with viral or cellular proteins embedded in the viral envelope. The rationale is that virus released from susceptible cells grown in the presence of radiolabeled sulfate, will carry the radioactive label in the modified protein.

3.4.1. Radiolabeling Virions With $[^{35}S]SO_4$

1. Grow 1×10^6 HEp-2 cells in a 75-cm^2 tissue culture flask for 24 h in Opti-MEM medium supplemented with 2% FBS and 100 mCi/mL $[^{35}S]SO_4$.
2. Remove medium and inoculate cells with 1×10^6 pfu of rgRSV in Opti-MEM.
3. Incubate the flask at 37°C for 2 h with occasional tipping to redistribute the virus inoculum and prevent the cell monolayer from drying.
4. Remove virus inoculum and rinse cells twice with PBS to remove unbound virus.
5. Feed infected cells with 10 mL Opti-MEM medium supplemented with 2% FBS and $[^{35}S]SO_4$ as in **step 1**.
6. Incubate cells at 37°C for 24 h.
7. Harvest medium and spin at 1500*g* for 5 min to remove cell debris.
8. Prepare linear sucrose density gradients in two ultracentrifuge tubes (weight/weight) sucrose in Hank's Balanced Salt Solution with Ca^{++} and Mg^{++}. Carefully layer 55%, 45%, 35%, and 25% and let stand at room temperature for 2 h or overnight at 4°C. The gradient should have a range from 25% to 55%.
9. Carefully layer equal portions of the harvested and clarified medium containing released rgRSV into each tube.
10. Centrifuge at 100,000*g* for 4 h at 4°C. Virus bands may not be visible.
11. Collect the gradient in 0.5-mL or 1-mL fractions in separate sterile tubes. Maintain sterility.
12. Test each fraction for virus infectivity by diluting them 1:10 in PBS (to dilute the sucrose, which is toxic for cultured cells if the concentration is too high), then 1:10 serially. Inoculate two wells of a 96-well tissue culture plate of HEp-2 cells. On the following day, count the number of green cells in wells with up to 200 green cells (or green cell doublets that represent local virus spread or infected cells that have divided).
13. Combine the fractions that contain the highest amount of infectious virus, dilute to fill the tube, and centrifuge at 20,000*g* for 90 min to pellet.

14. Discard supernatant and resuspend the virus pellet in 100 μL of PBS.
15. Denature the virus by adding 100 μL of Laemmli sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel loading buffer containing 1% β-mercaptoethanol.
16. Electrophorese samples on a 10% polyacrylamide gel.
17. Transfer the proteins onto a nitrocellulose membrane.
18. Dry the membrane and expose to X-ray film.

3.5. Assessment of Virus Directly From Patients for GAG Dependence

Although some viruses, such as herpes simplex virus, appear to use HS in vivo, many others are selected for HS usage during passage in cell culture. Viruses with clear evidence for adaptation include Sindbis, dengue fever, foot and mouth disease, Ross River, tick borne encephalitis, and human immunodeficiency viruses *(16,22,28,29,48–51)*. Sindbis virus is pathogenic for mice but grows poorly in cultured cells. Within a few passages in cultured cells, the virus grows to much higher titers but is no longer pathogenic in mice. This cell culture adapted virus binds heparin much better than the original mouse virus. When injected into mice intravenously, the heparin-binding virus is filtered out in the liver, in nonproductive associations *(16,28)*. It seems likely that there is a strong selection against HS binding in such a blood-borne virus in the animal, and a strong selection for HS binding in cultured cells.

It is important, therefore, early in the analysis of a virus-GAG interaction to determine whether laboratory-adapted virus accurately reflects wild virus. This analysis can be readily addressed by titrating the laboratory virus and several patient samples simultaneously in CHO K1 cells (expressing HS) and CHO PGSA-745 cells (lacking HS). Dividing the CHO K1 titer by the CHO PGSA-745 titer yields a GAG Dependency Index. For the lab strain that we generally use, rgRSV that has been adapted from the laboratory strain A2, the index is high—18 (**Fig. 4**). Interestingly, another laboratory virus strain, Long, has a much lower index—5.

We are presently performing such an analysis with clinical RSV samples. Although we have not yet tested the direct patient samples, we have serially passaged virus from patient samples 15 times in HEp-2 cells. The results for three representative examples are shown in **Fig. 4**. In two out of these three cases, the GAG Dependency Index increased between passage 5 and 15, indicating that RSV is readily selected for GAG dependence during passage in cultured cells (Kwilas, S. A. and Peeples, M. E., manuscript in preparation). We are presently testing earlier passages and the original patient sample, because it is possible that the GAG dependence at passage 5 may already represent a selection. We are also in the process of sequencing the glycoprotein genes of these viruses, before and after passage. Similar sequencing with cell culture adapted Sindbis virus found amino acid substitutions to basic amino acids, probably completing heparin binding sites.

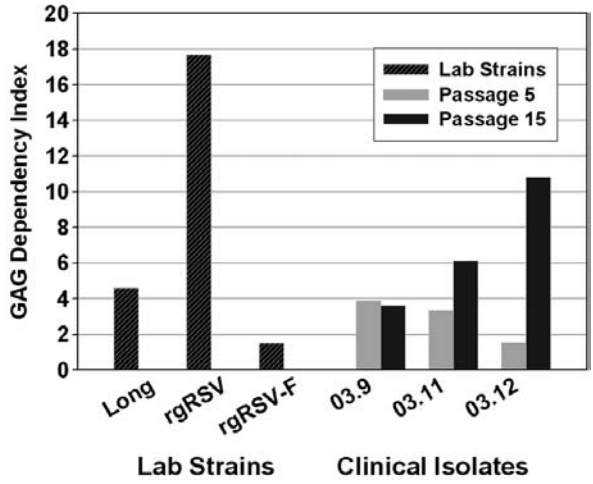

Fig. 4. Glycosaminoglycan (GAG) usage by respiratory syncytial virus (RSV) laboratory strains and clinical isolates. The GAG Dependency Index was calculated from the ability of these viruses to infect Chinese hamster ovary (CHO) K1 cells (expressing GAGs) divided by their ability to infect CHO PGSA-745 cells (severely deficient in GAG expression). Laboratory strains: recombinant green fluorescent protein-expressing (rg)RSV, a group A strain similar to A2; rgRSV-F, derived from rgRSV but with the SH and G genes deleted, leaving F as its only glycoprotein; and Long, another group A virus. The clinical viruses were each derived from a patient sample and passaged 15 times in HEp-2 cells. Their GAG Dependency Index was determined at passages 5 and 15.

3.5.1. Titration of Clinical Samples Containing Virus on CHO K1 and CHO psgA-745 Cells

1. For stocks of unknown titer, including clinical samples, make fivefold serial dilutions of the virus in serum-free Opti-MEM. Include available laboratory strains for comparison.
2. Inoculate wells of CHO K1 and CHO PGSA-745 cells growing in 96-well tissue culture dishes, and incubate for 24 h, as described under **Subheading 3.1.1.**
3. Fix cells with 4% paraformaldehyde for 20 min.
4. Permeabilize with 0.2% TritonX-100 in PBS for 30 min.
5. Block with 2% nonfat dry milk in PBS (blocking solution) for 30 min at 33°C.
6. Stain with an antibody to one or more of the viral proteins diluted in blocking solution for 30 min at 33°C.
7. Wash once with blocking solution.
8. Stain with a fluorescently labeled secondary antibody also in blocking solution for 1 h at 33°C. (Use rhodamine-labeled secondary antibody when staining expressing cells infected with a virus expressing GFP.)

9. Rinse twice with PBS.
10. Add PBS to wells and seal the edges with Parafilm, to enable short-term storage without sample drying.
11. Count stained cells. Divide the titer on CHO K1 by the titer on CHO PGSA-745 cells to derive the GAG Dependency Index.

4. Notes

1. Although we have used a GFP-expressing virus for these studies, unmarked virus can be used equally well, if infected cells are stained with antiviral antibodies, as described under **Subheading 3.5.1.**
2. The approach to determining GAG usage and characterizing the GAG(s) involved in virus infection that is described in this review is completely dependent on virus infectivity. Because GAGs are found on the surface of cells, the assumption is that the GAGs are used as a receptor by a virus. We have directly tested the receptor activity of GAGs by growing and purifying radiolabeled virus, and comparing their binding activity to their infectivity *(52)*. There is a good correlation between the efficiency of binding and the efficiency of infection of CHO K1 and CHO PGSA-745 cells, confirming that GAGs are involved in RSV binding to target cells.
3. Although flow cytometry is the ideal method for quantifying infected cells, they can also be counted with a fluorescence microscope, after inoculation of serial dilutions of the virus, as described under **Subheading 3.5.1.**
4. Trypsin treatment to release cells from the substrate sometimes results in cell aggregates that are difficult to analyze by flow cytometry and may clog the machine. To avoid these aggregates, work with subconfluent monolayers. An alternative to trypsin treatment to release cells from the culture pasticware, Versene may also help eliminate cell aggregates, although a longer treatment time is usually necessary. Including 10% fetal calf serum in the resuspension buffer also helps to prevent aggregation.
5. We have found that the GAG Dependency Index for one virus can vary somewhat from experiment to experiment, perhaps as a result of cell density. For this reason, it is important to compare viruses, including control viruses, in the same experiment.

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3

Expression of the Severe Acute Respiratory Syndrome Coronavirus 3a Protein and the Assembly of Coronavirus-Like Particles in the Baculovirus Expression System

Sehaam Khan, Mah-Lee Ng, and Yee-Joo Tan

Summary

The Bac-to-Bac Baculovirus expression system was used to generate a recombinant baculovirus capable of expressing the severe acute respiratory syndrome (SARS)-coronavirus (CoV) 3a protein. Using the same expression system, two structural proteins, membrane (M) and envelope (E), were co-expressed to form SARS-CoV virus-like particles (VLPs) within an insect cell. Expression of viral proteins was confirmed by Western blot analysis and the formation of VLPs was studied by transmission electron microscopy.

Key Words: Bac-to-Bac Baculovirus expression system; severe acute respiratory syndrome coronavirus; SARS-CoV; 3a protein; membrane protein (M); envelope protein (E); virus-like particles (VLPs); transmission electron microscopy; Western Blot.

1. Introduction

Baculovirus expression systems are widely used to allow for the expression of recombinant proteins *(1)*. The Bac-to-Bac Baculovirus expression system *(2)* is often preferred as it is a rapid system where purified recombinant baculoviruses can be positively identified within 2 wk *(3)*. The popularity of the system has further increased because the techniques used to isolate and purify the recombinant virus are relatively simple. A further advantage of insect cells is that they can fold, modify, traffic, and assemble newly synthesized polypeptides to form authentic, soluble end products *(4–6)*. However, although the baculovirus-insect cell system has protein processing capabilities similar to

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those of higher eukaryotes, the insect protein processing pathways are not necessarily equivalent to those of higher eukaryotes *(1)*. A good example of a similar but distinct processing pathway is the protein *N*-glycosylation pathway. Studies have shown that although insect cells could assemble *N*-glycans and transfer them to growing polypeptides, they have an unusual end-processing activity that trims an intermediate (common to both insect and mammalian pathways) to the insect-specific paucimannose end product *(7)*. Nevertheless, baculovirus expression of viral proteins has been successfully used for the study of numerous viruses *(1)*. This system has been particularly useful in the production of virus-like particles (VLPs) to study viral assembly processes and in several cases like the human papillomavirus and hepatitis C virus, such VLPs have been used in vaccine development *(8,9)*. Another important application is the production of glycosylated viral antigens for immunization and protection against viral infection, for example, influenza A viral antigens expressed using baculovirus have been evaluated as potential vaccine candidates *(10,11)* **.**

The recent severe acute respiratory syndrome (SARS) epidemic, which affected more than 30 countries across five continents, has profoundly disturbed social and economic activities globally. A novel coronavirus, termed the SARS-coronavirus (CoV), was identified as the etiological agent of SARS *(12)*. The SARS-CoV genome is nearly 30 kb in length and contains 14 potential open reading frames (ORFs) *(13,14)*. Five of these ORFs encode for genes that are homologous to proteins found in all known coronaviruses, namely the replicase gene 1a/1b and the four structural proteins, nucleocapsid, spike, membrane (M), and envelope (E), whereas the remaining nine ORFs encodes for accessory proteins, varying in length from 39 to 274 amino acids, which are unique to SARS-CoV. The largest of these accessory proteins is termed 3a (also known as U274, X1, or ORF3). Antibodies specific for 3a have been found in convalescent patients *(15,16)* and 3a has also shown to be expressed in SARS-CoV-infected cells *(17–19)*. 3a is a novel coronavirus structural protein as it is associated with virion purified from SARS-CoV-infected cells and it is incorporated into VLPs when co-expressed with M and E in the baculovirus system *(20,21)*. 3a is predicted to have three transmembrane domains *(13, 14)* and when it is expressed on the cell surface, its N-terminus is facing the extracellular matrix whereas the C-terminus is facing the cytoplasm *(19)*. It has also been reported that 3a is *O*-linked glycosylated, and this posttranslation modification may be important for its incorporation into virion *(22,23)*. The formation of a recombinant baculovirus expressing the 3a protein fused with a myc-tag at the N-terminus will be used to illustrate the methods used to express this protein using the Bac-to-Bac Baculovirus expression system.

The formation of VLPs of SARS-CoV using recombinant baculovirus technology has been demonstrated *(24,25)*. As has been observed for other

coronaviruses, the co-expression of two of the SARS-CoV structural proteins, M and E, is sufficient for the formation of VLPs. The M protein is a triple-spanning membrane glycoprotein that interacts with the nucleocapsid and spike protein during virion assembly *(26)*. The small E protein has more recently been recognized as an essential structural component of the coronavirion. A large portion of this protein is embedded within the viral membrane; only its hydrophilic carboxy terminus protrudes inside the virion *(27,28)*. Nal and co-workers recently showed that the SARS-CoV M protein is *N*-glycosylated, whereas the SARS-CoV E protein is not glycosylated *(29)*. Recombinant viruses expressing M and E respectively will be used to form VLPs. The co-expression will be shown by Western blot analysis, and the formation of VLPs will be shown by transmission electron microscopy.

2. Materials

1. Bac-to-Bac Baculovirus expression system (Invitrogen, Life technologies).
2. pFastBac1 vector (Invitrogen).
3. pXJ40myc-3a.
4. *Escherichia coli* strains DH5α and DH10Bac.
7. Restriction enzymes and T4 DNA ligase.
8. Luria-Bertani (LB) agar plates and media.
9. QIAprep Miniprep kit and QIAgen Midiprep kit.
10. Agarose and DNA sequencing gel equipment.
11. Oligonucleotide primers.
12. Isopropyl-β-D-thio-galactopyranoside (IPTG) and X-gal.
13. Ampicillin, kanamycin, gentamicin, bacitracin and tetracycline.
14. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) equipment.
15. 5% SDS lysis buffer: 0.3 *M* Tris-Cl, pH 6.8; 5% SDS; 50% glycerol; 0.1 *M* dithiothreitol (DTT); 0.1% bromophenol blue.
16. Whatman Filter paper.
17. *Sf* 9 insect cells.
18. Sf-900 II SFM insect medium.
19. 1% penicillin/streptomycin solution.
20. Cellfectin reagent.
21. Unsupplemented Grace's insect medium.
22. 5% nonfat milk.
23. 3a antiserum, anti-myc monoclonal, M antiserum, E antiserum.
24. Phosphate buffered saline with 0.05% Tween 20.
25. Supersignal West Pico.
26. X-ray film.
27. TEN buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, and 1 *M* NaCl with 1% Triton X-100.
28. Sucrose in TEN buffer.

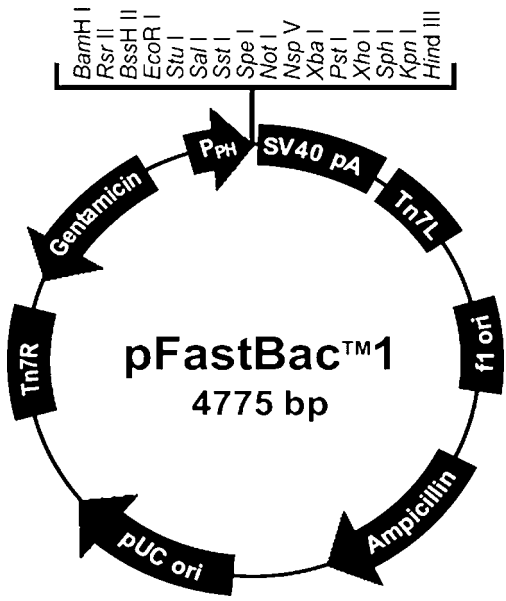

Fig. 1. Schematic drawing of pFastBac1 (Invitrogen).

29. Hybond-C Extra membrane.
30. Formvar coated copper grids and formvar coated nickel grids.
31. 2.5% gluteraldehyde.
32. Phosphotungsteic acid.
33. JEOL model: JEM1010 transmission electron microscope.
34. Incubation buffer: 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

3. Methods

The methods described below outline (1) the generation of the myc-3a recombinant virus, (2) the expression of the recombinant protein, (3) the co-expression of two structural proteins for VLPs formation, and (4) transmission electron microscopy staining and visualisation of the VLPs.

3.1. Generation of 3a Recombinant Virus

The vector utilised for this study, pFastBac1 (**Fig. 1.**), contains an expression cassette, which includes the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) promoter ***(30)*** (which allows for high level of expression in insect cells), a gentamicin resistance gene (for selection), and an SV40 polyadenylation signal to form a mini Tn7. This expression cas-

sette is flanked by the right and left arms of Tn7. In the Bac-to-Bac Baculovirus expression system, recombinant bacmid DNA is formed using the site-specific transposition properties of the Tn7 transposon *(32)*. Bacmid DNA (bMON14272) situated in *E. coli* DH10Bac cells, which allow for its propagation, contains the low-copy-number mini-F replicon, a kanamycin resistance marker, and *lac*Zα from pUC. The attachment site for the bacterial transposition Tn7 (mini-*att*Tn7) is inserted in the N-terminus of the *lac*Zα peptide. When these cells are grown in the presence of X-gal and the inducer IPTG, blue colonies are formed, because the *lac*Zα can complement a *lac*Z deletion present on the chromosome. The expression cassette present on the pFastBac1 (donor plasmid) is transposed to the mini-*att*Tn7 attachment site on the bacmid with Tn7 transposition functions provided in *trans* by a helper plasmid (pMON7124). The insertion of the mini-Tn7 of the pFastBac1 vector into the mini-*att*Tn7 attachment site of the bacmid disrupts the expression of the *lac*Zα peptide. Therefore, colonies containing recombinant bacmids will remain white in the presence of X-gal and IPTG because the *lac*Zα gene cannot express.

3.1.1. Cloning

1. The plasmid pXJ40myc-3a *(19)* was digested with restriction enzymes *Eco*RI and *Not*I as per the instructions of the manufacturer (New England Biolabs). This released a 0.822kb fragment, containing the 3a protein with the myc-epitope tag at the 5' end.
2. The fragment was ligated with pFastBac1 vector digested with the same combination of restriction enzymes. This was performed using T4 ligase enzymes according to the manufacturer's instruction and chemically transformed into *E. coli* DH5α cells by standard methods *(31)*.
3. The *E. coli* DH5α cells were then plated onto LB agar plates containing ampicillin (100 μg/mL) and incubated overnight at 37°C.
4. Single colonies were selected and grown in LB broth with ampicillin. The plasmid was then isolated following the instructions of the QIAprep Miniprep Handbook and checked for the presence of the insert and for the correct orientation using restriction enzyme digestions. Alternatively, PCR can be used to screen for positive clones (*see* **Note 1**).

3.1.2. Generating the Recombinant Bacmid

1. One nanogram of the pFastBac1-myc-3a plasmid was chemically transformed into the DH10Bac cells using standard methods *(31)*. The transformation mix was then incubated at 37°C at 225 rpm for 4 h.
2. After the 4-h incubation, 10-fold serial dilutions of the *E. coli* DH10Bac transformation mix were prepared to a dilution factor of 10^{-3}. One hundred microlitres of individual dilutions were plated onto LB agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal, and 40 μg/mL IPTG (*see* **Note 2**). Plates were then incubated for 48 h at 37°C.

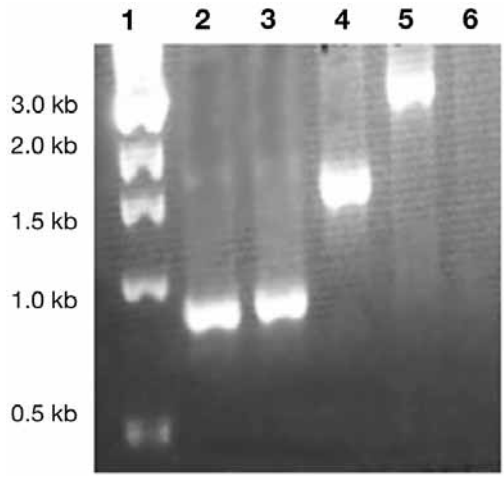

Fig. 2. PCR amplification of the recombinant bacmid DNA. Lanes 1, 1 kb marker (New England, Biolabs); lane 2, pFastBac1-myc-3a plasmid; lane 3, pXJ40myc-3a plasmid with gene specific primers (3a forward and 3a reverse); lanes 4 and 5, recombinant bacmid DNA with primer combinations of M13 Reverse and 3a forward, and M13 Forward (–40) and 3a reverse, respectively. Lane 6 was the water control.

3. To confirm the white phenotype, 10 white colonies were restreaked onto the same selection agar plates. These plates were incubated at 37°C for 16 h.
4. Once the phenotype was confirmed, a single colony was chosen and grown in LB media containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, and 10 μg/mL tetracycline.
5. The bacmid DNA was then isolated following the instructions of the QIAgen Midiprep Handbook.
6. Because of the large size of the bacmid DNA, restriction enzyme analysis is not recommended. The PCR using the M13 Forward (–40) and M13 Reverse primers is ideal to verify that the isolated bacmid DNA contain the gene of interest, because the bacmid contains these primer sites flanking the mini-*att*Tn7. PCR fragments were amplified using a combination of various primers: 3a reverse and 3a forward primers (**Fig. 2**, lanes 2 and 3), M13 Reverse and 3a forward primers (**Fig. 2**, lane 4), or 3a reverse and M13 Forward (–40) primers (**Fig. 2**, lane 5). The pFastBac1-myc-3a and the pXJ40myc-3a plasmids were used as positive controls for the PCR reaction and sterile distilled water was used as a negative control. For each primer set, a 50-μL standard PCR reaction was set up. The annealing temperature and extension time of a PCR cycle varied, depending on the primer combination. PCR fragments were visualized by agarose gel electrophoresis using standard procedures ***(31)*** (*see* **Note 3**).

3.1.3. Transfecton of Insect Cells With Bacmid to Produce Recombinant Virus

1. Two millilitres of 1 × 10^6/mL *Sf*9 insect cells, in Sf-900 II SFM medium (Invitrogen) containing a 1% penicillin/streptomycin solution (Sigma-Aldrich) (complete media), were seeded into six-well plates (*see* **Note 4**).
2. After the cells were allowed to attach for 1 h at 27°C, bacmid DNA which contained the inserted gene of interest was transfected into the *Sf*9 cells using Cellfectin reagent (Invitrogen). While the cells were attaching, 1 μg of Midiprep bacmid DNA was added to 100 μL of unsupplemented Grace's medium (Invitrogen) in a 1.5-mL microfuge tube (tube A). Six microliters of Cellfectin reagent was diluted in 100 μL of unsupplemented Grace's Medium in a separate 1.5-mL microfuge tube (tube B). The contents of tube A were added to the contents of tube B, and the solution was gently mixed, and incubated at room temperature for 30 min. Five minutes before the incubation time had expired, the Sf-900 II SFM medium was removed from the cells, and the cells were washed once with 2 mL of unsupplemented Grace's Medium after which the wash media was removed. A volume of 0.8 mL of unsupplemented Grace's Medium was added to the DNA–lipid complex, and this solution was added to the *Sf*9 cells.
3. The cells were incubated at 27°C incubator for 5 h, then the incubation media was removed and 2 mL of the complete growth media was added to the cells. The six-well plates were then incubated in a 27°C humidified incubator for 72 h (*see* **Note 5**).
4. The baculovirus infection cycle is characterized by a bi-phasic replication cycle during which two virion phenotypes are produced: (I) Occlusion derived virions (ODV) and (II) budded virus (BV) ***(33)***. Posttransfection, BV is usually released into the medium after 3 d. Depending on the transfection efficiency, a longer time period might be required to view the cytopathic effects (CPE). Some common CPE as time progresses, include enlarged nuclei, detachment, and finally cell lysis. Once CPE has been observed, the media from the wells were collected and transferred to a centrifuge tube.
5. The media was then centrifuged at 1500 rpm for 5 min at 4°C.
6. Aliquots of the supernatant, which constitute the P1 viral stock, was then transferred to sterile dark microfuge tubes, and stored at –80°C (*see* **Note 6**)
7. The P1 viral stock can be used to generate a viral stock of higher titer and volume. 1 × 10^7 *Sf*9 cells were added to 10 mL of Sf-900 II SFM medium and incubated for 1 h at room temperature to allow for cell attachment.
8. One milliliter of media was then removed and replaced with 1 mL of P1 viral stock, to allow for a final volume of 10 mL (*see* **Note 7**). The cells were then incubated for 48–72 h (until CPE was detected) in a 27°C humidified incubator.
9. After the incubation period, the media was collected and transferred to a centrifuge tube. The media was then centrifuged at 1500 rpm for 5 min at 4°C.
10. Aliquots of the supernatant were then transferred to sterile dark microfuge tubes, and stored at –80°C.
11. The P2 viral stock titer was determined by a plaque assay, as outlined **ref. *5***. Typically, the P2 virus stock has a 100-fold higher titer than P1.

3.2. Expression of the Recombinant Protein

The next step involved the confirmation of viral expression by Western blot analysis as outlined under **Subheadings 3.2.1–3.2.2**. This includes infection of cells with the recombinant P2 viral stock, *Sf*9 cell lysis, SDS-PAGE, and Western Blot analysis.

3.2.1. Infection of Sf9 Cells With the Recombinant P2 Viral Stock and Sf9 Cell Lysis

1. Viral stocks of the 3a recombinant virus were at a concentration of 1×10^8 plaque-forming units (pfu)/mL. 1×10^7 *Sf*9 cells were infected at an multiplicity of infection (MOI) of 1 and then incubated for 48–72 h (until CPE was detected) in a 27°C humidified incubator.
2. After the incubation period, cells were harvested by centrifugation at 1500 rpm for 5 min at 4°C.
3. The cells were then lysed with 5% SDS gel loading buffer (0.3 *M* Tris-Cl, pH 6.8; 5% SDS; 50% glycerol; 0.1 *M* DTT; 0.1% bromophenol blue). Because the 3a protein tends to form large aggregates when boiled, the lysate was incubated at 50°C for 15 min instead.
4. The lysate was then analyzed by a 15% SDS-PAGE.

3.2.2. Western Blot Analysis

1. Separated proteins were transferred onto Hybond-C Extra (Amersham Biosciences).
2. The membrane was blocked with 5% nonfat milk for 30 min and probed with primary antibodies, 3a rabbit antiserum (1:2000), or anti-myc monoclonal antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, California) with rolling at 4°C overnight (**Fig. 3A,B**). The antiserum used to probe the 3a protein was raised by immunizing rabbits with the amino acid fragment 134–274 of the 3a protein (***19***).
3. After three washes (15 min each) with PBS containing 0.05% Tween 20 (PBST) the blots were incubated in goat anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000, Pierce) at room temperature, with rolling for 1 h.
4. The blot was then washed with PBST three times for 15 min each, and visualised using Supersignal West Pico (Pierce) and developed on an X-ray film (Hyperfilm, Amersham Biosciences).

3.3. The Co-Expression of Two Structural Proteins for VLP Formation

The procedures outlined under **Subheadings 3.3.1.–3.3.3.** describe how the recombinant viruses expressing M and E, respectively, were co-expressed within insect cells to form VLPs. These procedures include the co-infection of insect cells with M and E recombinant viruses, the expression of viral proteins

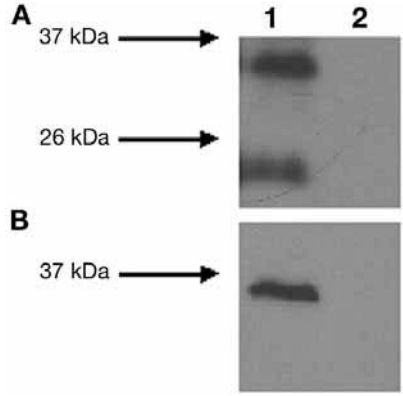

Fig. 3. Expression of severe acute respiratory syndrome (SARS)-coronavirus (CoV) 3a protein in insect cells. *Sf*9 cells were infected with a recombinant myc-3a baculovirus at a multiplicity of infection of 1 (lane 1). Cells were harvested at 72 h positinfection, lysed, and the cell lysate subjected to Western blot analysis using **(A)** anti-3a antibody and **(B)** anti-myc antibody. Two forms of myc-3a were detected by anti-3a antibody as previously reported *(19–21)*. Mock infected *Sf*9 cells were used as a negative control (lane 2).

by Western blot, and the purification of VLPs. The methods used to purify the VLPs were similar to that outlined in **ref.** *25*.

3.3.1. Infection of Insect Cells With M and E Recombinant Viruses

1. Triplicate 175-cm^2 tissue culture flasks with 2×10^7 cells of *Sf*9 insect cells were co-infected with the two recombinant viruses expressing M and E proteins at an MOI of 5:1. These recombinant viruses were kindly donated by Dr. Yu-Chan Chao *(24)*.
2. Once CPE was observed (usually 72 h postinfection) cells were harvested. Three rounds of infection were completed for a total of nine flasks.
3. To confirm that the co-infected cells were expressing both M and E, infected *Sf*9 cells were lysed with 5% SDS gel loading buffer. Even though the E protein can withstand temperatures up to 100°C, previous studies have shown that the M protein forms insoluble aggregates when boiled *(34)*. Thus, E:M lysate was heated at 50°C for 15 min.
4. The lysate was then subjected to Western blot analysis as described previously. The primary antibodies used were rabbit anti-M antibody (1:500) (anti-SARS virus PUPM C-term, purified Rabbit Pab, cat. no. AP6008b, ABGENT) and E polyclonal antibody (1:2000) at 4°C overnight, with rolling **(Fig. 4A,B)**. The antiserum used to probe the E protein was raised by immunising rabbits with amino acids 37–77 of the E protein (*35*).

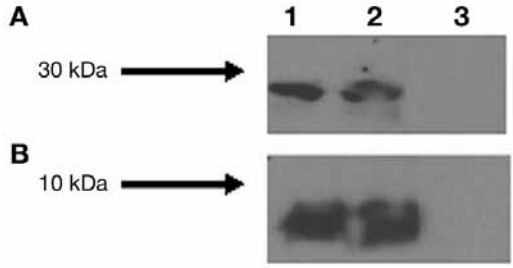

Fig. 4. Expression of severe acute respiratory syndrome (SARS)-coronavirus (CoV) M **(A)** and E **(B)** proteins in insect cells. *Sf*9 cells were infected with M only at a multiplicity of infection (MOI) of 10 (**A**, lane 1) and E only at an MOI of 2 (**B**, lane 1). The cells were also co-infected with the two recombinant baculoviruses, M and E, at an MOI of 5:1 respectively (**A,B**, lane 2). Cells were harvested at 72 h postinfection, lysed, and the cell lysate subjected to Western blot analysis using **(A)** anti-M and **(B)** anti-E antibody. Mock infected *Sf*9 cells were used as a negative control (lane 3).

3.3.2. Purification of VLPs

1. After the cells were sloughed in the growth media, the cells were separated from the media by centrifugation at 4000 rpm for 15 min.
2. The supernatant was then transferred to a sterile 50-mL centrifuge tube, while the cells were resuspended in 5 mL TEN buffer with 1% Triton X-100.
3. The cells were placed in liquid nitrogen (for quick freezing) and thawed at room temperature.
4. After the cells had thawed, they were sonicated at 2-min intervals for 10 min at 4°C. The lysed cells were then centrifuged at 3500 rpm for 30 min.
5. For each of the three rounds of amplification, the clarified supernatant of the triplicate flasks were pooled. The supernatant was then placed on a linear 30–45% (w/w) sucrose gradient in TEN buffer and centrifuged in a Beckman Ultracentrifuge at 27,000 rpm for 3 h. The opalescent band containing the particles was then collected at the interface (*see* **Note 8**).

3.4. Transmission Electron Staining and Visualization of the VLPs

Described as follows are the steps that can be used to stain and visualize the VLPs by transmission electron microscopy. These steps include negative staining and immunogold labelling. The immunogold procedure used was one modified from **ref.** *37*.

3.4.1. Negative Staining of Grid

1. Aliquots of purified VLPs were placed on a block of parafilm and the formvar-coated copper grid was placed over the aliquot (rough surface in contact with the

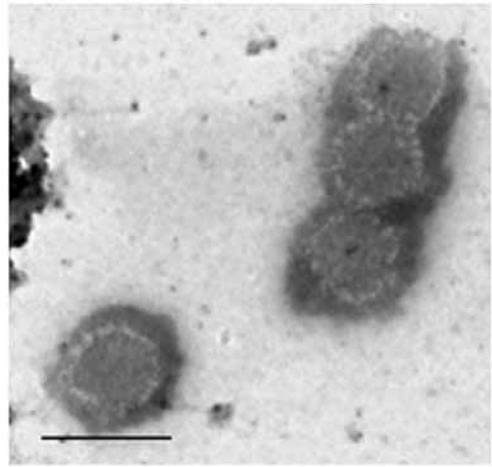

Fig. 5. Analysis of virus-like particles formed by co-infecting *Sf*9 cells with M and E and a multiplicity of infection of 5:1, respectively. Bar = 100 nm.

sample) for 1 min. The excess liquid was drained by touching the grid edge with a piece of filter paper.
2. To fix the sample onto the grid, the grid was placed onto a drop of 2.5% gluteraldehyde for 1 min. Once again, excess liquid was drained by touching the edge of the grid with a piece of filter paper.
3. To disseminate the sample evenly over the grid, the grid was placed on a drop of 30 μg/mL bacitracin for 1 min.
4. After the excess liquid was drained, the sample was negatively stained by placing the grid on a drop of phosphotungsteic acid (PTA), pH 6.0, for 1 min.
5. The excess liquid was finally drained, and the grid was allowed to dry thoroughly.
6. All samples were examined under a JEOL model: JEM1010 transmission microscope. As shown in **Fig. 5**, the VLPs of approx 100 nm can be detected and the size is slightly smaller than the spike-containing virions from SARS-CoV infected cells *(36)*.

3.4.2. Immunogold Labeling of Absorbed VLPs

1. An aliquot of 500 μL purified VLPs were centrifuged at 8000 rpm for 5 min and the pellet was resuspended in distilled water.
2. Ten-microliter drops of VLPs were absorbed onto formvar-coated nickel 300 mesh electron microscopy grids for 15 min and washed with water.
3. Grids were then floated onto incubation buffer for 15 min and then floated for 30 min on a droplet of the appropriate primary antibody (the same M and E antibodies as used under **Subheading 3.3.2.**) with different dilutions for M (1:10

and 1:100 diluted in incubation buffer) or E (1:50 and 1:200 diluted in incubation buffer),
4. **Step 3** was followed by three washes in incubation buffer.
5. Grids were floated for 30 min on a droplet of gold particles (diameter, 10 nm) (1:20 diluted in incubation buffer) conjugated to protein A, followed by three washes in incubation buffer.
6. Grids were floated for 5 min on 2.5% gluteraldehyde (prepared in PBS) to fix the sample, followed by two washes in PBS, followed by four washes in distilled water and,
7. Grids were floated for 1 min in 2% uranyl acetate, followed by four washes in distilled water.
8. After the distilled water washes, the grids were placed onto filter paper and allowed to dry. After each stage, the grids were carefully blotted onto filter paper by holding the gird perpendicular to the paper.
9. All samples were examined under a JEOL model: JEM1010 transmission microscope. Examples of M or E-immunogold labelled VLPs can be found in **refs.** *24*,*25*.

4. Notes

1. Colony PCR can be used as an alternative technique to screen for positive colonies. This could be done using a toothpick or sterile yellow tip to pick a colony and submerge the colony into the PCR mix. The fragment can then be amplified using a PCR program that is optimum for the primer combination, with the exception being an extended initial denaturation time. The primer combination used within the PCR can be specific for the gene of interest, or specific for priming sites on the pFastBac1 vector which flank the multiple cloning site (pFastBac_Fwd: 5'ACCATCTCGCAAATAAAG3' and pFastBac_Rev: 5'AACAACAATTGCATTCATTTT3').
2. When preparing the multiple antibiotic plates, the tetracycline concentration was increased from 10 μg/mL to 15 μg/mL. This was done to decrease the amount of satellites colonies obtained. After the 48-h incubation, plates were incubated at 4°C. This allowed for an enhancement of the blue colonies, which allowed for a more defined distinction between white and blue colonies.
3. The amplicon size between the two universal primers on the bacmid DNA is 2.3 kb when no gene is inserted. If the gene of interest together with the bacmid DNA is greater than 4 kb, it is recommended that a *Taq* polymerase such as the Expand High Fidelity PCR system (Roche) *(38)* is used, which will allow for amplification of larger fragments.
4. It is generally assumed that a confluent 25-cm^2 flask of *Sf*9 cells contains approx 1×10^7 cells. These cells, diluted in 10 mL of media, will then contain 1×10^6 cell/mL.
5. It is important to note that insect cells are not incubated in a CO_2 incubator but in a normal humidified incubator at 27°C. It is possible to obtain a slower growth at 19–22°C, but it is not advisable to exceed the temperature of 28°C, because the

insect cells do not grow well at higher temperatures. Also, it is extremely hard for the cells to recover once they have been placed under stress.

6. Repeated freeze–thaw cycles of virus samples are not recommended, as this can decrease titer. It is therefore recommended that a working stock of recombinant virus be stored at 4°C. Virus stocks can safely be stored this way, without loss of titer, for a least a year.
7. Assuming that the P1 viral stock has a titer of 1×10^6 pfu/mL, the number of cells to be infected is 1×10^7 cells/mL (1×10^6 cells in 10 mL of media) and the MOI required is 0.1 pfu/mL, it can be calculated that 1 mL of the P1 viral inoculum is required.
8. For a quick scan of VLPs, after the lysed cells have been separated from the cell debris, it is possible to place an aliquot of the clarified supernatant onto a formvar coated copper grid, stain the grid with PTA and view immediately by transmission electron microscopy.

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4

The C Type Lectins DC-SIGN and L-SIGN

Receptors for Viral Glycoproteins

Pierre-Yves Lozach, Laura Burleigh, Isabelle Staropoli, and Ali Amara

Summary

DC-SIGN and L-SIGN are C-type lectins that recognize carbohydrate structures present on viral glycoproteins and function as attachment factors for several enveloped viruses. DC-SIGN and L-SIGN enhance viral entry and facilitate infection of cells that express the cognate entry receptor (*cis*-infection). They are also able to capture viruses and transfer viral infections to other target cells (*trans*-infection). In this chapter, we will give an overview of protocols used to produce soluble viral glycoproteins at high levels and to study the molecular basis of viruses/DC-SIGN and L-SIGN binding and internalization. We will also describe techniques to investigate the molecular mechanisms by which DC-SIGN or L-SIGN spread viral infections.

Key Words: DC-SIGN; L-SIGN; dendritic cells; endothelial cells; viruses; envelope glycoproteins; endocytosis; viral entry; infection; viral transmission.

1. Introduction

DC-SIGN (CD209) and its homolog L-SIGN (also called DC-SIGN-R, CD209L) belong to the C-type (calcium-dependent) lectin family. This large group of proteins which includes the mannose receptor, DEC-205 or langerin, is specialized in the recognition of carbohydrate structures present on cellular and viral proteins and is implicated in several processes such as cell adhesion and antigen presentation *(1,2)*. DC-SIGN and L-SIGN are constitutively expressed by specific cell populations that play a key role in the activation of the innate and adaptive immune responses. DC-SIGN is highly expressed at the surface of dendritic cells (DCs) localized in the lymphoid tissues (thymus, tonsils, or lymph nodes), mucosal surface, and in the dermis *(3–5)*. DC-SIGN is not expressed by Langerhans cells, which are a unique DC subset

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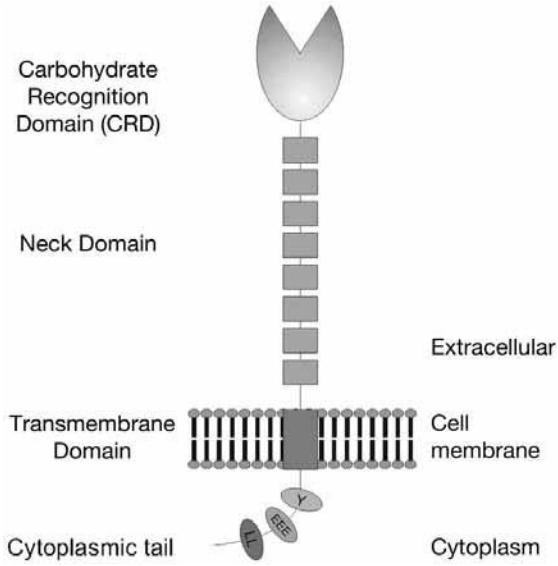

Fig. 1. Structure of DC-SIGN and L-SIGN proteins. The C-type lectins DC-SIGN and L-SIGN are type II transmembrane proteins. Their cytoplasmic tails contain internalization signals (di-leucine, tyrosine, and tri-acidic) which are involved in internalization of the lectin. The extracellular domain is composed of a carbohydrate recognition domain (CRD) and a neck domain (conserved in the case of DC-SIGN, variable for L-SIGN) implicated in the oligomerization of these lectins. The oligomerization is probably important for the orientation and subsequently for the function of the CRDs.

residing in epidermis. Certain macrophages such as Hofbauer cells in the placenta, Kupffer cells in the liver sinusoids, and alveolar macrophages have been shown to express DC-SIGN *(3,4)*. L-SIGN expression is restricted to endothelial cells such as those in lymph nodes, placenta, and, particularly, liver sinusoidal endothelial cells (LSECs) *(6,7)*.

DC-SIGN and L-SIGN share nearly 77% amino acid identity and are closely related in global architecture. Both lectins are type II transmembrane proteins composed of a short cytoplasmic tail responsible for signalling and internalization, a transmembrane region, a neck domain consisting of eight repeat regions of 23 amino acids and a carbohydrate recognition domain (CRD) which binds carbohydrate ligands in a calcium-dependent manner *(1,3,4)* (**Fig. 1**). The DC-SIGN CRD recognizes different mannose or fucose-based carbohydrates whereas the L-SIGN CRD appears to interact only with mannose resi-

Table 1
DC-SIGN and L-SIGN Are Receptors for a Broad Range of Viruses

	Virus family	Virus	Mechanisms	Viral envelope protein(s)*
DNA	*Herpesviridae*	CMV	*cis, trans, trans*-enh.	gB
RNA	*Coronaviridae*	SARS	*cis ?, trans*	Spike
	Filoviridae	Ebola	*cis ?, trans*	GP
		Marburg	*cis ?*	GP
	Flaviviridae	Dengue	*cis*	E
		HCV	*trans*	E1/E2
	Retroviridae	HIV-1/-2	*cis, trans, trans*-enh.	gp120
		SIV	*cis, trans, trans*-enh.	gp120
	Togaviridae	Sindbis	*cis ?*	E1 or E2 (?)

* Viral envelope protein(s) bound by DC-SIGN or L-SIGN. *cis*, *cis*-infection; CMV, cytomegalovirus. HCV, hepatitis C virus; HIV, human immunodeficiency virus; SARS, severe acute respiratory syndrome virus; SIV, simian immunodeficiency virus; *trans*, *trans*-infection; *trans*-enh, *trans*-enhancement.

dues on *N*-glycans (Man5GlucNac2 to Man9GlucNac2) *(8,9)*. The repeat regions within the neck domain permit the oligomerization of the lectins which is critical for their biological activities, because only tetramers efficiently capture glycosylated ligands *(10–12)*.

DC-SIGN was originally cloned for its ability to bind and internalize the heavily glycosylated human immunodeficiency virus (HIV) gp120 protein *(13)*. DC-SIGN strongly binds all HIV and simian immunodeficiency virus (SIV) strains examined to date and plays an important role in virus adhesion to DC *(14)*. These studies have paved the way for further investigations into interactions between DC-SIGN and pathogens and it has become clear that many viruses target DC-SIGN and L-SIGN to bind DCs and endothelial cells, respectively. Both lectins recognize high mannose oligosaccharides present on viral glycoproteins and thus function as attachment factors for several viruses including cytomegalovirus (CMV), dengue (DV), Ebola, severe acute respiratory syndrome (SARS), hepatitis C (HCV), Marburg and Sindbis viruses (**Table 1**) *(10, 15–27)*. Differential glycosylation of viral envelope glycoproteins strongly influences the efficiency of viral capture by DC-SIGN and L-SIGN *(10,19, 22)*. For example, the DV envelope glycoprotein E has two conserved *N*-linked glycosylation sites at Asn-67 and Asn-153 that mediate DV binding to DCs *(22)*.

Only mannosylated E glycoproteins (which are exposed at the surface of DV virions transmitted to humans by infected mosquitoes), and not E proteins with complex glycosylation (produced in mammalian cells), have been shown to interact with DC-SIGN-expressing cells *(22)*.

DC-SIGN and L-SIGN are endocytic receptors and their cytoplasmic tails carry putative internalisation signals such as a dileucine (LL) motif (which is present in both DC-SIGN and L-SIGN) and a tri-acidic cluster that is believed to be involved in intracellular trafficking (**Fig. 1**) *(3)*. Despite findings showing that a large fraction of viral particles captured by DC-SIGN are rapidly internalized and degraded *(28,29)*, viruses are nevertheless able to hijack DC-SIGN and L-SIGN functions to spread infection. For the viruses examined to date, DC-SIGN and L-SIGN have been shown to act as attachment factors rather than authentic entry receptors involved in membrane fusion. This does not exclude the possibility that some viruses, and particularly those that require targeting to acidified endosomes for membrane fusion, use these lectins as primary entry receptors. DC-SIGN and L-SIGN have also been shown to function as "*cis*-receptors" that enhance infection of target cells. This mechanism, known as *cis*-infection, has been described for DV, CMV, and HIV, and probably relies on the capacity of these lectins to concentrate viral particles at the cell surface, allowing optimal interaction with their cognate receptors and enhanced viral entry (**Table 1**) *(18,22,30)*. Viruses captured by DC-SIGN or L-SIGN can also be transmitted *in trans* to target cells expressing the entry receptors ("*trans*-infection"), as has been proposed for HIV, HCV, and SARS virus *(14,16,26,31)*. For HIV and CMV, DC-SIGN enhances infection of target cells at a low multiplicity of infection (MOI). DC-SIGN-bound viruses infect target cells more efficiently than free viruses and remain infectious for several days *(14,18)*. The molecular mechanisms underlying these processes remain poorly understood.

The contribution of DC-SIGN and L-SIGN to viral transmission and dissemination in vivo is currently unknown. Their role as principal attachment factors for a broad range of enveloped viruses and their restricted expression in anatomical site of virus exposure suggest that these two lectins dictate viral tropism for DCs and endothelial cells and consequently may influence viral pathogenesis. DCs are sentinel cells that capture pathogens entering skin or mucosal tissues and then migrate to the lymph nodes where they present processed antigens to T-cells, initiating adaptive immune responses. By interacting with DC-SIGN, viruses that are transmitted sexually (such as HIV) or through introduction into human skin by an insect vector (such as dengue virus or Sindbis virus) may hijack DC function either to modulate the immune response or to assure their dissemination from peripheral tissues to lymphoid organs *(32)*. LSECs also participate in the capture and processing of foreign antigens, in

addition to the elimination of undesirable macromolecules from the blood by transporting them to hepatocytes *(6,7)*. LSECs represent a barrier separating the liver and the blood and could be exploited by viruses such as HCV in order to gain access to hepatocytes *(16,33,34)*. We recommend the two following reviews for an overview of the physiological importance of LSECs in viral infections of the liver *(6,7)*.

In this chapter, we will provide general protocols to study the molecular interactions between viruses and DC-SIGN or L-SIGN and to investigate the functions of these two lectins in viral infection and transmission. We will first describe the methods used to obtain human dermal-like DCs and cells expressing DC-SIGN and L-SIGN. We next present protocols to produce soluble viral envelope proteins using a Semliki forest virus (SFV) vector *(35–37)* and to study the molecular basis of viral capture and internalization by DC-SIGN or L-SIGN. Finally, we will describe methods required to assess the contribution of DC-SIGN and L-SIGN to *cis*- and *trans*-infection.

2. Materials

2.1. Cell Culture and Antibodies

1. All of the products used for cell culture are purchased from Invitrogen (RPMI 1640, Glasgow's modified Eagle's medium [GMEM], fetal calf serum [FCS], penicillin/streptomycin, HEPES, and tryptose phosphate broth) except cysteine/methionine-free Dulbecco's modified Eagle's medium (DMEM) (ICN Biomedicals).
2. Phycoerythrin (PE)-conjugated mouse monoclonal antibodies (mAbs) directed against DC-SIGN (FAb161P), L-SIGN (FAb162P), or both lectins (FAb1621P) were purchased from R&D Systems. The anti-DC-SIGN mAb clone 1B10 (IgG2a, κ) has been developed in our laboratory and previously described *(18)*. The anti-DC-SIGN mAb1B10 is directed against the CRD and inhibits DC-SIGN activity *(18)*. The mAb1621 directed against both lectins is purchased from R&D Systems. The mAb1621 blocks the activity of both lectins. Differentiation of human DCs is assessed by fluorescence-activated cell sorting (FACS) analysis using fluorescein isothiocyanate (FITC)-conjugated mouse mAb anti-CD14 (MϕP9) and anti-CD1a (HI149) purchased from BD Biosciences.
3. HeLa and 293T cells are maintained in DMEM supplemented with 10% FCS and antibiotics (100 μg/mL^{-1} streptomycin and 100 U/mL^{-1} penicillin). Raji cells are grown in RPMI containing 10% FCS and antibiotics. BHK is cultured in GMEM with 5% FCS, 1% penicillin/streptomycin, 20 m*M* Hepes, and 10% tryptose phosphate broth.
4. Cell lines expressing DC-SIGN or L-SIGN are generated by transduction with the retroviral TRIP ΔU3 vector (a gift from Pierre Charneau, Pasteur Institute, France) encoding either DC-SIGN or L-SIGN *(16)*. Transduced cells are stained with PE-conjugated anti-DC-SIGN mAb and sorted for high expression level of DC-SIGN or L-SIGN.

2.2. Generation of Monocyte-Derived Dendritic Cells (see **Note 1**)

1. Human peripheral blood mononuclear cells (PBMC) are isolated from healthy donors by density gradient centrifugation through Ficoll-Paque Plus (Amersham Biosciences).
2. Lysis buffer: dissolve 8.3 g of NH_4Cl and 1 g of $NaHCO_3$ in 1 L of water complemented with 1 mL of EDTA (100 m*M*, pH ~8.0). Lysis buffer must be autoclaved and can be stored at 4°C for several months.
3. MACS buffer: phosphate-buffered saline (PBS) containing 2 m*M* EDTA (pH ~8.0) and 0.5% bovine serum albumin (BSA; Sigma Aldrich). This buffer can be kept at 4°C for several months.
4. Filters (cell strainer, 40 μm) used to eliminate cell aggregates are purchased from Falcon.
5. Monocytes are negatively selected using FCR blocking and biotin antibodies (Ab) cocktail and anti-biotin magnetic beads (Miltenyi Biotec).
6. Recombinant human interleukin (IL)-4 and recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF) are purchased from PeproTech and Gentaur respectively.

2.3. Recombinant Protein Expression

1. Soluble viral glycoproteins are produced using the SFV vector in BHK mammalian cells. SFV shuts off the cellular translation machinery and so transduced cells produce only the viral protein of interest, which is secreted and accumulates in the supernatant of infected cells. This allows the production of large amounts of soluble proteins with a high degree of purity. The SFV expression vector was originally described in reviews *(35–37)*. Briefly, the SFV genome is a single-stranded, positive RNA which encodes both structural and nonstructural viral proteins. A signal sequence in the 5' RNA domain permits specific viral genome packaging. The SFV vector is made of two cDNAs (pSFV-Δ*env* and pSFV-helper2) (**Fig. 2**). The first codes for the SFV RNA genome in which the sequence corresponding to structural proteins is replaced by the soluble glycoprotein of interest (pSFV-Δ*env*). The structural proteins are encoded by a second cDNA (pSFV-helper2) which lacks the packaging signal. cDNAs are transcribed in vitro and transfected into cells by electroporation. In this way, only RNAs encoding the protein of interest are packaged into new defective viral particles. The SFV envelope proteins are activated by the furin cleavage in the Golgi apparatus. Replacement of the furin site by a chymotrypsin site allows control of SFV particle activation.
2. pSFV-helper2 and pSFV-Δ*env* have been previously described *(35–37)*. Polymerase chain reaction (PCR) fragments coding for soluble glycoprotein of interest are usually digested either by BssH II (5') and Nsi I (3'), by BssH II (5') and Apa I (3') or by BamH I (5' and 3') and then introduced into pSFV-Δ*env* *(10,22,38)*.
3. Restriction enzymes Spe I and Sph I are purchased from New England Biolabs. The DNA purification kit (QIAquick PCR purification kit) is purchased from QIAgen.

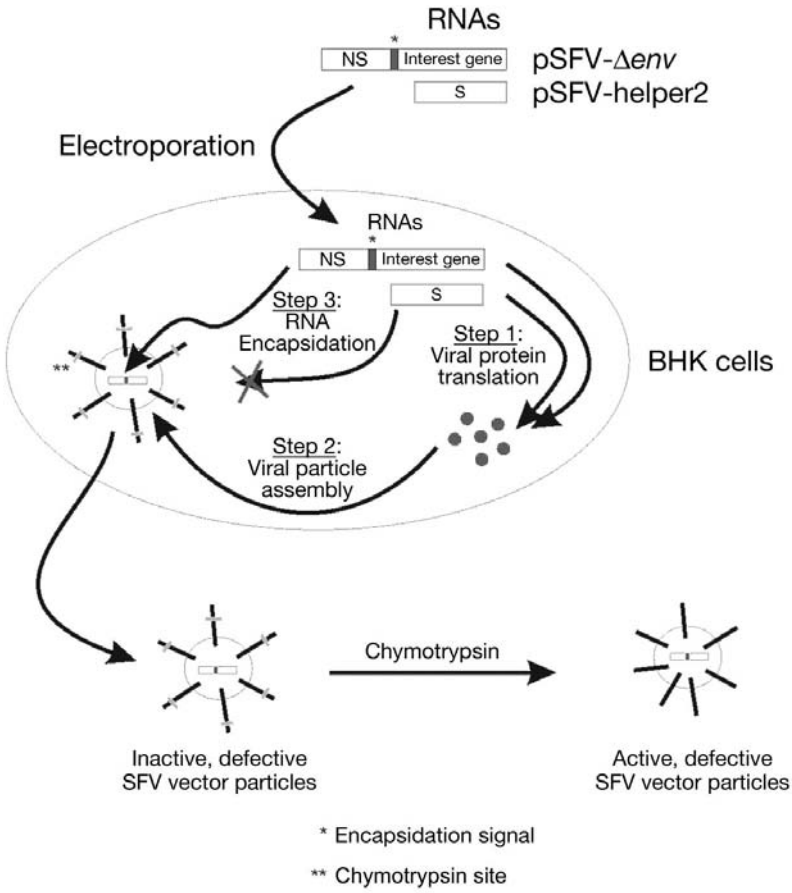

Fig. 2. Semliki forest virus (SFV) expression vector. The SFV vector is composed of two RNAs which are electroporated into BHK cells. New synthesized particles incorporate only the RNA coding for nonstructural proteins and the protein of interest (NS, nonstructural and S, structural) because it is the only one with an encapsidation signal. The furin site of the SFV envelope protein is replaced by a chymotrypsin site so the particles can be activated by chymotrypsin digestion.

4. SP6 RNA polymerase and RNAsin are purchased from Roche Applied Science and Promega, respectively.
5. 0.4-cm electroporation cuvets are purchased from Eurogentec.
6. TNE buffer: 12 m*M* TrisHCl, 2 *M* NaCl, and 0.2 *M* EDTA (pH ~8.0) are prepared in distilled water and can be stored at 4°C for several months.
7. Chymotrypsin and aprotinin are purchased from Sigma Aldrich.

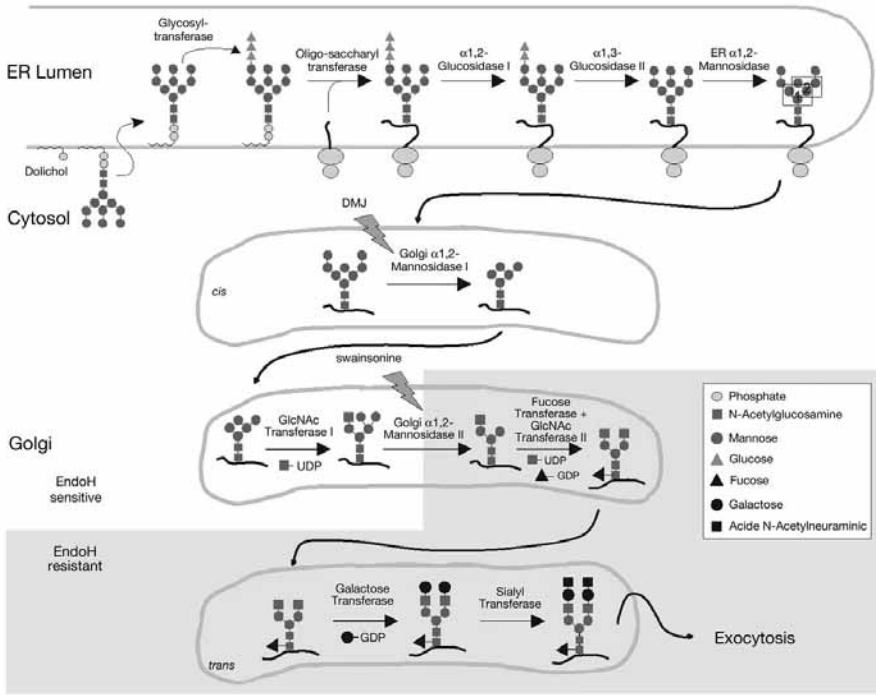

Fig. 3. Carbohydrate maturation in mammalian cells. Proteins with NXS or NXT sites that pass through the endoplasmic reticulum can be potentially glycosylated. Glycoproteins are sensitive to EndoH until they are modified by α1,2-mannosidase II. Swainsonine and 1-deoxymannojirimycin hydrochloride (DMJ) block maturation steps of glycoprotein carbohydrates. These drugs permit to produce mannosylated glycoproteins in mammalian cells. 1, internal tri-mannose branch recognized by DC-SIGN; 2, external tri-mannose branch; ER, endoplasmic reticulum; GDP, guanosine biphosphate; UDP, uridine biphosphate.

8. Peroxidase-conjugated mAb and diaminobenzidine (DAB) solution kit are obtained from Vector Laboratories (ABCYS Biologie). DAB solution is prepared by adding four drops of DAB solution, two drops of H_2O_2 solution, two drops of nickel solution, and two drops of buffer solution to 5 mL of water.
9. [^{35}S] cysteine and methionine (Pro-Mix ^{35}S) are obtained from Amersham Biosciences.
10. 1-deoxymannojirimycin hydrochloride (DMJ) and swainsonine are purchased from Calbiochem and Sigma respectively. These molecules specifically inhibit α-manosidase I and II and permit the production of proteins with mannose carbohydrate residues in mammalian cells (**Fig. 3**).

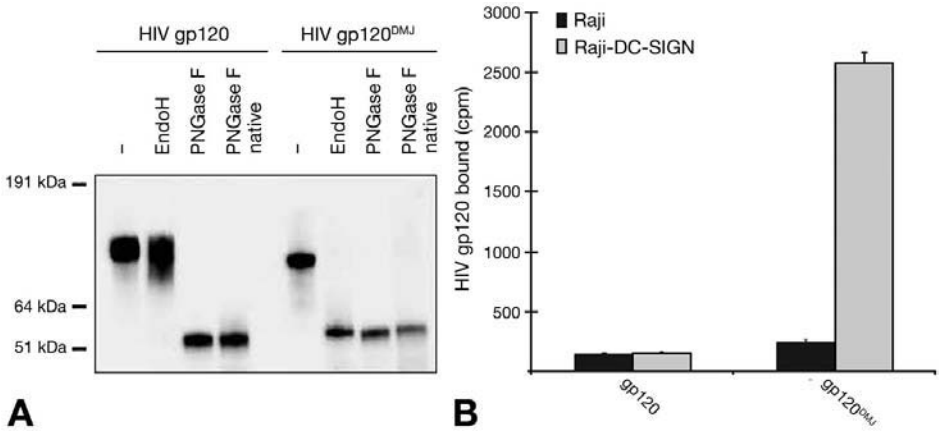

Fig. 4. Production of HIV gp120^{DMJ} and binding to DC-SIGN. **(A)** Soluble HIV gp120 is produced in BHK cells in the presence or absence of mannosidase inhibitors (1 m*M* DMJ and 5 μ*M* swainsonine) (HIV gp120^{DMJ} or HIV gp120, respectively) as described under **Subheading 3.2.3.** Secreted proteins were subjected to digestion with EndoH or PNGase F and analyzed by Western blot. Only HIV g120^{DMJ} is sensitive to EndoH confirming its high mannosylated glycosylation. **(B)** ^{35}S-labeled HIV gp120^{DMJ} and HIV gp120 (20 n*M*) are bound to Raji and Raji-DC-SIGN cells for 2 h at 4°C. Cells are washed three times before measuring cell-associated radioactivity. Note that only HIV gp120^{DMJ}, which carries only mannosylated *N*-glycans, binds to DC-SIGN.

11. Glycoproteins are concentrated through columns (Biomax, Millipore) with an appropriate molecular weight cut-off for the particular protein being produced.
12. Endoglycosidase H (EndoH) and peptide: *N*-glycosidase F (PNGase F) are purchased from Roche Applied Science and New England Biolabs respectively. The buffer provided with the PNGase F is used to treat the glycoproteins either with EndoH or with PNGase F. PNGase F is able to digest all *N*-glycans whereas EndoH digests only *N*-glycans unmodified by α-manosidase I and II (**Figs. 3** and **4A**).

2.4. Binding and Internalization Assays

1. Buffer A: PBS containing 1% BSA, 0.2% γ–globulin, 0.1% sodium azide, 1 m*M* $CaCl_2$, and 2 m*M* $MgCl_2$. Buffer A can be stored at 4°C for several months.
2. Buffer B: serum-free RPMI containing 1 m*M* $CaCl_2$ and 2 m*M* $MgCl_2$. Buffer B can be stored at 4°C for few months.
3. Mannan, EDTA (pH ~8.0) and EGTA (pH ~8.0) are all purchased from Sigma Aldrich. Mannan stock solution is dissolved in water (5 mg/mL^{-1}) and can be stored at 4°C for several months.
4. Scintillation solution is obtained from Wallac (optiphase supermix solution).

2.5. Viruses

Wild-type viruses or viral particles carrying the reporter genes firefly luciferase (Luc) or green fluorescent protein (GFP) can both be used to study DC-SIGN and L-SIGN-mediated *cis*-infection and *trans*-enhancement of target cell infection. These viruses can be prepared using standard methods, and an example is described in **Note 7**.

3. Methods

3.1. Generation of Dermal-Like DCs Expressing DC-SIGN

3.1.1. Isolation of PBMCs From Blood

1. Fresh blood (450 mL) is completed to 600 mL with PBS.
2. Prepare 20 50-mL tubes containing 15 mL of Ficoll. Slowly add 30 mL of blood into each tube, taking care to avoid mixing, then centrifuge for 20 min at 850*g* without brake at room temperature.
3. Aspirate the plasma and transfer the PBMCs (white ring) into new tubes containing 20 mL of PBS (two rings per tube). Complete each tube to 50 mL with PBS, and then centrifuge for 10 min at 300*g* at room temperature. Repeat this washing step twice, each time pooling two tubes.
4. Remove the supernatant and resuspend the pellet in 5 mL of lysis buffer for 4 min. Add 20 mL of PBS 2% FCS and centrifuge at 300*g* for 5 min at 4°C. Resuspend the pellet in 5 mL of cold MACS buffer.

3.1.2. Monocyte Isolation and Differentiation

1. Isolated PBMCs are passed through a filter placed inside a 50-mL tube (to eliminate cell aggregates) and the filter is rinsed twice with 5 mL of MACS buffer. Centrifuge at 300*g* for 5 min at 4°C.
2. Resuspend the cells at up to 10^7 cells per 30 µL of MACS buffer and add 10 µL each of FCR blocking reagent and biotin antibody cocktail per 10^7 cells. Incubate for 10 min at 4°C.
3. Add 30 µL of MACS buffer and 20 µL of anti-biotin microbeads per 10^7 cells and incubate for 15 min at 4°C.
4. Complete to 50 mL with MACS buffer and centrifuge 10 min at 300*g*, 4°C.
5. Resuspend the cells at 10^8 cells per 500 µL of MACS buffer. Wash the magnetized column with 3 mL of MACS buffer, then pass cells through the column.
6. Rinse the column three times with 3 mL of MACS buffer and collect the eluate containing monocytes. Cells are cultured at 10^6 cells/mL^{-1} in RPMI with 10% FCS, 1% penicillin/streptomycin, 50 ng/mL^{-1} IL-4, and 100 ng/mL^{-1} GM-CSF for 6 d. GM-CSF and IL-4 are added every 2 d. Differentiation of monocyte-derived DCs is assessed by FACS analysis (CD14 negative and CD1a- and DC-SIGN-positive).

3.2. Expression of Soluble Viral Glycoproteins

3.2.1. Production of Recombinant Semliki Forest Virus Particles (see **Note 2**)

1. Digest 2 μg of pSFV-helper2 and pSFV-Δ*env* with Spe I or Sph I for 2 h at 37°C. Linearized DNAs are purified with a QIAquick PCR purification kit and are then transcribed in vitro for 1 h at 37°C using SP6 RNA polymerase (20 UI) in buffer containing RNAsin and cap analog. After 1 h of incubation, 1 μL of SP6 RNA polymerase is added to the mixture and incubated for 30 min at 37°C (*see* **Note 3**).
2. pSFV-helper2 RNA is mixed with equal quantities of pSFV-Δ*env* RNA (usually 20 μL of each), added to 8×10^6 BHK cells in 800 μL of PBS (without calcium and magnesium) and immediately transferred to a 0.4-cm electroporation cuvet.
3. The RNA-cell mixture is subjected to two 0.4-ms pulses at 830 V and 25 μfarads in a Bio-Rad gene pulser and plated in 75 cm^2 flasks in 15 mL of GMEM medium containing 5% FCS.
4. Supernatants containing recombinant defective SFV particles are harvested 24 h later and cleared by centrifugation (850*g*, 10 min, room temperature). The supernatant is then concentrated by ultracentrifugation (100,000*g*, 4 °C, 1 h 45 min).
5. The pellet is covered with 200 μL of TNE buffer and incubated at 4°C for 1 h in a sealed tube to resuspend. The virus is stored in 20-μL aliquots at –80°C.

3.2.2. Titration of Recombinant Defective SFV Particles

1. Before infection, virus aliquots are activated by a chymotrypsin treatment (0.5 mg/mL^{-1} chymotrypsin, 1 m*M* $CaCl_2$) for 30 min at room temperature. To inhibit chymotrypsin activity, aprotinin (0.5 mg/mL^{-1}) is added to activated aliquots and incubated at 4°C for 10 min.
2. For infection, BHK cells (5×10^5 cells seeded in six-well plates 24 h before infection) are washed with serum-free GMEM and incubated with dilutions of viral particles in GMEM complemented with 2% FCS (500 μL of dilutions 10^{-2} to 10^{-7}) at 37°C. One hour later, 2 mL of GMEM complemented with 5% FCS are added.
3. At 7 h postinfection, cells are washed with serum-free GMEM medium and fixed with cold methanol for 5 min at –20°C follow by three washes with PBS.
4. Cells are incubated with 500 μL of primary antibody (directed against the soluble glycoprotein of interest) diluted in PBS for 1 h at room temperature or overnight at 4°C. Cells are washed twice with PBS prior to incubation with the secondary peroxidase-conjugated antibody (1/200 in 700 μL PBS per well) for 30 min at room temperature. Cells are then washed three times in PBS and incubated in DAB solution for 2 to 10 min and then rinsed three times in PBS. Stained cells are counted with a micrometric objective to determine the virus titer.

3.2.3. Production of Soluble Glycoproteins

1. Defective SFV particles are activated and cells are infected as described under **Subheading 3.2.2.** (*see* **Note 4**).

2. At 6 h postinfection, cells are washed five times in serum-free GMEM to eliminate BSA and then maintained in serum-free GMEM for protein production. For the production of radiolabeled protein, cells are starved for 1 h in serum- and methionine/cysteine-free DMEM prior to addition of 100 μCi/mL^{-1} [35S] cysteine and methionine.
3. Synthesis of proteins is continued up to 24 h postinfection in the presence or absence of α-mannosidase I and II inhibitors DMJ (1 m*M*) and swainsonine (5 μ*M*). This results in the production of soluble glycoproteins carrying only mannose *N*-glycans (**Figs. 3** and **4**). Supernatants are clarified by centrifugation (10 min, 850 g at room temperature) and concentrated (*see* **Subheading 2.3., item 11**).
4. The glycosylation pattern of the proteins produced can be verified by treatment with endoglycosidase H (endoH; 2 mU, Roche) or Peptide: *N*-Glycosidase F (PNGAse F; 1000 U, Biolabs). An example is given in **Fig. 4A**. Only HIV gp120 bearing mannose *N*-glycans (HIV gp120^{DMJ} produced in presence of DMJ and swainsonine) is sensitive to EndoH. In contrast, PNGaseF treatment allows complete de-glycosylation of HIV gp120 produced either in the presence or absence of inhibitors. The molecular weight of the exclusively mannosylated HIV gp120 is inferior to that of gp120 produced in the absence of mannosidase inhibitors. This is a result of the lower molecular weight of mannose residues compared to complex glycosylation.

3.3. Soluble Glycoprotein Binding to DC-SIGN or L-SIGN and Internalization Assays

1. Binding assays are performed in 96-well plates using 5 × 10^5 DC-SIGN- or L-SIGN-expressing cells in 100 μL of buffer A.
2. Cells are pelleted by centrifugation (300*g*, 4°C, 5 min) and buffer A is replaced by ^{35}S-labeled viral glycoprotein at desired concentrations in 100 μL of buffer A.
3. Binding was carried out for 2 h at 4°C with gentle agitation. Unbound radioactivity is removed by three washes with 200 μL of buffer A and cell pellets are resuspended in 25 μL of buffer A prior to addition of 175 μL of scintillate solution. Cell-associated radioactivity is counted in a 1450 Microbeta Trilux β counter (Wallac).
4. To assess the specificity of interactions between viral glycoproteins and DC-SIGN or L-SIGN, cells are preincubated for 30 min at 4°C with mannan (a CRD competitor), the neutralizing anti-DC-SIGN mAb (1B10) or anti L-SIGN mAb (mAb1621) (each at 20 μg/mL^{-1}), or the calcium chelator EDTA (5 m*M*) diluted in buffer A for before addition of labelled envelope proteins.
5. For internalization assays, ^{35}S-labeled proteins are bound to parental or DC-SIGN- or L-SIGN-expressing cells as described above except that the buffer A is replaced by the buffer B. Cells are washed three times and incubated for 30 min either at 4°C in 100 μL of cold buffer B or at 37°C in 100 μl of preheated buffer B to initiate endocytosis. To quantify glycoprotein internalization, cells maintained either at 4°C or 37 °C are treated with 200 μL RMPI containing 20 m*M* EDTA or mock treated in order to remove viral proteins bound to the lectin at the

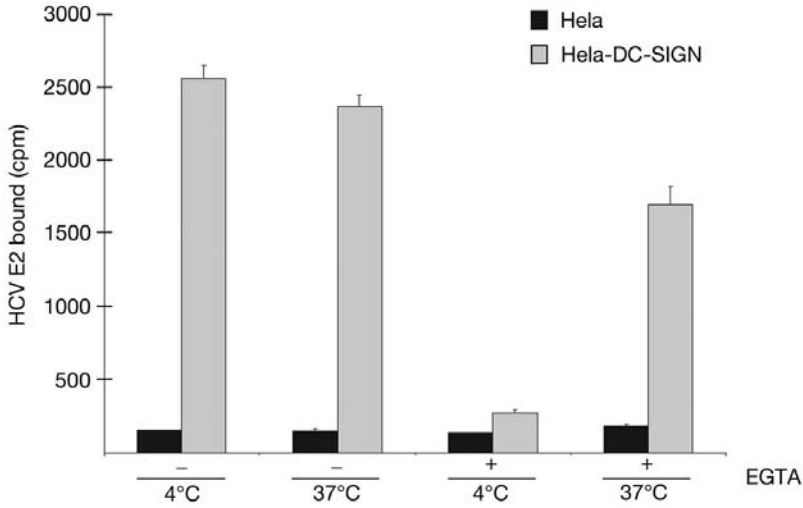

Fig. 5. DC-SIGN induces internalization of viral glycoproteins. HeLa and HeLa-DC-SIGN cells are incubated for 2 h at 4°C with ^{35}S-labeled hepatitis C virus (HCV) E2 glycoprotein. Cells are extensively washed to eliminate unbound material and incubated for 30 min either at 4°C or 37°C. Cells are treated with EDTA or mock treated to distinguish internalized (EDTA-resistant) from cell surface bound HCV-E2 glycoprotein (EDTA-sensitive).

cell surface (*see* **Fig. 5**). Cells are washed and resuspended in binding buffer prior to addition of optiphase supermix solution. ^{35}S activity is counted as described. For an alternative protocol, *see* **Note 5**.

3.4. DC-SIGN and L-SIGN-Mediated Infection and Viral Transmission to Target Cells

3.4.1. cis-Infection

DC-SIGN- or L-SIGN-expressing cells and their parental counterpart (10^5 cells) are exposed to viral particles for 2 h at 37°C at varying MOI in FSC-free medium supplemented with 1% penicillin/streptomycin, pH approx 7.5. Cells are washed three times with complete medium to remove excess virus and incubated at 37°C. Viral replication is evaluated 2 to 3 d later, depending on the readout used (*see* **Note 7**).

3.4.2. trans-Infection

DC-SIGN- or L-SIGN-expressing cells and their parental counterpart (10^5 cells) are incubated with viral particles at a high MOI for 2 h at 37°C in FCS-

free medium supplemented with 1% penicillin/streptomycin, pH approx 7.5 (*see* **Note 8**). Cells exposed to virus are washed extensively with cold PBS to remove unbound viral particles, resuspended in 100 µL of complete medium and co-cultured with an equal number of target cells in 96-well plates. Viral transmission is quantified two or 3 d later.

3.4.3. trans-*Enhancement of Target Cell Infection* (see **Note 8**)

DC-SIGN- or L-SIGN-expressing cells and their parental counterpart (10^5 cells) are incubated with viral particles at a low MOI (insufficient to directly infect target cells) for 2 h at 37°C and immediately co-cultured with target cells without washing. As an important control, viral particles are incubated with medium alone and 2 h later, transferred to target cells.

3.4.4. Retention of Viral Infectivity by DC-SIGN or L-SIGN

This assay permits to determine if viral particles bound to DC-SIGN or L-SIGN remain infectious for several days. The protocol is similar to that described under **Subheading 3.4.2.** or **3.4.3.** except that co-culture with target cells is started several days after exposure of cells expressing DC-SIGN or L-SIGN to virus. Multiple points can be tested to establish the kinetic of the virus protection by cells expressing these lectins.

4. Notes

1. Human primary LSECs expressing L-SIGN are difficult to obtain. For this reason, we use cell lines expressing L-SIGN and we describe only the generation of monocyte-derived DCs that constitutively express DC-SIGN.
2. Alternatively, the SFV particle production can be bypassed. The electroporation of BHK cells with pSFV-Δ *env* permits to directly produce the recombinant protein coded by the modified SFV plasmid. This alternative protocol is similar to **Subheading 3.2.1., steps 1** and **2**, except that the amount of pSFV-Δ *env* RNA transfected is doubled. The quantity of RNA electroporated may require adjustment depending on the gene of interest. The maximum volume of RNA we have electroporated is 150 µL. The next step of the recombinant protein production with this alternative protocol continues at **Subheading 3.2.3., step 2**. The advantage of production of viral particles is that cells can be infected at equal MOI and hence the quantity of protein produced is more reproducible than direct electroporation with pSFV-Δ*env* RNA.
3. The quality of transcribed RNA is verified by agarose gel (1%) electrophoresis. Transcribed RNAs can be stored at –20°C before electroporation.
4. Defective SFV particles are used at a MOI varying from 25 to 100. Some adjustments may be required depending on the gene of interest. The number of cells infected correlates with the quantity of protein produced and the efficiency of protein secreted. For information, the best production we observed is for HCV

envelope protein E2 *(10)*. We produce approx 20 μg of purified protein per 10^7 cells infected at a MOI of 50.

5. DC-SIGN- or L-SIGN-mediated viral internalization can also be investigated by confocal microscopy using purified viral glycoproteins (*see* **Note 6**) or whole virions. HeLa cells expressing DC-SIGN or L-SIGN (5×10^4 cells) are seeded on coverslips. The following day, cells are incubated with viral glycoproteins (5 $\mu g/mL^{-1}$) or wild-type viruses diluted buffer B for 1 h at 4°C. Cells are washed three times with ice cold PBS to remove unbound material and shifted to 37°C for different times to allow endocytosis. Cells are fixed with 3.2% paraformaldehyde for 15 min, washed twice in PBS and treated with PBS 0.2 *M* glycine for 10 min. Cells are then incubated with 500 μL of PBS containing 0.05% saponin and 0.2% BSA for 30 min. Both DC-SIGN and viral protein trafficking can be followed by using specific mAb. Cells are mounted in Vectashield containing DAPI (Vector Laboratories) and imaged on a Zeiss microscope using a Plan Apochromat ×63/1.4 oil immersion objective.
6. Optionally, the soluble glycoprotein can be purified by immuno-affinity when a tag peptide is introduced into its sequence. Several commercial tag peptides are available such as the Flag tag peptide which we used *(10)*.
7. Read-out is specific for the virus studied. We generally use wild-type viruses or viral particles carrying a reporter gene (GFP or Luc). For example, for HIV or HCV, we use single-cycle pseudotyped viral particles that are generated by co-transfecting 293T cells with an HIV-1 NL ΔEnv Luc or GFP (a HIV provirus lacking the Env gene and carrying the Luc or GFP gene in the place of Nef) and a cDNA plasmid encoding either HIV or HCV envelope proteins**.** Viral replication is evaluated by measuring luciferase activity in cell lysates (3 d postinfection using a luciferase reporter assay kit [Promega] and a Victor luminometer [Perkin Elmer]) or by counting the GFP-positive cells by FACS. For dengue virus, cell are infected with viral particles produced in insect cells. Viral replication is quantified by flow cytometry 2 d after using Ab specific of dengue virus antigens. For more examples, *see* **refs. *10*,*14*,*16*,*18*,*20–26*,*39*.**
8. DC-SIGN- or L-SIGN-expressing cells used for infection *in trans* should not be infectable by the virus studied, to allow evaluation of virus replication that occurs uniquely in the target cell.

Note Added in Proof

While this chapter was in press, four new studies were published concerning the interactions between DC-SIGN and viruses, and the role of DC-SIGN in HIV dissemination *(40–43)*.

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5

Functional Analysis of the *N*-Linked Glycans Within the Fusion Protein of Respiratory Syncytial Virus

Ping Li, Helen W. McL. Rixon, Gaie Brown, and Richard J. Sugrue

Summary

The respiratory syncytial virus fusion (F) protein is initially expressed as a single polypeptide chain (F0). The F0 subsequently undergoes posttranslational cleavage-by-cell protease activity to produce the F1 and F2 subunits. Each of the two subunits within the mature F protein is modified by the addition of *N*-linked glycans. The individual *N*-linked glycans on the F protein were selectively removed by using site-directed mutagenesis to mutate the individual glycan-acceptor sites. In this way the role of these individual glycans in targeting of the F protein to the cell surface, and on the ability of the F protein to induce membrane fusion, was examined.

Key Words: Respiratory syncytial virus; F protein; site-directed mutagenesis; glycosylation; metabolic labeling; fusion activity; surface expression.

1. Introduction.

Human respiratory syncytial virus (HRSV), classified in the genus *Pneumovirus* of the family *Paramyxoviridae*, has been recognised as the most common cause of severe respiratory tract infection in young children and in immunocompromised people. The HRSV fusion (F) protein, the main surface glycoprotein, plays a central role in virus entry and infection by mediating the fusion of virus membranes with those of host cells. The F protein also promotes the fusion of infected cell membranes with those of adjacent cells, resulting in syncytial formation and spread of RSV. The protein is synthesised as an inactive precursor (F_0) of 70 kDa that is cleaved posttranslationally by furin-like cellular proteases during its transport through the endoplasmic reticulum (ER) and the Golgi complex to the cell surface. This yields two disulphide-linked sub-

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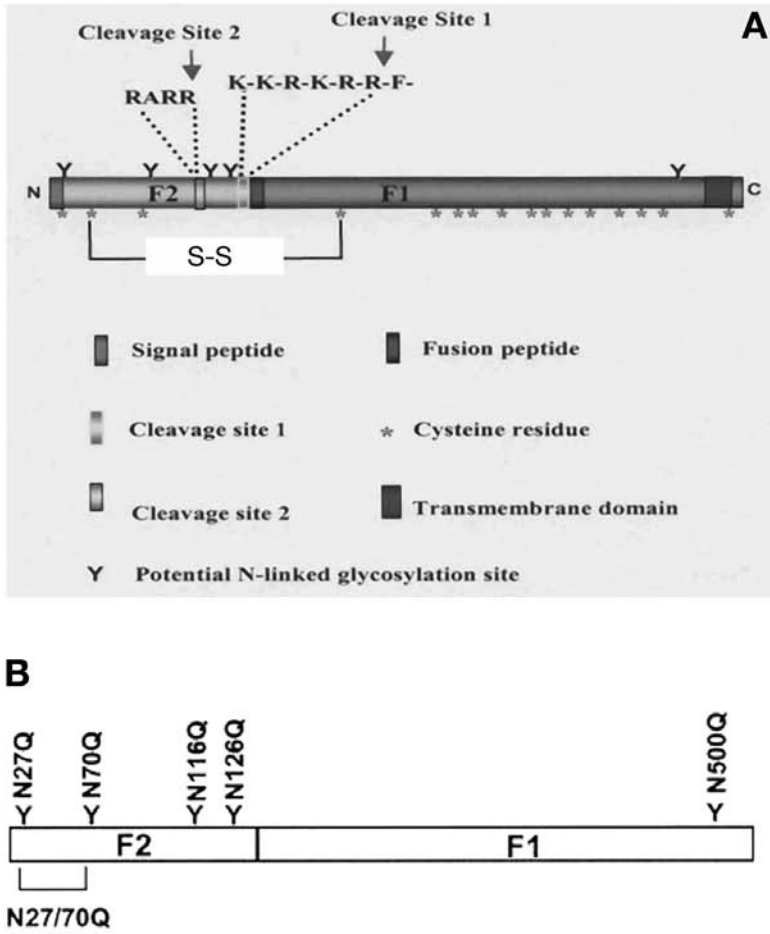

Fig. 1. **(A)** Location of the five potential *N*-linked glycosylation sites in the F protein amino acid sequence. Various aspects of the primary protein structure of the F protein are highlighted including the two cleavage sites. **(B)** The locations of the various F protein mutations.

units of 50 kDa (F_1) and 20kDa (F_2) (**Fig. 1A**), which are modified post-translationally by *N*-linked glycosylation and palmitoylation ***(1,2)***.

Many viral membrane proteins, including those of simian virus 5, human immunodeficiency virus (HIV), and influenza virus, are modified by the addition of *N*-linked oligosaccharide chains during protein maturation ***(3–5)***. *N*-linked glycosylation is associated with a wide variety of functions in viral

glycoproteins such as protein folding, intracellular trafficking, antigenic properties, and biological functions including fusion, hemagglutinin, and esterase activity ***(4,6–9)***.

N-linked glycosylation occurs only at a specific sequence motif, Asn-X-Ser/Thr (NXT/S), where X can be any amino acid except for a proline residue. The HRSV F protein (A2 strain) contains five potential *N*-glycosylation sites, four sites (N27, N70, N116 and N126) on the F_2 subunit, and only one (N500) on F_1 ***(10)***. To characterize *N*-linked glycosylation of the HRSV F protein and elucidate the possible role of each glycosylation site in protein folding, processing, and function, a series of *N*-linked glycosylation-deficient mutant F proteins was constructed by site-directed mutagenesis. cDNAs of the wild-type F protein sequence (Fwt) and each of the desired *N*-glycosylation site mutants were cloned into the expression vector pcDNA/3.1(–) (Invitrogen) downstream of the T7 promoter. The F protein *N*-glycosylation site mutants (N27Q, N70Q, N116Q, N126Q, N500Q, and N27/70Q), the names of which represent the positions of the sites mutated, were generated by replacing the asparagine residue (N) with glutamine (Q) at the NXT/S motif **(Fig. 1B)**. The F proteins were analyzed using the approach and techniques described in this chapter for determination of their *N*-linked glycosylation site usage, protein intracellular trafficking, cell surface expression, and fusion activity.

2. Materials

1. 35-mm and 60-mm cell culture dishes (Nunc).
2. Vero and HeLa cells.
3. Dulbecco¥s modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS), 100 U penicillin/mL, and 100 μg/mL streptomycin.
4. Opti-MEM (Gibco BRL)
5. Lipofectin (Invitrogen)
6. Methionine-free cell culture medium (Gibco, BRL).
7. [^{35}S]methionine (800 Ci/mmol), (Amersham)
8. Phosphate-buffered saline (PBS)
9. Complete™ Protease inhibitor cocktail tablets (Boehringer Mannheim)
10. Radio-immunoprecipitation assay (RIPA) buffer: 1% NP-40, 0·1% sodium dodecyl sulfate (SDS), 150 m*M* NaCl, 1 m*M* EDTA, 20 m*M* Tris–HCl, pH 7.5, protease inhibitor cocktail (diluted in accordance with the manufacturer's instructions).
11. SDS-polyacrylamide gel electrophoresis (PAGE) equipment.
12. SDS-PAGE gel fixative: 10% acetic acid.
13. Glucose-free cell culture medium (Gibco, BRL).
14. [^{3}H]glucosamine (24 Ci/mmol) (PerkinElmer Life Sciences).
15. Amplify (Amersham).
16. High-salt buffer: 1% Triton X-100, 650 m*M* NaCl, 1 m*M* EDTA, 10 m*M* sodium phosphate, pH 7.0.

17. Low-salt buffer : 1% Triton X-100, 150 m*M* NaCl, 1 m*M* EDTA, 10 m*M* sodium phosphate, pH 7.0.
18. Binding buffer: 0·5% NP-40, 150 m*M* NaCl, 1 m*M* EDTA, 0.25% BSA, 20 m*M* Tris–HCl, pH 8.0.
19. Protein A–Sepharose (Sigma).
20. Protein sample buffer: 1% SDS, 5% glycerol, 20 m*M* Tris, 1% β-mercaptoethanol, 0.2% bromophenol blue, pH 6.8.
21. Endoglycosidase H (Endo H) (500 units/μl) (New England Biolabs).
22. Denaturing buffer (0.5% SDS, 1% β-mercaptoethanol).
23. Endo H reaction buffer: 50 m*M* sodium citrate pH 5.5, supplied at 10X concentration with the enzyme by the manufacturer.
24. Becton-Dickinson FACScalibur (FACS) and Cell Quest software.
25. Cell Dissociation Solution (Sigma).
26. Primary antibody against the protein of interest: F antibody: monoclonal antibody (MAb)19 ***(11)***.
27. Secondary antibody conjugated to fluorescein isothiocyanate (FITC) or Cy5.
28. FACS buffer: PBS, 2% FCS, 0.05% sodium azide.
29. 4% paraformaldehyde (pH 7.4 in PBS).
30. 13 mm glass coverslips and 24-well cell culture plates.
31. Glass slides and Citifluor for mounting coverslips.
32. 0.1% saponin in PBS.
33. DAPI (4,6-Diamidino-2-phenylindole Dihydrochloride) (Sigma).

3. Methods

This chapter describes the methods and techniques used for characterization of the following aspects of the HRSV F protein:

1. Expression of the F protein and its *N*-glycosylation site mutants using the recombinant vaccinia virus T7 expression system.
2. Determination of *N*-linked glycosylation site usage.
3. Analysis of the maturation status of the glycans.
4. Measurement of protein intracellular trafficking and cell surface expression.
5. Fusion activity of the F protein.

3.1. Analysis of N-Linked Glycosylation-Site Usage in the HRSV F Protein

Site-directed mutagenesis is a powerful technique that can be used for characterization of *N*-linked glycoproteins. Knowledge of the nucleotide or amino acid sequence of the glycoprotein is a prerequisite for this technique. A common approach is to mutate a potential *N*-linked glycosylation site, and expressing the mutant protein in a eukaryotic expression system to determine the utilization of a specific *N*-linked glycosylation site. An average *N*-glycan has an approximate molecular mass of 2–3 kDa. Therefore, the usage of each glycosylation site can be determined by assessing the electrophoretic migration pat-

terns of the mutant proteins on SDS-PAGE and comparing the molecular weight of the mutant proteins with that of the wild-type glycoprotein. The methods used for expression of the F proteins, determination of *N*-linked glycosylation site usage, and characterization of the F protein *N*-glycosylation site mutants are described under **Subheadings 3.1.1.–3.1.4.** (*see* **Note 1**).

3.1.1. Transient Expression of HRSV F Proteins Using the Recombinant Vaccinia Virus T7 Expression System

1. Cells were grown either in plastic culture dishes or on 13-mm coverslips in 24-well plates using DMEM supplemented with 10% FCS and incubated at 37°C in 5% CO_2 in a humidified incubator. The cell monolayers should be at 70–80% confluence by the following day (*see* **Note 4**).
2. Wash the cell monolayers once with DMEM containing FCS and infect with recombinant vaccinia virus vTF7-3 (diluted in DMEM/FCS) at 5 plaque-forming units (pfu)/cell for 1 h at 37°C.
3. During the incubation period, prepare plasmid DNAs for transfection. In a 15-mL Falcon tube or a FACS tube, dilute the DNAs (0.5–1.5 μg) in 400–800 μL Opti-MEM and add 4 to 10 μL Lipofectin Reagent (Invitrogen). Gently mix the Lipofectin-DNA-Opti-MEM solution and incubate at room temperature for 20 min.
4. Wash the cell monolayers twice with PBS and once with Opti-MEM.
5. Remove the Opti-MEM and add the DNA-Lipofectin mix to the cells. Incubate the cells for 3 h at 37°C.
6. Remove the DNA-Lipofectin-containing medium. Add fresh DMEM containing 10% FCS and incubate the cells at 37°C for 9–24 h.

3.1.2. In Vivo [^{35}S]Methionine Pulse-Chase Labeling

1. For protein expression, transfect 35-mm dishes of 70–80% confluent cells as described under **Subheading 3.1.1.**
2. At 2 h posttransfection, rinse the cell monolayers twice with PBS and once with methionine-free medium.
3. Remove the medium and replace with 1 mL fresh methionine-free medium containing [^{35}S]methionine (50 μCi/mL).
4. Incubate the cells for 15 h at 37°C.
5. Remove the [^{35}S]methionine-containing medium and discard.
6. Rinse the cell monolayers twice with PBS. Add 1 mL fresh DMEM supplemented with 10% FCS and incubate for 2 h at 37°C.
7. Remove the medium and discard.
8. Rinse the cell monolayers twice with PBS and drain well. Add 300 μL RIPA buffer containing a protease inhibitor cocktail. Incubate the dishes on ice for 15–20 min.
9. Transfer the cell lysate to Eppendorf tubes and centrifuge for 1 min at 13,000*g* to pellet the cell debris and nuclei. Collect the supernatant .
10. Immunoprecipitate the F protein from the supernatant using specific antibodies (*see* **Subheading 3.1.4.**).
11. Separate the proteins by SDS-PAGE.

12. Fix the polyacrylamide gels in 10% acetic acid for 20 min.
13. Dry the gels on to 3MM paper under vacuum at 80°C.
14. Expose the dried gels on a phosphorimager screen and detect the radioactive protein bands using a personal FX phosphorimager.

3.1.3. In Vivo [^{3}H]Glucosamine Labeling.

1. For protein expression, transfect 35-mm dishes of 70–80% confluent cells as described under **Subheading 3.1.1.**
2. At 2 h posttransfection, rinse the cell monolayers twice with PBS and once with glucose-free medium.
3. Remove the medium and replace with 1 mL glucose-free medium containing [^{3}H]glucosamine (100 μCi/mL).
4. Incubate the cells at 37°C for 16 h.
5. Remove the [^{3}H]glucosamine-containing medium and discard.
6. Rinse the cell monolayers twice with PBS and drain well.
7. Add 300 μL RIPA buffer containing a protease inhibitor cocktail. Incubate the dishes on ice for 15–20 min.
8. Transfer the cell lysate to Eppendorf tubes and centrifuge for 1 min at 13,000*g* to pellet the cell debris and nuclei. Collect the supernatant .
9. Immunoprecipitate the F protein from the supernatant using the F antibody, MAb 19 (*see* **Subheading 3.1.4.**).
10. Separate the proteins by SDS-PAGE.
11. Fix the gels in 10% acetic acid for 20 min.
12. Incubate the gels in Amplify solution for 20 min.
13. Dry the gels and subject them to autoradiography at –70°C. Exposure time varies from a few days to several weeks depending on the protein being tested.

3.1.4. Immunoprecipitation

1. In a 1.5-mL Eppendorf tube, add the F antibody, MAb19 (previously assayed to determine the optimal working dilution), 100 μL cell lysate supernatant (from **Subheading 3.1.3., step 8**) and 400–600 μL binding buffer and incubate overnight at 4°C.
2. Incubate the mixture overnight at 4°C.
3. Isolate the immune complexes by adding 40 μL 50% protein-A–Sepharose beads and incubate with shaking for 2 h at 4°C.
4. Wash the immunoprecipitates six times with high-salt buffer and once with low-salt buffer by vortexing and centrifuging at 10,000*g* for 2 min, carefully discarding the supernatant each time.
5. Resuspend the protein A–Sepharose-bound immune complexes in 40 μL protein sample buffer and heat at 100°C for 5 min. Alternatively, use the protein A–Sepharose-bound immune complexes for endoglycosidase digestion (*see* **Subheading 3.2.1.**).
6. Centrifuge the samples (in protein sample buffer) at 10,000*g* for 2 min to remove the protein A–Sepharose and separate the proteins by SDS-PAGE. Complete the

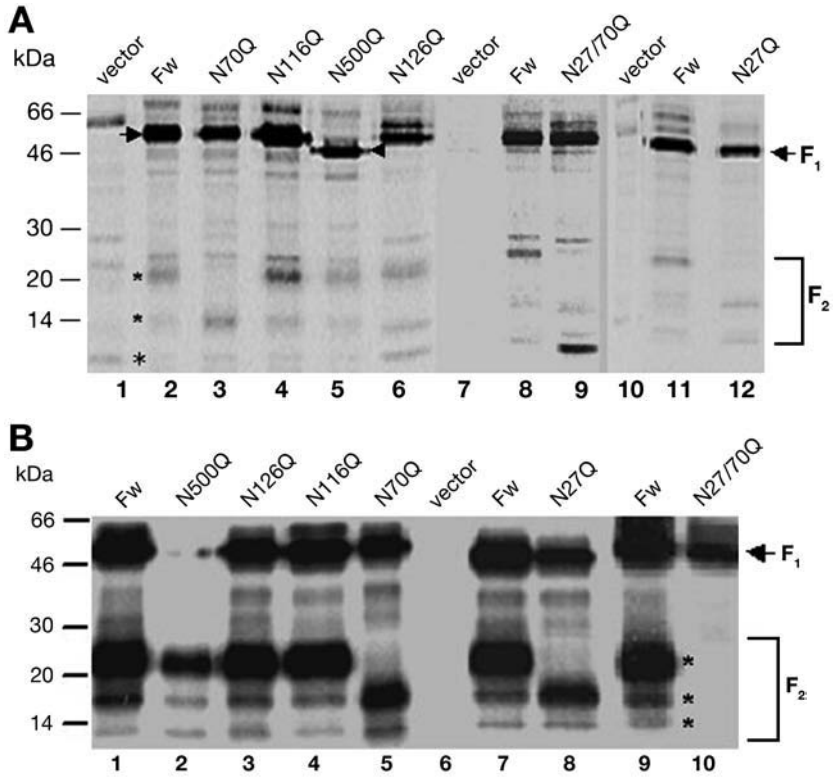

Fig. 2. Usage of the HRSV F protein *N*-linked glycosylation sites. Vero cells transfected with wild type F protein sequence and mutated F protein sequences in which selective glycosylation sites were removed. The cells were labelled with **(A)** [^{35}S] methionine or **(B)** [^{3}H]glucosamine (for glycan labelling) and the F protein isolated by immunoprecipitation using MAb19 and analysed by SDS-PAGE. The positions of the F1 (arrow) and F2 (asterisk) subunits are shown.

analysis by following **Subheading 3.1.2., steps 12–14** for [^{35}S]methionine-labeled samples or **Subheading 3.1.3., steps 11–13** for [^{3}H]glucosamine-labeled samples.

Figure 2A shows expression of the wild-type F protein (Fw) (lane 2) and its *N*-linked glycosylation site mutants using the recombinant vaccinia virus T7 expression system. Separation of the ^{35}S methionine-labeled F protein by SDS-PAGE following immunoprecipitation with anti-F MAb19 revealed an apparent reduction in molecular mass of the F_1 protein subunit from mutant N500Q (lane 5) and of the F_2 protein subunit from mutants N27Q (lane 12), N70Q (lane 3) and

N27/N70Q (lane 9). This suggests that the indicated site on the F protein is glycosylated. The SDS-PAGE migration pattern of F mutants N116Q and N126Q either from the [^{35}S]methionine protein-labeling experiment (**Fig. 2A**) or from the [^{3}H]glucosamine glycan-labeling experiment (**Fig. 2B**) was indistinguishable from that of Fw. These two *N*-glycosylation sites are located in a small peptide that is released from the mature F protein upon furin cleavage.

Separation of the glycan-labeled F protein from Fw and the mutant plasmids (**Fig. 2B**) by SDS-PAGE reveals a migration pattern which is very similar to that of the methionine-labeled F protein . No labeling of the F_1 subunit was observed in the N500Q mutant plasmid, as this site is the only *N*-glycosylation site on the F_1 protein. Similarly, no labeling of the F2 subunit was observed in the N27Q/N70Q mutant. This suggests that the *N*-linked glycosylation sites at N27, N70, and N500 are used.

3.2. N-Linked Glycan Analysis (The Maturation Status of the N-Linked Glycans)

Several endoglycosidases that cleave the *N*-linked oligosaccharides of glycoproteins are available for the study of *N*-linked glycan structure. Endo H cleaves *N*-linked glycans of high-mannose type in the ER. Thus, Endo H sensitivity is indicative of the maturation process and intracellular transport of the glycoproteins. PNGase F releases specifically *N*-linked glycans from the glycoproteins and therefore can be used to determine whether or not a protein is *N*-glycosylated. The HRSV F protein is modified by *N*-linked glycosylation and acquires Endo H resistance *en route* to the cell surface via the ER and Golgi complex ***(2,12)***. To determine the properties of each *N*-linked glycan of the *N*-linked glycosylation site mutants, cells expressing Fw and its *N*-glycosylation mutants were metabolically labeled with [^{35}S]methionine and the F proteins were immunoprecipitated with the F antibody, MAb 19. The F proteins were then subjected to Endo H digestion **(Subheading 3.2.1.)** and analyzed by SDS-PAGE.

3.2.1. Endoglycosidase H (Endo H) Digestion

In a 1.5-mL Eppendorf tube, denature the protein samples from the immunoprecipitation (IP) reaction (*see* **Subheading 3.1.4., step 5**) by adding 20 µL denaturing buffer (1X final concentration), and heating at 100°C for 10 min before cooling to room temperature. The protein A–Sepharose was removed by centrifugation.

1. The denatured protein sample was divided between two Eppendorf tubes.
2. For digestion with Endo H, add 10X EndoH reaction buffer to a final 1X concentration and 500 U Endo H. Mock-treat one protein sample by adding reaction buffer without adding Endo H.

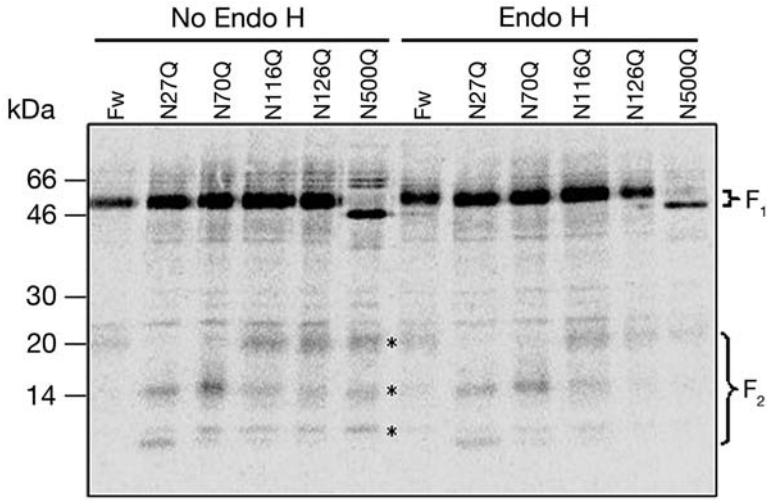

Fig. 3. Assaying the maturation of the mutant F protein sequences. [^{35}S]methionine-labeled F protein was isolated by immunoprecipitiation using MAb19 and mock-treated or treated with Endo H. The F protein was then analysed by SDS-PAGE. The positions of the F1 and F2 subunits are indicated.

3. Mix, pulse spin, and incubate for 20 h at 37°C.
4. Determine the molecular masses of both the treated and untreated protein samples by SDS-PAGE and autoradiography (*see* **Subheading 3.1.2., steps 11–14**).

Figure 3 shows that following digestion with EndoH and separation of the proteins by SDS-PAGE, both the F_1 and F_2 subunits of Fw and the F protein of the glycosylation mutants acquire Endo H resistance, as is demonstrated by their similar electrophoretic mobility when compared with untreated F proteins. This suggests that the *N*-linked glycan chains of the mutant proteins were processed from high mannose-type to complex sugars in the medial- or trans-Golgi regions of their host cells.

3.3. Analysis of Glycoprotein Transport to the Cell Surface

Glycoproteins are synthesised in the ER, where protein folding and co-translational modifications occur. Through cellular trafficking, many glycoproteins undergo further modifications and maturation (e.g., *O*- or *N*-linked glycosylation, palmitoylation, and proteolytic activation) in the Golgi complex, before being transported to the cell surface. It is known that *N*-linked glycans play a role in protein folding, sorting, and transport ***(10)*** and that the RSV F protein is transported to, and readily detected on, the cell surface of virus-

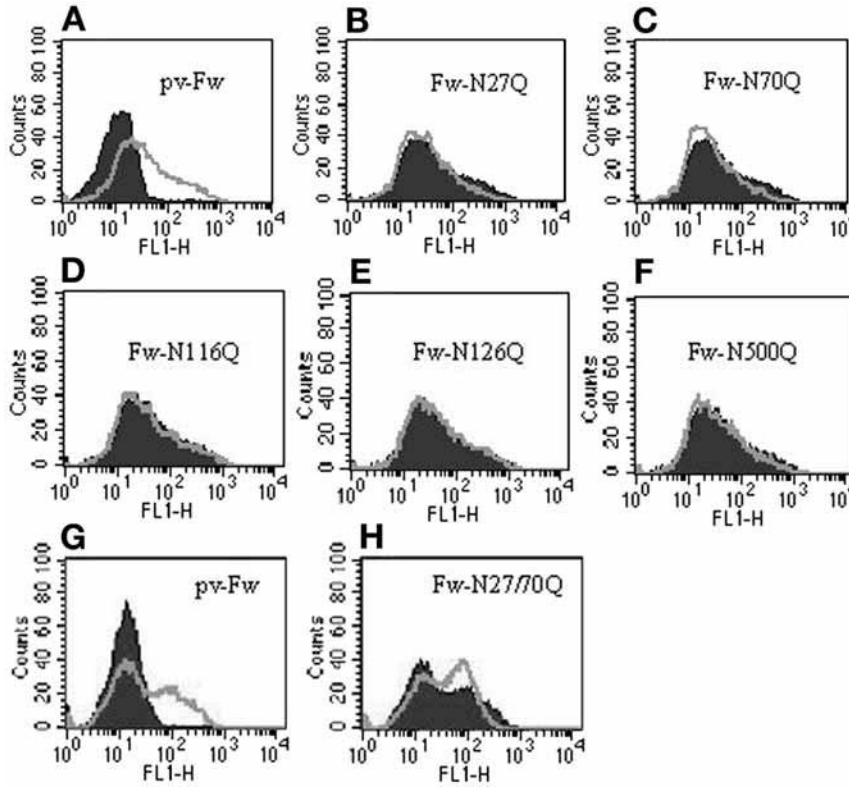

Fig. 4. Cell surface expression of the mutant F protein sequences. Surface transport of wild-type and mutant sequences was compared using flow cytometry. Histograms A and G represent a comparison of the surface fluorescence intensity of cells transfected with vector (filled curve) or pcDNA/Fw (open curve) and labeled with MAb19. Histograms B to F and H is a comparison of the surface fluorescence of cells transfected with the wild-type F protein sequence (filled curve) and specific mutant F protein sequences (open curve).

infected cells ***(2,13,14)***. This characteristic feature was used to assess the effect of *N*-linked glycosylation on F protein transport. A flow cytometry method for the quantitative analysis of cell surface F protein *N*-glycosylation mutants to determine whether or not the efficiency of F protein processing and transport is affected by the deprivation of *N*-glycans (*see* **Note 3**) is described under **Subheading 3.3.1.** **Figure 4** shows that the surface immunofluorescence intensity for mutants N27/70Q, N27Q, N70Q, and N500Q was reduced by varying amounts between 36% and 26% compared to that of Fw while mutants

N116Q and N126Q show an immunofluorescence intensity which is similar to that of the Fw control.

3.3.1. Flow Cytometry (FACS Analysis)

1. At 15–24 h posttransfection, wash the cell monolayers in 60-mm dishes three times with cold PBS.
2. Add 0.5 mL cell dissociation buffer to each dish and incubate at 37°C for 15 min. Gently tap the dish to detach the cells.
3. Transfer the cells to a FACS tube. Wash the cells three times with cold FACS buffer by centrifugation at 1000*g* for 10 min each time at 4°C and carefully discard the FACS buffer.
4. Fix the cells by adding 0.5 mL 4% paraformaldehyde to each tube and incubate on ice for 30 min.
5. Wash the cells three times with cold FACS buffer (*see* **step 3**) and incubate the cells with the F antibody, Mab19 (diluted 1 in 1000 with FACS buffer) for 30 min on ice, gently tapping the tube every 5–10 min to resuspend the cells.
6. Wash the cells three times with cold FACS buffer (*see* **step 3**) before staining with secondary antibody conjugated to FITC (diluted 1 in 200 with FACS buffer) for 30 min on ice, gently tapping the tube every 5–10 min to resuspend the cells.
7. Wash the cells three times with cold FACS buffer as in **step 3** and finally resuspend the cells in 0.5 mL FACS buffer.
8. Quantitate the fluorescence intensity using a Becton-Dickinson FACScalibur (FACS) and analyse the data with Cell Quest software. For each analysis, 10,000 –20,000 cells are automatically counted and scanned.

3.4. Fusion Activity Assay (Syncytial Formation)

Fusion of the viral envelope with cell membranes is an essential step in the virus life cycle of enveloped viruses, which enables penetration of the virus genome into the host cells. Fusion proteins of many Paramyxoviruses, including the F protein of RSV, can also promote fusion of the plasma membranes of infected cells with those of adjacent cells, resulting in giant, multinucleated cells, termed syncytia. Fusion occurs at neutral pH for members of the Paramyxoviruses. In this section, the effects of *N*-linked glycosylation on the fusion activity of F protein maturation was examined by confocal immunofluorescence microscopy, using the methods described under **Subheading 3.4.1.** (*see* **Notes 3** and **4**).

3.4.1. Indirect Immunofluorescence and Syncytial Plaque Formation Assay

1. Transfect 95–100% confluent HeLa cells grown on 13-mm glass coverslips in a 24-well plate (*see* **Subheading 3.1.1.**).

2. At 24 h posttransfection, wash the cell monolayers three times with cold PBS (*see* **Note 4**). Care should be taken to prevent the cells from drying in order to avoid any nonspecific fluorescence.
3. Remove the PBS and fix the cells with 4% paraformaldehyde in PBS for 30 min at 4°C.
4. Wash the cells three times with cold PBS containing 1% FCS.
5. Permeabilize the cells (for internal staining) by adding 0.5 mL 0.1% Saponin in PBS for 20–30 min at 4°C.
6. Wash the cells three times with cold PBS containing 1% FCS.
7. Remove the PBS and stain the cells with the F protein antibody, Mab19 (diluted 1 in 1000 with PBS containing 1% FCS) by incubating the cells in a humidified container at 20°C for 30 min.
8. Wash the cells three times with cold PBS containing 1% FCS and stain with the secondary antibody conjugated either to FITC or Cy5 (previously titrated for optimal dilution) by incubating the cells in a humidified container at 20°C for 30 min.
9. When assaying for the formation of syncytia, add DAPI (1/1000 dilution) during the preparation for staining with the secondary antibody conjugated to FITC or Cy5. DAPI is used for chromosome staining and therefore facilitates the identification of nuclei and hence, syncytial formation.
10. Wash the cells three times with cold PBS containing 1% FCS.
11. Mount the coverslips on slides with Citifluor and visualise using a Zeiss LSM Confocal Microscope. Analyze the images using LSM 510 v2.01 software.

The formation of syncytia was observed in cells transfected either with Fw or with each *N*-glycosylation mutant F protein (**Fig. 5**), indicating that the absence of a single *N*-glycan chain, or two *N*-glycans (at N27 and N70), does not completely abolish the fusion activity of the protein. However, the *N*-glycan chains at different locations on the F protein differentially affect the fusion activity of the protein in terms of size and frequency of syncytia induced in transfected cells. It has been reported that elimination of the single *N*-glycan at N500 in the HRSV Long strain reduces significantly syncytial plaque formation ***(15)***.

4. Notes

1. It should be noted that eukaryotic rather than prokaryotic expression systems should be used to achieve authentic protein glycosylation. Bacterial expression systems are commonly used for protein expression and subsequent purification when large quantities of proteins are required. However, proteins expressed in bacteria are not properly modified post-translationally as they require mammalian cells for accurate modifications.
2. There are a few commonly used techniques available for the detection of cell surface proteins, including immunofluorescence microscopy, flow cytometry (FACS analysis), and biotinylation ***(16)***.

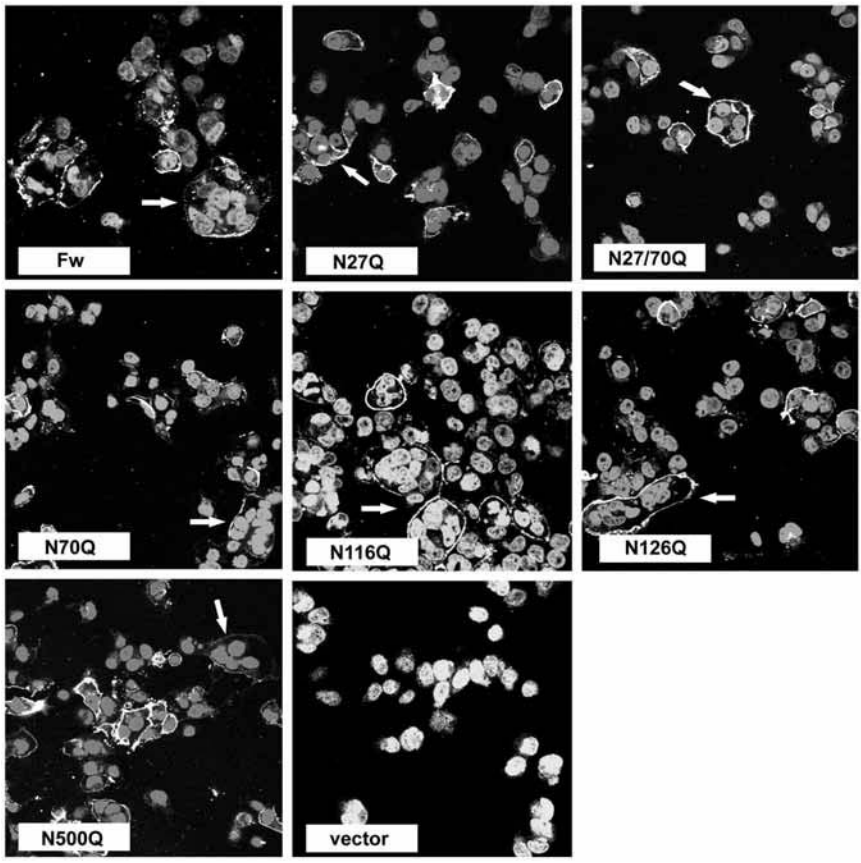

Fig. 5. Evaluation of mutant F protein sequences for fusion activity. Hela cells were transfected with plasmids containing wild type and mutant sequences. The F protein was detected using MAb19 and to better view syncytial formation the cells were stained with DAPI to reveal the nuclei. The presence of the syncytia are highlighted (white arrow).

3. Several assays and methods have been developed for the analysis of the fusion activity of fusion proteins. For example, syncytia can be observed directly using phase contrast microscopy, immunofluorescence microscopy, or microscopy to view cells after staining with crystal violet or Giemsa. Other methods used for assaying of fusion activity include transfer assays of lipid and aqueous dyes ***(17)***.
4. Various types of eukaryotic cells can be used for protein expression and characterization using the recombinant vaccinia virus T7 expression system. However, when observing syncytia, HeLa cells appear to be more suitable than Vero cells as they show delayed cytopathic effect after vaccinia virus infection. In addition,

the optimal expression time should be determined for the observation of clear syncytia induced by the expressed proteins, but at the same time, serious cytopathic effect produced by vaccinia virus should be avoided. In our experience, syncytia can be identified more easily at posttransfection times between 24 and30 h.

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6

Expression and Purification of Viral Glycoproteins Using Recombinant Vaccinia Viruses for Functional and Structural Studies

Zhu-Nan Li and David A. Steinhauer

Summary

Methods for generating recombinant vaccinia viruses for the expression of foreign viral glycoproteins in mammalian cell lines and the purification of expressed viral glycoproteins are described. These methods are based on many years of experience with the influenza hemagglutinin glycoprotein (HA). However, they are applicable for studies on other viral glycoproteins, and with slight modifications, could be useful for cellular proteins as well.

Key Words: Viral glycoproteins; recombinant vaccinia viruses; influenza; hemagglutinin; structure; function.

1. Introduction

Vaccinia viruses are able to infect a variety of mammalian cell lines. Since the original studies showing that these viruses can be utilized for the expression of foreign proteins by Moss and colleagues *(1)*, the techniques for generating recombinant vaccinia viruses have been modified and improved, and have been utilized extensively for a variety of purposes *(2)*. With these systems, the viral glycoproteins are glycosylated and expressed in a manner that is structurally and functionally representative of the homologous proteins expressed on viral and infected cell surfaces.

The main interest in our laboratory is the influenza hemagglutinin glycoprotein (HA). Although we have utilized many of the various recombinant vaccinia virus expression approaches over the years, the expression system of choice in our group is that developed by Blasco and Moss *(3)*. This method

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uses a plaque-size selection system, which makes the generation of recombinants rapid and efficient. It also allows for very good expression levels. Therefore, this chapter will concentrate on the generation of recombinants using the plaque-size selection system and some of the applications for which these recombinants can be utilized.

The methods to make the recombinant vaccinia viruses involve the virus strain vRB12, which is less efficient in the capacity for plaque formation compared to wild-type vaccinia *(4)*. The reason is that this strain lacks a functional vp37 gene, which is essential for efficient plaque formation. The plasmid pRB21 contains the vp37 gene, a promoter, and multiple cloning sites for insertion of the foreign protein genes *(3)*. These sites are flanked by thymidine kinase sequences to facilitate insertion of the vp37 and foreign protein coding sequences into the vaccinia virus genome by recombination. Following transfection of cells, a large percentage of plaques that develop to wild-type plaque size are formed by recombinant viruses that express the foreign protein.

The techniques described here are based on our experiences with the influenza HA, a type I glycoprotein that has been extensively studied. They are essentially the same as described by Blasco and Moss *(3)* in the original paper, possibly with slight modifications. The methods for making the recombinant vaccinia viruses, plaque purification, monitoring HA expression following infection of mammal cells, and preparation of working stocks will be addressed.

In our laboratory, expressed wild-type and mutant HA proteins have been useful for a variety of studies on receptor binding, membrane fusion, and antigenicity *(5–7)*. In addition, we were able to solve the high-resolution X-ray crystal structure of the proteolytic precursor form of the molecule using protein purified from recombinant vaccinia virus-infected cells *(8)*. This structure was shown to be identical to the known, cleaved structure, with the exception of about 20 residues surrounding the cleavage loop. We have also utilized proteins expressed using this system to solve the structure of the HA of the virus that caused the devastating influenza pandemic of 1918 *(9)*. Thus, the system is very versatile, and it is relatively easy to become skilled in the techniques involved.

2. Materials

1. vRB12 virus.
2. pRB21 plasmid.
3. CV-1 cells.
4. HeLa cells.
5. Serum-free Dulbecco's modified Eagle's medium (DMEM) (cellgro).
6. 2X DMEM (GIBCO).
7. 100X Penicillin/Streptomycin (cellgro).
8. 1.5% Noble Agar/DW (distilled water) (Difco).

9. 1% Neutral Red/DW (Sigma).
10. TPCK Tryspin (Sigma).
11. DNase (Promega).
12. 1% Crystal violet/20% Ethanol (Sigma).
13. 2% BSA/PBS (Sigma).
14. Opti-MEM (GIBCO).
15. Lipofectamine (Invitrogen).
16. *Clostridium perfringens* neuraminidase (Boehringer).
17. 25 m*M* Citrate pH4.5 (Sigma).
18. 3,3',5,5' tetramethyl benzidine hydrochloride (Sigma).
19. 30% H_2O_2 (Sigma).
20. 0.1 *N* H_2SO_4 (Sigma).
21. Dithiothreitol (DTT).
22. Coomassie Brilliant Blue (Sigma).
23. Enzyme-linked immunosorbent assay (ELISA) reader (Fisher).
24. Dounce homogenizer.
25. PM10 membrane filter (Millipore).
26. Q15 Sartorius ionexchange column.
27. ELISA buffer: (49 mL) 25 m*M* Citrate, pH 4.5, (1 mL) 7 mg/mL 3,3',5,5' tetramethyl benzidine hydrochloride (freshly made), and (50 μLl) 30% H_2O_2.
28. Plaque overlay agar: solution A, 1.5% Noble Agar/DW; solution B, 2X DMEM, 2X Penicillin/Streptomycin, 2% fetal bovine serum (FBS). Solution A and B mixed 50:50.

3. Methods

The methods described here outline the construction of the vaccinia recombinants, the screening of positive recombinants, the growth and quantitation of virus working stocks, and a couple of examples for which we have used recombinant vaccinia viruses for studies on HA.

3.1. Generation of Recombinant Vaccinia Viruses

The first step of the procedure is to grow stocks of the parental mutant virus vRB12. This virus is difficult to quantitate by plaque formation, but will form very small plaques that can be quantitated after 3–6 d. Grow a fairly large quantity depending on need and make aliquots for long-term use. Then, the best conditions will be optimized for the generation of recombinant vaccinia viruses. The gene of interest needs to be cloned into pRB21 ***(3)*** and purified by any of the well characterized techniques to standards suitable for transfection.

3.1.1. Infection of CV-1 Cells With vRB12

The CV-1 cells that are used for the generation and propagation of the viruses are grown in DMEM supplemented with 5% FBS and 1X penicillin/streptomycin.

1. CV-1 cells are plated in 35-mm Petri dishes or six-well plates 1 d prior to infection in a 2–3 mL volume. We prefer six-well plates for ease of handling (*see* **Note 1**).
2. On the day of infection/transfection select CV1 cells that are 60–80% confluent for infection and transfection.
3. Wash the cells twice with 2 mL of serum-free DMEM and infect with about 1×10^4 plaque-forming units (pfu) of vRB12 in 0.8 mL of Opti-MEM.
4. Incubate the cells at 37°C in a CO_2 incubator (unless specified, all incubations described in this chapter will be in a CO_2 incubator).

3.1.2. Transfection of vRB12 Virus-Infected CV-1 Cells With pRB21 Plasmids

1. At 2–4 h postinfection, the pRB21 plasmids are transfected into the vRB12 vaccinia-viruses infected cells.
2. Prepare the DNA such that it can be transfected within this 2–4 h time frame.
3. The procedure works regardless of the transfection reagent used. The procedure we use is to mix 0.5–2 μg plasmid DNA in 100 μL of Opti-MEM with 5 μL Lipofectamine in 100 μL of Opti-MEM, and incubate at room temperature for 30 min (*see* **Note 2**).
4. Add the lipid/plasmid complex to the CV-1 cells dropwise with gentle shaking.
5. Incubate at 37°C for 4–16 h, then add 1 mL of DMEM supplemented with 10% FBS and penicillin/streptomycin.
6. Resume the incubation at 37°C for 2–3 d.
7. At the point when the cytopathic effect (CPE) is apparent and cells are floating, compare with the negative controls (*see* **Note 2**), harvest the supernatants and cells by gentle disruption of the monolayer using a 1 mL pipetman.
8. Centrifuge at 2500*g* for 1 min. The supernatant will be used for the plaque analysis to look for large plaques as described below (*see* **Note 3**). The remainders will be kept at –20°C (*see* **Note 4**).

3.1.3. Plaque Purification of Recombinant Vaccinia Viruses

1. Plate CV-1 cells in six-well plates and incubate until they reach approx 90% confluency in 1 or 2 d.
2. Use the supernatants from the infected/transfected cells to do 10-fold dilutions on the CV-1 cells from undiluted to 10^{-5}. It is not necessary to wash the cells or change the tips, as this is not a true plaque assay. It is simply a way to find at least one well with clear, well-separated plaques for isolating and screening cloned viruses.
3. Allow the viruses to adsorb at 37°C for 1 h.
4. Aspirate the inoculum and gently apply 2 mL of agar overlay (DMEM, 1X penicillin/streptomycin, 1% FBS, 0.75% Noble Agar; *see* **Note 5**).
5. Allow the overlay to solidify in the hood for about 10 min. Do not try to do too many assays at once with the lids off. If the monolayer dries as a result of air circulation in the hood, patches of the cell monolayer can be damaged.

6. Incubate at 37°C for 2–3 d.
7. At day 2 or 3, the clear larger plaques containing inserts should be visible by comparison with negative control plaques (vRB12 virus) and should resemble positive control plaques (pRB21 only). In order to see the plaques more clearly, it is sometimes useful to add a second, 0.5–1 mL agar overlay supplement with 0.01% Neutral Red (*see* **Note 5**).
8. Incubate at 37°C for 1–4 h prior to picking the plaques.
9. Confirm the plaques by light microscopy and select and pick 4–6 of the larger well isolated plaques for each recombinant.
10. Amplify the plaques using CV-1 cells grown in 24-well plates (1 mL volume of media).
11. To pick the plaques, draw 0.3–0.5 mL of medium from CV-1 cell wells using a Pasteur pipet, insert it into the agar overlay over the plaque, and draw the agar plug into the pipet. Transfer the plug plus media to the CV-1 well from which the media was withdrawn and pipette up and down a few times to ensure that the plug does not remain in the pipet.
12. Incubate for 2–3 d to grow small virus stocks and monitor for CPE. Positive recombinants will display clear CPE and should be harvested by disruption of the monolayer by pipetting up and down with a 1-mL pipetman. Transfer this to a small tube. Use 0.2 mL for protein expression analysis and save the rest at 4°C or –20°C (*see* **Note 4**), if it is not expected that the protein expression analysis will be done within a week (*see* **Note 6**).

3.1.4. Protein Expression Analysis

The first assay that we usually carry out to screen the plaques for positive inserts is a Western blot assay.

1. The 0.1–0.2 mL from the small virus stock described above are added to confluent CV-1 cells in 24-well plates. It is a good idea to have the CV-1 cells ready at the time that the small virus stocks are harvested.
2. The next day, cell monolayers should display CPE, often showing a "spider web" type pattern.
3. Aspirate supernatants and replace with 1 mL PBS.
4. Harvest by pipetting the cells up and down and transfer to 1.5-mL Eppendorf tubes.
5. Spin in a microcentrifuge for 1 min at 9000*g*, and remove and discard supernatants.
6. Add 125 µL sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer (reducing).
7. Boil immediately for 2 min.
8. Load a portion on a 10–12% SDS polyacrylamide gel; the rest can be frozen and run again at any time. If the loading buffer is too viscous as a result of the amount of DNA present, freeze and boil again.
9. Immunoblot by standard procedures and choose positive recombinants that migrate at the appropriate molecular weight of your protein of interest and choose clones that display optimal expression.

10. In order to ensure that the original clone was not composed of overlapping plaques, a second plaque purification of the first positive plaques is desirable. To do this, take the small virus stocks from the first plaques (0.8 mL left) and repeat the steps described above under **Subheading 3.1.3.**
11. After confirming the protein expression as described under **Subheading 3.1.4.,** one mini-stock of this step will be used as seed virus stock (*see* **Note 4**) for growing large virus working stocks as described below.

3.1.5. Preparation and Titration of Virus Working Stocks

1. Passage CV-1 cells in 75-cm^2 flasks such that they are confluent in 1 or 2 d. CV-1 cells generally double about every 24 h.
2. Inoculate 200–500 μL of seed virus stock directly to the old medium (in our hands, the titers do not increase when original media is removed, virus adsorbed, and fresh media applied).
3. Incubate CV-1 cells at 37°C for 2–3 d until all cells show clear CPE; often, most cells will be floating.
4. Detach the cell monolayer with a sharp smack of the flask, or freeze the flask as well as the growth medium at –20°C or –80°C, and then thaw it.
5. Harvest the CV-1 cells and growth medium and disrupt the resuspended cells in a sonicating water bath by three cycle sonications of 1–2 min (generally, more than 95% of vaccinia virus is cell-associated). Alternatively, three rounds of freezing and thawing can also be employed for cell disruption (*see* **Note 7**).
6. Aliquot the recombinant vaccinia virus working stocks in volumes suitable for your purposes: 1-mL aliquots in cryotubes are often convenient. Store at –20°C or –80°C (*see* **Note 8**).
7. For plaque assays of stocks prepare CV-1 cells in six-well plates 1 or 2 d prior to virus titration.
8. Thaw virus stocks in 37°C water bath.
9. Make 10-fold dilutions of the stocks in serum-free DMEM. As opposed to the plaque purification described above, this is a quantitative plaque assay and each dilution must be mixed carefully and pipet tips changed between transfer to the next tube.
10. Wash confluent CV-1 cells with 2 mL of serum-free DMEM once.
11. Add 0.5 mL of 10^{-6}, 10^{-5}, 10^{-4} dilutions in duplicate in CV-1 cells.
12. Absorb virus at 37°C for 45–60 min, gently tilting back and forth every 15 min.
13. Aspirate the inoculum from higher dilution wells to lower dilution wells.
14. Add 2 mL of agar overlay (*see* **Subheading 3.1.3., step 4**) from higher dilutions wells to lower dilution wells.
15. Repeat **Subheading 3.1.3., steps 5** and **6** (*see* **Note 9**).
16. Count plaque numbers and calculate the virus titers. Ideally, choose wells with between 25 and 50 plaques. We normally achieve titers on the order of 10^7 pfu/mL. There are methods to increase viral titers at the expense of the final volume of your stocks. For example, as around 95% of the virus is cell associated, media can be withdrawn from the flask during growth of the virus stocks. Alternatively,

following the growth of the stocks, cells can be pelleted and resuspended in a smaller volume of media prior to cell disruption.

3.2 Detection of Cell Surface Expression and Conformation of Recombinant Vaccinia Viruses Expressed HA by ELISA

For the HA, there are various methods to measure transport to the plasma membrane. The two most common methods that we use to detect cell-surface expression are trypsin susceptibility and ELISA. The trypsin susceptibility assay is a modification of the Western blot assay described previously. In vaccinia-virus-infected cells, HA is expressed as a proteolytic precursor. If the surfaces of vaccinia-infected HA-expressing cells are treated with 2–5 μg/mL of trypsin, the HAs of mutants that are surface-expressed will yield two bands upon Western blot analysis under reducing condition. Mutants that do not reach the cell surface reveal only the band representing the precursor ***(6)***.

ELISA provides one of the most versatile assays for our work. HAs expressed in many cell lines such as CV-1, HeLa, and BHK21 by recombinant vaccinia viruses display the correct conformation as well as trypsin cleavage, low-pH conformational change, and fusion properties as those on the viral surface. Here, we describe the methods in which the expressed HA conformation can be examined by ELISA using a panel of well characterized conformational specific monoclonal antibodies ***(10)***.

1. Prepare HeLa cells in 96-well plates (1×10^4 cells/well).
2. Incubate at 37°C for 1 d, longer if needed, as the cells should not only be confluent, but very compact.
3. Infect HeLa cells with recombinant vaccinia virus at a multiplicity of infection (MOI) of 5 (1×10^5 pfu per well).
4. Incubate at 37°C for 8–18 h.
5. Wash cells twice with 100 μL of PBS.
6. Fix cells with 100 μL of 0.05% Glutaraldehyde/PBS at 4°C for 1 h or overnight.
7. Wash once and block with 100 μL of 2% BSA/PBS at 4°C for 1 h or overnight. It is acceptable to block at room temperature if only blocking for shorter periods.
8. Dilute the monoclonal antibodies (we use HC3, HC31, HC68, HC100, and HC263, which recognize non-overlapping epitopes on the HA) in 2%BSA/PBS to 1:1000.
9. Add 100 μL of diluted antibodies to each well.
10. Incubate at 37°C for 1 h.
11. Wash three times with 100 μL of 2% BSA/PBS.
12. Add 100 μL of 1:1000 diluted horseradish-conjugated *Staphylococcus aureus* protein A in 2%BSA/PBS. Anti-mouse secondary antibodies cross react with vaccinia virus proteins, so they are not desirable for these studies in some situations.
13. Incubate at 37°C for 1 h.
14. Wash five times with 100 μL of PBS.

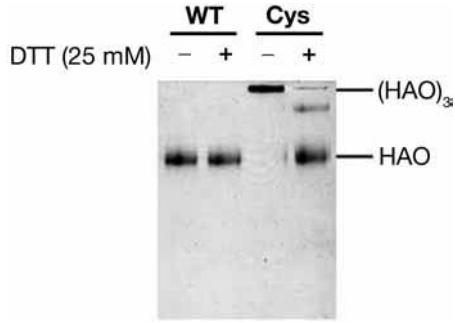

Fig. 1. This figure depicts experiments similar to those published by our group in 1992 *(7)*. The experiment involved a mutant in which HA1 residues 212 and 216 were changed to cysteine to introduce a novel disulfide bond at the membrane-distal region of HA (Cys in the figure). This covalently binds the three monomers of the HA trimer. This nonreducing gel shows that pre-treatment with 25 m*M* dithiothreitol can specifically reduce the introduced disulfide bond without cleaving disulfide bond between HA1 and HA2 (Cys14 HA1 and Cys137 HA2). The gel represents purified proteins stained with Coomassie Brilliant Blue.

15. Add 50 µL of ELISA substrate and shake the plate.
16. Stop the reaction by adding 50 µL of 0.1 *N* H_2SO_4 when blue/green color develops. The wells will then turn yellow.
17. Measure the OD 450 nm in an ELISA reader. Good controls for these studies include non-recombinant vaccinia with both antibodies, and no first antibody controls.

3.3. Purification of Recombinant Vaccinia Virus-Expressed HA Precursor (HAo) and the HA of the 1918 Influenza Viruses on Large Scale for X-ray Crystallography Studies

These types of studies require large quantities of infected cells, but offer advantages over other systems, such as bacterial or baculovirus approaches, as the proteins are derived from mammalian cells. Either HeLa cells grown in roller bottles or several large flasks of CV-1 cells can be used. We had more success with the CV-1 cells because we obtained high expression levels more reproducibly. However, other investigators may favor the HeLa cell system. As shown in **Fig. 1**, a large amount of high quality HA can be purified from the recombinant vaccinia virus infected CV-1 cells *(8,11)*. These proteins have been used for functional and structural studies *(8,9,11)*.

1. The first step is to obtain a large volume of stock virus up to 500 mL.
2. Infection of CV-1 cells with recombinant vaccinia virus expressing HA at an MOI of about 3 is optimal. However, lower MOI does not decrease HA output

dramatically, as the protein is very stable and will still be present when secondary infections lead to protein production.

3. Harvest the cells when they can be detatched from the plastic by a couple of sharp slaps to the flask.
4. Decant cells into a large centrifuge bottle and spin for 15 min at 4°C
5. Carefully pour off supernatants without spilling the cell pellet (use a clean safety receptacle underneath just in case).
6. Wash and resuspend the vaccinia-virus-infected CV-1 cells in 10 m*M* Tris HCl (Ph 8.0).
7. Homogenize in a Dounce homogenizer on ice. Cell disruption can be monitored by light microscopy. This can sometimes take as many as 100 dounces or more.
8. Add sucrose to bring the resulting solution to 70% w/v in sucrose. Overlay the tube with 10 m*M* Tris HCl (Ph 8.0).
9. The membranes are then floated by centrifugation at 82,700*g* in a Beckman SW28 rotor for 90 min at 4°C.
10. The membrane fraction separated by flotation centrifugation is pelleted by centrifuge at 82,700*g* for 60 min 4°C.
11. Resuspend the membrane fraction in 10 m*M* Tris HCl (pH 8.0), 10 m*M* calcium chloride, and 5 m*M* magnesium chloride (10^9 cell equivalents/10 mL) containing 50 mg/mL TPCK-trypsin (bromelain in the case of 1918 influenza HA) and 1 U/mL DNase. This is the step at which the ectodomains are solubilized from the membranes.
12. Incubate at 37°C for 30 min.
13. Pellet the membrane by centrifugation at 150,000*g* for 10 min in a Beckman TL100.
14. Harvest the supernatant and add *C. perfringens* neuraminidase to a final concentration of 25 μg/mL. This is to prevent HA receptor site–glycosylation site-mediated aggregation.
15. Incubate at 37°C for 30 min.
16. Overlay the mixture on a 10–30% sucrose gradient in 10 m*M* Tris HCl (pH 8.0) and centrifuge at 180,000*g* in a Beckman SW41 for 16 hours.
17. Collect HA-containing fractions and remove the sucrose using a Millipore PM10 membrane filter.
18. Purify HA further by binding to a Q15 Sartorius ionexchange column in 10 m*M* Tris HCl (pH 8.0) and eluting in 150 m*M* NaCl in 10 m*M* Tris HCl (pH 8.0).

4. Notes

1. Prepare two or three different densities of CV-1 cells, and choose the one with the optimal density for the infection/transfection experiments (60–80%).
2. We used many transfection protocols successfully. It is important to use negative controls (vRB12 only, vRB12+Lipofectamine, vRB12+other plasmid, etc.) and positive control (empty pRB21) so that the differences in CPE and plaque sizes can be determined.
3. Although more than 95% of vaccinia viruses are cell associated, we found the recombinant vaccinia viruses in the supernatant to be enough for this purpose.

4. It is good idea to keep the virus passage history clear.
5. Overlay for plaque assay. We make the agar overlay by boiling solution A and mixing with cold solution B (4°C) to use immediately. Alternatively, the mixture can be incubated in a 45–55°C water bath until needed. In the case of a second overlay, add 1 mL of 1% Neutral Red to Solution A; this can be reheated 5–10 times.
6. The viruses derived from the infected/transfected cells can be utilized immediately for plaque formation or they can be frozen for future use; they also remain viable for several days at 4°C.
7. Check the virus stock under the light microscope to confirm that the cells are disrupted.
8. Frozen vaccinia stocks can remain viable with little change in titer for as long as 5 yr or even longer.
9. Alternatively, aspirate the inoculum and overlay cells with 2 mL of serum-free DMEM supplemented with 1X Penicillin/Straptomycin. Incubate at 37°C for 2 d without moving the plate. Aspirate, wash once with PBS, and fix with 2 mL of 0.25% Glutaraldehyde/PBS. Aspirate, and stain with 1 mL of 1% Crystal violet in 20% ethanol for 15–30 min. Wash with tap water and count the plaques.

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7

The Use of Two-Dimensional SDS-PAGE to Analyze the Glycan Heterogeneity of the Respiratory Syncytial Virus Fusion Protein

Terence P. McDonald and Richard J. Sugrue

Summary

The respiratory syncytial virus (RSV) fusion (F) protein is synthesized as an inactive precursor (F0), which subsequently undergoes post-translational cleavage to give the disulphide bond-linked F1 and F2 subunits. The methodology detailing the use of two-dimensional electrophoresis, endoglycosidases, and α-mannosidase inhibitors, as applied to investigating F protein glycan maturation, is given. Examples are used to show how this methodology was used to provide evidence for glycan heterogeneity within the mature F protein.

Key Words: Proteomics; F protein; respiratory syncytial virus; deoxymannojirimycin; Swainsonine.

1. Introduction

The mature and infectious human respiratory syncytial virus (RSV) particle comprises a ribonucleoprotein (RNP) core that is surrounded by a viral envelope in which three different integral membrane glycoproteins, the attachment (G), small hydrophobic (SH), and fusion (F) proteins, are located ***(1–6)***. The F protein plays a central role in cell entry and infection by mediating fusion of the virus and host-cell membranes. The F protein is synthesized as an inactive precursor (F0) of 70 kDa, which undergoes cleavage at two conserved furin consensus sequences during its transport through the secretory pathway ***(7–9)***. This yields the mature, and active, form of the protein, consisting of the F1 (50 kDa) and F2 (20 kDa) subunits, which are linked by disulfide bonding ***(10)***. The F1 subunit is glycosylated at a single site, N500, whereas the F2 subunit is glycosylated at two sites, N27 and N70 ***(11–15)***. Initially, each attached glycan

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chain exists in a form that has a mannose core to which chains of mannose residues are attached. These mannose chains are removed subsequently by Golgi resident α-mannosidases 1 and 2, and replaced with other terminal glycans, such as *N*-acetyl glucosamine and fucose. This maturation process changes the structure of the attached glycans from relatively simple structures to those that exhibit a high degree of complexity ***(16)***.

This work details the methodology applied to the characterization of the F protein glycan maturation and heterogeneity. Pulse-chase [^{35}S]methionine labeling of virus-infected cells, and endoglycosidase treatment of the labeled F protein, allowed the maturation process of the F protein to be monitored. The glycosylation status of the mature F protein was examined using specific endoglycosidase and α-mannosidase inhibitors, together with two-dimensional (2D) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the latter providing the tools to resolve different glycosylated forms of the mature F protein.

2. Materials

1. Human RSV A2 strain.
2. Mammalian cells: Hep2 and Vero cells.
3. Dubecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS) and 100 U penicillin/mL and 100 μg/mL streptomycin.
4. Methionine-free DMEM (Gibco).
5. [^{35}S]methionine (800 Ci/mmol from Amersham).
6. Deoxymannojirimycin (DMJ) (Calbiochem).
7. Swainsonine (SW) (Calbiochem).
8. 60- and 160-mm cell culture flasks (Nunc).
9. Phosphate-buffered saline (PBS).
10. TM buffer: 10 m*M* Tris, pH 7.4 and 1 m*M* $MgCl_2$.
11. Radio-immunoprecipitation (RIP) buffer: 1% NP-40, 0.1% SDS, 150 m*M* NaCl, 1 m*M* EDTA, 20 m*M* Tris-HCl, pH 7.5.
12. Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals).
13. Denaturation buffer: 0.5% SDS and 1% β-mercaptoethanol, supplied with PNGase F.
14. Peptide: *N*-glycosidase F (PNGase F) (500,000 U/mL) (New England Biolabs).
15. PNGase F reaction buffer: 50 m*M* sodium phosphate, pH 7.5, 1% NP-40, 5% SDS, 1% β-mercaptoethanol, supplied with PNGase F.
16. Endoglycosidase H (EndoH) (5000 U/μL) (New England Biolabs)
17. EndoH reaction buffer: 50 m*M* sodium citrate pH 5.5, 0.5% SDS, 1% β-mercaptoethanol, supplied with EndoH.
18. Protein precipitation reagents: 6% TCA and 60 m*M* sodium deoxycholate.
19. 2D Clean-Up buffer (supplied in 2D Clean-Up Kit from Amersham Biosciences).
20. Urea (PlusOne from Amersham Biosciences).
21. Thiourea (ACS reagent from Sigma).
22. (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate) (CHAPS) (Ultra grade from Sigma).

23. IPG Buffer, pH 3.0–10.0 (Amersham Biosciences).
24. Urea/CHAPS buffer: 8 *M* urea, 2.6 m*M* thiourea, 4% CHAPS, 20 m*M* Tris pH 7.4, 30 m*M* dithiothreitol (DTT), 2% IPG buffer pH 3.0–10.0, 2 m*M* phenylmethylsulfonylfluoride (PMSF), EDTA-free complete protease inhibitors. Prepare freshly.
25. Rehydration solution: 8 *M* urea, 2% (w/v) CHAPS, trace bromophenol blue (30 m*M* DTT and 2% IPG buffer pH 3.0–10.0 are added just before use. Store in aliquots at –20°C
26. Binding buffer: 0.5% NP-40, 150 m*M* NaCl, 1 m*M* EDTA, 10 m*M* sodium phosphate, pH 8.0.
27. Low-salt buffer: 1% Triton X-100, 150 m*M* NaCl, 1 m*M* EDTA, 10 m*M* sodium phosphate, pH 7.0.
28. High-salt buffer: 1% Triton X-100, 650 m*M* NaCl, 1 m*M* EDTA, 10 m*M* sodium phosphate, pH 7.0.
29. Immobiline DryStrip gels 7 cm pH 3.0–10.0 linear (Amersham Biosciences).
30. SDS equilibration buffer: 50 m*M* Tris pH 8.8, 6 *M* urea, 30% (v/v) glycerol, 2% SDS. Just prior to use, add 100 mg DTT per 10 mL SDS equilibration buffer.
31. Strip holders for use with Immobiline DryStrip gels (Amersham Biosciences).
32. IPGphor isoelectric focusing unit (Amersham Biosciences).
33. SDS-PAGE Protein sample buffer: 1% SDS, 5% (v/v) glycerol, 20 m*M* Tris, 1% β-mercaptoethanol, 0.2% bromophenol blue.
34. SDS Electrophoresis buffer: 25 m*M* Tris base, 192 m*M* glycine, 0.1% SDS.
35. Agarose sealing solution: SDS electrophoresis buffer (discussed previously), 0.5% agarose, trace bromophenol blue.
36. SDS-PAGE gel fixing solution: 10% methanol, 7% glacial acetic acid.
37. Western blotting polyvinylidene difluoride (PVDF) membranes (Problot from Applied Bioscience).
38. Western blot transfer buffer: 50 m*M* Tris base, 50 m*M* glycine, 20% methanol, 0.01% SDS.
39. Block solution: 1% Marvel (nonfat dried milk, Premier Brands), 0.05% Tween-20 (Sigma) in PBS.
40. Secondary antibody against anti-mouse (whole molecule) peroxidase conjugate (Sigma).
41. Monoclonal primary antibody (MAb169 and MAb19) against the F1 subunit ***(17)***.
42. ECL™ Western blotting detection reagents (Amersham Biosciences).
43. Silver staining protein kit (Amersham Biosciences).
44. Gel drying film (Promega).

3. Methods

This chapter briefly describes the methods and techniques used for analysis of the glycan status of the RSV F protein:

1. F protein processing in virus-infected cells
2. Examination of glycan status in the F protein by 2D SDS-PAGE
3. The effect of α-mannosidase inhibitors on F protein maturation.

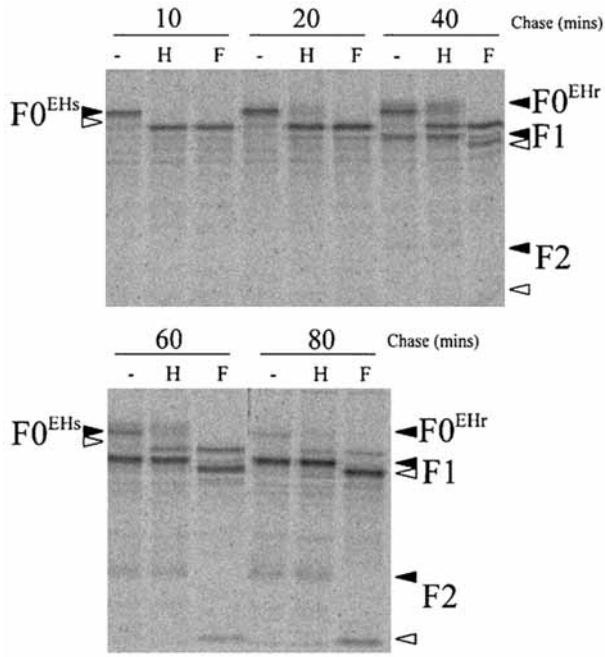

Fig. 1. Processing and intracellular transport of the respiratory syncytial virus (RSV) fusion (F) protein. Virus-infected Vero cell monolayers were labeled for 5 min with [^{35}S]methionine, washed with phosphate-buffered saline, and then incubated in chase medium containing nonradioactive 1 m*M* methionine. At between 0 and 80 min lysates were prepared, and the F protein isolated by immunoprecipitation using MAb19. The immunoprecipitates were treated with EndoH (H), PNGase F (F) or untreated (–) prior to analysis by SDS-PAGE. The positions of the glycosylated (closed triangle) and deglycosylated (open triangle) forms of $F0^{Ehs}$, $F0^{EHr}$, F1, and F2 are indicated. $F0^{Ehs}$ and $F0^{EHr}$ are the EndoH-sensitive and -resistant forms of F0, respectively.

3.1. Pulse-Chase Labeling of the RSV F Protein.

RSV-infected cells were pulse-chase labelled with [^{35}S]methionine and the F protein isolated by radio-immunoprecipitation (RIP) using the F protein monoclonal antibody MAb19 (**Fig. 1**). This allowed the posttranslational cleavage of the F protein to be monitored. The F protein is initially expressed as a 70 kDa precursor which is subsequently cleaved into the F1 (50 kDa) and F2 (20 kDa) subunits. The different F protein species were subsequently examined for their sensitivity to the endoglycosidases, peptide-*N*-glycosidase F (PNGase F) and endoglycosidase H (EndoH). Removal of the attached *N*-linked glycans is shown by a reduction in apparent mass in SDS-PAGE analysis.

1. Vero cells (90% confluent) in 60-mm dishes were infected with RSV [using a multiplicity of infection [MOI] = 1] in DMEM with 2% FCS.
2. At 18 h postinfection, rinse the cell monolayers twice with PBS and once with methionine-free medium.
3. Remove the medium and replace with 1 mL fresh methionine-free medium containing [^{35}S]methionine (100 μCi/mL).
4. Incubate the cells for 5 min at 33°C.
5. Remove the [^{35}S]methionine-containing medium and discard.
6. Rinse the cell monolayers twice with PBS. Add 1 mL fresh DMEM supplemented with 1 m*M* methionine and incubate at 33°C.
7. At specific time intervals (*see* **Fig. 1**) remove the medium and discard.
8. Rinse the cell monolayers twice with PBS and drain well. Add 300 μL radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail. Incubate the dishes on ice for 15–20 min.
9. Transfer the cell lysate to a microcentrifuge tubes and microcentrifuge for 10 min at 13,000*g* to pellet the cell debris and nuclei. Collect the supernatant.
10. Immunoprecipitate the F protein from the supernatant using the F protein antibody (*see* **Subheading 3.3.**)
11. Resuspend the protein-A-sepharose in 0.5% SDS and 1% mercaptoethanol in distilled water, and heat at 100°C for 10 min.
12. Microcentrifuge the samples for 2 min at 13,000*g* to remove the protein A–sepharose.
13. Harvest the supernatants, make up to either 50 m*M* sodium phosphate, 1% NP-40, pH 7.5, or 50 m*M* sodium citrate, pH 5.5, and incubate with 1000 U PNGase F or 1000 U EndoH (NEB), respectively
14. Separate the proteins by SDS-PAGE.
15. Fix the polyacrylamide gels in 10% acetic acid for 20 min.
16. Dry the gels on to 3 MM paper under vacuum at 80°C.
17. Expose the dried gels on a phosphorimager screen and detect the radioactive protein bands using a personal FX phosphorimager.

Figure 1 shows the identification of two forms of the F protein precursor. Initially (0–20 min), a single noncleaved and EndoH-sensitive F protein species ($F0^{EHs}$) was observed. However, at longer chase times, a second form of noncleaved F protein was detected that was fully resistant to deglycosylation by EndoH ($F0^{EHr}$). Levels of $F0^{EHr}$ (73 kDa) were maximal at 40 min and coincided with the appearance of the F1 and F2 subunits. The F1 and F2 subunits were resistant to EndoH treatment but were sensitive to PNGase F treatment. Following PNGase F treatment, both $F0^{EHs}$ and $F0^{EHr}$ were deglysosylated to a single 58 kDa product, while the F1 and F2 subunits migrated with an apparent mass of 45 and 10 kDa, respectively. The data show that posttranslational cleavage of the F protein correlates with the acquisition of EndoH resistance and suggests that posttranslational cleavage of the F protein occurs at the trans-Golgi complex.

3.2. Analysis of F Protein Glycosylation Using 2D SDS-PAGE

2D SDS-PAGE is a powerful technique that allows species of similar apparent molecular mass to be resolved by sorting proteins according to two independent properties. In the first dimension, isoelectric focusing separates proteins according to their isoelectric point (pI). In the second dimension, SDS-PAGE separates proteins according to their apparent molecular mass. Excellent sample preparation is absolutely essential for good 2D SDS-PAGE separation. This is especially so with the analysis of membrane proteins, as their hydrophobic nature is a major obstacle to achieving good resolution of protein species. The sample preparation procedures detailed below allowed good resolution of the membrane-associated proteins by 2D SDS-PAGE.

3.2.1. Isolation of Membranes

1. Maintain the Hep2 cell line with DMEM containing 10% FCS, 100 U penicillin/mL and 100 μg/mL streptomycin at 37°C in 5% CO_2.
2. Grow cells (5×10^7) to 80–90% confluence. Remove growth media, infect cells with RSV A2 at MOI of 1, and incubate in 10 mL DMEM containing 2% FCS for 2 h at 33°C.
3. Wash the cell monolayer twice with PBS and continue incubation for 16 h.
4. Wash the cell monolayer twice with PBS and twice with TM buffer at 4°C.
5. Scrape cells into 3 mL of TM buffer supplemented with Complete EDTA-free protease inhibitors. Dounce homogenise with 80 strokes at 4°C.
5. Remove unbroken cells and nuclei by centrifugation at 1000*g* for 5 min.
6. Collect the total membranes by subjecting the supernatant to further centrifugation at 45,000*g* for 15 min.
7. Wash the total membrane pellet twice in TM/protease inhibitor buffer.

3.2.2. Sample Preparation for 2D SDS-PAGE

1. Resuspend the total membrane pellets in 50–100 μL double-distilled water.
2. Denature the protein by adding denaturation buffer (1X final concentration) and incubate at 100°C for 10 min.
3. Add 10X PNGase F reaction buffer to give a final concentration of 1X, and then add 2500 U of PNGase F. Incubate for 18 h at 37°C. Treating as above without the addition of PNGase F provides an untreated control.
4. Add sodium deoxycholate (to final concentration of 60 m*M*) to the membranes and precipitate the protein with 6% TCA at 4°C for 15 min.
5. Collect the precipitate by microcentrifugation at 12,000*g* for 10 min, then wash twice in 2D Clean-up Kit buffer. The protein pellet should then be gently air dried for a few minutes. It is important not to overdry the pellet.
6. Resuspend the precipitated protein in urea/CHAPS buffer, and incubate at room temperature overnight (*see* **Note 1**).
7. Assay protein using the Amersham PlusOne 2D Quant kit.
8. Dilute each protein sample into rehydration fluid to give a final concentration of 40 μg/125 μL (*see* **Note 2**).

3.2.3. 2D SDS-PAGE

1. Use protein sample described under **Subheading 3.2.2.** to hydrate each 7 cm pH 3.0–10.0 IPG DryStrip gel overnight at 21°C (*see* **Note 3**).
2. Separate the proteins by isoelectric focusing using the IPGPHOR isoelectric focusing system with the following program. Step 1: 500 V for 30 min (250 Vh); step 2: 1000 V for 30 min (500 Vh); step 3: 8000 V for 1 h (8000 Vh) (*see* **Note 4**).
3. After completion of the first dimension, incubate the gel strips in 3 mL SDS Equilibration buffer for 15 min at room temperature.
4. Place the gel strip at the top of a 1-mm thick 8–15% SDS-polyacrylamide gel. No stacking gel is required.
5. Fix the gel strip by adding melted agarose (1 mL) to prevent it from moving or floating in the electrophoresis buffer.
6. Separate the proteins in the second dimension by SDS-PAGE at 5 mA/gel for 15 min and 10 mA/gel for 1 h 30 min.
7. Transfer the proteins to PVDF membranes by Western blotting.
8. Wash in PBS and block for 18 h at 4°C in Block solution.
10. Wash twice in PBS prior to incubation with primary antibody, MAb169 diluted 1 in 1000 in Block solution, for 1 h.
11. Wash the membrane four times in PBS/0.05% Tween-20 prior to incubation for 1 h with the secondary antibody, anti-mouse immunoglobulin (Ig)G peroxidase conjugate diluted 1 in 1000 in Block solution.
12. Visualize using the ECL protein detection system.

Figure 2A shows the silver-stained polyacrylamide gel of membrane proteins from RSV-infected cells after 2D SDS-PAGE (*see* **Note 5**). After 2D SDS-PAGE separation, the proteins were transferred by Western blotting on to PVDF membranes and the F1 protein subunit detected using MAb169 ***(17)*** (**Fig. 2B**). Two major F1 protein spots were detected in 2D SDS-PAGE analysis, each spot corresponding to a single F1 protein species in the sample. This technique provided the means to resolve different forms of F1 protein subunit. In a parallel analysis, prior treatment of the sample with PNGase F, which removed the *N*-linked glycans, resulted in the appearance of a single F1 protein spot. This provided evidence that the multiple F1 protein spots were due to differences in the glycan structure.

3.3. α-Mannosidase Inhibitors

F protein glycan maturation was analysed using α-mannosidase inhibitors. Deoxymannojirimycin (DMJ) inhibits the activity of α-mannosidase-1, whereas swainsonine (SW) inhibits the activity of α-mannosidase-2. The use of these α-mannosidase inhibitors was complemented by analyzing the sensitivity of the F protein to EndoH treatment. EndoH is able to remove *N*-linked glycans of high mannose type from proteins, while mature glycan chains containing complex sugars remain unaffected. The effect of these specific inhibitors and EndoH sensitivity of the F-protein glycans were monitored by SDS-PAGE.

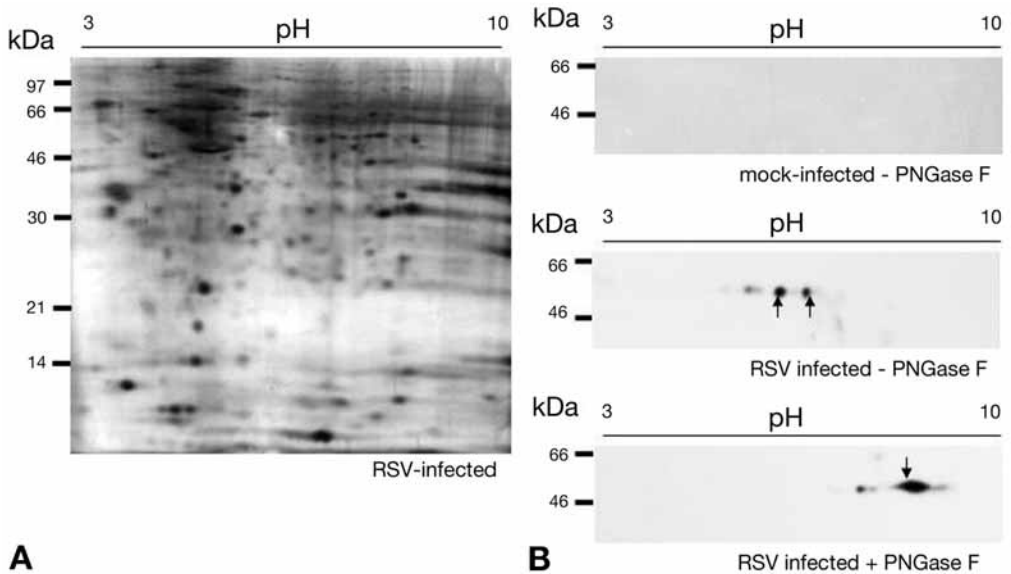

Fig. 2. Analysis of fusion (F) protein glycosylation using two-dimensional (2D) SDS-PAGE. Total membrane proteins were isolated from mock and RSV-infected Hep2 cells at 20 h postinfection, and incubated in the presence or absence of PNGase F. Proteins were separated by 2D SDS-PAGE using a pH gradient of 3.0–10.0 in the first dimension, and 12.5% SDS-PAGE in the second dimension. Nontreated (–) and PNGase F-treated (+) samples are indicated: **(A)** Silver-stained polyacrylamide gel, after 2D SDS-PAGE. **(B)** The membrane proteins were next transferred by Western blotting onto a PVDF membrane and probed with MAb169. The different F protein species are indicated (↓). Also shown is a polyvinylidine difluoride membrane in which proteins from mock-infected cells were probed with MAb169.

1. Grow cells in 60-mm cell culture dishes to 80–90% and infect with RSV A2 as described under **Subheading 3.1.**
2. Remove the medium and wash twice with PBS.
2. Incubate cells in methionine-free DMEM containing 2 m*M* DMJ and 0.4 μ*M* SW for 1 h.
4. Add [^{35}S] methionine to 100 μCi/mL and continue incubation for 16 h.
5. Remove the radioactive medium and wash the cell monolayer twice in PBS.
6. Add 1 mL of RIP buffer to the cells and incubate on ice for 10 min.
7. Transfer the cell lysate to micro-centrifuge tubes and remove cell debris and nuclei by microcentrifugation for 1 min at 13,000*g*.
8. In a 1.5-mL microcentrifuge add 100 μL cell lysate, 600 μL binding buffer, and 1 μL F antibody MAB19. Incubate 16 h at 4°C.
9. Add 50 μL 50% protein A–sepharose and incubate for 1 h with continuous shaking.
10. Wash the immune complexes five times with high-salt buffer and once with low-salt buffer by microcentrifugation at 1000*g* for 1 min.
11. For digestion with EndoH, denature the bound protein by incubation at 100°C for 10 min in 1X denaturation buffer.
12. Add EndoH reaction buffer to a final 1X concentration and 500 U of EndoH. Incubate for 18 h at 37°C.
13. Add protein loading buffer and heat at 100°C for 5 min.
14. Separate protein products by SDS-PAGE
15. Detect [^{35}S]methionine labelled proteins by phosphorimaging.

Figure 3A shows the expression of different forms of the F protein subunits in the presence or absence of α–mannosidase inhibitor treatments. The F protein subunits differ in their apparent mass, which correlates with different degrees of glycan maturation. The effect of these specific inhibitors on the EndoH sensitivity of the F protein glycans were monitored by SDS-PAGE (*see* **Fig. 3B**). In the presence of DMJ and SW, the F1 and F2 subunits were sensitive to EndoH treatment. This clearly shows that the F protein is expressed in an immature form in the presence of these inhibitors. Evidence of the loss of F protein glycan maturation following DMJ treatment was supported by 2D SDS-PAGE (**Fig. 2C**). In this analysis, the separation pattern of the F1 subunit isolated from nontreated and DMJ-treated cells was compared. In nontreated cells, several F1 subunit species were detected, being similar to that described under **Subheading 3.2.** In contrast, the F1 protein isolated from DMJ-treated cells migrated as a single species, which had undergone a shift to a more basic isoelectric point, and which is presumably a result of the absence of complex glycans. This suggests that during virus infection, the F1 protein species contain different terminal carbohydrates.

4. Notes

1. In order to achieve good isoelectric focusing, protein samples must be completely disaggregated, solubilised and denatured. Urea is always used as the denaturant together with a non-ionic detergent. Various detergents were tried such as NP-40,

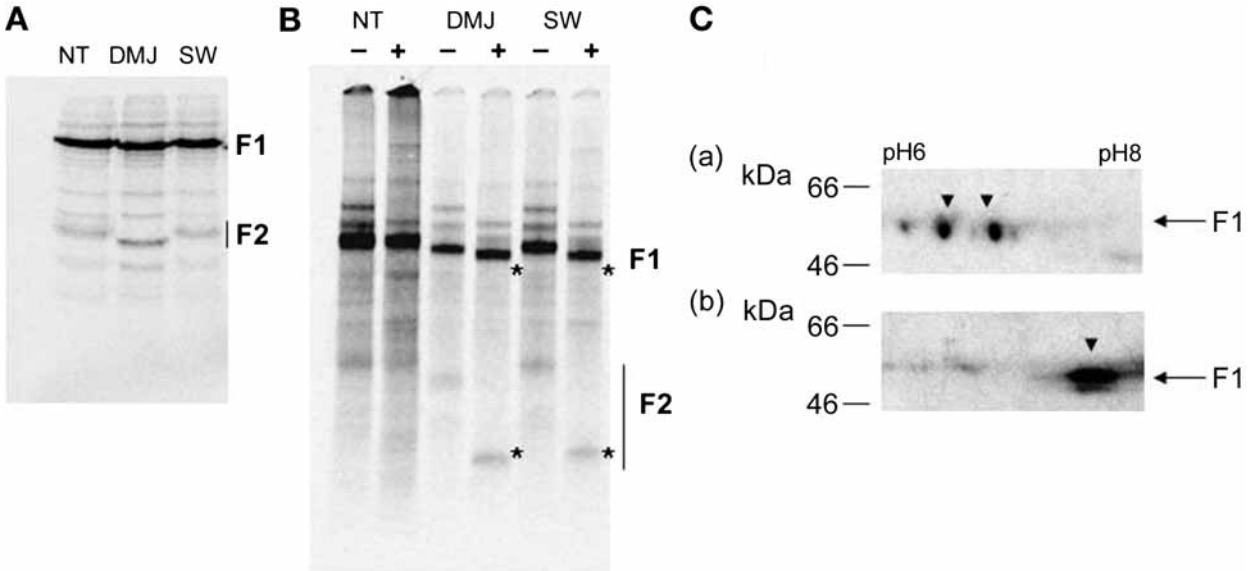

Fig. 3. Fusion (F) protein heterogeneity in glycan maturation is demonstrated with the use of α-mannosidase inhibitors. **(A)** Respiratory syncytial virus (RSV)-infected Hep2 cells were labelled with [^{35}S]methionine in the absence (NT) or presence of either deoxymannojirimycin (DMJ) or swainsonine, and the F protein was isolated by immunoprecipitation using MAb19. **(B)** The F protein was then either nontreated (–) or EndoH-treated (+) and analyzed by 15% SDS-PAGE. The positions of the deglycosylated (*) F protein species are indicated. **(C)** The proteins from **(a)** nontreated or **(b)** DMJ-treated cells were separated by two-dimensional SDS-PAGE and transferred by Western blotting onto polyvinylidine difluoride membranes, which were then probed with MAb169. In all panels, the positions of the F protein species are indicated.

Triton X-100, and octylglucoside. However, CHAPS proved to be the best detergent for this purpose. The most suitable constituents, and optimal concentration, of the solubilization buffer were determined empirically. For example, 2% IPG buffer (rather than 0.5%) and the omission of Tris base provided the best results. Time and temperature of protein solubilization was important, with overnight incubations at room temperature found to be the best combination.

2. Accurate quantification of the protein sample for 2D SDS-PAGE analysis is important. However, the reagents used to prepare and solubilize samples are incompatible with many common protein assays. The PlusOne 2D Quant kit enables the quantitative precipitation of sample leaving interfering contaminants in solution.
3. In order to separate proteins with a similar pI, it was necessary at times to use DryStrip gels with shorter pH gradients, e.g., pH gradients 4–7 and 6–11. These were successfully used with the protocols described here. The IPG buffer with the pH range identical to that of the gel strip must be used in all the buffers.
4. The protocols described are for use with 7 cm IPG dry strips. Scaling up of the procedure for use with 24 cm strips was not problematic with the use of the following adaptations.
   a. Dilute protein samples in rehydration fluid to give a final concentration of 400 μg/375 μL for hydration of the dry strip.
   b. Separate the proteins by isoelectric focusing with the following program: step 1, 500 V for 1 h (500Vh); step 2, 1000 V for 1 h (1000Vh); step 3, 8000 V for 1 h (32000 Vh).
   c. To separate with the second dimension, use Ettan Dalt*six* electrophoresis system. This system allows the simultaneous casting and electrophoresis of six gels. Electrophoresis at 4 W per gel takes about 5 h.
5. Samples prepared as described in this chapter were found to be suitable for labelling with fluorescent dyes (CyDye™) for analysis by 2D difference gel electrophoresis (2D-DIGE).

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8

The Use of Monoclonal Antibodies and Lectins to Identify Changes in Viral Glycoproteins That are Influenced by Glycosylation

The Case of Human Respiratory Syncytial Virus Attachment (G) Glycoprotein

Joanna Rawling and José A. Melero

Summary

The influence of viral envelope glycans is often overlooked, but one should bear in mind that variable glycosylation may affect the properties of viral envelope glycoproteins and potentially alter the course of an infection. Hence, there is a need for simple methods that can be use to identify changes in the glycosylation pattern of viral glycoproteins in a large number of samples. We describe here methods for the analysis of cell-line specific changes in glycosylation of the respiratory syncytial virus (RSV) attachment glycoprotein (G), which involve the use of lectins and anti-carbohydrate antibodies. Given the role of the G glycoprotein in RSV antigenicity, we also describe procedures based on Western blotting to determine the effect of G protein glycosylation changes on reactivity with human sera. We found that glycosylation of the C-terminal domain of the G protein reduces reactivity with human sera, indicating that variable glycosylation may contribute to evasion of the humoral immune response by RSV.

Key Words: Respiratory syncytial virus; G protein; lectin; V8 protease.

1. Introduction

Enveloped viruses are characterized by a lipid envelope in which one or more types of virally encoded integral-membrane proteins are embedded, giving the appearance of "spikes" on the virion surface. Because viral envelope proteins are synthesized by the cellular secretory pathway, enveloped viruses are able to exploit the glycosylation apparatus of the host cell. Preformed oligosaccharides

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($Glc_3Man_9GlcNAc_2$) are added co-translationally to Asn residues of viral envelope proteins at consensus sequences (N-X-S/T) encoded by the viral genome. Following enzymatic removal of the terminal glucose groups, these *N*-linked glycans may be subjected to further processing, depending on their accessibility to a variety of host cell glycosyltransferases and glycosidases expressed in the ER/Golgi compartments *(1)*. As a result, many enveloped viruses are extensively glycosylated by a sheath of heterogeneous *N*-linked oligosaccharides of cellular origin. *O*-linked glycosylation of some viral glycoproteins also takes place as the proteins traverse the Golgi, by addition of a GalNAc sugar to S/T residues. Subsequent elongation of the sugar chain is carried out by specific glycosyltransferases. Although there is no consensus *O*-glycosylation sequence *per se*, potential *O*-glycosylation sites may be predicted based on flanking amino acids, which are usually rich in Ser, Thr, Pro, Val, Ala, and Gly, and on surface exposure of Ser/Thr residues *(2)*. This has led to the development of an internet-based tool (NetOglc) for the prediction of *O*-glycosylation sites (http://www.cbs.dtu.dk/services/netOglc/), which was used by Hansen et al. *(2)* to identify potential *O*-glycosylation sites in the HIV-1 gp120 glycoprotein. *O*-linked sugars are even more diverse than *N*-linked glycans, and may range in size from simple monosaccharides up to large, sulfated polysaccharide chains.

Glycosylation makes up approx 50% of the molecular weight (MW) of a number of medically important viral envelope glycoproteins. These include Env gp120 of human immunodeficiency virus (HIV), a lentivirus and causative agent of AIDS, the glycoprotein (GP) of Ebola virus, a filovirus which causes lethal hemorrhagic disease, and the attachment (G) glycoprotein of respiratory syncytial virus (RSV), a pneumovirus which represents the most important cause of severe lower respiratory tract infections in children worldwide (for a review, *see* **ref.** *3*). HIV-1 gp120 contains a median of 24 *N*-glycosylation sites *(4)*, whereas RSV G protein (Long strain) contains 8 *N*-glycosylation sites and up to 70 potential *O*-glycosylation acceptor sites, which are clustered within two domains (*see* **Fig. 1**). Such extensive *O*-glycosylation of viral envelope proteins is somewhat unusual, and the clustering of *O*-glycans is thought to confer mucin-like properties on RSV G protein by stabilizing an elongated, extended conformation *(5)*. Glycoproteins of other enveloped viruses that contain similar clusters of *O*-glycosylation include herpes simplex virus glycoprotein C and the Ebola virus GP protein.

1.1. Effect of Glycosylation on Viral Envelope Glycoproteins

The properties of viral glycoproteins are heavily influenced by the nature, position, and extent of glycosylation. This is not surprising if one considers that the average molecular weight of an *N*-linked glycan is more than 20 times that of an amino acid residue, and that glycans cover a greater volume of space, with

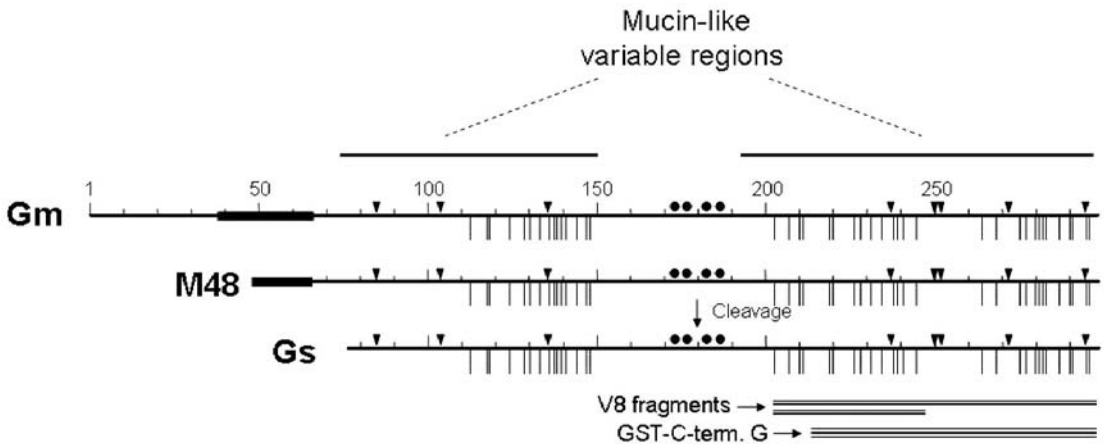

Fig. 1. Schematic diagram of the G protein primary structure. A straight line of 298 amino acids denotes the Gm polypeptide of the Long strain of human respiratory syncytial virus (RSV), in which the hydrophobic transmembrane region is indicated by a thick solid line (residues 38 to 66). The potential *N*-glycosylation sites (black triangle), the *O*-glycosylation sites (vertical line) predicted with the NetOGlyc software *(9,12)*, and the cluster of four cysteines (black circle) are also indicated. Formation of soluble G protein (Gs) occurs by translation initiation at Met48, and subsequent cleavage after residue 65 *(27)*. The locations of Gs fragments partially resistant to *Staphylococcus aureus* V8 protease and the C-terminal 85 amino acids fused to glutathione-*S*-transferase, both mentioned in this Chapter, are indicated below the protein diagrams.

the surface area of the $Man_3GlcNAc_2$ pentasaccharide covering the same surface area as an antibody footprint *(6)*. Moreover, glycans exhibit far greater structural complexity than amino acids. The position of viral envelope glycoproteins on the external surface of the virion means that they are involved in host cell binding and entry and are major targets for neutralizing antibodies produced by the host immune response. Hence differential glycosylation has the potential to affect not only the folding *(7)* and stability *(8)* of viral glycoproteins, but also binding to host cell components, including those of the innate immune response *(9,10)* and virus receptors *(11–13)*, which in turn may influence viral infectivity *(14)* and tropism. Finally, altered glycosylation can influence the immunogenicity of viral envelope proteins *(15)*, facilitate escape from the cellular immune response *(16)*, or affect antigenicity *(17)*. Thus, it is important to consider the effect of glycosylation changes when working with glycoproteins.

1.2. Analyzing Glycosylation Changes

Numerous factors exist which lead to differential glycosylation of glycoproteins. Viral envelope proteins expressed by different viral strains may be differentially glycosylated, as demonstrated for the Ebola virus by Lin et al. *(10)*. Variability in the sequence of viral envelope proteins among different isolates may directly lead to loss of oligosaccharide acceptor sites or may alter protein conformation, thereby preventing access of the cellular glycosylation machinery to potential glycosylation sites *(2)*. Secondly, changes in culture conditions or producer-cell type may result in the expression of a number of different glycoforms, because different cell types may differ in the repertoire of glycosyltransferases and glycosidases expressed, and in the time taken for proteins to transit the secretory pathway. For instance, the glycosylation of HIV gp120 varies significantly between CD4+ T cells and macrophages, the two cellular targets of HIV infection in vivo, with Env produced in macrophages containing more complex carbohydrates than Env produced in peripheral blood mononuclear cells (PBMCs) *(10,18)*. Finally, it may be desirable to deliberately alter glycosylation of viral glycoproteins through mutation of glycosylation acceptor sites, digestion of glycans with panels of glycosidases, or via treatment with various drugs that inhibit glycosylation (i.e., tunicamycin), in order to investigate the effect of viral envelope oligosaccharides on infectivity, immunogenicity, or antigenicity.

Although the glycan structures have previously been elucidated for a number of viruses by mass spectrometry or high-performance liquid chromatography (HPLC), these procedures often require relatively large quantities of purified starting material and give results that require extensive interpretation. Hence, these methods are not applicable to the analysis of a large number of

Table 1
Glycan Specificities of Plant Lectins Used in This Study

Lectin	Abbr.	Plant source	Binding specificity	Class of sugars recognized
Lens culinaris	LCA	Lentil	Internal α-linked man	*N*-linked
Arachis hipogaea	PNA	Peanut	Galβ1-3GalNAc	*O*-linked
Ricinus communis	RCA	Castor bean	Galactose	*O*-linked and *N*-linked
Sambucus nigra	SNA	Elderberry	NeuAcα2-6Gal	*O*-linked and *N*-linked

Table 2
Glycans Recognized by Anti-Carbohydrate Antibodies

Antibody	Epitope *	Sequence of sugar epitope	Reference
AM-3	sialyl Lewis x	NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc	*35*
FH4	Lewis x	Galβ1-4(Fucα1-3)GlcNAc	*36*
T174 57/27	Lewis a	Galβ1-3(Fucα1-4)GlcNAc	*37*,*38*
T218	Lewis b	Fucα1-3Galβ1-3(Fucα1-4)GlcNAc	*38*
77/180	Lewis y	Fucα1-3Galβ1-4(Fucα1-3)GlcNAc	*37*

* Note that the Lewis-based epitopes recognized by the anti-carbohydrate antibodies described may be present on either *N*-linked or *O*-linked sugars.

distinct viral envelope protein glycoforms. Thus, more rapid procedures that can be carried out using only partially purified material or even whole-cell extracts have been developed in order to facilitate analysis of changes in glycosylation of viral glycoproteins.

Lectins preferentially bind to specific sugar configurations, representing versatile reagents for glycan analysis, which may be achieved by employing panels of plant, algal, or animal lectins to probe for a variety of carbohydrate structures. The specificities of some commonly-used lectins that bind to O-linked or *N*-linked sugars are indicated in **Table 1**. Unlike mass spectrometry or HPLC, lectins can be used to analyze differential glycosylation without the need for first releasing the sugars from the glycoprotein. This is particularly advantageous when analyzing changes in *O*-linked glycosylation, because release of *O*-linked sugars can be problematic, in particular where *O*-glycans are clustered together *(19)*.

Carbohydrate-specific antibodies also represent analytical tools for the analysis of changes in glycosylation (**Table 2**). In particular, a number of glycan-specific Abs are available which recognize *O*-glycan (mucin) epitopes,

and which have been widely used in cancer research *(20)*. Anti-carbohydrate antibodies may display greater specificity than lectins, because some lectins recognize more than one sugar structure, and may be combined with fluorescence-based techniques to accurately detect specific sugar epitopes. Given the mucin-like structure of RSV G protein *(21)*, Abs reactive against mucins may be used to analyze changes in glycosylation.

1.3. Effect of Glycosylation Changes on the Interaction of Viral Envelope Glycoproteins With the Immune System

Carbohydrates are generally regarded as poorly immunogenic because (1) identical glycan epitopes are also found on host cell glycoproteins, thus are recognized as "self" by the immune system, (2) glycoproteins display considerable microheterogeneity, and (3) carbohydrates are extensive structures that may mask potential protein-based epitopes *(22)*. Indeed the glycosylation of HIV gp120 is thought to act as an evolving "glycan shield," whereby changes in *N*-glycosylation acceptor sites due to escape mutations in gp120 enable HIV to evade the host immune response by shielding underlying epitopes with variable glycosylation *(23,24)*. Similarly, the acquisition of *N*-glycosylation sequons in the influenza H3 HA1 glycoprotein is also thought to protect from the binding of neutralizing antibodies *(25)*. Recent studies of antibodies, produced in monkeys inoculated with SIV gp120 glycosylation mutants, indicate that *N*-linked glycosylation influences immunogenicity in addition to antigenicity *(15)*.

1.4. The RSV Attachment (G) Glycoprotein

The G protein of human respiratory syncytial virus is a type II glycoprotein of 295-315 amino acids (depending on the virus strain), with a signal/anchor hydrophobic domain between residues 38 and 66 (**Fig. 1**). The G molecule is synthesized as a 32 kDa polypeptide precursor, which is extensively modified by the addition of *N*- and *O*-linked oligosaccharides, and is also palmitylated, probably at a single cysteine residue located in the *N*-terminal cytoplasmic tail *(26)*. In addition to the membrane-bound form of G (Gm), a soluble form (Gs) lacking the signal/membrane-anchor region is also produced in RSV-infected cells by alternative initiation from a second in-frame AUG codon in the G open reading frame (Met48) *(27)*, followed by N-terminal proteolytic processing. While Gm forms oligomers (probably tetramers), Gs remains monomeric *(28)*. However, Gm and Gs cannot be distinguished by their glycosylation profiles or reactivity with monoclonal antibodies (MAbs). Thus, Gm is thought to represent the attachment protein of RSV virions, whereas Gs is likely to have some immunomodulatory role.

The C-terminal ectodomain of the G protein contains a central region (aa 164-176) and four cysteines (residues 173, 176, 182, and 186), which are con-

served in all human RSV isolates. Flanking this region, there are two protein segments that have a high level of sequence variation and high serine and threonine content, with an overall amino acid composition similar to that of the mucins secreted by epithelial cells.

RSV has been classified into two antigenic groups (A and B), based mainly on the reactivity of MAbs with the G protein, the most variable gene product among RSV isolates. By testing the reactivity of MAbs with a large panel of viral strains, three types of epitopes have been identified in the G protein: (1) *conserved* epitopes that are present in all human RSV isolates, (2) *group-specific* epitopes shared by all viruses of the same antigenic group, and (3) *strain-specific* or *variable epitopes* that are present in certain isolates of the same antigenic group ***(29)***. The antigenicity of RSV G glycoprotein is influenced by variation in *O*-glycosylation ***(30)***, in particular within the C-terminal domain, where epitopes recognized by strain-specific antibodies are located. In contrast, conserved or group-specific epitopes are located within the central, conserved domain (**Fig. 1**), which lacks glycosylation acceptor sites ***(12)***. Moreover, there is evidence that positive selection of certain *O*-glycosylation sites operates in response to selective immune pressure ***(31)***. Hence, glycosylation changes in the G glycoprotein may enable the virus to "hide" from the host immune response.

First, we describe methods for the analysis of *glycosylation changes* in the G glycoprotein that result from the production of RSV in distinct cell lines (*see* **Subheadings 3.3.** and **3.4.**). The methods outlined are based on Western blotting using specific MAbs, or on reactivity of G protein glycoforms with certain lectins or carbohydrate-specific antibodies. Second, the influence of carbohydrates on G protein *antigenicity* (*see* **Subheadings 3.5.** and **3.6.**) is analyzed by the reactivity of antibodies with segments of Gs. The procedures described are carried out on either glycosylated Gs fragments, resulting from *Staphylococcus aureus V8 protease* digestion, or on Gs that is deprived of carbohydrates, achieved by bacterial expression of a glutathione-*S*-transferase (GST)-fusion construct of the Gs C terminal domain. Although a focus is placed on the RSV G protein, these methods may be easily adapted to other highly glycosylated viral glycoproteins.

2. Materials

1. Extraction buffer: 0.1 mL/60 mm dish of 10 m*M* Tris-HCl, pH 7.5, 140 m*M* NaCl, 5 m*M* EDTA, 1% Triton X-100, and 1% sodium deoycholate.
2. 3X sodium dodecyl sulfate (SDS) sample buffer: 240 m*M* Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, and 0.03% bromophenol blue.
3. 1X SDS sample buffer: 80 m*M* Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue.

4. High-salt buffer: 1.5 *M* glycine and 3 *M* NaCl, adjust the pH to 8.9 with NaOH..
5. Transfer buffer: 25 m*M* Tris, 192 m*M* glycine, 20% methanol, and 0.1% SDS.
6. Block solution: 0.2% I-Block (Tropix), 0.1% Tween-20 in phosphate-buffered saline (PBS).
7. PNS: PBS containing 0.5% NP40.
8. Pre-equilibration buffer: 10 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, and 1 m*M* EDTA.
9. Bacterial wash buffer: 9.1 m*M* HEPES, 55 m*M* $MgCl_2$, 15 m*M* $CaCl_2$, and 250 m*M* KCl, adjusted to pH 6.7.
10. Bacterial lysis buffer: 10 *mM* Na_2HPO_4, 1.8 m*M* KH_2PO_4, pH 7.4, 140 m*M* NaCl, 2.7 m*M* KCl.
11. Dialysis buffer: 0.1 *M* sodium bicarbonate pH 8.3, 0.5 *M* NaCl.
12. Resin wash buffer 1: 0.1 *M* sodium acetate pH 4.0, 0.5 *M* NaCl.
13. Resin wash buffer 2: 0.1 *M* Tris-HCl pH 8.0, 0.5 *M* NaCl.

3. Methods

3.1. Preparation of Extracts of Human RSV-Infected Cells (see **Note 1**)

1. Seed the cells in Petri dishes at a density of 10^5 cells/cm^2 in Dulbecco's medium supplemented with 5–10% fetal calf serum (FCS), 2 m*M* glutamine, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and place in an incubator set at 37ºC with an atmosphere of 5% CO_2 and 95% humidity.
2. Twenty-four hours later, remove the medium and wash the monolayer twice with the same medium supplemented with 2.5% FCS. Add the virus inoculum to the cell monolayer (MOI 0.1–1.0 plaque-forming units [pfu]/cell) in a small volume of medium with 2.5% FCS (e.g., 0.25 mL for a 60-mm dish). Return the cells to the incubator for 1 h and then add new medium with 2.5% FCS (e.g., 5 mL/60 mm dish), and incubate the cells for 2–3 d. Syncytia should start to develop after 24 h and maximal effect should be observed after 2–3 d.
3. Scrape off the cells into the medium with a rubber policeman. Transfer the cells and medium to universal tubes and spin down the cells at 5000*g* for 5 min. Wash the cell pellet with PBS and finally resuspend the pellet in extraction buffer (0.1 mL/60 mm dish)
4. Vortex the cells thoroughly and spin down cell debris in a minifuge at top speed for 10 min. Collect the supernatant and keep it at –20ºC (or –80ºC) until used.

3.2. Preparation of Immunoaffinity Columns for Purification of the Gs Glycoprotein (see **Note 2**)

1. Pack 5 mL of protein A–Sepharose CL4B (Amersham) in a syringe or a small column, wash with PBS, and equilibrate with at least 5 volumes of high-salt buffer.
2. Load the antibody sample (diluted 1:1 with high-salt buffer) onto the column (*see* **Note 2**). Collect the flow-through and re-load onto the column.
3. Wash the column with high salt buffer until the $OD_{280\ nm}$ values of the flow through are lower than 0.1.

4. Elute the antibody with 0.1 *M* sodium citrate, pH 3.0. Collect 1-mL fractions and follow the $OD_{280\,nm}$ readings. The column can be regenerated by equilibration in PBS.
5. Pool the eluted fractions with the highest values of $OD_{280\,nm}$ and dialyze against a large volume of dialysis buffer.
6. Weigh 1 g of CNBr-activated Sepharose 4B (Amersham) and wash, following the manufacturer's instructions (1 g of Sepharose is enough for a column of 3.5 mL bed volume).
7. Mix the purified antibody with the washed resin and incubate overnight at 4ºC in a rotating wheel (10 mg of antibody/g of resin).
8. Spin down the resin and measure the $OD_{280\,nm}$ value of the supernatant (it should be less than 0.1).
9. Add 20 mL of 0.1 *M* Tris-HCl, pH 8.0 per gram of resin in order to block the activated groups that did not react with the antibody. Incubate for 3 h at room temperature in a rotating wheel.
10. Wash the resin with three alternating cycles of 10 mL of resin wash buffer 1 and resin wash buffer 2.
11. Pack the resin in a small column or syringe and equilibrate with 10 volumes of PBS.

3.3 Detection of Cell-Specific Changes in Glycosylation of the G Glycoprotein by Western Blotting With Anti-G Specific Antibodies (see **Note 3**)

1. Prepare an SDS-polyacrylamide gel electrophoresis (PAGE) gel with several lanes.
2. Dilute cell extracts (made as indicated under **Subheading 2.1.**) in 3X sample buffer (e.g., 20 µL of cell extract plus 10 µL of 3X SDS sample buffer). Boil the samples for 3 min.
3. Load the samples into the lanes, placing molecular markers in the outer lanes. It is convenient to use colored markers.
4. Run the electrophoresis at constant voltage (100–200 V) until the front is near the bottom of the gel.
5. Activate an immobilon membrane (Millipore) by soaking in methanol for 30 s, followed by three washes with distilled water. Then soak the membrane in transfer buffer for a few seconds (*see* **Note 4**).
6. Place the gel on top of the immobilon membrane and sandwich the gel and membrane between several pieces of filter paper (Whatmann 3MM Chr) soaked in transfer buffer.
7. Mount a submarine blotting cell with the gel in a vertical position in the following order: cathode/filters/gel/membrane/filters/anode. Fill the cell with sample buffer to cover the gel. Transfer for 1 h at 0.8 mA/cm^2 of gel.
8. Dismount the blotting cell. Place the membrane in a plastic tray and saturate the protein binding sites with block solution for 2–16 h at 4ºC.
9. Incubate the membrane with anti-G specific MAbs for 2 h and develop the membrane with anti-mouse peroxidase and ECL, according to the manufacturer's

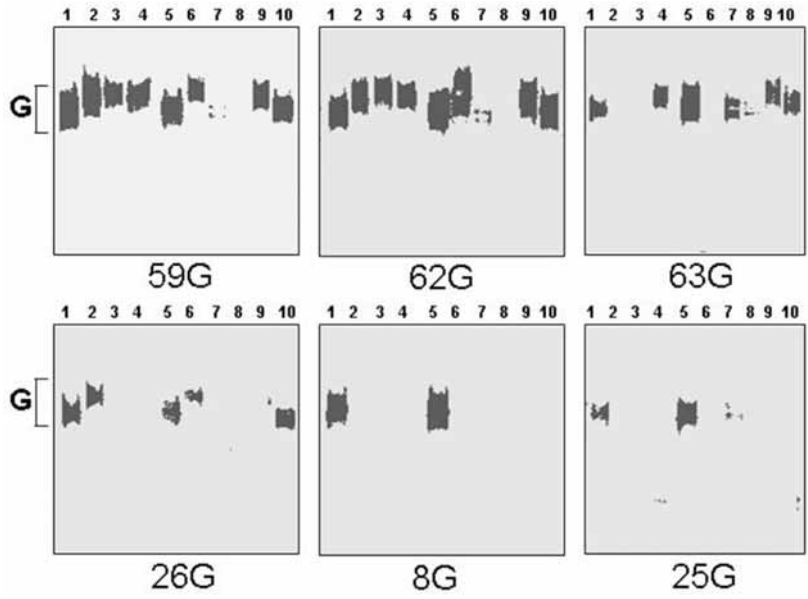

Fig. 2. Western blot of respiratory syncytial virus (RSV)-infected cell extracts, visualized by monoclonal antibodies specific for human HRSV G protein. The following cell lines were infected with the Long strain of RSV: HEp-2 (lanes 1 and 5), HT29 (lane 2), M6 (lane 3), Caco-2 (lane 4), M3 (lane 6), SKLC.1 (lane 7), SKLC.13 (lane 8), KNS.62 (lane 9), and L-132 (lane 10). Extracts were prepared and analyzed by Western blots using the antibodies indicated below each panel. Note the altered mobility of the G protein band, depending on the infected cell line. These cell-type specific glycosylations affect not only the electrophoretic mobility, but also reactivity with certain monoclonal antibodies.

instructions (Amersham Biosciences). Each incubation step should be followed by two to three washes with PBS-0.1% Tween 20, with the exception of the last wash before adding the ECL substrate, which is done with PBS (*see* **Fig. 2** for representative results).

3.4. Immunoprecipitation of G Glycoprotein With Lectins or Anti-Carbohydrate Antibodies

1. Make a 1:10 dilution of cell extracts (prepared as indicated under **Subheading 2.1.**) with PNS and incubate with biotinylated lectins (Sigma) for 2 h at 4ºC in a rotating wheel.
2. Meanwhile, wash the streptavidin-agarose beads (*see* **Note 5**) three times with PNS by centrifugation for 2 min in a minifuge (top speed).
3. Add the extract-lectin mix to the pellet of streptavidin-agarose beads. Resuspend by vortexing and incubate for another hour at 4ºC in the rotating wheel.

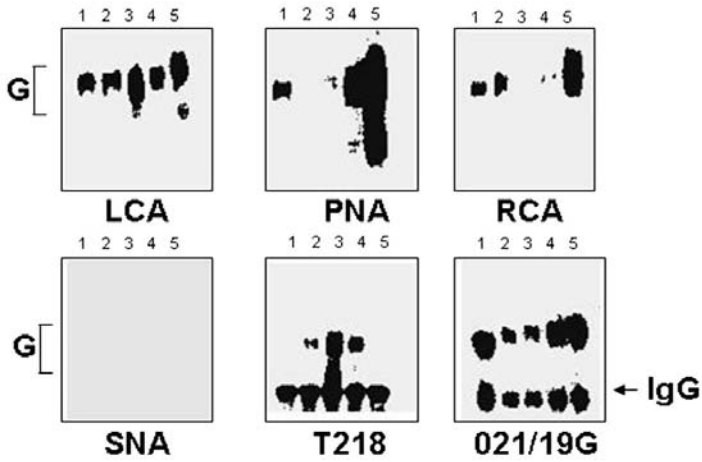

Fig. 3. Western blot of the G glycoprotein immunoprecipitated from respiratory syncytial virus (RSV)-infected cell extracts by sugar-specific reagents. Lectins LCA, PNA, RCA, and SNA and the antibody T218 (*see* **Tables 1** and **2** for specificities) were used to immunoprecipitate RSV Long-infected extracts of the following cell lines: HEp-2 (lane 1), HT29 (lane 2), M6 (lane 3), M3 (lane 4), and Caco-2 (lane 5). G protein present in the immunoprecipitates was visualized by Western blot with antibody 021/19G. In order to compare G protein expression between different cell lines, the extracts were first immunoprecipitated with the G-specific antibody 021/19G, and Western blots developed with the same antibody (bottom right panel). Note the presence of a band corresponding to the heavy chain of the antibodies T218 and 021/1G (indicated by an arrow), which reacted with the anti-mouse Ig antiserum used for Western blotting.

4. Spin down the beads in the minifuge and wash three times with a large excess of PNS, followed by a final wash with PBS.
5. Add 20–50 μL of 1X SDS-sample buffer, boil the samples for 2 min to dissociate the proteins bound to the beads, and spin down the beads for 5 min in minifuge.
6. Analyze the immunoprecipitated proteins by Western blot with an anti-G specific antibody, as indicated in the previous section (*see* **Note 6**) (**Fig. 3**).

3.5. Purification of Soluble G Protein (Gs) and Generation of Protease Resistant Fragments

3.5.1. Purification of Gs by Immunoaffinity Chromatography

1. Collect the supernatant of HEp-2 cells infected with human RSV (48-72 h post-infection) (*see* **Note 7**) and clarify by centrifugation at 15,000*g* for 15 min.
2. Concentrate the supernatant by filtration through polyethensulfone membranes of 50 kDa exclusion pore size (Vivaflow; Sartorius), and buffer exchange to PBS by several cycles of dilution and concentration.

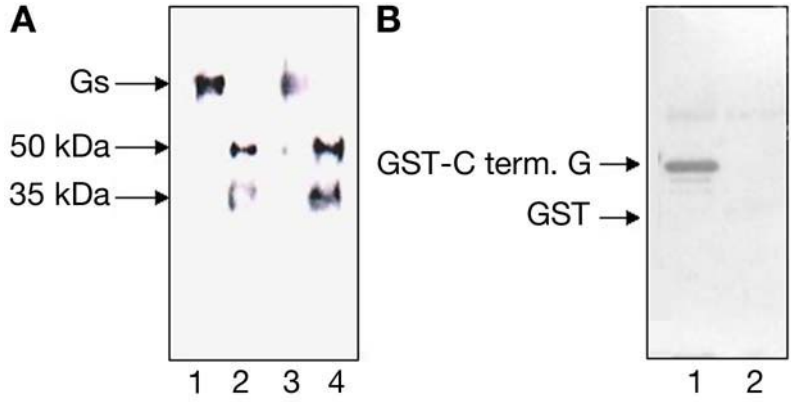

Fig. 4. Western blot of purified soluble G protein (Gs), protease V8-resistant fragments of Gs and chimeric glutathione-*S*-transferase (GST)-C terminal G protein. **(A)** Purified Gs from the Long strain of human respiratory syncytial virus (RSV) was visualized by Western blot with a pool of anti-GMAbs (lanes 1 and 2), or with a polyclonal anti-RSV antiserum (lanes 3 and 4), either before (lanes 1 and 3) or after digestion (lanes 2 and 4) with V8 protease. **(B)** Western blot of purified GST protein fused to the C-terminalt 85 amino acids of the G protein (Long strain) (lane 1), or purified GST (lane 2), visualized by human serum.

3. Load the concentrated sample onto a Sepharose column conjugated to anti-G MAbs, pre-equilibrated with PBS.
4. Wash the column with 20 volumes of PBS and elute the material retained by the column with 10 volumes of 0.1 *M* glycine-HCl, pH 2.5, collecting fractions of 0.5–1.0 mL. Neutralize the fractions with saturated Tris.
5. The presence of Gs in the eluted fractions is revealed by Western blotting, as indicated under **Subheading 3.1.** Pool the fractions with the highest concentration of Gs and concentrate if necessary with Vivaspin (50 kDa exclusion pore size) (**Fig. 4A**).

3.5.2. Digestion of Gs With S. aureus *V8 Protease* (see **Note 8**)

1. Add increasing amounts of *S. aureus* V8 protease to a fixed amount of purified Gs pre-equilibration buffer .
2. Incubate in a water bath at 37ºC for 1 h and stop the reaction with 3X sample buffer.
3. The extent of digestion can be visualized by Western blot (**Fig. 4A**).

3.6 Expression and Purification of the C-Terminal 85 Amino Scids of the G Protein Fused to GST (see **Note 9**)

1. Grow *Escherichia coli* that harbor the pGEX plasmid, until the $OD_{600\ nm} = 1.0$. Induce synthesis of the GST-fusion protein by adding IPTG 1 mM, then grow the bacteria for an additional 3–4 h.
2. Harvest the bacteria by centrifugation (3000*g*, 30 min at 4ºC) and wash the pellet with bacterial wash buffer.

3. Resuspend the bacterial pellet in bacterial lysis buffer containing 1 mg/mL of lysozyme.
4. Lyse the cells by three freeze/thaw cycles, and spin down the lysates at 70,000*g* for 25 min at 4ºC. If necessary, spin down the lysate at 300,000*g* for 60 min at 4ºC, in order to remove insoluble fragments.
5. Load the supernatant onto a glutathione–Sepharose 4B column (Amersham Biosciences), pre-equilibrated with PBS, and wash the column with the same buffer until the $OD_{280\ nm}$ reading reaches background levels.
6. Elute the bound material with 10 m*M* Tris-HCl, pH 8.0 and 10 m*M* reduced glutathione, and follow the $OD_{280\ nm}$ readings. Pool fractions with the highest absorbance. The presence of fusion protein, consisting of the C-terminal 85 amino acids of Gs fused to GST, can be visualized by Western blot (Fig. 4B) (*see* **Note 10**).

4. Notes

1. Human RSV can productively infect a large range of mammalian cell lines. García-Beato et al. ***(32)*** described a series of human cell lines of epithelial or fibroblast morphology that could be infected with this virus. Hep-2 (a human cell line derived from an epidermoid carcinoma of the larynx), is commonly used as a cell substrate for the propagation of human RSV in tissue culture. The Long strain of human RSV (antigenic group A) is widely used as the prototype RSV strain; however other strains, including A2 (group A) or CH18537 (group B), are commonly used in many laboratories
2. The source of monoclonal antibodies may be ascitic fluids or hybridoma culture supernatants. It is advisable to concentrate the supernatant of hybridoma cultures by filtration with membranes of 50 kDa exclusion pore size (Vivaflow, Sartorius) in order to reduce the volume and the time taken to load the antibody onto the protein A-Sepharose column. The column binding capacity is specified by the manufacturer.
3. G protein glycosylation depends on the specific set of glycosyltransferases and glycosidases that are present in a given cell. This cell-specific glycosylation leads to changes in the electrophorectic mobility of the G protein, and in its reactivity with anti-G specific MAbs, as visualized by Western blot (**Fig. 2**).
4. It is essential that the transfer buffer contains 0.1% SDS, in order to ensure a uniform electrotransfer of the fully glycosylated G protein as well as the glysolylation intermediates and the unglycosylated precursor.
5. If anti-carbohydrate antibodies are used instead of lectins for the immunoprecipitation of the G proteins, replace streptavidin–agarose by protein A–agarose beads.
6. The reactivity of MAbs that bind to the central region of the G protein ectodomain (deprived of carbohydrates) is not influenced by changes in glycosylation. Thus, these antibodies can be used to visualize the G protein immunoprecipitated from cell extracts by lectins or by anti-carbohydrate antibodies (**Fig. 3**).
7. Gs represents about 10–15% of the G protein produced in RSV-infected cells. Gs is secreted into the culture supernatant, which can therefore be used as the source

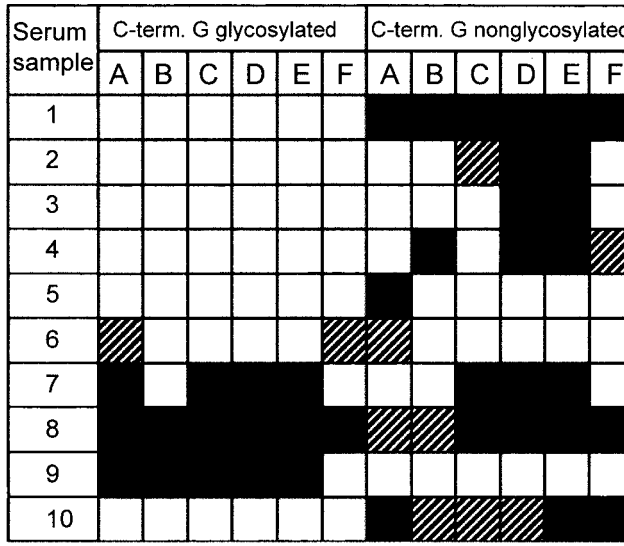

Fig. 5. Reactivity of human sera with the C-terminal segment of the G protein from six different human respiratory syncytial virus isolates. The reactivity of 10 human sera with the G protein fragment resistant to V8 protease (50 kDa) (C-terminal G glycosylated) or with the last 85 aminoacids of G fused to glutathione-*S*-transferase (C-terminal nonglycosylated) was evaluated by Western blot. Black square, strong reactivity; gray square, weak reactivity; white square, no reactivity. The G segments were derived from the following viral strains: Mon/3/88 **(A)**, Mad/2/88 **(B)**, Mad/3/89 **(C)**, Mad/5/92 **(D)**, Mad/8/92 **(E)**, and Long **(F)** *(34)*.

material for the purification process. However, recombinant vaccinia viruses have been described that secrete much larger amounts of Gs into the culture supernatant of infected cells *(27,28)*. The use of these vaccinia viruses increases the yield of purified Gs severalfold.

8. The amount of V8 protease used for digestion of Gs should be calculated separately for each protein batch. Other proteases (e.g., papain or bromelain) generate Gs fragments similar to those generated by V8 (**Fig. 4A**).
9. Cloning of protein segments fused to GST is best achieved using the pGEX vector system (Amersham Biosciences). The reader is referred to the manufacturer's instructions for the cloning strategy. A detailed description of the production of chimeric proteins with the C terminal 85 amino acids of the G protein fused to GST can be found in Cane et al. *(33)*.
10. The chimeric GST-C terminal G protein contains most of the amino acid sequence included in the large fragment of the G protein that is partially resistant to V8 protease (*see* **Fig. 1**); however, the GST fusion protein is not glycosylated since it is expressed in bacteria. **Figure 5** shows the reactivity of 10 human sera *(1–10)*

with the C-terminal fragment of Gs, generated by V8 protease digestion (glycosylated), or expressed as a GST fusion protein (nonglycosylated), from six different human RSV isolates. Note that each serum reacted differently with the virus panel, and that this reactivity was influenced by the presence of carbohydrates in the G protein segment. In general, sera were less reactive with the glycosylated C-terminal segment of Gs than with the unglycosylated counterpart.

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9

Expression, Glycosylation, and Modification of the Spike (S) Glycoprotein of SARS CoV

Shuo Shen, Timothy H. P. Tan, and Yee-Joo Tan

Summary

The spike (S) glycoprotein of coronaviruses is known to be essential in the binding of the virus to the host cell at the advent of the infection process. To study the maturation pathway of the S glycoprotein of the severe acute respiratory syndrome (SARS)-coronavirus (CoV) within the host cell, a T7/vaccinia virus-based expression system coupled to immunoprecipitation with anti-S antibodies was used to test and analyze different forms of the S glycoprotein. The state of maturity of the S glycoprotein can be deduced from its sensitivity to hydrolysis by endoglycosidase H (EndoH) or *N*-glycosidase F (N-Gly F). A fully matured S glycoprotein will be modified with complex oligosaccharides which makes it resistant to cleavage by EndoH but not by N-Gly F. By exploiting this characteristic, it is then possible to determine which forms of the immunoprecipitated S protein are properly processed by the host cell. With this system, many different constructs of the S glycoprotein can be analyzed in parallel thus providing another method by which to study the functional domains of S involved in membrane fusion event that occurs during viral infection.

Key Words: Severe acute respiratory syndrome (SARS); coronavirus; spike glycoprotein; maturation; membrane fusion; endoglycosidase H.

1. Introduction

Many enveloped viruses encode a membrane fusion glycoprotein for the entry of cells through receptor binding and viral-cell membrane fusion, including the newly emerged severe acute respiratory syndrome (SARS)-coronavirus (CoV) ***(1,2)***. Their *N*-linked glycans are needed for proper folding to reach the native conformation and to pass ER quality control ***(3)***. The spike (S) glycoprotein of coronaviruses is responsible for receptor binding and membrane fusion. It shares similarity with class I viral fusion proteins and is a typical type I integral mem-

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brane protein. The N-terminal S1 contains the receptor-binding site whilst the C-terminal S2 is a fusion subunit and is anchored to the viral envelope through a transmembrane domain. The S protein of SARS CoV is co-translationally *N*-glycosylated in the ER and trimerized if folded properly. One of the essential steps in the *N*-linked glycosylation is the transfer of a preformed, 14-core-unit-oligosacharide to a specific Asn residue in the sequence Asn-X-Ser/Thr where X is any residue except Pro, Asp, and Glu. The oligosaccharide chain is trimmed down in the ER and the *cis*-Golgi. Different external sugars are then added to the trimmed chain in the medial- and *trans*-Golgi. Glycoprotein with high mannose oligosaccharides in the ER and *cis*-Golgi remain sensitive to endoglycosidase H (EndoH) treatment. They become EndoH resistant after being processed by the medial- and *trans*-Golgi resident enzymes to glycoproteins with complex oligosaccharides. Only the mature S glycoprotein is readily assembled into virions and transported to the cell surface, where it partakes in cell–cell membrane fusion ***(4)*** and facilitates the rapid spread of virus infection. Therefore, the acquisition of the EndoH resistance and the cell surface expression is an indication that the S glycoprotein has been properly processed and transported through the constitutive secretory pathway. The pulse-chase labeling and deglycosylation techniques are widely used to analyze the glycosylation and modification process of viral glycoproteins. Here, we describe methods for characterization of the spike glycoprotein of SARS CoV.

2. Materials

1. The monkey kidney cell lines Cos7 and Vero E6 (American Type Culture Collection [ATCC], Manassas, VA).
2. Dulbecco's modified Eagle's medium (DMEM) and methionine/cysteine free DMEM (ICN Biochemicals, Ohio).
3. Streptomycin, penicillin, fetal bovine serum (HyClone, UT).
4. SARS-CoV strain Sin2774 (GenBank accession no. AY283798) ***(5)***.
5. Recombinant vaccinia virus vTF7-3 ***(6)***.
6. [^{35}S]-methionine/cysteine (Expre ^{35}S^{35}S-Protein Labeling Mix, 7.0 mCi/632 μL, 1175 Ci/mmol) (NEN).
7. Effectene Transfection Reagents (Qiagen).
8. Protein A–sepharose beads (Roche Diagnostics)
9. Radio-immunoprecipitation assay (RIPA) buffer: 150 m*M* NaCl, 50 m*M* Tris-HCl (pH 7.5), 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 m*M* phenylmethylsulfonylfluoride (PMSF). One tablet of Complete™ Protease Inhibitor Cocktail Tablet (Roche) is added to every 50 mL of RIPA buffer.
10. Lysis buffer: 50 m*M* Tris-HCl (pH 7.6), 1% NP-40.
11. 1X SDS gel loading buffer: 50 m*M* Tris-HCl, pH 6.8, 2% SDS, 100 m*M* dithiothreitol (DTT), 10% glycerol, and 0.1% bromophenol blue.
12. Rabbit anti-S antibodies ***(7)***.

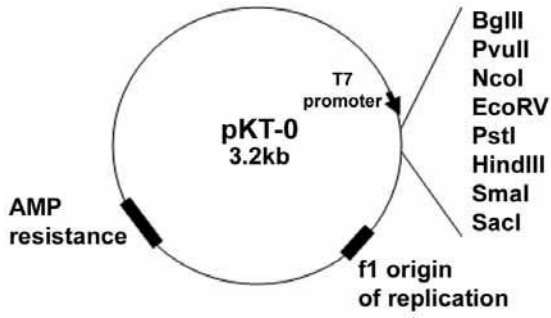

Fig. 1. Restriction map and multiple cloning site of pKT-0. pKT-0 is a mammalian expression vector that allows a gene of interest to be highly expressed if it is inserted into the multiple cloning site, as shown on the map.

13. SDS-polyacrylamide gel electrophoresis (PAGE) reagents and equipment (Bio-Rad).
14. Gel fixing solution: 45% methanol and 10% acetic acid in distilled water.
15. Amplify solution (Amersham Bioscience, UK).
16. Endoglycosidase H (Roche Diagnostics).
17. *N*-glycosidase F (Roche Diagnostics).

3. Methods

The methods described below include a T7/vaccinia virus expression system and construction of the plasmid **(Subheading 3.1.)**, the expression of the viral glycoprotein in mammalian cells and immunoprecipitation of radiolabeled viral protein **(Subheading 3.2.)**, and treatment of viral glycoprotein with EndoH and *N*-glycosidase F (*N*-Gly F) **(Subheading 3.3.)**.

3.1. T7/Vaccinia Virus Expression System and Recombinant pKT-S Plasmid Construction

3.1.1. A T7/Vaccinia Virus Expression System

The pKT-0 plasmid was developed by Liu ***(6)***. Expression of a target gene, inserted into the multiple cloning site region, is under the control of a T7 promoter (**Fig. 1**). A vaccinia/T7 recombinant virus vTF7-3 expressing bacteriophage T7 RNA polymerase was used to infect cells and to drive the expression of a target gene controlled by a T7 promoter.

3.1.2. Amplification of the S Gene by Reverse-Transcription PCR and Construction of the pKT-S Plasmid

1. Viral RNA was extracted from the SARS-CoV strain 2774-infected Vero E6 cells in a bio-safety level 3 laboratory using the RNeasy Mini Kit (Qiagen).

2. Reverse-transcription (RT)-PCR was performed using the Expand Reverse Transcription and High Fidelity PCR Kits (Roche) with specific primers (*see* **Note 1**).
3. The plasmid pKT-0 was digested with BamHI/EcoRV, and was treated with 1 U of alkaline phosphatase per 3 μg vector in a volume of 100 μL at 37°C for 30 min (*see* **Note 2**)
4. The PCR product was digested with BamHI/StuI and was ligated into BamHI/EcoRV-cut pKT-0. Ligation was performed at 16°C overnight with a molar ratio of vector to insert of 1:3 to 1:10. This results in plasmid pKT-S, where expression of the S gene insert is controlled by the T7 promoter.
5. The ligation product was transformed into competent DH10B cells.
6. Insert-positive cDNA clones were obtained by screening with restriction analysis.
7. The integrity of the S gene insert was confirmed by sequencing analysis.

3.2. Expression Analysis of the Viral Glycoprotein in Mammalian Cells and Immunoprecipitation of Radiolabeled Viral Proteins

The next step involves a pulse-chase labeling experiment to investigate the maturation of SARS-CoV S protein. This includes infection of Cos7 cells with vaccinia virus vTF7-3 followed by transfection with pKT-S **(Subheading 3.2.1.)**, radiolabeling of cells with [^{35}S]-methionine/cysteine and chasing with cold methionine/cysteine for 0.5, 1, 2, 4, and 6 h **(Subheading 3.2.2.)**, and immunoprecipitation of the S protein with rabbit anti-S antibodies followed by separation of protein in SDS-PAGE and visualization by autoradiography **(Subheading 3.2.3.)**.

3.2.1. Infection of Cells With Vaccinia Viruses Followed by Transfection With Plasmid pKT-S

1. Infect Cos7 cells (grown to 40-80% confluency in 60 mm Petri dishes) with vTF7-3 vaccinia viruses at a multiplicity of infection (MOI) of 1 plaque-forming units (pfu) per cell in a total volume of 200 μL per dish.
2. Mix 2 μg of pKT-S plasmid with 280 μL of DNA condensation buffer and 8 μL of Enhancer (Effectene transfection reagents, Qiagen) in a 2-mL tube for each dish. Vortex for 1 s and incubate at room temperature for 5 min.
3. Add 20 μL of Effectene Transfection Reagent to the DNA-Enhancer mixture. Vortex for 10 s and incubate at room temperature for 10 min.
4. Gently aspirate viral inoculate supernatant from dishes 1 h postinfection (refer to **step 1**). Add 1 mL of DMEM medium to cells.
5. Add 0.7 mL of DMEM medium to the tube containing the transfection complex. Mix by pipetting twice and add the mixture dropwise onto the cells in the dish. Gently swirl the dishes.
6. Incubate cells at 37°C in 5% CO_2 incubator.
7. For control experiments, infect cells with vTF7-3 vaccinia viruses and mock-transfect cells with empty vector, pKT-0.

3.2.2. Metabolic Labeling of Viral Protein With [^{35}S]-Methionine/Cysteine Pulse-Chase Methods

1. Gently aspirate the transfection mixture (from **Subheading 3.2.1.**) from each dish 3 h after transfection. Wash the cells once with PBS at room temperature.
2. Incubate cells in 1 mL of methionine/cysteine-free DMEM for 30 min to deplete cellular stores of methionine and cysteine.
3. Thaw [^{35}S]-methionine/cysteine for 30 min before use behind protection shield in an area designated for radioactive experiments.
4. Replace the depleting medium in each dish with 1 mL of fresh methionine/cysteine-free DMEM containing 2 μL of [^{35}S]-methionine/cysteine (*see* **Note 3**)
5. Incubate cells in the dishes for 15 to 30 min for pulse labeling.
6. Replace the labeling medium with 1 mL depleting medium complemented with cold methionine and cysteine (final concentration: 5 m*M* each) to stop pulse labeling and to begin chasing the viral protein.
7. At each time point, remove the medium and wash cells once with PBS (*see* **Note 4**). Add 1 mL of RIPA buffer to cells in each dish.
8. Leave the dish on ice for 10 min and swirl occasionally.
9. Cell lysate may be used immediately in immunoprecipitation or frozen and stored at –80°C.

3.2.3. Immunoprecipitation and SDS-PAGE Analysis of the Viral Protein

1. Transfer the fresh or thawed cell lysates into 2-mL Eppendorf tubes. Centrifuge the tubes in a 4°C room at 13,000 rpm for 10 min.
2. Carefully transfer 0.3 mL of the supernatant of the cell lysate to each fresh tube (*see* **Note 5**).
3. Add 5 μL of rabbit anti-S serum to each tube.
4. Place the tubes in a plastic container and shake on a rotator in a 4°C room for 1 h.
5. Pipet 30 μL of 50% suspension of Protein A–sepharose beads into each tube using a blunted yellow tip to ensure an equal transferred quantity of beads each time.
6. Incubate the tubes on a shaker in a 4°C room for 2 h or overnight.
7. Centrifuge the tubes for 1 min and remove the RIPA buffer by aspiration with a 26.5-gauge needle attached to a vacuum line (*see* **Note 6**).
8. Add 1 mL of RIPA buffer to wash the beads in each tube. Centrifuge the tubes for 1 min and remove the wash buffer as previously described in **step 7**. Repeat this wash step four times.
9. After the final wash, add 15 μL of 1X SDS gel loading buffer to the beads in each tube and vortex briefly.
10. Heat the samples at 100°C for 5 min and then centrifuge the tubes for 2 min at 13,000 rpm at room temperature.
11. Load the sample onto an 8% SDS-PAGE gel to resolve the proteins.
12. Fix the gel in gel fixing solution for 30 min.
13. Soak the fixed gel in Amplify solution for 15 min.

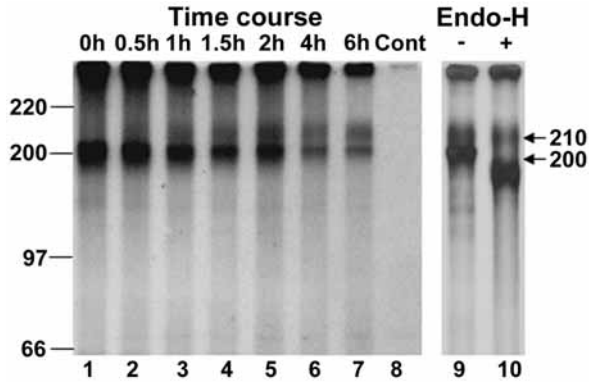

Fig. 2. Time-course of S protein maturation. Cos7 cells transfected with pKT-S were radiolabeled and chased for 0 h, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, and 6 h respectively (lanes 1–7). Cos7 cells transfected with plasmid without insert are harvested at 6 h as negative control (lane 8). All the cell lysates were immunoprecipitated with rabbit α-SΔ10 antibodies and then separated on SDS-PAGE gels. In a separate experiment, the immunoprecipitated proteins (6 h posttransfection) were either treated (+) with EndoH (lane 10) or left untreated (–) as a control (lane 9). The S-specific bands and their molecular masses (in kDa) were indicated on the right. High-Range Rainbow Molecular Weight Markers (Amersham) were used to assess protein mass, as indicated on the left.

14. Dry the gel at 80°C for 1 h on a gel dryer.
15. Visualize the viral protein by autoradiography (**Fig. 2**).

Over the time-course of the experiment, the maturation of the 200-kDa form of S to the 210-kDa form could be observed as there was a gradual increase in the 210-kDa band accompanied by a reciprocal decrease in the 200-kDa band (**Fig. 2**). The 210-kDa band is the mature glycosylated S protein as it was resistant to EndoH while the 200-kDa band is the immature glycosylated S protein as a result of its sensitivity to both *N*-Gly F and EndoH (*see* **Subheading 3.3.**).

3.3. Treatment of Viral Glycoprotein With EndoH or N-Gly F

EndoH cleaves high-mannose and hybrid structures on *N*-linked oligosaccharides of glycoproteins but does not act on more complex sugars. *N*-Gly F hydrolyzes all types of *N*-glycan chains from glycoproteins unless they carry α-1-3 linked core fucose residues which are normally present only in insect and plant glycoproteins.

3.3.1. EndoH Treatment of the Immunoprecipitated Viral Glycoprotein

1. Infect the cells in 60-mm dishes with vTF7-3 vaccinia viruses and transfect the cells with plasmid pKT-S as described under **Subheading 3.2.1.**
2. Radiolabel the cells as described under **Subheading 3.2.2.**
3. Immunoprecipitate the viral protein in the cell lysates as described under **Subheading 3.2.3., steps 1–8**.
4. Add 20 μL of denaturing buffer (Roche Diagnostics) to the beads after the fourth wash with RIPA buffer.
5. Heat the samples in the tubes at 100°C for 5 min to release the viral proteins from the antibody-antigen complex on protein A–sepharose beads.
6. Equally divide 20 μL of the viral protein in denaturing buffer into two fresh tubes.
7. Add 10 μL of digestion buffer (Roche Diagnostics) containing 1 U of EndoH to one tube and add 10 μL of digestion buffer without EndoH to the other tube.
8. Incubate both aliquots at 37°C for 3 h.
9. Add 5 μL of 5X SDS gel loading buffer to each tube and heat the samples at 100°C for 5 min.
10. Separate samples on an 8% SDS-PAGE mini-gel and visualize the viral protein by autoradiography (**Fig. 2**).

3.3.2. EndoH Treatment of the Viral Glycoprotein in Nonlabeled Cell Lysates

1. Infect the cells in 60-mm dishes with vTF7-3 vaccinia viruses and transfect the cells with plasmid pKT-S as described inder **Subheading 3.2.1.**
2. Incubate the cells at 37°C in a 5 % CO_2 incubator for 12 to 16 h.
3. Wash the cells twice with ice-cold PBS and scrape the cells into 1 mL of cold PBS with a cell scraper.
4. Transfer cell suspension into a 1-mL tube and spin the cells at 4000 rpm for 5 min in a 4°C room.
5. Discard the supernatant and resuspend the cell pellet in 0.2 mL of lysis buffer and then leave the tube on ice for 20 min.
6. Spin the cell lysate at 13,000 rpm in a 4°C room for 5 min.
7. Transfer 20 μL of supernatant to a new tube and mix with 1 μL of 4% SDS and 1 μL of 20% β-mercaptoethanol.
8. Incubate the mixture at 100°C for 5 min to denature the proteins.
9. Divide the mixture into two fresh tubes. Add 10 μL of digestion buffer containing 1 U of EndoH or *N*-Gly F to one tube and add 10 μL of digestion buffer without the enzymes to the other tube.
10. Incubate the tubes at 37°C for 1 h and run the samples on an 8% SDS-PAGE mini-gel.
11. Transfer proteins to a nitrocellulose membrane using a Biorad mini-transfer tank and block the membrane with 5% nonfat milk in PBS containing 0.05 % Tween-20 for 1 h at room temperature.

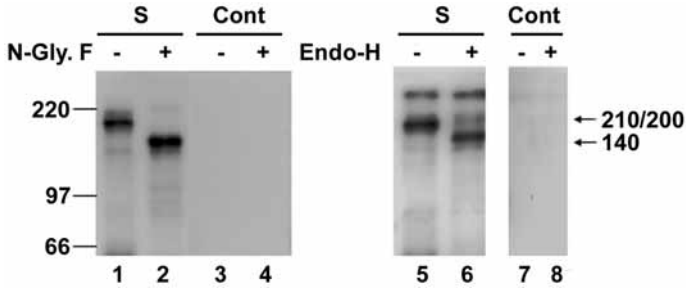

Fig. 3. Treatment of the S-derived proteins in cell lysates with *N*-glycosidase F (*N*-Gly-F) and endoglycosidase H (EndoH). The full-length S was expressed in Cos-7 cells. Cells were resuspended in lysis buffer. The samples were either treated (+) with **(A)** *N*-Gly-F and **(B)** EndoH or mock-treated (–). Proteins were separated on SDS-PAGE gels. Western Blot was performed with rabbit-anti-S and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies. Lysates from mock-transfected cells were used as negative controls (lanes 3, 4, 7, and 8). Molecular masses of specific proteins are indicated on the right and masses of markers are indicated on the left in kilodalton.

12. Incubate the membrane with rabbit anti-S primary antibodies (1:10,000 to 1:60,000) at 4°C overnight.
13. Wash the membrane four times with PBS-0.05 % Tween-20.
14. Incubate the membrane with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce, Rockford, IL) at a dilution of 1:2000 for 1 h at room temperature.
15. Wash the membrane four times and then visualize the resolved proteins with an enhanced chemiluminescence reagent (Pierce, Rockford, IL) (**Fig. 3**).

4. Notes

1. To facilitate efficient translation initiation, a Kozak consensus sequence (CCACC) was introduced in the forward PCR primer immediately upstream of the AUG initiation codon of the S gene and downstream a unique restriction enzyme site of the vector.
2 Dephosphorylation of the vector after double digestion with two restriction enzymes will reduce the likelihood of re-ligation of single cut plasmids, which can arise from incomplete digestion. This will decrease the transformation background when screening for the insert-positive cDNA clones.
3. For 15 to 30 min pulse-labeling of the cells in a 60-mm dish, 2 μL of [^{35}S]-methionine/cysteine (22 μ*M*) in 1 mLof medium is needed. For longer labeling, 4 μL (44 μ*M*) is required.
4. The amount of fully glycosylated proteins in cells will peak after chasing for 4 h. After that, the yield of glycoprotein decreases due to cell death caused by vTF7.3

infection, however the ratio of mature to immature glycoprotein is increasing after 4 h chasing (**Fig. 2**).

5. To prevent high background, avoid pipetting out the cell debris. Add more lysis buffer (0.5 to 1 mL) if the lysate is too viscous after centrifugation.
6. If there is no aspirator available, the "radioactive" RIPA buffer may also be removed by carefully pipetting it out and discarding it into a properly designated waste container.

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10

Analysis of Glycoproteins of Viruses in the Family *Bunyaviridae*

Xiaohong Shi and Richard M. Elliott

Summary

The membrane glycoproteins (Gn and Gc) of viruses in the family *Bunyaviridae* form projections on the virion envelope and are involved in virus entry and eliciting protective immunity. The glycoproteins are modified by N-linked glycosylation and accumulate in the Golgi complex where virions mature and bud. In this chapter, we describe the methods that have been used in our laboratory for the study of the glycoproteins of Bunyamwera virus, the prototype of the family. The protocols cover the expression of viral glycoproteins, examination of intracellular localization by immnunofluorescent confocal microscopy, radiolabeling, immunoprecipitation, and SDS-PAGE analysis of the proteins, and the improved reverse genetic system to rescue recombinant viruses that contain mutations at *N*-linked glycosylation sites.

Key Words: *N*-linked glycosylation; *Bunyaviridae*; Bunyamwera virus; bunyavirus glycoprotein; protein expression; virus rescue and reverse genetics.

1. Introduction

The family *Bunyaviridae* contains more than 300 mostly arthropod-borne viruses that share biochemical and morphological characteristics; the family is classified into five genera (*Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus and Tospovirus*) ***(1,2)***. Several members of the family cause encephalitis or hemorrhagic fever in humans, e.g., La Crosse, Hantaan, Rift Valley fever, and Crimean-Congo hemorrhagic fever viruses, and are recognized as posing an increasing threat to human health ***(3)***. All viruses have a tripartite negative-sense RNA genome that encodes four structural proteins. The largest segment (L) codes for an RNA-dependent RNA polymerase (L protein), the medium segment (M) for a precursor containing the two glycoproteins (Gn and Gc), which

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are associated with the envelope and form spikes, and the smallest segment (S) codes for the nucleoprotein (N). Some viruses also produce non-structural proteins from the M (called NSm) and S (called NSs) segments.

Both Gn and Gc proteins encoded by viruses of this family are type I integral membrane glycoproteins *(4)* and are modified by *N*-linked glycosylation *(5–10)*. The two glycoproteins (and NSm where present) are cotranslationally cleaved from a polyprecursor encoded by the M segment. The glycoproteins of this family usually target and accumulate in the Golgi complex where virus assembly and budding occurs *(6,11–14)*.

In recent years, we have studied the glycoproteins of Bunyamwera (BUN, the prototype of the family) and Hantaan (HTN) viruses, including the interaction between Gn and Gc and the role of *N*-linked glycosylation on correct protein folding and intracellular trafficking. By using reverse genetics we generated *N*-glycosylation site deficient mutant BUN viruses and used them to investigate the role of *N*-glycosylation of the viral envelope proteins in virus replication and infectivity.

2. Materials

1. Dulbecco's modified Eagles's medium (DMEM) (Invitrogen).
2. Glasgow minimal essential medium (GMEM) (Invitrogen).
3. 10% tryptose phosphate broth.
4. Fetal bovine serum.
5. Geneticin (200 mg per milliliter stock solution, stored at –20°C).
6. Opti-MEM (Invitrogen).
7. 13 mm-diameter glass coverslip.
8. 35 mm- and 60 mm-diameter Petri dishes.
9. 24-well plates.
10. 5 mL polystyrene round-bottom tube (BD Falcon™ REF 352054).
11. Vero E6 (ATCC C008) or HeLa $T4^+$ *(15)* cells.
12. BSR-T7/5 cells, a BHK derivative that stably expresses T7 RNA polymerase *(16)*.
13. Vaccinia virus vTF7-3, a recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase *(17)*.
14. Transfection agents, such as Lipofectin (Invitrogen) or FuGENE Transfection reagent (Roche).
15. Phosphate-buffered saline (PBS).
16. Citifluor (Citifluor Ltd. Leicester) or other reagents for mounting coverslips
17. 4% w/v paraformaldehyde in PBS.
18. 0.1% w/v Triton X-100 in PBS.
19. Primary antibodies. Anti-BUN rabbit serum and anti-BUN Gc monoclonal antibody (MAb) 742 were described in other publications *(18,19)*. Anti-GM130 *(20)* was gift from Martin Lowe (School of Biological Science, University of Manchester, UK) and antihuman golgin-97 *(21)* was purchased from Molecular Probes Inc. (Leidein, The Netherlands).

20. Secondary antibodies. Goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) (Sigma). Goat anti-mouse antibody conjugated with Cy5 (Amersham Phamacia Biotech, Buckingham, UK).
21. Zeiss LSM Confocal Microscope, LSM 510 v2.01 software.
22. DMEM without methionine and cysteine (REF D0422, Sigma).
23. [^{35}S]methionine (800 Ci/mmol, Amersham)
24. Non-denaturing RIP buffer: 50 m*M* Tris-HCl pH 7.4, 1% Triton X-100, 300 m*M* NaCl, 5 m*M* EDTA. Add protease inhibitor cocktail before use according to manufacturer's instruction.
25. Protease inhibitor cocktail (1 697 498, Roche)
26. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) reagents and equipment.
27. SDS-PAGE gel fixing solution (10% acetic acid).
28. RIP wash buffer: 50 m*M* Tris-HCl pH 7.4, 0.1% Triton X-100, 300 m*M* NaCl, 5 m*M* EDTA.
29. Protein A–Agarose (P0932, Sigma).
30. Protein sample buffer: 1% SDS, 5% glycerol, 20 m*M* Tris HCl, pH 6.8, 1% β-mercaptoethanol, 0.2% bromophenol blue.
31. Plasmids that generate full-length antigenome RNA transcripts pT7riboBUNL(+), pT7riboBUNM(+), pT7riboBUNS(+) have been described previously *(22)*. Three *N*-glycosylation-site mutant constructs pT7riboBUNM-N60Q, pT7riboBUNM-N624Q, and pT7riboBUNM-N1169Q, in which the asparagine residue (N) at a potential *N*-glycosylation site was substituted with glutamine (Q), were generated from pT7riboBUNM (+), using a site-directed PCR mutagenesis approach *(14, 23)*.
32. DAC-30 (Eurogentec) or FuGENE 6 Transfection reagent (1 814 443, Roche).

3. Methods

The protocols described below outline (1) the transient expression of glycoproteins using the vTF7-3 vaccinia virus system, (2) examination of the intracellular localization of BUN glycoproteins by confocal microscopy and immunofluorescent antibody staining, (3) radiolabeling, immunoprecipitation and SDS-PAGE analysis of BUN glycoproteins, and (4) generation of recombinant virus containing *N*-glycosylation site mutations by reverse genetics.

3.1. Transient Expression of Glycoproteins Using vTF7-3 Vaccinia Virus System

The advantage of using the recombinant vaccinia virus vTF7-3 for expressing a foreign gene is that the bacteriophage T7 RNA polymerase expressed by the virus can drive the expression of the target gene that was simply cloned into a plasmid vector under control of a T7 promoter without the need to produce a recombinant vaccinia virus *(17,24)*. This system has been successfully used for expression of viral proteins in the *Bunyaviridae* family on studies of

N-linked glycosylation, processing, protein folding, and intracellular transport *(6,12,14,19,25)*. vTF7-3 was also used previously to establish the reverse genetics system for recovery of infectious BUN virus from cDNA clones *(22)*.

3.1.1. Expression Plasmid

The coding regions of the bunyavirus glycoproteins were cloned into expression vectors, such as pTM1 *(24)* or pGEM (Promega) under control of the bacteriophage T7 promoter (*see* **Note 1**).

3.1.2. Infection and Transfection of Cells

1. Seed cells onto coverslips in 24-well plates at a density of 0.5×10^5/coverslip or onto 35-mm Petri dishes at 5×10^5 cells/dish. Incubate cells in 5% CO_2 at 37°C overnight (Cells will be 90% confluent).
2. Infect cells with vTF7-3 diluted in OptiMEM at 5 plaque-forming units (pfu)/cell (100 μL diluted virus for each coverslip and 200 μL for each 35-mm Petri dish).
3. Incubate the cells for 60 min with gentle shaking every 10 to 15 min.
4. Prepare the mixture of DNA and transfection regent (during the incubation time). For cells on coverslips, 0.5 μg plasmid DNA and 3 μL of Lipofectin are diluted in 125 μL of OptiMEM. For cells on 35-mm dishes, 2 μg DNA and 10 μL of Lipofectin are diluted in 250 μL OptiMEM.
5. Mix the diluted DNA and liposome. Incubate for 10 min at room temperature.
6. Remove the vTF7-3 from coverslips or dishes and wash cells once with 0.5 mL OptiMEM.
7. Add DNA-liposome mixture to the cells. Incubate for 3 h at 37°C.
8. Add 0.5 mL (for cells on coverslips) or 2 mL (for cells on dishes) of DMEM containing 10% FBS. Continue incubation for further 3 h to overnight.
9. Examine expression of protein of interest by using either immunofluorescent staining **(Subheading 3.2.)** or SDS-PAGE analysis of radiolabelled protein **(Subheading 3.3.)**.

3.2. Co-Immunofluorescent Staining of Bunyavirus Glycoproteins and Golgi Markers

In general, the confocal microscope will always give better results for colocalization studies than can be obtained with a conventional immunofluorescence microscope. It has the capability to simultaneously detect two or more different emitted colors, and can reject the out-of-focus interference. It thus can reveal a sharper detail of cellular structure or the localization of proteins of interest when they are probed with immunofluorescent conjugates, especially when examining the intracellular distribution in cells or the colocalization of different proteins to specific organelle.

Here we describe the method for double staining of BUN glycoproteins and Golgi markers, such GM130, a cis-Golgi matrix protein *(20)*, or anti-golgin 97,

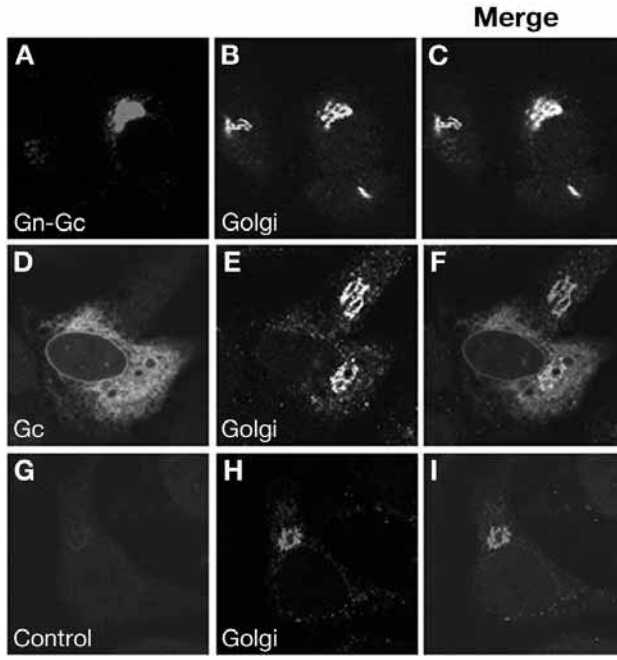

Fig. 1. Intracellular localization of co-expressed and separately expressed of BUN Gc proteins. HeLaT4+ cells were infected with vTF7-3 followed by transfection with BUN cDNAs as indicated. Gc proteins were co-expressed with Gn from the whole M segment cDNA (**A–C**) or expressed from just the Gc coding region cDNAs (**D–F**). Cells were doubly stained with anti-Gc monoclonal antibody (MAb) 742 and anti-GM 130 serum (**A–C**), or anti-BUN serum and anti-golgin-97 (**D–F**). **G–I** are the control of vTF7-3 infected cells co-stained with anti-BUN and anti-golgin-97. Merged confocal microscopic images are shown in the right column. It was clearly shown that translocation of BUN Gc protein to the Golgi complex requires the co-expression of its Gn counterpart. Gc expressed alone is retained in the ER and failed to target to the Golgi complex.

a novel 97-kd Golgi complex autoantigen (***21***). The colocalization of BUN glycoprotein Gc and Golgin markers (GM130 and human golgin-97) are shown in **Fig. 1**.

3.2.1. Sample Preparation for Confocal Microscopy and Immunofluorescence

The transfected or virus infected cells grown on 13 mm-diameter glass coverslips are fixed with 4% paraformaldehyde-PBS and permeabilized with 0.1 % Triton X-100 before reacting with antibodies.

1. Wash the cell monolayers grown on coverslip twice with cold PBS.
2. Fix the cells with 0.5 mL of 4% paraformaldehyde in PBS for 30 min at room temperature.
3. After fixing, wash the cells five times with cold PBS.
4. For staining of internal antigens, permeabilize the cells with 0.5 mL of 0.1% Triton X-100 in PBS for 20 min at room temperature.
5. Wash the cells three times with cold PBS. The cells are ready for antibody staining or can be stored at 4°C in PBS.

3.2.2. Reaction with Primary and Conjugated Secondary Antibodies

For co-staining with more than two antigens in one sample, the primary antibodies must be raised in different species and there is must be no immune-cross reactivity between them. In the case of co-staining of BUN Gc protein and Golgi marker, we use either a combination of anti-BUN rabbit serum and anti-golgin-97 mouse MAb, or anti-Gc mouse MAb M742 and anti-GM130 rabbit serum (*see* **Note 2**).

1. Incubate the cells with the primary antibody dilution for 30 min at room temperature (*see* **Note 3**).
2. At end of the incubation period, transfer the coverslips (cell side up) into wells of a 24-well plate. Wash the cells five times with cold PBS.
3. Incubate with the secondary antibody conjugated to either FITC or Cy5.
4. Repeat **step 2**.
5. Mount the coverslips on slides using Citifluor and visualize using a confocal microscope. Analyse the images using appropriate software.

3.3 Analysis of BUN Glycoproteins by Radiolabeling, Immunoprecipitation and SDS-PAGE

Radiolabeling followed by immunoprecipitation is useful to investigate the co- and posttranslation modifications of glycoproteins, such as carbohydrate side chain processing, protein folding, oligomerization, and immunoreactivity. The two glycoproteins can be immunoprecipitated with specific antibodies from either virus-infected cells or cells transfected with M segment cDNA. The methods described in protocols 3 and 4 work well to analyse the glycoproteins of both HTN and BUN viruses. **Figure 2** showed the protein folding and maturation process of BUN glycoproteins Gn and Gc expressed in Vero E6 cells transfected with BUN M cDNA.

3.3.1. Metabolic [^{35}S]methionine Radiolabeling of BUN Virus Proteins

1. At the appropriate time after transfection or virus infection, remove the culture medium from the 35-mm dishes.
2. Rinse the cell monolayers once with PBS and once with methionine-deficient medium.

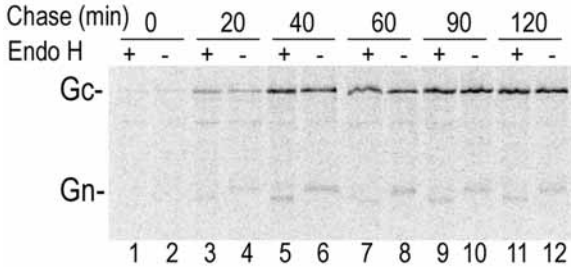

Fig. 2. Analysis of *N*-linked glycosylation of BUN glycoproteins by treatment with Endo H. The vTF7-3 infected Vero E6 cells were transfected with BUN M cDNA and then labeled with [^{35}S]methionine for 20 min. Cells were lysed for immunoprecipitation at the time points indicated post protein labeling. Equal volumes of cell lysate were immunoprecipitated with anti-BUN Gc MAb 742, a conformational sensitive antibody. The resulting precipitates were subjected to Endo H digestion and analyzed by SDS-12.5 % PAGE under reducing conditions. The figure shows that properly folded Gc protein was detectable just at end of protein labeling (lanes 1 and 2), but reached a peak after 40 min of chase (lanes 4 and 6). The folded protein acquired endo H resistance after 60 min (lanes 7 to 12), a measure of the time required for complete maturation of BUN glycoproteins. (From **ref. *23***.)

3. Add 2 mL fresh methionine-deficient medium to each dish and incubate for 40 min (to starve of methionine).
4. At the end of the starvation period, replace the medium with protein labelling medium containing [^{35}S]methionine (30 to 100 μCi/mL).
5. Incubate the cells for 10 min to 15 h at 37°C according to the different application (*see* **Note 4**).
6. At the end of the incubation, remove the labelling medium, rinse the cells once with growth medium and chase up to 2 h at 37°C with 2 mL of growth DMEM supplemented with 10% FCS and methionine (15 μg/mL).

3.3.2. Preparation of Cell Lysate for Direct SDS-PAGE Analysis

1. Add 100 μL of protein dissociation buffer to each dish. Swirl the dish to ensure dissociation buffer covers all the cell monolayer.
2. Scrape the lysed cells and transfer cell lysate to a microfuge tube.
3. Pass the sample through a 4-gauge needle with a syringe to shear the DNA and make it less viscous.
4. Denature the sample by heating, usually at 100°C for 3 min. However, to detect bunyavirus Gn the sample is heated at 37°C for 10 min before loading. The sample is ready for SDS-PAGE analysis or can be storeed at –20°C.

3.3.3. Preparation of Cell Lysate for Immunoprecipitation

To prevent protein proteolysis and degradation, all procedures are carried out on ice or at 4°C.

1. Remove the medium and rinse the cells once with cold PBS.
2. Add 300 μL RIP buffer containing protease inhibitor cocktail (*see* **Note 5**).
3. Leave the dishes on ice for 10 min.
4. Harvest the cell lysate to microfuge tubes. Vortex for 5 s and incubate on ice for another 10 to 15 min.
5. Centrifuge the tubes for 10 min at 16,000*g* to remove the cell debris and nuclei.
6. Transfer the supernatant to new microfuge tube and stand on ice or store at –20°C.

3.3.4. Preparation of Antibody Conjugated Protein A–Agarose Beads.

1. To prepare 50% Protein A–Agarose slurry, mix 100 mg beads with 800 μL RIP buffer and incubate for at least 30 min at room temperature before use. (The rehydrated beads can be stored at 4°C for 4 wk.)
2. Combine 30 μL of 50% protein A–agarose beads, 1 μL antibody and 0.5 mL ice-cold PBS (*see* **Note 6**).
3. Incubate on rotating wheel for 2 h to 24 h at 4°C.
4. Spin for 5 s at 16,000*g*, 4°C. Remove supernatant carefully.
5. Wash the beads three times with 1 mL of ice-cold RIP wash buffer.
6. Wash once with RIP buffer.

3.3.4. Immunoprecipitation

1. Add 10 μL of 10% BSA and 300 μL of cell lysate to antibody-conjugated beads.
2. Incubate the mixture for 2 to 24 h at 4°C on rotating wheel.
3. Spin 5 s at 16,000 rpm. Remove supernatant carefully.
4. Wash the beads four times with 1 mL of ice-cold RIP wash buffer.
5. Wash beads once with 1 mL of cold PBS. Remove supernatant completely.
6. Add 30 μL of 2X protein sample buffer and boil for 3 min. The immunoprecipitates is ready for SDS-PAGE analysis or can be used for endoglycosidase digestion, following the protocol detailed in Chapter 6.

3.4. Generation of the Recombinant Virus by Reverse Genetics

Study of the molecular biology of negative-strand RNA viruses has been revolutionized by the development of reverse genetic techniques, which enable researchers to recover (rescue) infectious virus from cDNA copies of the viral genomes. A rescue system was previously reported to recover infectious Bunyamwera virus (genus *Orthobunyavirus*) entirely from cloned cDNA utilizing a recombinant vaccinia virus expressing bacteriophage T7 RNA polymerase to drive intracellular transcription of transfected T7 promoter-containing plasmids *(22)*. Since then, the system has been dramatically improved in our laboratory by transfecting BSR-T7/5 cells (a BHK-derived cell line that constitutively

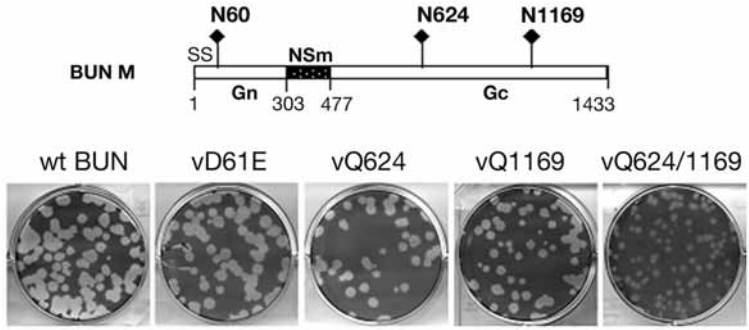

Fig. 3. Plaque morphology of mutant BUN viruses containing mutations at *N*-glycosylation sites. Cell monolayers were fixed with 4% formaldehyde and stained with Giemsa's staining solution 4 d after infection. The locations of the potential *N*-glycosylation sites is indicated as lollipops on the schemamtic of the BUN glycoprotein precursor with gene order of Gn (residues 1 to 302), NSm (303 to 476) and Gc (477 to 1433). The *N*-glycosylation site-deficient mutants were generated by substitution of one (for single mutation) or two (for double mutations) of the asparagines (N) residues with glutamine (Q) by using site directed PCR mutagenesis. (From **ref. *23***.)

expresses T7 RNA polymerase ***[16]***) with just three ribozyme plasmids without the need for separate helper-protein-expressing plasmids ***(26)***. By site-directed PCR mutagenesis of the M segment cDNA, we introduced *N*-glycosylation site mutations into infectious recombinant Bunyamwera virus ***(23)***. The plaque morphologies of the recombinant BUN viruses bearing *N*-glycosylation site mutations are shown in **Fig. 3**. Mutant virus vQ624/1169, in which two glycosylation sites were mutated, showed the smallest plaques (**Fig. 3**).

3.4.1. Plasmids

Plasmid constructs pT7riboBUNL(+), pT7riboBUNM(+), pT7riboBUNS(+), which generate full-length antigenome RNA transcripts, were described elsewhere ***(22)***. Three N-glycosylation-site mutant constructs pT7riboBUNM-N60Q, pT7riboBUNM-N624Q and pT7riboBUNM-N1169Q, in which the asparagine residue (N) at a potential *N*-glycosylation site (**Fig. 3**) was substituted with glutamine (Q), were generated from pT7riboBUNM (+), using a site-directed PCR mutagenesis approach ***(14,25)***.

3.4.2. Preparation of BSRT7/5 Cells for Virus Rescue

1. Seed BSR-T7/5 cells onto 60-mm diameter petri dish (1×10^6 cells per dish) and incubate overnight at 5%, CO_2, 37°C incubator (Cells should be 90% confluent before tranfection).

On the next day:

2. Dilute separately in two polystyrene round-bottom tubes 1 μg of each plasmid and 10 μL of transfection regent in 350 μL of OptiMEM (Total volume of 700 μL).
3. Mix diluted DNA and liposome (transfection solution) and shake mixture gently.
4. Incubate for 30 min at room temperature.
5. Remove medium from dish and rinse the cells with 2 mL of OptiMEM.
6. Add transfection solution to cells.
7. Incubate cells for 5 h.
8. Add 3 mL of GMEM growth medium and continue incubation for 4 to 5 d at 37°C.
9. Harvest supernatant and clarify at low speed centrifugation to remove cell debris. Store supernatant at –20°C or –70°C for plaque assay using Vero or BHK-21 cells.

4. Notes

1. Plasmid construction. For the purpose of expressing proteins of interest using the vTF7-3 system, the coding region of the relevant gene can be cloned into variety of the expression vectors under the control of bacteriophage T7 promoter. For expressing proteins in BSRT7/5 cells without vTF7-3 infection, pTM1 vector *(24)* is recommended.
2. Antibodies. The dilution of antibody should be optimized by titration in same cell line and under the conditions same for immunofluorescence assay.
3. We usually disperse approx 20 μL of antibody solution on the lid of a 24-well-plate and then put the coverslip face down on the drop of antibody. The same procedure is used for staining with the secondary antibodies.
4. The amount of isotope used and the time of protein labelling depend on the purpose of experiment and the expression system used. For pulse labelling to examine protein folding and intracellular transport, we used 100 to 200 μCi of [^{35}S]methionine and labelled for 10 to 30 min. For general examination of expression of a particular protein, we used less amount of isotope, such as 30–50 μCi and labeled for a longer time (a few hour to overnight).
5. We usually use 300 μL of RIP buffer to lyse cells grown on 35 mm-diamiter Petri dishes, but a larger volume is applied if the cell lysate will be used for more than one immunoprecipitation assay.
6. The amount of antibody used depends on its titre and affinity. For antibodies of high titer, 1 μL or less is enough to immunoprecipitate specific radiolabeled proteins.

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Secretion of the Respiratory Syncytial Virus Fusion Protein From Insect Cells Using the Baculovirus Expression System

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Summary

Sequences derived from the respiratory syncytial virus (RSV) fusion (F) protein were expressed in insect cells as recombinant glutathione-*S*-transferase (GST)-tagged proteins. The sequence covering the F_2 subunit (GST-F2), and a truncated form of the F protein in which the transmembrane domain was removed (GST-F_2/F_1), were cloned into the baculovirus pAcSecG2T secretory vector. These virus sequences also had the endogenous virus signal sequence removed and replaced with a signal sequence derived from the baculovirus gp67 glycoprotein, which was present in pAcSecG2T. The recombinant RSV glycoproteins were successfully detected in expressing cells by immunofluorescence assay and in the tissue culture medium by western blot analysis. The secreted recombinant GST-F_2/F_1 protein was further analysed using glycosidases. Our results showed that the GST-F_2/F_1 protein were sensitive to peptide:*N*-glycosidase F (PNGase F) treatment, but not to Endoglycosidase H (EndoH) treatment. This indicates that the secreted recombinant proteins were modified by the addition of mature *N*-linked glycan chains.

Key Words: Respiratory syncytial virus; Baculovirus; GST-F_2 protein; GST-F_2/F_1 protein.

1. Introduction

The baculovirus expression system has become one of the most widely used systems for the routine production of recombinant proteins (for a recent review, *see* **ref. *1***). A range of recombinant proteins, from cytosolic enzymes to membrane-bound proteins, including glycoproteins, have been successfully expressed. However, the insect protein processing pathway is somewhat distinct from that of the higher eukaryotes. Studies have indicated that insect cells could assemble

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N-glycans, transfer them to nascent polypeptides and trim the *N*-glycan precursors to produce high mannose or paucimannose end products *(2,3)*. However, these insect cells had little or none of the galactosyltransferase and sialyltransferase that are present in the higher eukaryotes. These enzymes are required for the elongatation of trimmed *N*-glycans to produce complex products containing terminal galactose and/or sialic acid residues. Although studies have shown that most glycoproteins expressed in insect cells generally do not contain complex modifications *(4)*, there are insect cell lines available which have been engineered successfully to mimic the mammalian glycoslyation processes *(5–8)*.

However, the baculovirus expression system remains a convenient method by which to generate large quantities of glycosylated proteins, which can then be subsequently used for a variety of downstream investigations.

The fusion (F) protein of the human respiratory syncytial virus (RSV) plays an important role during virus infection by promoting the fusion of virus membrane to that of the host cells. The precursor protein (F_0) is approx 70 kDa, and is cleaved by furin-like cellular proteases to yield two disulfide-linked subunits of 50 kDa (F_1) and 20 kDa (F_2). The F protein is further modified by palmitoylation and *N*-linked glycosylation *(9,10)*. In this chapter, the F protein was expressed in insect cells using a baculovirus secretory expression system. The methods used to analyze the secreted form of the recombinant protein, and to determine its glycosylation status, will be described.

2. Materials

1. Parent plasmid containing F_2/F_1 gene of RSV, subtype A2.
2. Baculoviral transfer vector, pAcSecG2T (Pharmingen).
3. Agarose gel electrophoresis apparatus.
4. BL21-competent cells.
5. Ampicillin.
6. Qiagen miniprep DNA kit.
7. DNA sequencing apparatus.
8. 35-mm tissue culture dishes.
9. 24-well tissue culture plate.
10. *Spodotera frugiperda* (*Sf*21) cells (Invitrogen).
11. 28°C incubator.
12. TC100 medium, Invitrogen.
13. Fetal calf serum (FCS).
14. BaculoGold DNA (Pharmingen).
15. Transfection reagents A and B (Pharmingen).
16. Low melting agarose (Invitrogen).
17. Neutral red.
18. Vortex.
19. Rotator.
20. 12-mm coverslips.

21. Phosphate-buffered saline (PBS), pH 7.2.
22. 4% paraformaldehyde.
23. 0.1 % saponin.
24. Monoclonal antibody to glutathione-*S*-transferase (GST) (Sigma).
25. Monoclonal antibody to RSV (NovacastRA).
26. Anti-mouse conjugated to fluorescein-isothiocyanate (FITC).
27. Citifluor.
28. Florescence microscope.
29. Benchtop centrifuge for low-speed spinning.
30. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) apparatus.
31. Glutathione sepharose 4B resin (Pharmacia).
32. DEN solution: 0.5% SDS and 1% mercaptoethanol.
33. Peptide:*N*-glycosidase F (PNGase (New England Biolabs).
34. PNGase F reaction buffer: 50 m*M* sodium phosphate, pH 7.0, containing 1% NP40.
35. Endoglycosidase H (EndoH) (New England Biolabs).
36. EndoH reaction buffer: 50 m*M* sodium citrate, pH 5.5.
37. 1X SDS sample buffer: 80 m*M* Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue.

3. Methods

This chapter describes the methods and techniques used for the expression of secreted F protein sequences derived from RSV :

1. Generation of recombinant baculoviruses.
2. Expression of GST-F_2 and GST-F_2/F_1 proteins in insect cells.
3. Analysis of *N*-linked glycosylation sites in GST-F_2/F_1 proteins.

3.1. Generation of Recombinant Baculoviruses

The bacuovirus glycoprotein gp67 is transported through the secretory pathway into the virus envelope during infection of insect cells ***(11)***. The signal sequence that encodes the secretion of gp67 is thus deployed in the pAcSecG2T transfer vector (**Fig. 1**). The signal sequence precedes the GST gene before the multiple cloning site in the vector ***(12)***. The gene of choice, cloned into the multiple cloning site downstream of the GST, will be expressed under the strong control of the polyhedron promoter, and secretion will be under the control of the gp67 signal sequence. The recombinant protein will be forced into the secretary pathway, after which, the signal sequence will be cleaved off. The GST protein will be secreted into the serum-free insect culture media, and the GST tag allows single-step purification of the recombinant proteins using glutathione agarose beads ***(13)***. This strategy is ideal for purifying proteins which are normally processed through the secretory pathway.

The RSV F_2 and F_2/F_1 genes were inserted into the vector and fused with the GST coding sequence. Expression of this sequence was under the strong poly-

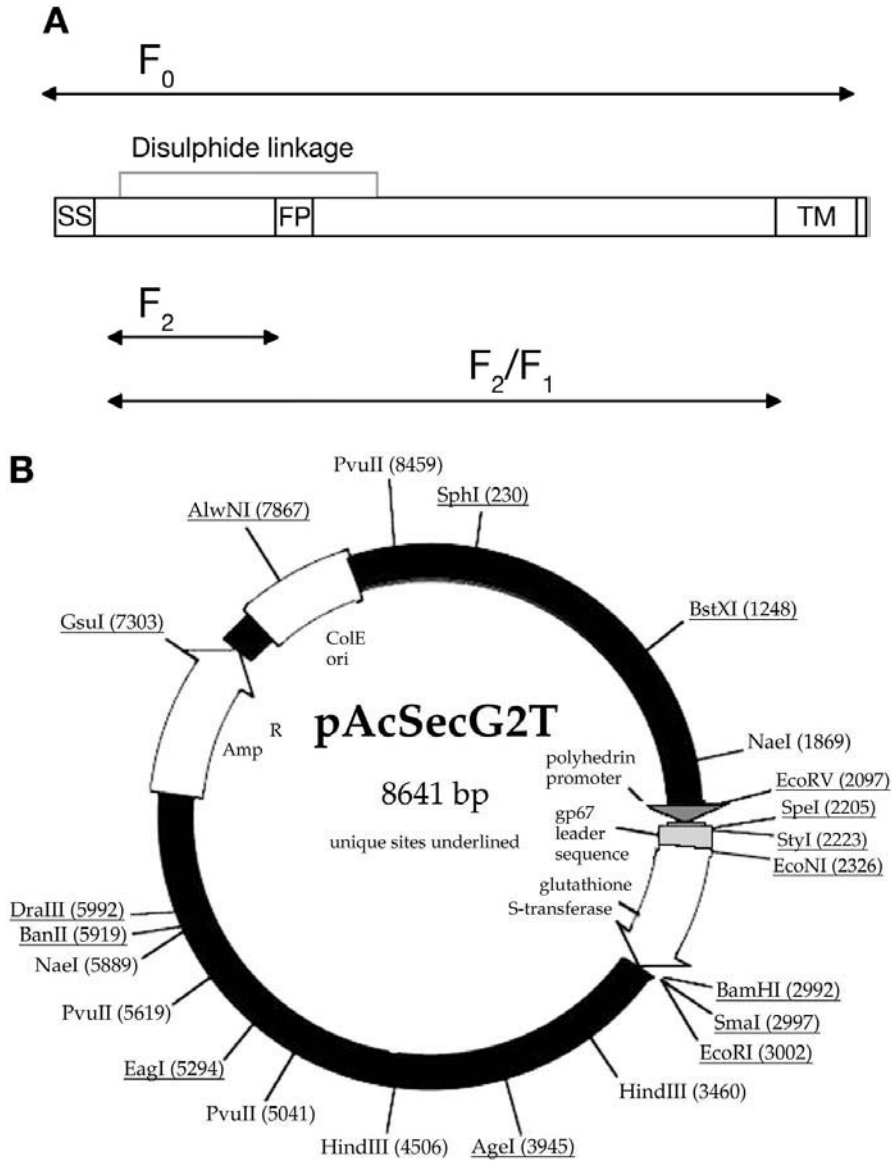

Fig. 1. Construction of the baculovirus transfer vectors containing the respiratory syncytial virus (RSV) fusion (F) protein sequences. **(A)** The RSV F_2 and F_2/F_1 genes gene sequences were synthesised by PCR. The F protein sequence (Fo) was used as the template for the PCR cloning. **(B)** These sequences were then inserted into the baculovirus transfer vector (pAcSecG2T). Expression of the F_2 and F_2/F_1 genes is under the control of the polyhedrin promoter. SS represents the signal peptide, FP the fusion peptide, and TM represents the transmembrane domain.

hedrin promoter in the pAcSecG2T vector. The proteins were expressed as gp67-GST fusion proteins, and secreted into the insect culture media. The methods used for the construction of recombinant transfer vectors, and their transfections to generate recombinant baculoviruses, are described under **Subheadings 3.1.1.** and **3.1.2.**

3.1.1. Construction of the pAcSecG2T Transfer Vectors Containing the RSV F_2 and F_2/F_1 Genes

1. Amplify the F_2 and F_2/F_1 coding regions from its parent plasmid with specific primers using PCR in the conventional thermal cycler.
2. Using standard molecular biology methods, digest the purified products of the correct size with the appropriate restriction enzymes and ligate into the same sites in pAcSecG2T to generate recombinant vectors, pGST-F_2 and pGST-F_2/F_1 (*see* **Note 1**).
3. Transform the ligation reaction into chemically competent BL21 bacterial cells, and plate out the transformants on LB agar with selection in the presence of ampicillin (100 μg/mL).
4. Select colonies, and grow overnight at 37°C in 5 mL of Luria-Bertani broth with ampicillin.
5. Extract the recombinant plasmids pGST-F_2 and pGST-F_2/F_1 using the Qiagen miniprep kit, and analyze the recombinant vectors for the presence of the respective inserts in agarose gel with ultraviolet illumination.
6. Sequence the F_2 and F_2/F_1 constructs present in the recombinant vectors using the dideoxy sequencing method before proceeding to **Subheading 3.1.2.** for the generation of recombinant baculoviruses.

3.1.2. Construction of the Recombinant Baculoviruses

1. Seed 1.8×10^6 *Sf*21 cells onto 35-mm tissue culture dishes and grow at 28°C in TC100 medium supplemented with 10% FCS (*see* **Note 2**).
2. Combine 0.5 μg of BaculoGold DNA, and 2 to 5 μg of recombinant pGST-F_2 and pGST-F_2/F_1 vectors in a tube. Mix well by vortexing.
3. Incubate the mixture for 5 min before adding 1.0 mL of transfection reagent B.
4. Discard the medium from the *Sf*21 cells, and add 0.5 mL of transfection reagent A.
5. Add 0.5 mL of mixture from step 3 drop by drop to the *Sf*21 cells. Incubate the transfected *Sf*21 cells at 28°C for 4 h, discard the transfection mixture, add fresh medium and continue incubation for 5 d.
6. Harvest the baculoviruses by keeping the supernatant of the insect cell culture at 4°C in the dark. The supernatant will contain two types of baculoviruses, wild-type and recombinant baculoviruses (which contain the gene of interest).
7. To obtain single recombinant baculoviruses, plaque-purify the transfected supernatant before amplifying the virus. Seed the cells as described under **Subheading 3.1.2., step 1**. Prepare serial dilution of the transfected supernatant at 1×10^{-3}, 10^{-4}, and 10^{-5}. Add 100 μL of each dilution onto the *Sf*21 cells, and incubate

at 28°C for 1 h. After which, remove the inoculum, and add 1 mL of Agarose mix (an equal volume of 2% low melting agarose to 2X TC100 medium). Once the agarose solidifies, add another ml of TC100 medium supplemented with 2% FCS (*see* **Note 3**).

8. On the fifth day postinfection, visualize the virus plaques by staining with 0.2% neutral red.
9. Remove an agarose plug directly over each plaque using a sterile pasteur pipet and place the plug in 1 mL of 1X TC100 medium supplemented with 10% FCS in a tube. Elute the virus particles out of the agarose plug by either vortexing the tube, or rotating the tube overnight at 4°C. Inoculate about 200 µL of the virus suspension onto *Sf*21 cells seeded as described under **Subheading 3.1.2., step 1**, and incubate for 4 to 5 d.
10. Harvest the virus supernatant. Determine the virus titre by repeating **steps 7** and **8**.
11. This virus stock can be used to infect *Sf*21 cells, and the infected cells screened for the presence of recombinant GST-F_2 and GST-F_2/F_1 proteins using immunoflorescence reactions with antibodies to GST (*see* **Subheading 3.2.1.**).

Schematic diagrams depict the construction of RSV F_2 and F_2/F_1 **(Fig. 1A)** genes in the baculovirus transfer vector, pAcSecG2T **(Fig. 1B)**. The full-length of F_2 gene was cloned without its endogenous signal sequence, downstream of the GST gene in the pAcSecG2T vector. The F_2/F_1 gene was also likewise cloned without either its signal sequence or transmembrane region.

3.2. Analysis of Recombinant Protein Expression.

There are two procedures commonly used for the analysis of recombinant protein expression in insect cells, namely immunoflorescence assay and western blotting. Recombinant protein expression can be detected using an antibody that is either specific to the protein of interest, or a tag that is fused to the protein. The recombinant baculoviruses generated under **Subheading 3.1.** will contain the RSVF_2 and F_2/F_1 constructs with the signal sequence of the gp67 protein at its 5' end. The baculovirus derived signal sequence will allow the recombinant proteins to be secreted into the cell culture media as GST fusion proteins. In this section, the presence of recombinant GST-F_2 and GST-F_2/F_1 proteins within the infected insect cells are detected using immunoflorescence. Fusion proteins that are secreted into the cell culture media were screened using SDS-PAGE gel with western blotting. These methods are described under **Subheadings 3.2.1.** and **3.2.2.**

3.2.1. Immunofluorescence Assay (see **Note 4**)

1. Seed *Sf*21 cells on 12-mm coverslips in a 24-well plate. Infect with the recombinant baculoviruses at a multiplicity of infection (MOI) of 1 plaque-forming unit (pfu). Incubate at 28°C for 5 d.
2. On the fifth day postinfection, wash cell monolayer 3 times with cold PBS.

3. Remove PBS and fix cells with 4% paraformaldehyde (prepared in PBS) for 30 min at 4°C.
4. Wash the cells three times with cold PBS.
5. Permeabilise the cells by adding 0.5 mL of 0.1% saponin in PBS for 20–30 min at 4°C.
6. Wash the cells three times with cold PBS.
7. Remove the PBS and stain the cells with antibody against the GST protein (diluted in PBS), for 1 h at 37°C in a moist chamber.
8. Wash the cells three times with PBS.
9. Stain the cells with secondary antibody conjugated to FITC, and incubate further for 1 h at 37°C.
10. Wash the cells three times with PBS.
11. Mount the coverslips on slides with Citifluor, and examine with florescence microscopy.

3.2.2. Detection of the Recombinant Proteins in the Culture Supernatant

1. Seed *Sf*21 cells on 35-mm dishes as under **Subheading 3.1.2., step 1**. Infect with recombinant baculoviruses at MOI of more than 1 pfu. Incubate at 28°C for 5 d.
2. On the fifth day postinfection, remove the tissue culture supernatant carefully.
3. Clarify the culture supernatant by low-speed centrifugation at 2000*g* for 10 min.
4. Add 40 μL glutathione sepharose 4B beads suspension (50% w/v, prewashed with PBS) to the culture supernatant.
5. Incubate at 4°C for 60 min with gentle shaking.
6. Wash the protein bound to the glutathione sepharose 4B resin six times with PBS.
7. Add 1X SDS sample buffer. Incubate at 100°C for 10 min, and cool to room temperature.
8. Analyse the proteins by 12% SDS-PAGE and Western blotting using monoclonal antibodies raised to either the GST or the F protein.

Positive staining of the monoclonal antibody to *Sf*21 cells infected with baculovirus carrying both the GST-F_2 and GST-F_2/F_1 constructs was observed (**Fig. 2**). No staining was observed in the mock-infected *Sf*21 cells alone. To detect the secreted form of recombinant proteins, the insect cell culture media was harvested, clarified, and the protein allowed to bind to glutathione sepharose 4B resin. The bound proteins were analysed by Western blotting using a monoclonal antibody against GST (**Fig. 3**). **Figure 3A** shows the expression of secreted GST-F_2 protein from *Sf*21 cells infected with four different baculoviruses carrying the F_2 construct (lanes 2 and 4 to 6). **Figure 3B** shows the expression of secreted GST-F_2/F_1 protein from *Sf*21 cells infected with six different baculoviruses carrying the F_2/F_1 construct (lanes 2 to 7). Probing the membranes with anti-GST showed species corresponding in size to the GST-F_2 and GST-F_2/F_1 proteins. The untagged F_1 domain was not detected with this antibody. Lane 1 shows the expression of the 26 kDa GST protein from a virus

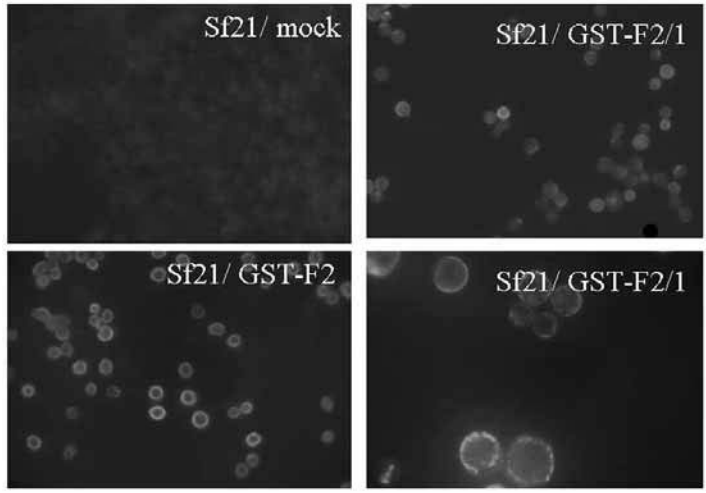

Fig. 2. Immuno-fluorescence staining of recombinant respiratory syncytial virus protein F in *Sf*21 cells. *Sf*21 cells were either mock-infected or infected with the recombinant baculoviruses expressing the glutathione-*S*-transferase (GST)-F_2 and GST-F_2/F_1 proteins. The cells were fixed and stained using anti-GST.

expressing GST only. Our results suggest that both the RSV F_2 and F_2/F_1 proteins can be expressed in insect cells and secreted into the cell culture supernatant.

3.3. Analysis of the Glycosylation Status of the GST-Tagged Proteins

As discussed previously, both mammalian and insect cells have different glycosylation pathways. The two systems process glycoproteins into a common intermediate, the *N*-glycan precursor, after which the glycosylation processes differ. In mammalian cells, these precursors are elongated to produce complex products containing terminal carbohydrates (e.g. galactose and sialic acid). The same precursors are not elongated in insect cells but produced paucimannose structures ($Man_3GlcNAc_2Fuc$). However, the same procedures can be used to analyse the glycosylation properties of recombinant proteins produced in both cell lines. The protein of interest is digested with two common endoglycosidases: peptide:*N*-glycosidase F (PNGase F) and Endoglycosidase H (EndoH). PNGase F is able to remove the entire carbohydrate moiety from proteins modified by *N*-linked glycoslyation, and EndoH specifically removes high mannose chains. The electrophoretic migration pattern for the digested and undigested

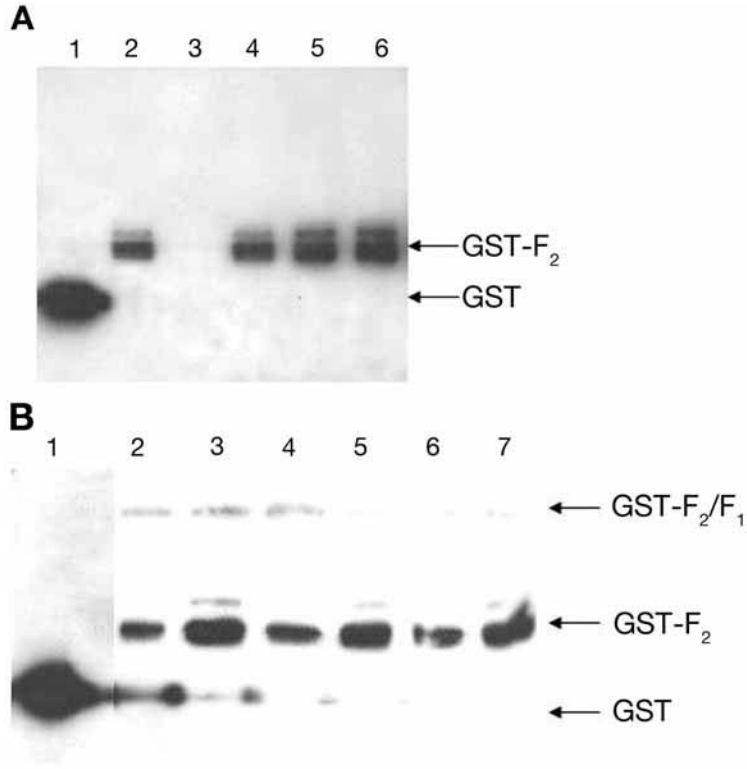

Fig. 3. Detection of the secreted form of recombinant glutathione-*S*-transferase (GST)-F_2 and GST-F_2/F_1 proteins. The culture supernatants of the virus-infected *Sf*21 cells, expressing GST-F_2 and GST-F_2/F_1 proteins, were incubated with glutathione sepharose 4B resin. The proteins bound to the resin were separated by 12% SDS-PAGE, and transferred by Western blotting onto polyvinylidine difluoride membranes. The membranes were then probed with anti-GST. **(A)** Western blot analysis of secreted recombinant GST-F_2 protein. Lanes 2 and 4–6 represent secreted GST-F_2 expressed in *Sf*21 cells from four different baculoviruses carrying the F_2 gene construct. Lane 3 is a similar analysis from mock-infected cells. **(B)** Western blot analysis of secreted recombinant GST-F_2/F_1 protein. Lanes 2–7 represent secreted GST-F_2/F_1 proteins expressed in *Sf*21 cells from six different baculoviruses carrying the F_2/F_1 gene construct. Lane 1 in both **A** and **B** is the same analysis performed using cells infected with a baculovirus expressing GST only. The respective protein bands are highlighted.

proteins is then assessed by SDS-PAGE gel and Western blotting. The method used to determine the *N*-linked glycosylation status of the recombinant GST-F_2/F_1 protein is described under **Subheading 3.3.1.**

3.3.1. Glycosidase Digestion

1. *Sf*21 cells are infected as described in **Subheading 3.2.2., steps 1–3**.
2. Add DEN solution to the clarified lysates, incubate at 100°C for 10 min, and cool to room temperature.
3. Aliquote the denatured proteins into four portions.
4. For EndoH digestion, make up the lysates in EndoH reaction buffer and either add 2000 U of EndoH or mock-treat without adding Endo H.
5. For PNGase F digestion make up the lysates in PNGase F reaction buffer and either add 2500 U of PNGase F or mock-treat without adding PNGase F.
6. Mix, and incubate reactions at 37°C for 20 h.
7. Analyze the proteins by 12% SDS-PAGE and western blotting using antibodies either against GST or the F protein.

Figure 4 shows the results of the analysis of the glycosylation status for the GST-F_2/F_1 protein expressed in insect cells. The clarified lysate containing the GST-F_2/F_1 protein was subjected either to PNGase F (lane H) or EndoH digestion (lane F). The digested proteins were analysed by Western blotting using antibodies against GST (**Fig. 4A**) and the F-protein (**Fig. 4B**). Lanes containing mock-treated proteins are indicated (–). There was a shift in the migration pattern after digestion with PNGase F (lane F), but not with EndoH (lane H) reactions. Our results showed that the GST-F_2/F_1 was sensitive to PNGase F treatment, indicating that the protein had been modified by the addition of *N*-linked glycans. The negative reaction with the digestion of EndoH suggested the absence of immature glycan chains. The results confirmed that it was possible for recombinant proteins expressed in insect cells to be modified by the addition of mature *N*-linked glycans. This strategy represents a useful method to produce recombinant expressed virus glycoproteins for use in other types of investigation (e.g., structural analysis).

4. Notes

1. The vector pAcSecG2T, contains a signal sequence which enables any gene of interest to be cloned in the vector for secretion into the tissue culture media. However, a new baculovirus expression system from Invitrogen, the Baculodirect system, allows the gene of interest to be cloned into one transfer vector. Transfection with different forms of linearised baculoviral DNAs will result in the expression of proteins in insect cells with either a His-tag at the N- or C-terminal, as well as for secretion into the media. The generation of recombinant baculoviruses using the Baculodirect system takes about 2 wk, and is much faster than the traditional way of generating viruses.
2. Generally *Sf*9 cells are recommended for the generation of recombinant baculoviruses. In this chapter, we found that *Sf*21 cells worked just as well for the transfection of baculoviral vectors. When infecting the insect cells for protein expression, we tend to use *Sf*21 cells. We have also used another insect cell line,

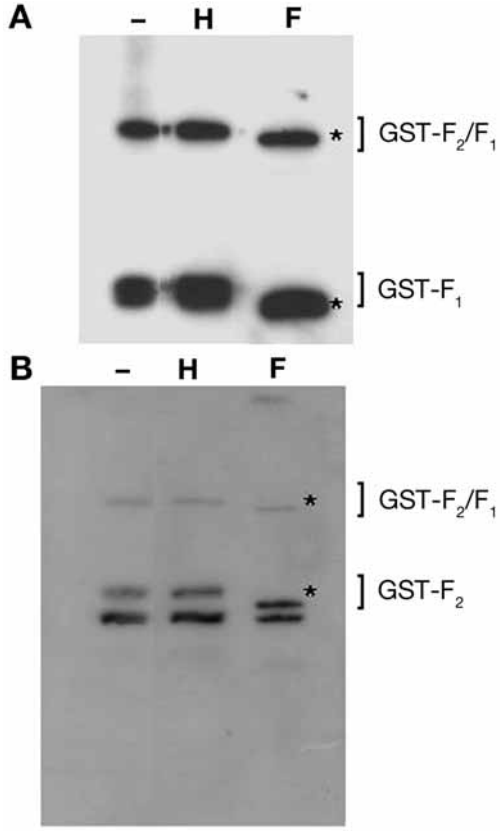

Fig. 4. Glycosylation analysis of recombinant glutathione-*S*-transferase (GST)-F_2/F_1 proteins. The tissue culture medium from sf21 cells infected with a bacuolovirus expressing GST-F_2/F_1 protein was harvested. The GST-F_2/F_1 protein was isolated using glutathione sepharose 4B resin and the bound protein was either mock-treated (–) or treated with either EndoH (lane H) or PNGase F (lane F). The RSV proteins were separated by 12% SDS-PAGE and transferred by Western blotting onto polyvinylidine difluoride membranes, which were then probed either with **(A)** GST or **(B)** F protein antibodies The respective protein bands are highlighted. The migration of the F protein species after glycosidase digestion is highlighted (*).

High Five cells, which are derived from *Trichoplusia ni*. The expression level for each recombinant protein in the different insect cells has to be optimised in terms of MOI, and postinfection time. A good range of MOI is 1 to 10, with postinfection varying from 24 to 96 h.

3. The plaque assay is used to titer the infectivity of the recombinant baculovirus generated. It is also used frequently to purify recombinant baculovirus from any contaminating wild-type virus. At least primary and secondary stocks are prepared from the initial seed stocks before the working virus stock is prepared. The working stock is then used for infecting insect cells for further experiments.
4. Immunofluorescence assays can be used to screen for protein expression in insect cells, especially when they are infected with virus prepared from the secondary and working stocks. By tagging the protein expression with a fusion tag such as histidine (His) or GST at either the C-terminal or N-terminal, the infected insect cells can still be detected with anti-His or anti-GST, in the absence of specific antiserum to the protein of interest.

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12

Characterization of the Dengue Virus Envelope Glycoprotein Expressed in *Pichia pastoris*

Boon-Huan Tan, Jian Lin Fu, and Richard J. Sugrue

Summary

The full-length and truncated forms of recombinant envelope (E) glycoprotein from Dengue virus type 1, Singapore strain S275/90 were expressed in the yeast, *Pichia pastoris,* using a secretory vector. A truncated form of the E protein in which the transmembrane domain was deleted was secreted successfully into the culture medium. The E protein was also co-expressed with C and prM proteins using a non-secretory yeast vector. The co-expression of C, prM and E proteins resulted in the spontaneous formation of virus-like particles (VLPs), which were confirmed by sucrose gradient analysis and transmission electron microscopy. Furthermore, the VLPs were used to immunise rabbits, and shown to be immunogenic by immunofluorescence staining of dengue virus-infected Vero cells. The yeast-expressed E protein was treated with PNGase F, which showed that although the protein was modified by the addition of *N*-linked glycans, the recombinant expressed E protein was not hyperglycosylated.

Key Words: Dengue; *Pichia pastoris*; E glycoprotein; CprME expression; secretion; glycosidase digestion; virus-like-particles; sucrose gradient.

1. Introduction

Dengue (DEN) virus represents the most important flavivirus causing human disease, threatening up to 2.5 billion people globally ***(1,2)***. The virus is about 40 nm in diameter and belongs to the family *Flaviviridae,* genus *Flavivirus*. It contains a positive-strand RNA genome of about 11 kb in size, which is translated into a single polyprotein encoding ten viral proteins. The gene order is C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The viral structural proteins (capsid [C], envelope [E] and membrane [M] proteins) occupy the first one-third of the genome, which are then followed by seven non-structural (NS) proteins, designated NS1 to NS5.

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The prM and E proteins are modified by *N*-linked glycosylation and they are the only two DEN structural proteins that are glycoslyated. The nucleocapsid, comprising the C protein and genomic RNA, acquires the prM and E proteins during the budding process in the endoplasmic reticulum lumen. The role that the prM protein plays during virus infection is currently unclear. However, during virus morphogenesis the prM is cleaved in the cell to generate the M and pr proteins, an event which is required for the virus to attain its full infectivity. The viral E protein binds the virus to the cell-surface receptor, and mediates fusion to the host-cell membranes during virus entry (for a review on the roles of DEN proteins, *see* **ref.** *3*).

Expressions of flaviviral glycoproteins have been successfully shown in different mammalian systems. Yeast represents an alternate eukaryotic host for the expression of flaviviral glycoproteins. For example, Japanese Encephalitis virus E protein has been successfully expressed in *Saccharomyces cerevisiae* *(4)*. However, recombinant proteins expressed in *S. cerevisiae* have been shown to exhibit hyperglycoslyation, with the addition of a terminal 1,3-linked mannose *(5,6)*. These undesirable modifications are not present in recombinant proteins expressed in the *Pichia pastoris* expression system (for a recent review, *see* **ref.** *7*). This book chapter describes the expression of full-length and truncated recombinant E protein sequences, derived from the DEN virus type 1, Singapore strain S275/90 *(8)*, in *P. pastoris*. In addition, co-expression of the E protein with C and prM proteins lead to the production of virus-like particles (VLPs) *(9,10)*. The methods used to determine the absence of hyperglycosylation in these recombinant proteins, and the confirmation of their antigenic and immunogenic properties, are described.

2. Materials

1. Primer CRGSTX 5'-GAA ACA GCT CGA GTG TCC CCT ATA CTA GGT-3'.
2. Primer CLANKS 5'-GAG TAG GCC TAC GTA GTC TAG AAT TCC ACC-3'.
3. Primer DIR899E 5'-GCT AGG AAT TCC ATC CAT GGC CAT GCG - 3'.
4. Primer DIFE401A 5'-TTA CGA ATT CCT ATT ACG CTT GAA CCA T-3'.
5. Primer DIF2XOOX 5'-CAC ATC TCG AGT CCG CCT GAA CCA TGA-3'.
6. Primer DIR81E 5'- AGC AGA ATT CTG ATG AAC AAC -3'.
7. Primer DIFYE 5'-TTA CGA ATT CCT ATT ACG CTT GAA CCA-3'.
8. Vector, pGEX-KG.
9. Plasmid pAD97 containing DEN1 (S275/90) E gene.
10. pGEX-KG/EX20.
11. *P. pastoris* expression vectors pHIL-S1 and pHIL-D2 (Invitrogen).
12. Plasmid pFA/1 containing DEN1 (S275/90) structural genes, CprME.
13. Thermal cycler.
14. Agarose gel electrophoresis apparatus.
15. Sequenase Version 2.0 (United States Biochemical).
16. DNA sequencing apparatus.

17. Qiagen gel elution kit.
18. Restriction enzymes: *Bgl*II, *Eco*RI, *Not*I, *Sma*I, *Stu*I, *Xba*I, *Xho*I.
19. T4 polymerase.
20. 35 mm dishes and 90 mm culture plates.
21. Spheroplasts of *P. pastoris* strain GS115 (his 4) for transformation.
22. Regeneration dextrose agar: 1 *M* sorbitol, 2% dextrose, 0.002% biotin, 0.005% L-glutamic acid, 0.005% L-methionine, 0.005% L-lysine, 0.005% L-leucine, 0.005% L-isoleucine, and 1% agar.
23. Minimal methanol agar: 1.34% yeast nitrogen base, 0.002% biotin, 0.5% methanol, and 1.5 % agar.
24. Minimal dextrose agar: 1.34% yeast nitrogen base, 0.002% bioitn, 2% dextrose, and 1.5 % agar.
25. BMGY: 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.002% biotin, 1% glycerol, and 100 m*M* sodium phosphate, pH 6.0.
26. BMMY: 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.002% biotin, 0.5% methanol, and 100 m*M* sodium phosphate, pH 6.0.
27. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) apparatus.
28. Polyclonal antibodies to purified DEN-1 virus.
29. Polyclonal antibodies to bacterial-expressed DEN E protein.
30. Developing solution: 100 μg/mL 4-chloro-1-naphthol, 0.01%hydrogen peroxide, 150 m*M* NaCl, 50 m*M* sodium phosphate, pH 7.0.
31. Glutathione sepharose 4B resin (Pharmacia).
32. Phosphate-buffered saline (PBS), pH 7.2.
33. Denaturing buffer: 0.5% SDS and 1% mercaptoethanol.
34. Peptide *N*-glycosidase F (PNGase F)(New England Biolabs).
35. PNGase F reaction buffer: 50 m*M* sodium phosphate, pH 7.0, containing 1% NP40.
36. Endoglycosidase H (EndoH) (New England Biolabs).
37. EndoH reaction buffer: 50 m*M* sodium citrate, pH 5.5.
38. 5 to 50% sucrose solutions, prepared in 1X PBS.
39. Beckman Ultracentrifuge and SW 41 Ti rotor.
40. SW 41 Ti ultracentrifuge tubes (344059).
41. Electron microscopy grids (Copper grids, coated with formvar).
42. 2% glutaraldehyde.
43. Uranyl acetate: one part of 2% uranyl acetate to nine parts of 1.8% methyl cellulose.
44. Transmission electron microscope.
45. DEN 1 virus-infected Vero cells (African Green monkey cells; ATCC, CCL-81) in monolayer.
46. Acetone.
47. Preimmune and immune sera to virus-like particles.
48. Donkey anti-rabbit immunoglobulin (Ig)G conjugated to fluorescein isothiocyanate (FITC).
49. Florescent mounting fluid, DAKO.
55. Florescence microscope.

3. Methods

The methods and techniques used for the expression and characterization of DEN viral glycoproteins are as follows:

1. Construction of E gene in *P. pastoris* expression vectors.
2. Expression of DEN E glycoproteins in *P. pastoris*.
3. Determination of N-linked glycosylation sites.
4. Formation of VLPs.

3.1. Construction of the Recombinant P. pastoris *Expression Vectors*

The virus gene sequences were first inserted into the appropriate yeast transfer vector. In these vectors, the expression of the recombinant protein is under the control of the alcohol oxidase (AOX 1) promoter *(11,12)*. The AOX 1 promoter is inducible by the presence of methanol in the absence of any other carbon source. As *P. pastoris* is a methylotrophic yeast, it consumes methanol readily, thus generating large quantities of the alcohol oxidase to as much as >30% of the total soluble protein. In this way, high-level expression of the genes of interest could be driven from the AOX 1 promoter. In addition, these vectors also contain selectable markers, different types of affinity tags for easy protein purification, and also the use of secretion signals to target the recombinant protein into the growth medium.

The DEN E glycoproteins were constructed under the AOX 1 promoter in two expression vectors, pHIL-S1 and pHIL-D2 (Invitrogen). Both vectors carry the *HIS*4 gene for selection in *his*4 strains, and the 3' AOX 1 sequences for integration into the host genome. In addition, the pHIL-S1 vector contains the *P. pastoris* alkaline phosphatase signal sequence, which targets transport of the recombinant protein into the yeast secretory pathway. The methods used for the expression of recombinant proteins from *P. pastoris* transformed with pHIL-S1 and pHIL-D2 are described under **Subheadings 3.1.1.–3.1.3.**

3.1.1. Construction of DEN E Gene in pHILS1 Expression Vectors

1. Amplify the GST coding sequence with GST-specific forward primer CRGSTX and reverse primer CLANKS, from vector pGEX-KG with PCR.
2. Analyze and elute the 0.7-kbp PCR product from 1.2 % agarose gel electrophoresis using standard methods.
3. Digest the purified product with *Xho*I and *Stu*I, and ligate into the same sites in pHIL-S1 to generate pHIL-S1/GST (*see* **Note 1**).
4. Design specific primers to clone the E gene of different lengths (1 to 495 amino acids, 1 to 401 amino acids, and 1 to 213 amino acids), and engineer restriction enzyme sites into the 5' end of the sequences.
5. PCR amplify the full-length of E gene (1 to 495 amino acids) with primers DIR899E and DIFYE, using plasmid pAD97 as the template.

6. PCR amplify truncated E genes (1 to 410 amino acids, and 1 to 213 amino acids) with primers CRGSTX and DIFE401A, and CRGSTX and DIF2X00X respectively, using vector pGEX-KG/EX20 as the template.
7. Analyze all PCR products in a 1% agarose gel electrophoresis and size-fractionate the PCR products of interest from the gel.
8. Sequence all PCR product completely with dideoxy sequencing method using Sequenase Version 2.0.
9. Perform *EcoR*I digestion on the purified PCR product containing the full E gene (1 to 495 amino acids), and ligate to the *EcoR*I-digested pHIL-S1/GST.
10. Perform *Xho*I/*Stu*I digestion on the purified PCR product obtained for the truncated E gene (1 to 401 amino acids), and ligate to the same sites of pHIL-S1.
11. Digest the PCR product obtained for the truncated E gene (1 to 213 amino acid) with *Xba*I, blunt-ended with T4 polymerase, and ligated to *Xho*I/*Sma*I digested pHIL-S1.
12. Linearise the recombinant vectors with *Bgl*II digestion and proceed with the transformation into spheroplasts (*see* **Subheading 3.2.1.**).

3.1.2. Construction of DEN E Gene in pHIL-D2 Expression Vectors

1. Design DEN 1 specific primers covering the genes CprME, and engineer *EcoR*I sites into the 5' end of the sequences.
2. PCR amplify CprME with primers DIR81E and DIFYE, using plasmid pFA/1 as the template.
3. Analyze the PCR product in a 1% agarose gel electrophoresis.
4. Size-fractionate the 2.3 kbp PCR product of interest from the agarose gel.
5. Sequence the PCR product completely using the dideoxy sequencing method to confirm the sequence.
6. Perform *EcoR*I digestion on the purified PCR product, as well as the *P. pastoris* expression vector pHIL-D2.
7. Ligate the *EcoR*I digested PCR product to the same sites on pHIL-D2.
8. Linearise the recombinant vector, pHIL-D2/CprME, with *Not*I digestion and proceed with the transformation into spheroplasts (*see* **Subheading 3.2.1.**).

Figure 1 is a schematic diagram depicting the construction of expression vectors. DEN E proteins of different length were constructed under the AOX1 promoter in the two expression vectors. Different lengths of the E gene, fused at its 5' end with a GST tag for affinity purification later, were expressed in *P. pastoris* using pHIL-S1 (**Fig. 1A**). Full-length E proteins (GST E495, representing 1-495 amino acids) and truncated forms (GST E213, and GST E 401 representing 1-213 and 1-401 amino acids respectively from the N-terminal) were successfully expressed. Using a second vector, pHIL-D2, which is designed for intracellular protein expression but containing the same AOX1 promoter, the E protein was co-expressed as part of the CprME construct (**Fig. 1B**). In the latter case, the DEN virus glycoproteins are targeted into the yeast secretory pathway by the endogenous virus signal sequences which are present in the virus glycoproteins.

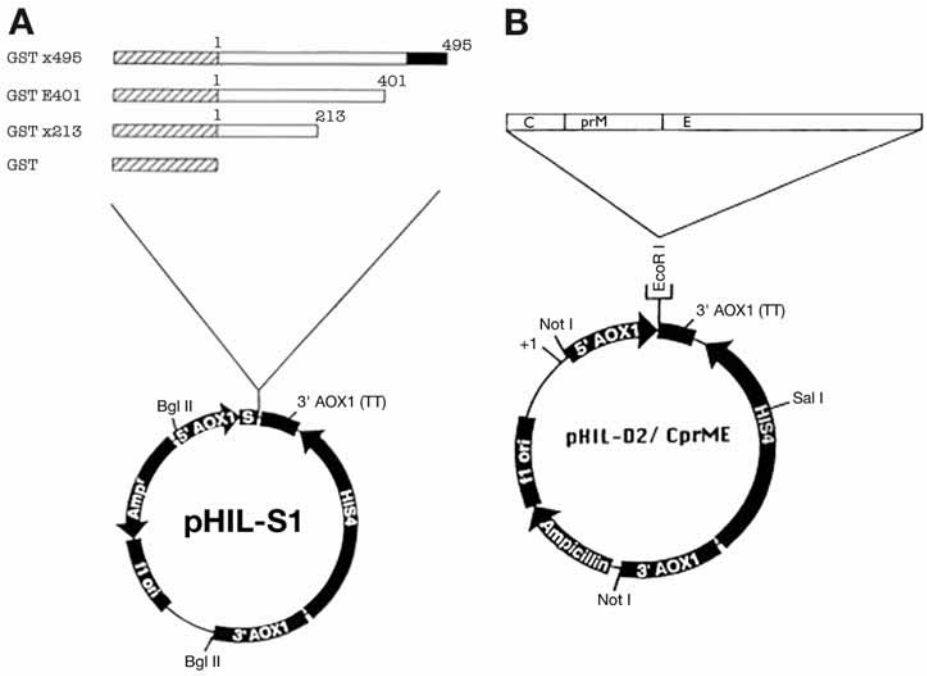

Fig. 1. Construction of the *Pichia pastoris* transfer vectors containing the DEN virus protein sequences. **(A)** PCR products containing the glutathione-*S*-transferase (GST) and regions of the E protein sequence were inserted into pHIL-S1. The GST (hatched) and E protein (clear) coding regions and E protein transmembrane regions (black) are highlighted. S represents the signal sequence. **(B)** The DEN virus sequence containing the C, PrM, and E protein were inserted into pHIL-D2. In all cases the protein expression was under the control of the AOX1 promoter.

3.2. Analysis of Recombinant E Protein Expression

The methods used for the transformation of yeast recombinant vectors generated under **Subheading 3.1.**, and the expression of the DEN E recombinant proteins, including its secreted forms, are described under **Subheadings 3.2.1.** and **3.2.2.**

3.2.1. Yeast Transformation, Growth and Induction

1. Linearize the yeast recombinant vectors under **Subheadings 3.1.1.** and **3.1.2.** with *Bgl*II.
2. Transform the linearized yeast recombinant vectors with spheroplasts of *P. pastoris* strain GS115 (his 4) (*see* **Note 2**).

3. Incubate at 30°C for 6 d in regeneration dextrose agar.
4. Select histidine prototrophs and regenerate yeast colonies by replica plating on minimal methanol and minimal dextrose agar plates.
5. Incubate plates at 30°C for 2 d.
6. Select yeast colonies which grow on minimal dextrose agar but not minimal methanol agar plates.
7. Grow selected yeast transformants in BMGY at 30°C for 48 h.
8. Induce protein expression by resuspending the culture in BMMY and incubating at 30°C for another 96 h.
9. Analyze the proteins by 12% SDS-PAGE and western blotting with polyclonal antibody to DEN-1 virus.
10. Visualize protein bands using developing reaction.

3.2.2. Secretion of Recombinant E Protein

1. Induce the transformant in BMMY at 30°C for 96 h (*see* **Subheading 3.2.1., step 8**).
2. Clarify the culture supernatant by low-speed centrifugation.
3. Add 50 µL of glutathione sepharose 4B beads 50% (w/v, prewashed with PBS) to 0.25 mL of the culture supernatant.
4. Incubate at 4°C for 60 min with gentle shaking.
5. Wash the E protein bound to the glutathione sepharose 4B resin 6X with PBS.
6. Analyze the proteins by 12% SDS-PAGE and western blotting with polyclonal antibody to E protein (*see* **Subheading 3.2.1., steps 9** and **10**).

The results showing the expression of recombinant DEN E proteins in truncated forms (115, 213, and 401 amino acids from the N-terminal) and full-length (495 amino acid), as well as co-expression with C and prM proteins, are shown in **Figs. 2** and **3**. The yeast transformants were harvested by centrifugation and lysates prepared by vortexing the cells with glass beads. The lysate was clarified to remove the unbroken cells. The intracellular expression of DEN E proteins in *P. pastoris* was analyzed by Western blotting with antisera specific to bacterial-expressed E protein (**Fig. 2**, lanes 2 to 4) and the GST control with antiserum to bacterial-expressed GST (**Fig. 2**, lane 1). Yeast transformants representing GST E213 (lane 2), GST E401 (lane 3) and GST E495 (lane 4) were able to express truncated E proteins of the expected size in a single antigenic species, and the full-length E proteins respectively. The expression of the GST E495 fusion protein in *P. pastoris* was accompanied by extensive proteolytic degradation as shown by the major species of protein at size 50 kDa.

Although all the DEN E sequences were expressed using pHIL-S1, which was designed to secret recombinant protein into the growth media, only the truncated E protein which had its transmembrane region deleted (from its C-terminal) was secreted successfully. A 68-kDa band representing the recombinant GST-E401 protein was the only E protein species detected (**Fig. 3**, lanes 3 and 4).

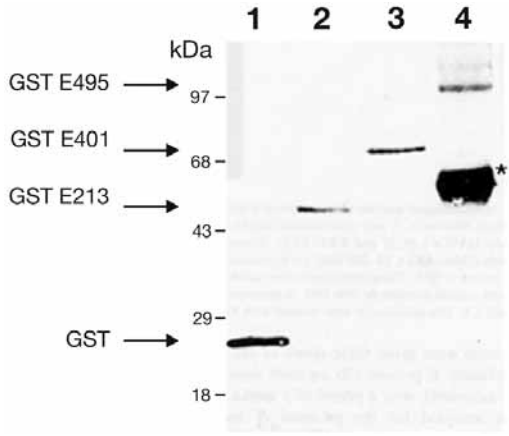

Fig. 2. Expression of recombinant DEN E protein in *Pichia pastoris*. The yeast transformants were harvested and lysates prepared by vortexing the cells with glass beads. The lysates were then clarified and analysed by Western blotting. The expression of DEN E proteins was probed with antisera specific to bacterial-expressed E protein. The various E protein species are indicated as glutathione-*S*-transferase (GST) E213 (lane 2), GST E401 (lane 3) and the full-length E protein, by GST E495 (lane 4). The transformant expressing GST was probed with antiserum to bacterial-expressed GST (lane 1). *Indicates possible intracellular degradation of GST E495.

3.3 Analysis of the Glycosylation in E Protein

Some viral glycoproteins expressed in *P. pastoris* have been reported to be expressed in a hyperglycosylated state ***(6,13)*** (*see* **Note 3**). This in turn has the potential to significantly change both the antigenicity and immunogenicity of the proteins expressed. One procedure that can be used to determine the glycosylated state of the recombinant protein, is to digest the protein with two common endoglycosidases that cleave *N*-linked glycans, namely peptide *N*-glycosidase F (PNGase F) and Endoglycosidase H (EndoH). PNGase F is able to remove the entire carbohydrate moiety from proteins modified by *N*-linked glycoslyation, and EndoH specifically removes high mannose chains. The electrophoretic migration pattern for the digested and undigested proteins could be assessed by SDS-PAGE gel and Western blotting with the specific antisera. The method used to determine the glycosylation status of the expressed recombinant E protein is described under **Subheading 3.3.1.**

3.3.1. Glycosidase Digestion

1. Induce the transformant containing the CprME construct (*see* **Subheading 3.2.1., step 8**).

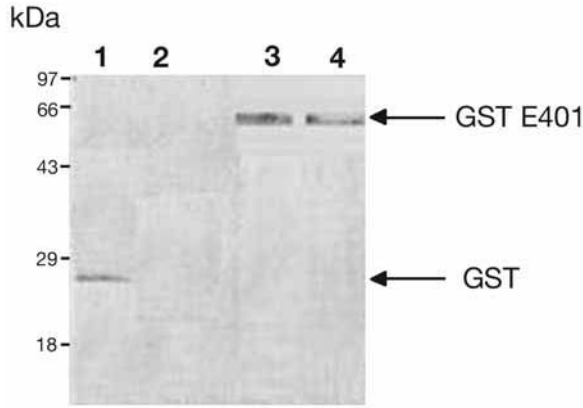

Fig. 3. Secretion of recombinant DEN E proteins. The culture supernatants of yeast transformants expressing either glutathione-*S*-transferase (GST) (lane 1) or GST E401 (lanes 3 and 4) were incubated with Glutathione Sepharose 4B resin and the bound proteins were separated using 12% SDS-PAGE and analysed by Western blotting. Also shown is the culture supernatant from a yeast tranformant produced using pHIL-S1 only (lane 2). Lanes 1–3 were probed with anti-GST while lane 4 was probed with antiserum raised against bacterial-expressed E protein.

2. Clarify the culture supernatant by low-speed centrifugation.
3. Add denaturing buffer to the clarified lysates, incubate at 100°C for 10 min, cool to room temperature.
4. Aliquote the denatured proteins into four portions.
5. For EndoH reaction, make up the lysates in 50 m*M* sodium citrate, pH 5.5 and add 2000 U of EndoH or mock-treat without adding EndoH.
6. For PNGase F reaction, make up the lysates in 50 m*M* Sodium phosphate with 1% NP-40, and add 2500 U of PNGase F or mock-treat without adding PNGase F.
7. Analyze the proteins by 12% SDS-PAGE and western blotting with polyclonal antibody to E protein (*see* **Subheading 3.2.1., steps 9** and **10**).

Figure 4 shows the analysis for hyperglycosylation in the E protein expressed in *P. pastoris*. The yeast transformant expressing the CprME sequence was induced, the cells lysed and the clarified lysate subjected to PNGase F (lanes 1 and 2) and EndoH digestion (lanes 3 and 4). The DEN E proteins were analyzed by Western blotting and the membranes probed with antiserum raised to bacterial-expressed E protein. There was a change of approx 6 kDa in the electrophoretic migration of the E protein after digestion with both PNGase F (lane 2) and EndoH (lane 4) reactions. Our results showed that the DEN E protein was sensitive to EndoH-treatment, further indicating that the protein had been modified by the addition of small mannose chains via *N*-linked glycoslyation,

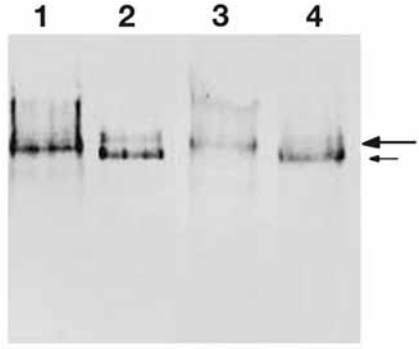

Fig. 4. Glycosylation analysis of the recombinant E proteins. The E protein co-expressed with C and prM was subjected to PNGase F (lanes 2) and EndoH digestion (lanes 4). The DEN E proteins were separated by SDS-PAGE and transferred by Western blotting onto PVDF membranes. The membranes were then probed with antiserum raised to bacterial-expressed E protein. Lanes 1 and 3 represent mock-treated reactions. The long and short arrows represent a change in the migration pattern before and after digestion.

but without the addition of complex sugars. The size difference of 6 kDa between glycoslyated and nonglycoslyated DEN E protein is consistent with the modification of both the potential sites in the E protein. Our results confirmed that there was no hyperglycoslyation following the expression of the E protein.

3.4. Formation of Virus-Like Particles

Co-expression of the flaviviral E and prM proteins have been reported in a variety of animal cell lines to result in the formation of VLPs. These VLPs have been reported to induce neutralizing antibodies against the wild-type virus particles, indicating their potential as possible sub-unit vaccine candidates *(14,15)*. Biophysical methods could be used to determine the presence of VLPs by performing a density gradient, and analyzing each fraction using western blotting with antisera to the specific protein of interest. The morphology and size of the VLPs could also be visualized under the transmission electron microscopy (TEM). The methods used to confirm VLP formation with recombinant DEN E protein in *P. pastoris* are described under **Subheadings 3.4.1.–3.4.4.**

3.4.1. Sucrose Gradient Centrifugation

1. Induce the transformant containing the CprME construct (*see* **Subheading 3.2.1., step 8**).
2. Prepare a clarified yeast lysate.

3. Prepare a gradient in the centrifuge tube from 5 to 50% of sucrose solutions made up in PBS (*see* **Note 4**).
4. Leave the tube overnight at 4°C or 4 h at room temperature.
5. Layer the clarified lysate, containing the CprME proteins over the gradient.
6. Centrifuge at 4°C for 16 h at 100,000*g* in the Beckman SW 41 Ti rotor.
7. Fractionate the gradient into 13 fractions.
8. Analyze each fraction with Western blotting with polyclonal antibody to E protein (*see* **Subheading 3.2.1., steps 9** and **10**), and analysis by transmission electron microscopy (*see* **Subheading 3.4.2.**).

3.4.2. Electron Microscopy Analysis

1. Place 10 µL of each sucrose fraction onto the EM grid. Drain dry.
2. Wash in PBS, pH 7.4 for 10 min at room temperature.
3. Fix in 2% glutaraldehyde for 5 min at room temperature.
4. Wash in PBS, pH 7.4 for 5 min at room temperature. Repeat four times.
5. Wash grid in distilled water for 5 min at room temperature. Repeat four times.
6. Stain suspension with uranyl acetate for 5 min at room temperature.
7. Air-dry.
8. Examine with a transmission electron microscope.

3.4.3. Immunoflorescence Assay

1. Fix virus-infected Vero cells in prechilled acetone for 10 min.
2. Incubate the virus-infected cells with preimmune and immune sera for 1 h at 37°C.
3. Wash cells three times with PBS.
4. Stain with donkey anti-rabbit IgG conjugated to FITC, and incubate further for 1 h at 37°C.
5. Wash cells three times with PBS.
6. Mount cells with florescent mounting fluid, and examine with florescence microscopy.

To determine whether the co-expression of C, prM, and E could form VLPs, the yeast transformmant containing the CprME construct was induced, the cells lysed, and the clarified lysate applied to a 5 to 50% sucrose gradient, which was centrifuged for 16 h at 100,000*g* (**Fig. 5A**). Our results indicate that fraction 3, collected from the top of the gradient, indicates the presence of E protein when analysed by Western blotting with the antiserum raised to bacterial-expressed E protein (lane 3). When the peak fraction 3, was further analyzed by TEM, VLPs were observed at a high magnification of ×60,000 (**Fig. 5B**).

The immunogenic nature of the VLPs was further examined by immunizing rabbits with the purified VLPs harvested from fraction 3 (six doses of approx 25 µg per dose were administered to each rabbit under standard procedures). DEN 1 virus-infected Vero cells showed positive florescence staining when reacted with the antisera raised against the VLPS fraction, but not with the preimmune serum (**Fig. 6**).

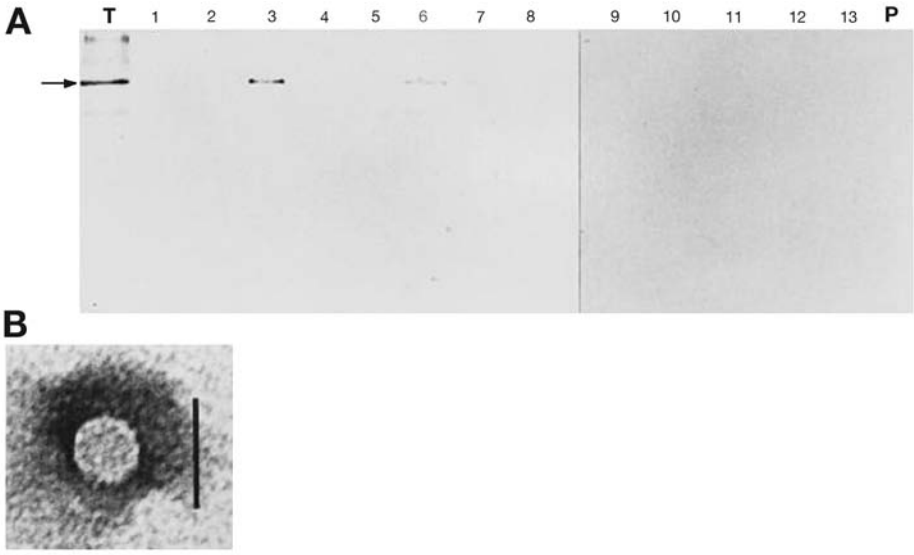

Fig. 5. Biophysical analysis of recombinant E proteins. Protein expression in the yeast transformant containing the CprME construct was induced, the cells lysed and the clarified lysate applied to a 5 to 50% sucrose gradient. **(A)** The sucrose gradients were fractionated and the individual fractions analyzed by Western blotting with E protein antiserum. Lane T represents the total sample applied to the gradient before centrifugation, 1 to 13 fractions collected from the top of the gradient to the bottom, and P, the pellet obtained after the centrifugation. The arrow indicates the E protein. **(B)** The peak fraction, 3, was further analyzed by TEM, at a high magnification of ×60,000. Bar represents 50 nm.

4. Notes

1. The pHIL-S1 vector was the original vector for *Pichia* expression. In order to express the protein of interest with a tag for easy purification, we inserted the GST gene at the 5' end of the DEN E gene. In this way, different lengths of the E gene were expressed, fused with GST. However, the pPICZ series of vectors (Invitrogen) contains a 10 amino acid epitope, derived from c-myc, as well as a C-terminal polyhistidine tag. These vectors allow the gene of interest to be easily cloned, with the fusion tag of both the c-myc and poly-His, for easy purification and identification.
2. The original *Pichia* vectors described in this book chapter were transformed by spheroplasting. The protocols for chemical transformation and electroporation of vectors into *Pichia* are available now, and this reduce the time to obtain transformants from 8 d to 2–4 d.

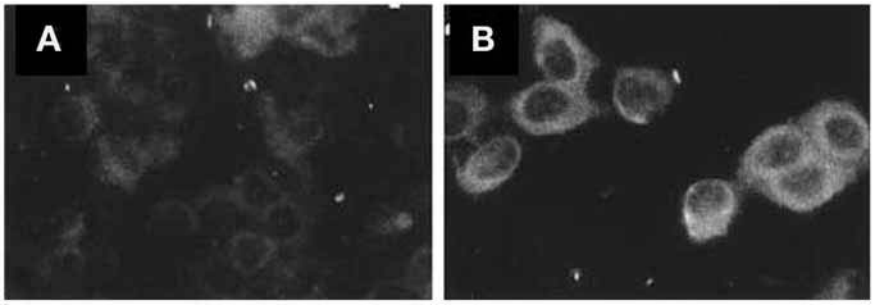

Fig. 6. Immunogenic analysis of the recombinant E proteins. The purified VLPs in fraction 3 were used to immunize rabbits. Preimmune **(A)** and VLP antisera **(B)** were then used in an immunofluoresecence assay on DEN 1 virus-infected Vero cells. The positive staining of DEN virus infected cells can be seen in **(B)**.

3. The secreted recombinant glycoproteins produced in *Pichia* may resemble that of glycoproteins produced in mammalian system as they are not hyperglycosylated. The *Pichia* does not have core oligosaccharides with terminal α1,3 glycan linkages. At the posttranslational level, the *N*-linked glycoslyation sites in *Pichia* are modified with short mannose residues, 8–14, as compared to the 50–150 residues added in *S. cerevisae*, causing hyperglycosylation in the latter.
4. Sucrose gradient is commonly used to investigate viral protein complexes. The sucrose gradient needs to be fractionated after the centrifuging, and other methods are required to analyse the protein complexes. In this book chapter, we used electron microscopy to provide a visual observation of the morphology for the VLPs produced. Western blotting of the VLPs with specific antisera would also provide further identification of the protein expressed, which was not shown in this chapter. Instead, we used the VLPs purified from the sucrose fraction to raise antisera in rabbit. The immunogenicity of this antiserum was confirmed using by immunofluorsecnce staining of DEN virus-infected cells

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13

Cloning, Expression, and Functional Analysis of Patient-Derived Hepatitis C Virus Glycoproteins

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Summary

Hepatitis C virus (HCV) infection is a major cause of severe chronic liver disease including cirrhosis and hepatocellular carcinoma. HCV has been classified into six major genotypes that exhibit extensive genetic variability, particularly in the envelope glycoproteins E1 and E2. Knowledge of genotypic and quasispecies variation on viral glycoprotein properties is important in understanding the structure–function relationship of the proteins. Through their perceived role as components of the virion and mediators of virus attachment and entry, HCV glycoproteins are primary targets for the development of antiviral agents. In this chapter, we describe methods optimized to extract E1E2-encoding sequences of all the major genotypes from HCV-infected patient sera, and their amplification, cloning, expression, and biochemical characterization. Furthermore, we describe a method to generate retroviral nucleocapsid pseudotyped with HCV E1E2 of diverse genotypes (HCVpp) whereby infectivity of the retroviral particle is conferred by HCV glycoproteins. Finally, we show how the HCVpp can be used in an infection assay to determine the viral glycoprotein function at the level of the host–pathogen interface and subsequent events leading to virus infection.

Key Words: HCV; HCVpp; E1E2 glycoproteins; virus entry; CD81; SR-B1; RT-PCR; antibodies.

1. Introduction

Hepatitis C virus (HCV), a member of the *Flaviviridae*, is an enveloped virus containing a positive strand genomic RNA which encodes a single polyprotein of approx 3010 amino acids that is processed co- and posttranslationally by host and viral proteases into at least 10 different proteins (C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) ***(1)***. HCV exhibits a high degree of genetic

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variability—it can be classified into 6 genetically distinct genotypes and further subdivided into at least 70 subtypes, which differ by approx 30% and 15% at the nucleotide level, respectively *(2)*. The viral proteins responsible for cell attachment and entry of hepatitis C virus (HCV) are the glycoproteins E1 and E2 *(3)*. In vitro expression experiments have shown that E1 and E2 proteins form a noncovalent heterodimer, which is proposed to be the functional complex on the virus surface *(4–7)*. As such, the expression of these glycoproteins has important application in vaccine discovery and drug targeting. Initial studies of glycoprotein structure and function have focused on a limited number of molecular clones. However, recently we have developed techniques to enable recovery of functional E1 and E2 genes from patient samples, and their expression in mammalian cells *(8,9)*. These samples have allowed characterization of common entry pathways of divergent strains of HCV, and analysis of the differences in the glycoprotein phenotype during the progress of disease.

The function of HCV E1E2 in cell attachment and entry has been investigated using the recently developed retrovirus-based pseudo-particle (pp) assay whereby infectivity of the retroviral particles is conferred by HCV E1E2 envelope proteins *(10,11)*. This HCVpp assay has been particularly useful in the functional analysis of HCV E1E2 derived from patients infected with diverse genotypes and subtypes of the virus, in dissecting the role in virus entry of key E2 receptors, CD81 and SR-B1, and in measuring the capacity of antibodies and patient sera to neutralize infection of target cells by HCVpp *(8,9,12–15)*.

Here, we describe methods optimized for recovery, amplification and expression of HCV glycoproteins, and their functional analysis using the HCVpp system.

2. Materials

1. pcDNA3.1 V5-DTOPO cloning vector (Invitrogen, UK).
2. HCV antibody and/or HCV RNA positive plasma or serum samples.
3. Viral RNA Isolation Kit, QIAPrep™ spin miniprep kit, QIAquick™ Gel Extraction kit (all from Qiagen, UK).
4. Oligonucleotide primers.
5. Restriction enzymes, T4 DNA ligase (Roche, UK), Expand High Fidelity DNA polymerase (Roche, UK), HotStarTaq (Qiagen, UK), Thermoscript cDNA synthesis kit (Invitrogen, UK).
6. *Escherichia coli* One-Shot™ TOP10F' cells (Invitrogen, UK).
7. Agarose gel, sodium dodecyl sulfate (SDS)-polyacrylamide gel, and Western blot equipment.
8. ABI PRISM® 3100 sequencer, ABI "Big Dye" DNA sequencing reagents.
9. Tissue culture flasks and dishes (Nunc), Minisart Single use syringe filter (Sartorius, UK).

10. Human hepatoma cells Huh-7 ***(16)*** and human epithelial kidney (HEK-293T) cells (ATCC CRL-1573).
11. 2.5 *M* $CaCl_2$, 2x HeBS and sterile distilled water supplied with the calcium phosphate transfection kit (Sigma, UK).
12. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), Pen/Strep, Glutamine (Life Technologies, UK).
13. Flowcytometer FACSCalibur.
14. Antibodies to HCV E2, and MLV capsid; Protein A-HRP, anti-mouse IgG-HRP and Protein A-Sepharose (Sigma, UK).
15. L-[^{35}S] Redivue™ Pro-Mix™ (Amersham Biosciences, UK).
16. Nunc Maxisorp ELISA plates.
17. GNA (*Galanthus Nivalis*) lectin (Sigma, UK).
18. BD Vacutainer collection tubes (Becton Dickinson, UK).
19. Lysis buffer 2 : LB2; 20 m*M* Tris-HCl, pH 7.4; 20 m*M* iodoacetamide; 1 m*M* EDTA; 150 m*M* NaCl; 1% Igepal C630.
20. Reducing protein gel loading buffer: 200 m*M* Tris-HCl, pH 6.7; 0.5% SDS, 10% glycerol, 20 m*M* dithiothreitol (DTT).
21 Nonreducing protein gel loading buffer: 200 m*M* Tris-HCl, pH 6.7; 0.5% SDS, 10% glycerol.
21. Western blotting blocking solution: 5% milk solution in phosphate-buffered saline (PBS) 0.05% Tween-20.
22. PBS-T: PBS, 0.05% Tween-20.
23. TBS-T: Tris-buffered saline 0.5% Tween-20.
23. Wash buffer 1: 10 m*M* Tris-HCl, pH 7.4; 1 m*M* EDTA; 150 m*M* NaCl; 0.2% Igepal C630.
24. Wash buffer 2: 10 m*M* Tris-HCl, pH 7.4; 1 m*M* EDTA; 150 m*M* NaCl; 0.2% SDS; 0.2% Igepal C630.
25. Wash buffer 3: 10 m*M* Tris-HCl, pH 7.4; 1 m*M* EDTA; 500 m*M* NaCl; 0.2% Igepal C630.
26. FPBS: PBS plus 2% FCS and 0.01% sodium azide.

3. Methods

The methods described in outline (a) the isolation and cloning of HCV glycoprotein-encoding cDNA from patient sera, (b) the characterization of the glycoproteins, and (c) the generation of HCVpps and their functional analysis.

3.1. Isolation of HCV E1E2-Encoding Sequences From Different Genotypes and Their Cloning in a Mammalian Expression Vector

3.1.1. Collection of Serum for Extraction of Viral Genomic RNA

1. Obtain patient blood collected in BD Vacutainer collection tubes from clinic and isolate serum using red top serum separation tubes (SST). Recover serum after centrifugation at 2000*g* for 10 min and store at –80°C (*see* **Note 1**).

2. If possible, ensure the genotype of the HCV present in the serum/plasma samples has been determined. Because of the variability in nucleotide sequence observed across the envelope genes, particularly in E2, specific primers are required for successful amplification of the majority of clinical samples. Use a genotyping assay that is capable of resolving all six genotypes of HCV. Genotyping can be performed using commercially available assays, for example Inno-LiPA (Innogenetics, Belgium) or using in house PCR methods *(17)*.
3. Isolate viral RNA from 140 µL of serum using a Viral RNA Isolation Kit. Bring serum to room temperature in 1.5-mL microcentrifuge tube, vortex for 10 s and add 560 µL of lysis buffer AVL (*see* **Note 2**).
4. Vortex the mixture for 15 s and incubate at room temperature for 10 min, before pulse centrifuging the sample to recover all of the solution. Add 560 µL of molecular grade 100% ethanol, vortex for 15 s and pulse-centrifuge.
5. Place a QIAamp spin column into a 2-mL collection tube, pipet 630 µL of sample carefully onto the membrane, and centrifuge the tube for 1 min at 6000*g*. Discard the flow through and add the remaining sample to the column, repeating the centrifugation step.
6. Place the spin column into a clean collection tube, add 500 µL AW1 wash buffer to the column, and centrifuge for 1 min at 6000*g*. Discard the flow through again and add 500 µL AW2 wash buffer. Centrifuge at 20,000*g* for 3 min to prevent residual AW2 buffer remaining on the column. To ensure complete removal of wash buffer, place the column once again into a clean collection tube and centrifuge for 1 min at 20,000*g*.
7. Cut off the lid of a 1.5-mL microcentrifuge tube using scissors, and insert the column. Add 60 µL of elution buffer AVE and centrifuge at 6000*g* to recover purified RNA. Smaller volumes of elution buffer can increase the concentration of recovered RNA, but generally result in lower yield.
8. Store the recovered RNA at –80°C in small aliquots. RNA stored at this temperature is stable for prolonged periods (over 1 yr).

3.1.2. cDNA Synthesis

To generate appropriate template for PCR amplification of the E1 and E2 genes, cDNA is generated with a primer specific for HCV RNA. Primers used are complementary to the conserved regions of the p7 gene, to generate negative-sense cDNA. The cDNA acts as template for PCR of the E1 and E2 genes. Using an appropriate volume of RNA, typically 8 µL, cDNA is synthesized with the addition of primer designed specifically for each genotype of virus (*see* **Note 3**). These primers are listed in **Table 1**. In each case, the outer antisense primer is used for cDNA synthesis.

1. Denature the RNA template in the presence of 15 pmol of primer and 2 µL of a 10 m*M* stock of dNTPs, in a final volume of 12 µL, by heating to 65°C for 5 min and then rapidly cool on ice.

Table 1
Primer Sequences Used for the Generation of cDNA and PCR Amplification of the E1 and E2 Regions of Hepatitis C Virus Genomes of all Genotypes of Virus*

Sense primers		
• All genotypes	OUTER	GGACGGGGTAAACTATGCAACAGG
	INNER	CACCATGGGTTGCTCTTTTTCTATC
Antisense primers		
• Genotype 1	INNER	AAAGTTTCTAGATTASGCCTCAGCYGTGGMTA
	OUTER(1a)	GGGATGCTGCATTGAGTA
	OUTER(1b)	CCGGCCACGGACGCCGCATTG
• Genotype 2	INNER	AAACTTTCTAGATTACGCTTCGGCTTGGCCCA
	OUTER	RGACCATTGGMRCTAGCAGC
• Genotype 3	INNER	AAGATAAGCTTATGCTTCCGCCTGWGAWATC
	OUTER	TGCGCTGAGGGCGTTCAG
• Genotype 4	INNER	GACAGTTACGCCTGAACTTGACTTACCATAAACATC
	OUTER	CACCAGCGGGTGAAGCAGCATTGA
• Genotype 5	INNER	TTATGCTTCGGCCTGACAAACCAAG
	OUTER	GCCAAGCGAAGCAAATAACGAGCGAACCCCAGAAAA
• Genotype 6	INNER	TTATGCCTCTACCTGGCCGATGATCAACATGA
	OUTER	GCAGGGCCAGGATTAGCAGGAGGAGCGGCCA

*Outer antisense primers are utilized for cDNA synthesis, and nested outer and inner pairs of primers used in PCR reactions.

2. Add 4 μL of 5X reaction buffer, 1 μL of 100 m*M* DTT, 40 U of RNaseOUT, 1 μL of Thermoscript reverse transcriptase and 1 μL of RNase-free water. Centrifuge briefly to collect the sample, and mix gently with the tip of a pipet.
3. Incubate the reaction at 50°C for 1 h, then inactivate the polymerase by heating to 85°C for 5 min. Add 2 U of RNase H to remove the template RNA and leave single-stranded cDNA product, and incubate the sample at 37°C for 20 min.
4. The cDNA produced is stable at 4°C for short-term storage, or –20°C for prolonged storage. This single-stranded DNA serves as template for amplification of the E1 and E2 genes by PCR.

3.1.3. Full-Length E1E2 Polymerase Chain Reaction

1. Thaw the template at 4°C, and prepare PCR reaction mixtures (*see* **Note 3**). Amplification of E1E2 from patient samples requires two nested rounds of amplification. In our experience, the Expand High Fidelity Polymerase mixture performs better than most other polymerases. Use 0.5 U of polymerase for the amplification of E1 and E2 in a 25 μL reaction containing 5 pmol each of an appropriate sense and antisense primer, 200 μ*M* concentration of the four dNTPs, and Expand high fidelity buffer containing a final concentration of 1.5 m*M* $MgCl_2$.
2. The primers used for the amplification of E1 and E2 were designed based on knowledge of existing sequence data for these genes. The outer primers are placed in the flanking regions in the HCV core (upstream) and p7 (downstream) coding sequence, and correspond to a highly conserved region of each genotype. The inner primers are located such that encoded ORF following amplification encompassing amino acids 170–746 (with respect to the polyprotein of strain H77c; Genbank accession number AF011751). The primers artificially introduce a start codon at the 5' end of the proposed signal peptide of E1, and a stop codon following the last amino acid of the mature E2 protein. This permits expression of the genes in mammalian cell culture, and incorporation of their products into retroviral pseudo-particles. The inner sense primers also include the sequence CACC at the 5'-end to facilitate directional cloning into the TOPO family of cloning vectors (Invitrogen).
3. To ensure amplification of a representative number of variants present in any given sample, a volume of cDNA possessing 25–50 copies should be used as template for PCR amplification. Copy number can be assessed by limiting dilution PCR *(**18**)*. In brief, twofold serial dilutions of cDNA are amplified, in triplicate, using the full length nested primer E1E2 amplification protocol. The cDNA copy number in the original sample is estimated using the Poisson formula $N = (-\ln f)\ (1/d)$, where N is the viral titre per input volume, f is the frequency of negative tubes at the end-point dilution, and d is the dilution.
4. Amplification cycle parameters are:

1X	94°C for 2 min
25X	94°C for 45 s
	50°C for 45 s
	72°C for 90 s (extending 5 s/cycle)
1X	72°C for 7 min

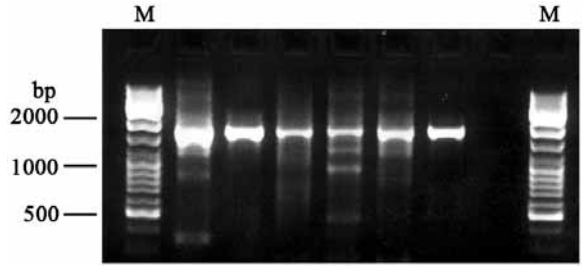

Fig. 1. PCR amplification of a selection of E1E2 sequences from sera of patients infected with hepatitis C virus (HCV) genotype 1a. RNA was extracted from patient sera and cDNA generated using a specific primer (Table 1). The E1E2 sequences were then amplified following two rounds of nested PCR. M, DNA size markers

5. One microliter of the first-round product is then used as template in a second-round PCR amplification using the genotype-specific inner sense and antisense primers. Reaction conditions and cycling parameters are the same as for the first round PCR.
6. Analyze the second-round PCR products on a 2% agarose gel stained with ethidium bromide. Correct PCR amplification results in an amplification product of between 1734 and 1752 bp (**Fig. 1**). In most circumstances, amplification yields a single band. However, in our experience some clinical samples yield multiple PCR products. The desired band can be excised from a gel and purified using a Gel Purification Kit. Once pure product is obtained, these products are accurately quantified using a spectrophotometer. We use a Nanodrop spectrophotometer (Nanodrop Technologies), designed for analyzing small quantities of sample. To obtain an accurate reading, use a negative PCR reaction as a blank sample. From the absorbance recorded, calculate the correct amount of product for use in a cloning reaction.

3.1.4. Cloning of Amplified E1E2 Genes

1. The E1E2 PCR products are cloned into the mammalian expression vector pcDNA3.1D/V5-His-TOPO vector (Invitrogen) (**Fig. 2**), according to the manufacturer's protocol. In brief, add 50 ng of the PCR product to 1 μL of plasmid, 1 μL of salt solution and distilled water to a final volume of 6 μL, and incubate at room temperature for 15 min.
2. Add the entire ligation reaction to a 50-μL aliquot of One-Shot™ TOP10F cells, and incubate the mixture on ice for 20 min. Heat-shock the cells to 42°C for 30 s and return to ice. Add 250 μL of SOC medium to the cells and incubate at 37°C for 1 h. Spread the culture onto a Luria-Bertani (LB) agar plate containing 100 μg/mL ampicillin to select competent cells that have taken up plasmid. Colonies are selected by incubating the plate overnight at 37°C.

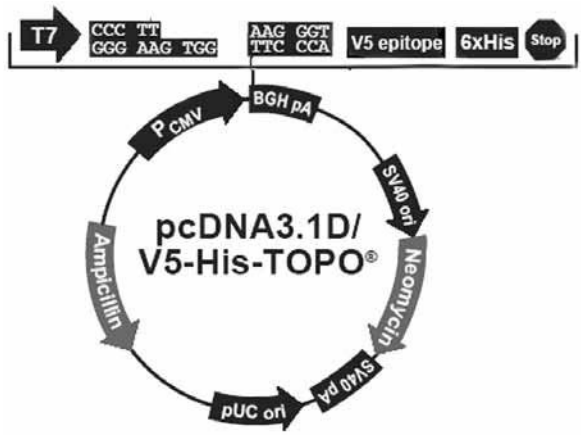

Fig. 2. Schematic drawing of the mammalian expression plasmid adapted from Invitrogen (UK).

3. To identify bacteria harboring cloned E1E2 genes, use individual colonies as a source of template DNA for screening PCR reactions. Prepare PCR reactions containing 5 pmol of each vector-specific primers T7 (TAATACGACTCACTA TAGGG) and BGH (TAGAAGGCACAGTCGAGG), 0.15 μL of HotStarTaq, 200 μ*M* concentration of each dNTP in a 25-μL reaction volume.
4. Pick a portion of each bacterial colony using a sterile, nuclease-free pipet tip and insert into the PCR reaction mix. Use the following thermal cycle for amplification:

1X	95°C, 15 min
25X	94°C, 45 s
	50°C, 45 s
	72°C, 3 min
1X	72°C, 7 min.

5. Following amplification, resolve the products on a 2%, ethidium bromide-stained agarose gel—clones harboring E1E2 will yield a product of approx 1.9 kb (**Fig. 3**). Inoculate 3 mL LB medium containing 100 μg/mL ampicillin with colonies containing E1E2 inserts, and incubate shaking at 225 rpm overnight at 37°C.
6. Aliquot the cultures into two 1.5-mL microcentrifuge tubes, and pellet the cells by centrifugation at 13,000*g* for 2 min
7. Discard the supernatant from one of the tubes and add 400 μL of clean LB medium. Resuspend the pellet thoroughly by vortexing then add 100 μL of sterile glycerol. Mix and store at –80°C for long-term storage.
8. Extract plasmid from the other aliquot of cells using the Plasmid Miniprep Kit as follows. Resuspend the cell pellet in 250 μL of buffer P1 (containing RNase), add 250 μL of P2 buffer, and mix gently by inverting five times to lyse the cells.

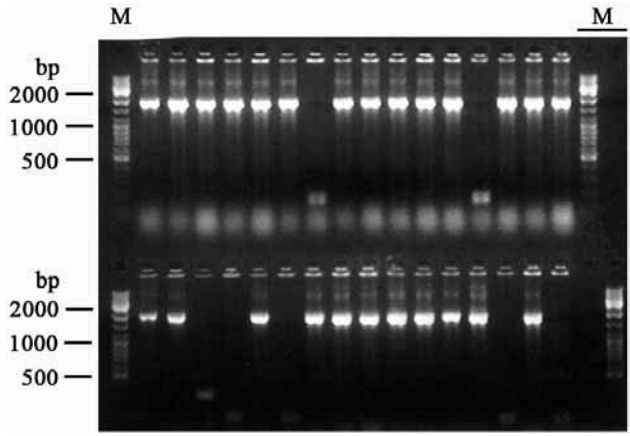

Fig. 3. Screening of transformed bacterial colonies for presence of cloned E1E2 genes. In each sample, a single transformed bacterial colony was used directly as template in a PCR reaction using appropriate vector-specific primers. A PCR product of approx 1900 bp indicates the presence of a cloned E1E2 gene. M, DNA size markers.

Add 350 μL of buffer N3 and mix immediately by inverting two times to precipitate proteins and genomic DNA.

9. Centrifuge the tubes at 10,000*g* for 10 min and remove the supernatant to a Qiaquick column. Spin the sample at 10,000*g* for 1 min, allowing plasmid DNA to bind to the column membrane.
10. Add 700 μL of buffer PE to the spin column and spin for 1 min at 10,000*g*. Discard the flow through and place the tube back into the collection tube. Remove the residual wash buffer by centrifuging at 14,000*g* for 2 min.
11. Transfer the spin column to a clean 1.5-mL microcentrifuge tube without a lid. Add 50 μL of nuclease-free distilled water and centrifuge at 10,000*g* for 1 min to elute the purified DNA.
12. Quantify the plasmid using a spectrophotometer. This DNA is used as template for sequencing reactions, and also for expression and functional studies in mammalian cells described later.

    Determine the nucleotide sequence of individual clones using Big Dye chemistry (ABI). To obtain nucleotide sequence for an entire E1E2 cDNA, three primer sequencing runs are generally required. The primers used are the vector-specific T7 and BGH primers together with an E1-specific primer E1IS (TGG GAT ATG ATG ATG AAC TGG). The E1IS primer is located in a highly conserved region of the HCV genome and therefore works in most instances. In the unlikely event that this primer fails in sequencing runs, a variant specific primer can be designed based on the sequence determined using the vector-specific T7 and/or BGH primers.

13. Approximately 500 ng of plasmid is used in a sequencing reaction containing 3.2 pmol of primer, 2 μL of big dye and 2 μL of dilution buffer (Applied Biosystems) in a total volume of 10 μL. Perform the sequencing reaction in a thermal cycler with the parameters 94°C for 20 s, 50°C for 20 s, 72°C for 6 min, repeated 25 times. Transfer the reaction mix to a 0.5 mL microcentrifuge tube, and add 2 μL of 3 *M* sodium acetate (pH 5.2) and 50 μL of 100% molecular grade ethanol to precipitate the labeled DNA. Incubate the DNA at room temperature for at least 20 min and then centrifuge at 14,000*g* for 45 min. Discarded the supernatant and wash the pellet twice with 250 μL of 70% ethanol, centrifuging at 14,000*g* for 15 min each time. Air-dry the pellet at room temperature and analyze the DNA using an ABI PRISM 3100 sequencer.
14. Sequence assembly and editing is carried out using appropriate DNA analysis software, such as Lasergene (DNAStar Inc), or the freely available Bioedit software (http://www.mbio.ncsu.edu/BioEdit/BioEdit.zip).
15. Phylogenetic and molecular evolutionary analysis of samples can be performed with the MEGA3 software ***(19)*** (www.megasoftware.net), using an implementation of the ClustalX algorithm.

3.2. Characterization of HCV Glycoproteins

3.2.1. Transfection

1. Grow the human epithelial kidney (HEK) 293T cells (ATCC CRL-1573) in T175 tissue culture flask in DMEM (GIBCO BRL) supplemented with 10% FCS (heat-inactivated at 56°C for 30 min), 5% nonessential amino acids, and penicillin/streptomycin (EFC10). Split the cells when they reach 80% confluence by trypsinizing using trypsin-versene solution (e.g., Life Technologies) following removal of the medium and washing once with versene. Incubate cells with trypsin-versene at 37°C until they round up and detach (*see* **Note 4**). Add 10 mL of EFC10 medium to inactivate the trypsin and spin the cell suspension by low-speed centrifugation (5 min at 50 g). Resuspend cell pellet in fresh EFC10.
2. Approximately 24 h prior to transfection, seed 2×10^6 cells in 100-mm tissue culture dishes each containing 15 mL of EFC10 medium.
3. The next day, prepare DNA transfection precipitate as follows: mix 3 μg HCV E1E2 expression plasmid with sterile distilled water to a volume of 400 μL. Add 100 μL of 2.5 *M* $CaCl_2$ and mix. Add the DNA/$CaCl_2$ mix dropwise to 500 μL 2X HeBS in a 2-mL plastic Bijou (Sterilin) while aerating the HeBS using a sterile plastic Pasteur pipet. Vortex and incubate at room temperature for 20 min (*see* **Note 5**).
4. Add the precipitate dropwise directly to cell medium such that it is distributed evenly over the cell monolayer. Mix gently and incubate overnight at 37°C (approx 16 h).
5. Gently replace medium with 5 mL of fresh EFC10 medium containing 10 m*M* HEPES and incubate cells for further 24 h.

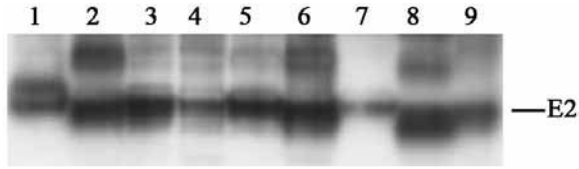

Fig. 4. Western blot of E2 glycoprotein from different genotypes of hepatitis C virus, as detected with the anti-E2 MAbs ALP98 and AP33. Lanes 1 to 3, different isolates of genotype 1; lanes 4 and 5, isolates of genotype 2; lane 6 to 9, isolates of genotype 3, 4, 5, and 6, respectively.

3.2.2. Glycoprotein Analysis

The E1 and E2 proteins expressed in transfected HEK 293T cells are assayed by Western blot, ELISA, and immunoprecipitation for reactivity to antibodies with defined, conserved epitopes.

3.2.2.1. Western Blot

1. After completing **Subheading 3.2.1., steps 1–5**, lyse the transfected HEK cells in 1 mL per dish of lysis buffer 2 for 30 min on ice, spin at 13,000 rpm for 5 min, and collect the clarified supernatant. The clarified lysate may be stored at –20°C.
2. Mix 15 µL volume of transfected cell lysates with an equal volume of protein gel loading buffer, and fractionate the proteins on a SDS-polyacrlyamide gel electrophoresis (PAGE) gel with a 9% resolving gel. Load a molecular weight marker (Rainbow Full-Range Marker, GE Healthcare) alongside to assess the apparent molecular weight of proteins expressed. Transfer the proteins from the SDS-PAGE gel to nitrocellulose membranes using a semi-dry blotting apparatus (Bio-Rad) at 25 V for 40 min.
3. Block the membrane for 1 h with Western blotting blocking solution and then wash three times with 50 mL of PBS-T.
4. Incubate the membrane with a mixture of primary mouse monoclonal antibodies (MAbs) AP33 and ALP98 to HCV E2 *(20)*, each at a concentration of 1 µg/mL in 5 mL of blocking buffer for 1 h at room temperature. Wash the membrane three times each for 10 min with PBS-T.
5. Incubate the secondary antibody, goat anti-mouse immunoglobulin (Ig)G conjugated to horseradish peroxidase, at a dilution of 1/1000 in blocking buffer at room temperature for 1 h. Finally, wash the membrane three times each for 10 min with PBS-T.
6. Visualize the proteins using enhanced chemiluminescence (ECL Plus, GE healthcare). The luminescence is detected using Kodak Light-1 film, typically exposing the film for 1 min and developing with Kodak GBX developer. An example of a protein blot of E2 of diverse HCV genotypes is shown in **Fig. 4**.

3.2.2.2. GNA Capture ELISA

To assess their relative quantities in transfected cells, the HCV glycoproteins in serially diluted cell lysates are first captured in an ELISA dish wells coated with the lectin *Galanthus nivalis* (GNA) followed by detection using an anti-E1 or -E2 MAb. Comparison of protein quantities is determined from the 50% binding dilution for each sample.

1. Coat the wells of ELISA plate (Nunc Maxisorp) with 100 μL 0.5 μg/mL GNA lectin in PBS, overnight at 4°C.
2. Block coated wells with 200 μL of 5% milk PBS-T, incubating for 1 h at room temperature.
3. Wash four times with 300 μL of PBS-T. The plates may be stored at this stage at –20°C for several months or 4°C for several days. Dilute transfected cell lysates (prepared as described in **Subheading 3.2.2.1.**) threefold serially in PBS-T containing 5% milk powder and add samples between neat and 1/81 dilutions to GNA-coated wells.
4. Following incubation for 4 h at room temperature, wash wells four times with PBS-T.
5. Add 100 μL/well anti-E2 MAb ALP98 diluted at a concentration of 1 μg/mL in PBST-milk and incubate for 1 h at room temperature.
6. Wash wells four times with TBS-T, add 100 μL/ well of rabbit anti-mouse IgG-alkaline phosphatase conjugate at a dilution of 1/1000, and incubate for 1 h at room temperature.
7. Wash four times with TBS-T, add 100 μL/well of p-nitrophenol phosphate (pNPP) substrate, incubate in dark at room temperature for up to 30 min, and read absorbance at 405 nm wavelength using a molecular devices Vmax plate reader. An example is shown in **Fig. 5**.

3.2.2.3. Immunoprecipitation

1. Transfect HEK cells as described above **(Subheading 3.2.1.)**.
2. The next day, wash cells once with PBS, add methionine- and cystine-free DMEM (Sigma) medium supplemented with 4% dialyzed heat-inactivated FCS, and penicillin/streptomycin (EDFC4) and 25 μCi/ml L-[^{35}S] Redivue Pro-Mix for 48 h.
3. Incubate at 37°C for 2 d.
4. If analyzing HCVpps (*see* **Subheading 3.3.1.**), collect medium, spin at 850*g* for 5 min to remove cell debris, and collect the clarified medium.
5. Lyse cells in LB2 on ice for 30 min, spin at 14,000*g* for 5 min, and collect the clarified supernatant.
6. Add a mixture of anti-E2 MAbs ALP98 and AP33 to the clarified medium and cell extract and incubate for 2 h (or longer) at 4°C.
8. Equilibrate protein A–Sepharose beads in LB2 to give 50% slurry.
9. Add 25 μL of the slurry to the MAb-containing clarified medium or cells extracts and incubate at 4°C for 2 h rotating.

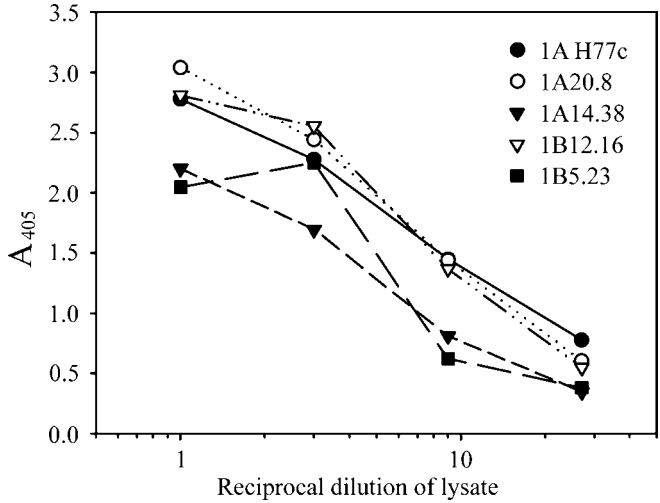

Fig. 5. Titration of 293T cell lysates transfected with E1E2 genes derived from hepatitis C virus genotype 1a (H77.20, 1A20.8, 1A14.38) and 1b (1B12.16 and 1B5.23) isolates. Diluted protein samples were detected with the anti-E2 MAb ALP98.

10. Wash the beads with 1 mL each of the following buffers by spinning at low speed for 2 min:

    Twice with LB2;
    Twice with wash buffer 1;
    Twice with wash buffer 2;
    Twice with wash buffer 3;
    Once with distilled water.

11. Resuspend the final pellet in reducing or nonreducing protein gel loading buffer and analyze by SDS-10% PAGE. Dry the gels and expose to a phosphor screen and visualize the radiolabeled proteins with a Bio-Rad Personal FX phosphorimager. HCV E1E2 glycoproteins are known to form a noncovalent heterodimer (thought to be the functional, prebudded form) and disulfide-linked high molecular weight aggregate *(7)*. The later can be visualized on SDS-PAGE performed under non-reducing conditions. An example is shown in **Fig. 6.**

3.3. Functional Analysis by HCVpp Assay

Because they incorporate envelope glycoproteins from heterologous viruses and integrate and express reporter genes from defective genomes, retrovirus vectors have been very useful in investigating the mechanisms by which various viruses attach and enter their target cells *(21–23)*. The retrovirus-based HCVpp assay described in this chapter, developed by Bartosch et al. *(10)*,

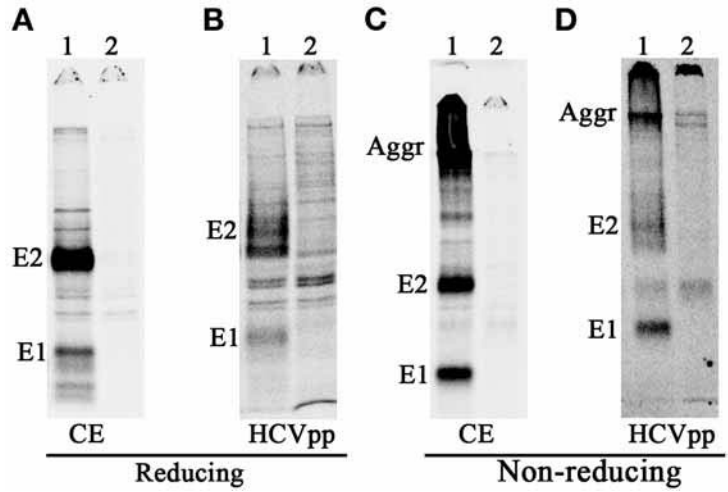

Fig. 6. Immunoprecipitation of the E1E2 hetrodimer. Radiolabeled proteins from the cell extracts (CE, panels **A** and **C**) and medium (HCVpp, **B** and **D**) of HEK 293-T cells co-transfected with the MLVgag-pol + MLV-GFP (lane 2) + a plasmid expressing E1E2 derived from HCV genotype 1a (lane 1) were immunoprecipitated using an anti-E2 MAb AP33 and the immune complexes analyzed under reducing and non-reducing conditions as shown. Aggr, aggregate.

involves co-transfecting HEK-293T cells with plasmids expressing the HCV glycoproteins, the murine leukaemia virus (MLV) Gag-Pol, and the MLV transfer vector carrying the green fluorescent protein (GFP) reporter (*see* **Notes 6** and **7**). Upon expression, the MLV gag-pol particles encapsidate the replication-defective genome carrying the GFP sequence and acquire the HCV glycoprotein-containing envelope before being released into the medium. The HCVpp released in the medium are then used to infect the human hepatoma cells (Huh-7), and the infection measured by detection of GFP following incubation at 37°C.

3.3.1. Infection of Target Cells With HCVpp

1. Co-transfect HEK-293T cells in 100-mm tissue culture dish with three plasmids, carrying sequences encoding MLV gag-pol (8 μg), MLV GFP (8 μg), and HCV glycoproteins (3 μg), and change the medium exactly as described under **Subheading 3.2.1**. Following incubation at 37°C for 24 h, collect the medium containing HCVpp as described in **step 3**.
2. Twenty-four hours before the HCVpp are ready for harvest, prepare target cells as follows. Trypsinize Huh-7 cells as described for HEK-293T under **Subhead-**

ing 3.2.1. Seed in six-well dishes at 1×10^5 cells per well in 3 mL of EFC10 medium. (Alternatively, use 12-well dishes, reducing cell number and volumes accordingly.)

3. Collect the medium containing HCVpp from the HEK-293T cells from **step 1**, spin at 850*g* for 10 min, and filter through a 0.45-μm pore-size Minisart singe-use syringe filter. The HEK-293T cells should be no more than 80% confluent at this stage.
4. Remove medium from Huh-7 target cells and add 0.6 mL per well of HCVpp.
5. Incubate at 37°C for 3 to 4 h, then remove the inoculum and re-feed the cells with 3 mL of fresh EFC10 medium.
6. After 3 to 4 d incubation at 37°C, analyze the infected cells for GFP expression. The level of GFP in HCVpp-transduced cells is too low to be seen using a fluorescence microscope, so the cells must be analyzed by flow cytometry, using a fluorescence activated cell analyzer (FACSCalibur, Beckton Dickinson). This provides accurate and quantitative data even when very low numbers of cells have been effectively transduced.
7. Rinse the cell monolayer in each well with versene, then add a few drops of trypsin/versene and incubate at 37°C for 15 min.
8. Resuspend thoroughly in 1 mL FPBS to obtain a single-cell suspension, and transfer to a suitable tube.
9. Determine the transduction efficiency as the percentage of GFP-positive cells (following subtraction of the number of GFP-positive Huh-7 cells "infected" with the no-envelope control which is typically 0.05%). Calculate the infectious titres, expressed as transducing units per ml, from the transduction efficiency. The relative infectivities of HCVpp incorporating viral glycoproteins from diverse genotypes and subtypes are shown in **Table 2**.

3.3.2. Sucrose Gradient Fractionation of HCVpp

1. Co-transfect HEK-293T cells with plasmids encoding MLV gag-pol, MLV GFP, and HCV glycoproteins as described under **Subheadings 3.2.1.** and **3.3.1.**
2. Collect the medium containing HCVpp from the HEK cells, spin at 800*g* for 10 min, and filter through a 0.45-μm pore-size Minisart singe-use syringe filter (Sartorius).
3. Put 5 mL of 20% sucrose in PBS into a suitable centrifuge tube (of approx 33 mL volume).
4. Gently layer 25 mL filtered supernatant medium over the sucrose cushion.
5. Spin for 3 h at 116,000*g* in a swinging bucket rotor (e.g., Sorval AH-629) at 4°C.
6. Pour off all the liquid, leave tube inverted to drain for a few minutes.
7. Add 0.5 mL PBS to the pellet. Cover with parafilm and leave overnight at 4°C.
8. Gently resuspend the pellet and layer onto a 20 to 60% gradient of sucrose in PBS in a suitable centrifuge tube (of approx 12 mL volume).
9. Spin for 18 h at 270,000*g* in a swinging bucket rotor (e.g., Sorval TH-641) at 4°C.
10. Divide the gradient into 1-mL fractions (about 12) using a cut-off Gilson pipet tip.

Table 2
Infectivity of HCVpps Incorporating E1E2 of Diverse Genotypes

Genotype	Strain or construct	%GFP-positive cells*	TU/mL (10^{-4})
1a	1a H77c	11.80	3.93
1a	1A14.8	12.47	4.16
1b	1B12.6	29.33	9.78
2a	2A2.4	20.51	6.84
2b	2B1.1	34.99	11.66
2b	2B2.8	11.61	3.87
3a	3A13.6	0.30	0.10
3a	3A13.7	0.95	0.32
4	4.21.16	31.62	10.54
4	4.21.17	24.76	8.25
5	5.15.7	0.75	0.25
5	5.15.11	0.70	0.23
6	6.5.340	1.14	0.38

*The transduction efficiency was calculated after subtracting the number of green fluorescent protein (GFP)-positive cells resulting from "infection" with no-envelope control.

11. Dilute each fraction in 10 mL PBS, and spin for 1 h at 153,000*g* in a swinging bucket rotor (e.g., Sorval TH-641) at 4°C.
12. Add 0.5 mL EFC10 medium to each pellet, cover with parafilm, and leave overnight at 4°C.
13. Gently resuspend the pellet and infect target cells with an aliquot as described under **Subheading 3.3.1.** Use a second aliquot of each fraction for Western blot analysis to identify HCV E1 and E2, and MLV capsid. An example is shown in **Fig. 7**.

3.3.3. Antibody-Mediated Neutralization of HCVpp Infection

1. To test for neutralizing capability of antibodies directed against HCV glycoproteins, pre-incubate antibody with an aliquot of HCVpp in a closed sterile tube at 37°C for 30 min, then proceed as described under **Subheading 3.3.1.** For unequivocal results, the purified antibody should be tested over a range of concentrations. **Figure 8** shows neutralization of diverse HCVpps by the broadly reactive anti-E2 MAb AP33 ***(9)***, with its concentration required to achieve 50% inhibition of infection (IC_{50}) ranging from 0.8 μg/mL for genotype 6 to 11 μg/mL for type 2b. Similarly, patient sera can be screened for the presence of neutralizing antibodies.
2. To test for neutralizing capability of antibodies directed against cell surface receptors of HCV, CD81, and SR-B1, add 0.3 mL of antibody appropriately diluted in

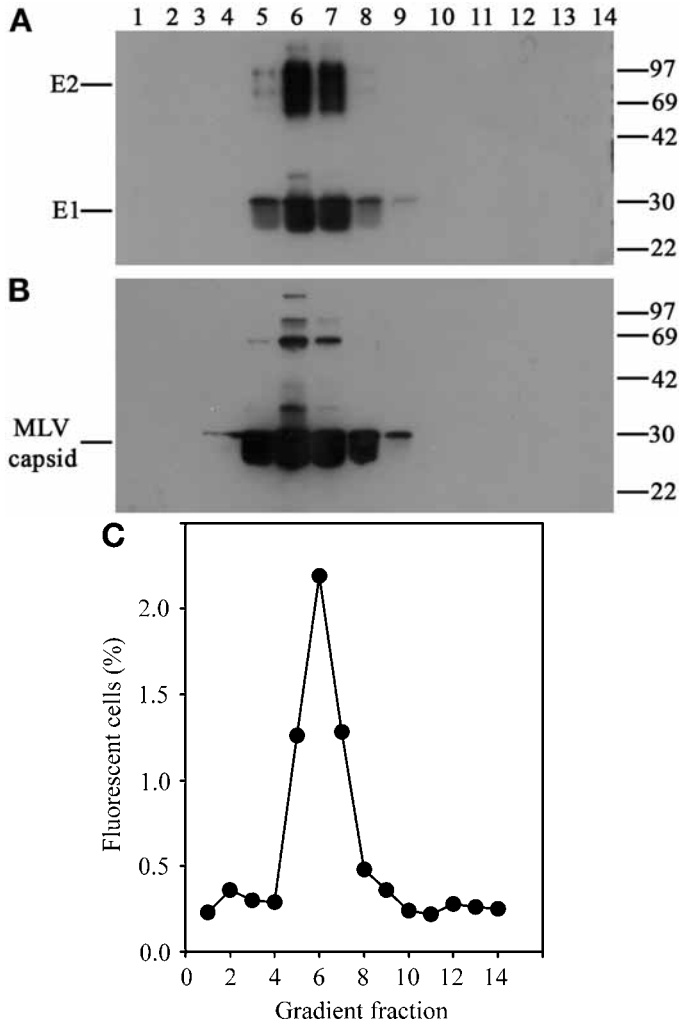

Fig. 7. Sucrose gradient fractionation of HCVpp incorporating genotype 1a E1E2 glycoproteins. The fractions collected from the sucrose gradient centrifugation of HCVpp preparation were Western blotted for E1 and E2 **(A)** or for the MLV capsid **(B)**. The presence of infectious HCVpp in each fraction was determined by measuring number of green fluorescent protein-positive Huh-7 cells **(C)**. As shown, the HCVpp infectivity completely correlates with the relative levels of HCV E1E2 and MLV capsid.

EFC10 to the target cells, incubate at 37°C for 30 min, then add an equal volume of HCVpp and proceed as in **Subheading 3.3.1.** above. An example of neutralization of diverse HCVpps by anti-CD81 and anti-SR-B1 antibodies is shown in **Fig. 9A,B**, respectively.

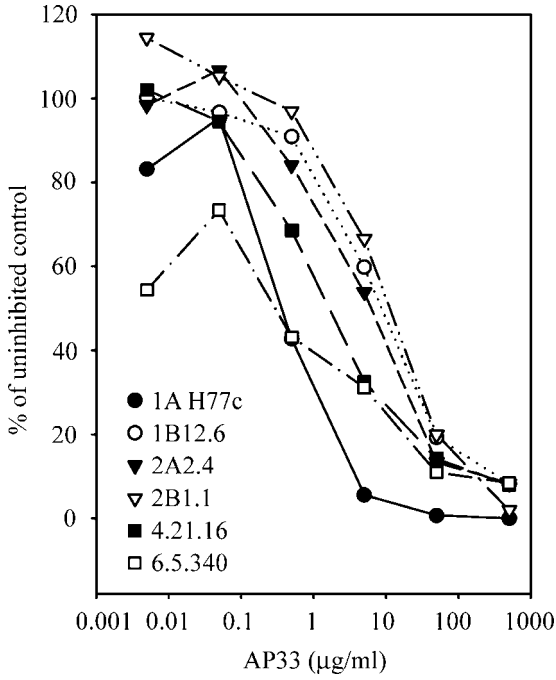

Fig. 8. Neutralization of diverse HCVpps by anti-E2 MAb AP33. HCVpp incorporating E1E2 derived from genotype 1a (1A H77c), 1b (1B12.6), 2a (2A2.4), 2b (2B1.1), 4 (4.21.16), or 6 (6.5.340) were pre-incubated with different concentrations of purified MAb AP33 prior to infection of Huh-7 cells. The neutralizing activity of the antibody is expressed as percentage of inhibition of the infectious titers.

4. Notes

1. Repeated freeze-thawing (but not long-term storage at –80°C) of patient sera significantly reduces the recovery of viral RNA from these samples.
2. The serum RNA isolation method **(Subheading 3.1.1.)** allows efficient, rapid recovery of HCV RNA, and avoids potentially hazardous phenol/chloroform extraction. Serum must be equilibrated to room temperature before processing in a class I hood. The AVL lysis buffer must be prepared beforehand with the addition of appropriate carrier RNA, and the suspension solubilized by heating to 80°C and cooling to room temperature. Prepared AVL buffer must be stored at 4°C and resuspended before use.
3. The recovery of HCV E1 and E2 genes and their expression poses a significant challenge. The genes display significant heterogeneity, E2 containing the most divergent sequence identified amongst HCV isolates. This diversity is also apparent at each end of the coding region, from the signal peptide of E1 to the cytoplas-

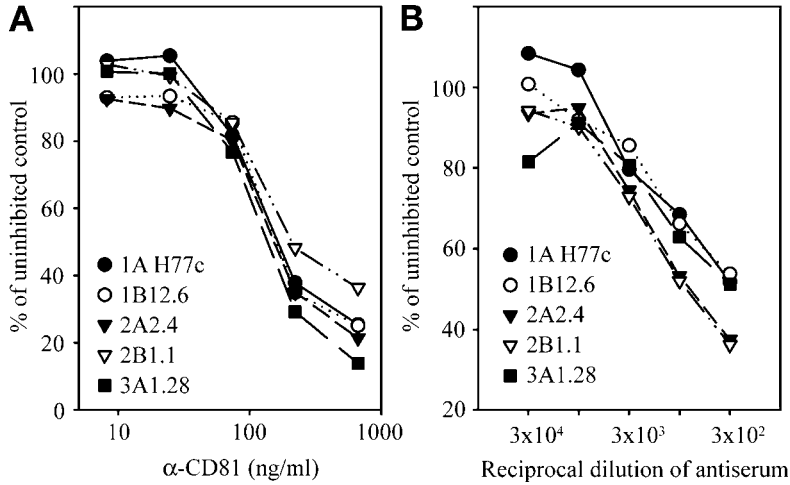

Fig. 9. Neutralization of diverse HCVpps by antibodies to virus receptors. Huh-7 cells were pre-incubated with different concentration of anti-CD81 **(A)** or anti-SR-B1 **(B)** antibody prior to infection with HCVpp incorporating E1E2 derived from genotype 1a (1A H77c), 1b (1B12.6), 2a (2A2.4), 2b (2B1.1), or 3 (3A1.28). The neutralizing activity of the antibody is expressed as percentage of inhibition of the infectious titers.

mic domain of E2. This has implications for successful PCR amplification of this region, requiring sensitive, specific PCR protocols. In addition, high efficiency recovery of HCV RNA from clinical samples is difficult, requiring sensitive extraction and purification. At each stage, particular attention must be paid to preventing contamination, with the use of filter tips, nuclease-free reagents and appropriate decontamination of surfaces. In particular, amplified products must never be handled in areas used for pre-PCR manipulation of samples, as contaminating aerosols are a significant problem.

4. The HEK-293T cells should be split between 1:20 and 1:30 every 3 to 4 d. They should not be allowed to grow above 80% confluence. To achieve maximum efficiency of transfection, it is essential to handle cells as described under **Subheading 3.2.1.**
5. Other commercially available transfection reagents can also be used to efficiently transfect cells instead of the calcium phosphate system described here.
6. An alternative to the MLV system described here, HIV pseudotypes incorporating HCV glycoproteins can be used ***(11)***. This system requires two plasmids; one encoding envelope-defective HIV-1 proviral genome expressing a reporter gene and the other expressing HCV glycoproteins.

7. A transfer vector carrying the luciferase gene can be used as an alternative to GFP as a reporter for HCVpp generation and their subsequent analysis. This allows the use of smaller volumes of HCVpp-containing inoculum to infect target cells in 24-, 48-, or 96-well plates, although variability between samples tends to be greater. If GFP-expressing MLV transfer vector is used for HCVpp generation, the cells should be examined under an ultraviolet microscope (after collection of the medium following transfection) to get an indication of transfection efficiency. The best titres of HCVpp are obtained when {GT}90% cells are transfected.

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