Human Respiratory Syncytial Virus Glycoproteins Are Not Required for Apical Targeting and Release from Polarized Epithelial Cells

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Human respiratory syncytial virus (HRSV) is released from the apical membrane of polarized epithelial cells. However, little is known about the processes of assembly and release of HRSV and which viral gene products are involved in the directional maturation of the virus. Based on previous studies showing that the fusion (F) glycoprotein contained an intrinsic apical sorting signal and that N- and O-linked glycans can act as apical targeting signals, we investigated whether the glycoproteins of HRSV were involved in its directional targeting and release. We generated recombinant viruses with each of the three glycoprotein genes deleted individually or in groups. Each deleted gene was replaced with a reporter gene to maintain wild-type levels of gene expression. The effects of deleting the glycoprotein genes on apical maturation and on targeting of individual proteins in polarized epithelial cells were examined by using biological, biochemical, and microscopic assays. The results of these studies showed that the HRSV glycoproteins are not required for apical maturation or release of the virus. Further, deletion of one or more of the glycoprotein genes did not affect the intracellular targeting of the remaining viral glycoproteins or the nucleocapsid protein to the apical membrane.

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In the present study, we investigated the roles of the SH, G, and F glycoproteins in the directional sorting and release of HRSV. We chose to concentrate on the glycoproteins for numerous reasons, the first being that all three HRSV glycoproteins contain N-linked glycans, and the G protein also contains O-linked glycans. N- and O-linked glycans can act as apical sorting signals, as has been demonstrated for erythropoietin and neurotrophin receptor and for other apically targeted proteins (reviewed by Potter et al. [36]). Second, HRSV glycoproteins have been shown to associate with lipid rafts (19, 26, 30), which are characterized by triton-insoluble membranes that localize to the apical surface of polarized epithelial cells (reviewed by Schuck and Simons [40]). F glycoprotein has also been shown to interact with RhoA (35), a host cell GTPase that localizes to lipid rafts, and has been shown to be required for the fusion activity of the F protein (16). Finally, it was demonstrated that the HRSV F glycoprotein contained an intrinsic apical targeting signal (7). Based on these previous studies, we investigated whether there was a role for the HRSV glycoproteins in determining the directional release of virus from the apical membrane of polarized epithelial cells. To carry out these studies, we recovered engineered viruses lacking the glycoprotein genes either individually or in groups and examined the directional release of these viruses, as well as the directional sorting of the remaining viral proteins in polarized epithelial cells.

MATERIALS AND METHODS

Cells and antibodies. Vero 76 (Vero) and HEP-2 cells were acquired from the American Type Culture Collection (MDCK) cells were kindly provided by S. VandePol (University of Virginia). All three cell types were grown in Dulbecco minimal essential medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum. Vbac cells (Vero cells expressing the baculovirus GP64 protein carrying the F protein COOH-terminal residues 563 to 573) were described previously (33). MBs 6 and 19 were provided by Geraldine Taylor (Institute for Animal Health, Compton, United Kingdom), MAB L9 was provided by Ed Walsh (University of Rochester School of Medicine, Rochester, NY), and MAB 15 was provided by James Stott (Institute for Animal Health, Compton, United Kingdom). Goat anti-transferrin receptor (TfnR) antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Bovine anti-goat conjugated secondary antibodies were from Molecular Probes (Carlsbad, CA).

Construction of cDNAs and recovery of infectious HRSVs. All cDNAs were based on the HRSV A2 strain. cDNAs engineered for these studies were constructed as described previously (31). The construction and recovery of virus RSASH was reported previously (31). Briefly, using standard cloning techniques, shuttle vectors were constructed lacking the G or F transmembrane glycoprotein open reading frame (ORF) and containing the green fluorescent protein (GFP) ORF instead, as shown in Fig. 1. All HRSV ORFs were maintained in their original genome positions to maintain expression profiles similar to that of a wild-type virus. For generation of the recombinant wild-type virus, the shuttle vectors were then used to recover virus RSASH, RSAG, RSF, and RSASH,G,F, which have the two glycoprotein ORFs deleted and replaced with ORFs encoding marker proteins GUS and GFP. Finally, virus RSASH,G,F has the ORFs encoding each of the three HRSV glycoproteins replaced with those of reporter genes GFP, CAT, and GUS, respectively.

FIG. 1. Schematic of the gene content of engineered viruses. All engineered viruses were generated from a cDNA of the A2 strain of HRSV. Viruses RSASH, RSAG, and RSF have the missing ORFs of the SH, G, and F genes replaced with that of GFP. Viruses RSASH,G, RSASH,F, and RSAG,F have the two glycoprotein ORFs deleted and replaced with ORFs encoding marker proteins GUS and GFP. Finally, virus RSASH,G,F has the ORFs encoding each of the three HRSV glycoproteins replaced with those of reporter genes GFP, CAT, and GUS, respectively.

Virus infections. Infections of cell monolayers by HRSV were carried out by adsorbing virus to the apical chamber for 1.5 h at 37°C, followed by removing the inoculum and washing the cells once with growth medium and then continuing incubation at 37°C for 48 h or 72 h, depending on the assay. Infections of cell monolayers by VSV were carried out by adsorbing virus to cells in the basolateral chamber for 1 h at 37°C, removing the inoculum, washing the cells once with growth medium, and further incubating the cells at 37°C for 6 h.

Plaque assays. Vero cells were used for assays with wild-type virus and viruses retaining the F protein. Vbac cells, which constitutively express baculovirus GP64 protein (31), were used to assay viruses lacking the F protein. Cells were plated in six-well plates near confluence and incubated at 33°C overnight, a temperature conducive for proper folding of the GP64 protein. Virus was serially diluted in growth medium, and the dilutions were used to infect cultures in duplicate. Virus was adsorbed to cells for 1.5 h at 33°C, when it was replaced by an agar overlay solution consisting of Dulbecco modified Eagle medium, 25 mM HEPES (pH 7.4), and 0.5% sea plaque agar. Plates were put at 4°C for 10 min for the agarose to harden and then moved to 33°C to incubate for 6 to 8 days. Cells were fixed with 4% paraformaldehyde (PFA) for 45 min at room temperature. The agar overlay was removed, and the cells were fixed again for 10 min in 4% PFA. Cells were washed twice with phosphate-buffered saline (PBS) and incubated with anti-F MAb19 at 1:2,500 or with anti-G L9 at 1:5,000 antibody for 45 min at room temperature. Cells were washed once with PBS and incubated with goat anti-mouse β-Gal antibody at 1:500 for 45 min at room temperature. Cells were washed twice with PBS and incubated with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate solution consisting of 0.5 mM potassium ferriyride (31), 0.5 mM potassium ferrocyanide, 0.1 mM MgSO4, and X-Gal at 1:50 mg/ml in PBS overnight at room temperature in the dark. The next day, cells were washed twice with distilled H2O and dried, and the plaques were counted on a light box.

Immunofluorescence staining and confocal microscopy. MDCK and HEP-2 cells were plated on 12-mm, 3-μm-pore-size polycarbonate filter inserts (Millipore, Billerica, MA) and incubated at 37°C overnight. Cells were then infected with engineered viruses at a multiplicity of infection (MOI) of 0.1 and further incubated at 37°C for 48 h. At day 2 postinfection, the transepithelial electrical resistance (TEER) was measured to ensure polarization of the monolayer. Cells were washed twice with PBS and fixed with freshly prepared 4% PFA in PBS for 15 min at room temperature. Cells were washed twice with PBS, permeabilized with 0.02% Triton X-100, blocked in 1% bovine serum albumin (BSA) for 10 min, and stained with the following primary antibodies diluted in 0.1% BSA: amplified by reverse transcription-PCR, and selected areas were verified by bulk nucleotide sequence analysis. No changes were found. Virus stocks at passages 3 and 5 were used for the experiments described.
anti-F MAB19 at 1:5,000, anti-G L9 at 1:15,000, and anti-N MAB15 at 1:15,000 for 1 h at room temperature. Cells were washed twice with PBS, blocked with 1% BSA, and incubated with secondary anti-mouse antibody conjugated to Alexa Fluor 594 at a dilution of 1:1,000 for 30 min at room temperature. Cells were washed twice with PBS and stained with Hoechst stain (Molecular Probes, Carlsbad, CA) at 0.05 mg/ml in PBS. The cells were washed three times with PBS, mounted on slides with coverslips, and stored at 4°C in the dark. Images were taken on a Zeiss LSM 510-U confocal microscope in the x-z plane using a ×100 objective lens.

Cell ELISA analysis. MDCK cells were plated on 12-mm, 3-μm-pore-size polycarbonate filter inserts and incubated at 37°C overnight. Cultures were infected in duplicate with engineered viruses at an MOI of 0.25 and incubated at 37°C for 72 h. At day 3 postinfection TEER measurements were taken to ensure polarization of the cell monolayer. Cells were washed twice with PBS and fixed in freshly prepared 4% PFA for 20 min at room temperature. Cells were washed twice with PBS, blocked in 1% BSA for 10 min and incubated with the primary antibodies anti-F MAB19 at 1:5,000, anti-G L9 at 1:15,000, or anti-TfnR at 1:10 for 1 h at room temperature. One filter of each duplicate was incubated with antibody in the apical chamber, and the other was incubated with antibody in the basolateral chamber. Cells were washed and incubated with secondary antibody, goat anti-mouse antibody conjugated to HRP at 1:3,000 or bovine anti-goat antibody conjugated to HRP at 1:2,500, for 1 h at room temperature. Cells were washed twice with PBS and incubated with o-phenylenediamine (OPD; Sigma-Aldrich, St. Louis, MO) substrate. At various times after the addition of substrate, 50-μl aliquots were collected and added to 2 ml sulfuric acid in a 96-well plate to stop the reaction. The optical density at 490 nm was determined in an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA).

Anti-N ELISA on released virus. MDCK and HEP-2 cells were plated on 24-mm, 3-μm-pore-size polyester filter inserts (Millipore) in six-well plates and incubated overnight at 37°C. The next day, the cells were infected with engineered viruses at an MOI of 0.25 or 1.0 for single or double/triple glycoprotein-deleted viruses, respectively, and incubated at 37°C for 48 h. The medium was changed 24 h prior to supernatant harvest. At day 3 postinfection, TEER measurements were taken to ensure polarization of the cell monolayer. The apical and basolateral supernatants were carefully removed from the cell monolayer and put into 15-ml tubes (BD Biosciences, San Jose, CA). The supernatants were centrifuged at 750 × g for 4 min to pellet any cell debris. The supernatant was harvested to a new 15-ml tube and centrifuged at 1,500 × g for 30 min to pellet the virus. The viral pellets were resuspended in 0.1 ml of PBS, and the entire volume was used to coat a 96-well ELISA plate that was incubated at 4°C overnight. The next day, the wells were fixed with 80% acetone in PBS for 20 min and then washed twice with PBS, permeabilized in 0.02% Triton X-100 for 10 min, and blocked in 0.1% BSA for 30 min at room temperature. The samples were incubated with primary antibody, anti-N MAB6 at a 1:15,000 dilution for 1 h at room temperature and then washed twice with PBS and incubated with secondary antibody, goat anti-mouse antibody conjugated to HRP, at 1:3,000, for 1 h at room temperature. The samples were washed with PBS and incubated with OPD substrate for 5 to 15 min, at which point 2 M sulfuric acid was added to stop the reaction. The optical density at 490 nm was determined in an ELISA plate reader.

RESULTS

Construction of cDNAs and recovery of engineered viruses. To examine the involvement of each glycoprotein in the directional targeting and release of HRSV, we deleted each individual glycoprotein gene in a full-length cDNA clone of the HRSSV genome and replaced its ORF with that of a reporter gene (Fig. 1). Since HRSSV transcription is obligatorily sequential, replacement of any deleted genes is necessary to maintain wild-type levels of viral gene expression. In the case of individual deletions, GFP was used to replace the HRSSV gene. When two glycoproteins were deleted, the genes were replaced with GFP and β-glucuronidase (GUS), and when three genes were deleted they were replaced with GFP, GUS, and chloramphenicol acetyltransferase (CAT) reporter genes (Fig. 1). All of the cDNAs described above maintained not only the same number of genes as in wild-type HRSSV but also the genuine intergenic junctions to preserve authentic transcription levels. These viruses were tested for N and M2-1 protein expression levels by Western blotting, and all engineered viruses were found to express comparable amounts of these two proteins (data not shown). Viruses were recovered from engineered cDNAs as described in Materials and Methods. Vero cells were used to propagate viruses retaining the F protein. Vbac cells, which constitutively express baculovirus GP64 protein (33), were used to propagate viruses lacking the F protein due to the fact that F protein is essential. Viruses grown in Vbac cells incorporate GP64 into their membranes by trans complementation as they are released. The GP64 complemented virions can infect new cells that do not express GP64, and the virus will replicate and be released (but will not be able to reinfet since they lack a glycoprotein that allows for cellular entry).

Detecting directional release of virus from polarized MDCK cells. HRSSV is released apically from polarized epithelial cells (6, 38, 45, 46) while VSV, another enveloped negative strand virus, is released basolaterally (5). We cultured the polarized epithelial cell line, MDCK, on transwell filter inserts and determined the time course for establishment of a polarized monolayer. Two criteria were used to determine whether the monolayer was confluent and polarized: TEER and tight-junction staining by immunofluorescence microscopy. To test that our cell culture system recapitulated the directional release of HRSSV and VSV, we used polarized epithelial MDCK cells on filter inserts and infected them with either wild-type HRSSV or wild-type VSV, as described in Materials and Methods. To test whether HRSSV was able to pass through the filter insert into the basolateral media for detection, we also infected the non-polarized epithelial cells, HEP-2, on filter inserts with HRSSV. The TEER of the MDCK monolayer and the HEP-2 monolayer was measured. As expected, MDCK cells consistently gave a high normalized TEER reading (90 to 120 Ωcm²), indicating polarization, while HEP-2 cells gave a low normalized reading (<1 Ωcm²), indicating no polarization. Both the apical and the basolateral media were collected from the infected cell cultures, and a plaque assay was used to determine the location of released virus. The data in Fig. 2A show that the majority of HRSSV was released apically from the polarized MDCK cells, whereas VSV was found only in the basolateral media. HRSSV was released from the nonpolarized HEP-2 cells into both the apical and the basolateral supernatants, indicating that the filter inserts did not inhibit the passage of the virus into the basolateral chamber.

For these studies, we generated viruses that lacked not only the SH and G proteins but also the F glycoprotein, which is required for viral infectivity. In previous work we developed a method to recover infectious HRSSV lacking the essential F gene by complementing the deleted virus in trans with the baculovirus GP64 attachment and fusion protein (33). Due to the trans-supplied GP64 functional substitute, these viruses will infect MDCK and replicate. However, they lack F or a functional complement in the genome, precluding an assay based on infectivity such as the plaque assay. Therefore, to detect released virus, we used an anti-N ELISA that quantitated the amount of released HRSSV N protein in apical and basolateral media that had first been clarified of any cell debris. MDCK and HEP-2 cells were infected with wild-type HRSSV on filter
inserts. The apical and basolateral supernatants were collected daily for 4 days, and virions were concentrated by centrifugation. The resuspended viral pellets were used to coat a 96-well ELISA plate, which was then assayed for the amount of N protein released as described in Materials and Methods. The anti-N ELISA confirmed the plaque assay results of Fig. 2A (Fig. 2B): HRSV was released apically from polarized MDCK cells infected with wild-type virus, and at 1 to 4 days postinfection the apical and basolateral supernatants were analyzed for released HRSV by using an anti-N ELISA as described in Materials and Methods. The bars represent the percentage of absorbance measured at 490 nm in the apical and basolateral chambers of total absorbance.

**FIG. 2.** Directional release of virus from polarized epithelial cells. (A) Release of wild-type VSV and HRSV from polarized epithelial cells quantified by plaque assay. Polarized MDCK or nonpolarized HEp-2 cells grown on filter inserts were infected with either wild-type VSV or HRSV viruses. The apical and basolateral media were collected at 3 days postinfection for HRSV and at 6 h postinfection for VSV and analyzed for infectivity by plaque assay on Vero cells as described in Materials and Methods. Error bars represent standard deviations. (B) Release of wild-type HRSV quantified by anti-N ELISA. Polarized MDCK cells or nonpolarized HEp-2 cells were infected with virus, and at 1 to 4 days postinfection the apical and basolateral supernatants were analyzed for released HRSV by using an anti-N ELISA as described in Materials and Methods. Error bars represent standard deviations from at least three experiments.

**FIG. 3.** Directional release of HRSV with single glycoprotein gene deletions. Polarized MDCK cells and nonpolarized HEp-2 cells grown on filter inserts were infected with wild-type or engineered viruses at a multiplicity of 0.25. At 3 days postinfection the apical and basolateral supernatants were analyzed for released HRSV by using an anti-N ELISA. Error bars represent standard deviations from at least three experiments.

Methods. At day 3 postinfection, the apical and basolateral supernatants were collected, and the virus was concentrated and used to coat an ELISA plate. The anti-N ELISA was used to quantify the amount of HRSV N protein found in the apical and basolateral media from each infected monolayer. The data in Fig. 3 show that ca. 80% of viruses lacking the SH, G, or F proteins were released apically, similar to wild-type virus, where 90% of virus was released from the apical membrane. In contrast to the polarized MDCK cells, wild-type virus was released from nonpolarized HEp-2 cells in equal amounts from the apical and basolateral surfaces.

**Effect of single glycoprotein deletion on the directional sorting of viral proteins.** We next determined whether the intracellular trafficking and localization of the viral proteins to the apical membrane was influenced by the glycoproteins. Using confocal microscopy, the cellular localization of N, F, and G proteins was examined. To visualize the distribution of the viral proteins in an epithelial monolayer, polarized MDCK or nonpolarized HEp-2 cells were plated on filter inserts and infected with engineered viruses at a multiplicity of 0.1. At 2 days postinfection, the cells were fixed, permeabilized, and stained with anti-N, -F, and -G antibodies. The nuclei were visualized with Hoechst stain. Images of infected cells were taken by confocal microscopy at a magnification of ×100 as described in Materials and Methods. As can be seen in Fig. 4A, the N protein was evenly distributed on the cell surface in nonpolarized HEp-2 cells, while in polarized MDCK cells infected with wild-type HRSV, ΔSH, ΔG, and ΔF viruses the N protein was observed only at the apical membrane. Similarly, the localization of F protein in MDCK cells infected with wild-type HRSV, ΔSH, and ΔG viruses was also apical, while in HEp-2 cells F protein was found on all surfaces of the plasma membrane (Fig. 4B). Finally, HRSV G protein also localized toward the apical membrane in polarized epithelial cells infected with wild-type, ΔSH, and ΔF viruses (Fig. 4C). In all images of MDCK cells infected with wild-type or engineered HRSVs, a lifting up of the infected cells from the monolayer was observed. This phenomenon for HRSV infection has also been described recently using human airway epithelial cells, a polarized ciliated cell system (Ray Pickles, unpublished data), as
well as in MDCK cells expressing only F glycoprotein fused to GFP (11). In these studies, it was shown by TEER or microscopy that extruded cells were replaced and that the integrity of the monolayer remained intact. In our study, to ensure that the cells were not losing their polarization due to this lifting effect, the TEER was monitored for both mock-infected and HRSV-infected monolayers. The measurements for both uninfected and infected cells were in a range found in previous studies using MDCK and F-GFP (90 to 120 Ω·cm²) (11), suggesting that the cell monolayer maintained its polarization. Also, the localization of viral proteins was entirely apical, which would be expected only in cells maintaining polarization.

**Quantitation of viral protein targeting when a single glycoprotein is deleted.** The directional localization of the F and/or G glycoproteins in MDCK cells infected with engineered HRSV was analyzed quantitatively using a cell-based ELISA described previously (32). MDCK cells were plated on filter inserts in duplicate and infected with engineered viruses at an MOI of 0.25. At 3 days postinfection, the cells were fixed and permeabilized, and stained for HRSV N protein (A), F glycoprotein (B), and G glycoprotein (C), as shown in red. Engineered viruses lacking a particular glycoprotein gene were still stained for that protein as a control. The nuclei were stained with Hoechst as shown in blue. All images were taken on a Zeiss confocal at ×100 magnification in the x-z plane.

**Figure 4.** Intracellular localization of HRSV proteins expressed by viruses with single glycoprotein deletions. HEp-2 and MDCK cells grown on filter inserts were infected with wild-type or engineered viruses at a multiplicity of 0.1. At 2 days postinfection the cells were fixed, permeabilized, and stained for HRSV N protein (A), F glycoprotein (B), and G glycoprotein (C), as shown in red. Engineered viruses lacking a particular glycoprotein gene were still stained for that protein as a control. The nuclei were stained with Hoechst as shown in blue. All images were taken on a Zeiss confocal at ×100 magnification in the x-z plane.

and the percentage of apically and basolaterally localized F or G proteins was quantified. The data in Fig. 5A show that 85 to 90% of cell surface F proteins localized apically in wild-type HRSV, ΔSH, and ΔG HRSV-infected cells. Similarly, the data in Fig. 5B show that ca. 80% of cell surface G protein localized to the apical membrane for wild-type HRSV, ΔSH, and ΔF-infected cells. As a control, to determine whether the filter might hinder antibody binding to the basolateral cell membrane, endogenous TfR, a known basolaterally sorted protein (14), was also analyzed. The data in Fig. 5C show that 90% of TfR localized basolaterally, confirming that basolateral detection was efficient in this assay.

**Effect of simultaneous deletion of two or three glycoprotein genes on the directional release of HRSV.** To determine whether there was redundancy of function between the HRSV glycoproteins in the apical release of the virus, we engineered viruses in which two or three of the glycoprotein genes were deleted and replaced with reporter genes (Fig. 1). Viruses ΔSH,G, ΔSH,F, ΔG,F, ΔSH,G,F, and wild-type HRSV were used to infect polarized MDCK or nonpolarized HEp-2 cells plated on filter inserts at an MOI of 1.0. It was necessary to increase the MOI of HRSV with multiple deletions to detect released virus due to decreased virus spread throughout the monolayer compared to wild-type virus or virus with a single glycoprotein deleted. On day 3 postinfection, the apical and basolateral superna-
tants were collected, concentrated, and used to coat an ELISA plate as described in Materials and Methods. The amount of HRSV N directionally released into the medium was quantified by an anti-N ELISA. The data in Fig. 6 show that 70 to 80% of the viruses with two or three glycoproteins deleted were released apically from MDCK cells, similar to wild-type virus release. In comparison, 50% of wild-type HRSV was released apically from nonpolarized HEp-2 cells.

HRSV protein localization when multiple glycoproteins are deleted. To determine how multiple deletions affected directional sorting due to possible redundant targeting signals in more than one glycoprotein, we first examined the intracellular localization of N, F, and G by confocal microscopy. MDCK cells were plated on filter inserts and infected with recombinant viruses lacking two or all three viral glycoproteins at an MOI of 0.1. At 2 days postinfection the cells were fixed, permeabilized, and stained for the three structural proteins. Images were obtained as described in Materials and Methods. Figures 7A, B, and C represent the localization of the N, F, and G proteins, respectively, and show that despite double and triple glycoprotein deletions, all components still sort apically.

We next quantified the directional sorting of F and G proteins by cell ELISA to extend and corroborate our microscopy data. MDCK cells were plated on filter inserts in duplicate and infected at an MOI of 0.25 with SH,G and SH,F recombinant viruses, both of which retained either F or G glycoproteins. At 3 days postinfection the cells were fixed and incubated either apically or basolaterally with anti-F or anti-G primary antibodies, followed by anti-mouse HRP-conjugated secondary antibody. After incubation with OPD substrate, the amount of F or G protein on the apical and basolateral cell surfaces was quantified as described in Materials and Methods. Approximately 85 to 95% of the glycoproteins localized to the apical membrane of polarized epithelial cells infected with virus with either the SH and F proteins deleted or the SH and G proteins deleted (Fig. 8).

DISCUSSION

We examined the requirements for the viral glycoproteins in the directional targeting and release of HRSV in the context of whole infectious virus. We chose to investigate the viral glycoproteins because all three glycoproteins were shown to interact with the apically localized lipid rafts (19, 26, 30), and the HRSV F protein, when expressed in the absence of other viral proteins, had been reported to have an independent apical
sorting signal (7). Also, the only currently known apical targeting signals in polarized cells are glycosylphosphatidylinositol anchors, some transmembrane domains, and N- and O-linked glycans (reviewed by Potter et al. [36]). HRSV proteins are not known to contain glycosylphosphatidylinositol anchors, and the only viral proteins that meet the remaining known criteria for apical targeting are the viral glycoproteins.

To carry out our studies, we recovered viruses genetically engineered to have one or more of the HRSV glycoprotein genes deleted and replaced with reporter genes. The replacement of the deleted genes was important for maintaining the transcriptional regulation of all downstream genes, since deletion of a gene or genes without replacement would result in an upregulation of the expression levels of downstream genes (3, 44). This system allowed us to analyze the contributions of each glycoprotein in its most natural state.

Since viral proteins must first traffic to the site of assembly and release, we examined both the directional release of the engineered viruses from polarized epithelial cells and the intracellular targeting of individual viral proteins to the apical or basolateral membranes. To study the intracellular localization of the viral proteins, we used two different approaches: a quantitative cell ELISA, which allowed examination of transmembrane proteins with an extracellular domain, and confocal microscopy, which allowed the visualization of the overall localization of numerous viral structural proteins in permeabilized cells.

We first studied the effects of deleting individually each of the three glycoprotein genes to determine whether the directional release of the virus was dependent on the presence of any one of the glycoproteins. We analyzed both the release of the virus from the polarized epithelial cell line, MDCK, and the intracellular directional targeting of the viral proteins N, F, and G as a function of the deletions. The results of these assays showed that the apical targeting and release of HRSV was not dependent upon signals in any one glycoprotein since the deletion of the SH, G, or F genes did not alter the apical release of virus. We next examined the viruses with pairs of glycoprotein genes deleted—ΔSH,G, ΔSH,F, and ΔG,F—to determine whether there was redundancy between glycoproteins. The results of these assays also showed that the apical targeting and release of HRSV was not affected by the deletion of any two of the glycoproteins, suggesting that all three glycoproteins contained sorting signals that were able to direct the apical release of the virus independently or that the glycoproteins were not the directional mediators. To determine whether the latter case was possible, we analyzed the directional maturation of a virus with all three glycoprotein genes deleted (ΔSH,G,F) and found that the triple-deletion virus was released and targeted apically, indicating that the glycoproteins are not required for the directional targeting and release of HRSV from polarized epithelial cells.

Directional budding of enveloped viruses has a long and varied history. It was originally thought that viral glycoproteins determined the site of assembly and release (4, 34). This idea was based on observations that viral glycoproteins were found to localize at the site of virus budding even when expressed in the absence of other viral proteins. An example is Semliki Forest virus (SFV) that buds apically from Fischer rat thyroid cells but basolaterally from Caco-2 cells. Coincidentally, the glycoproteins of SFV, when expressed alone, localize apically in Fisher rat thyroid cells and basolaterally in Caco-2 cells (48). Exceptions to the hypothesis that glycoprotein’s mediate viral release were uncovered when it was found for measles and...

FIG. 7. Intracellular localization of HRSV proteins expressed by viruses having multiple glycoprotein deletions. MDCK cells grown on filter inserts were infected with wild-type or engineered viruses at a multiplicity of 0.1. At 2 days postinfection cells were fixed, permeabilized, and stained for HRSV N protein (A), F glycoprotein (B), and G glycoprotein (C), as shown in red. Engineered viruses missing a glycoprotein were still stained for that protein as controls. The nuclei were stained with Hoechst as shown in blue. All images were taken on a Zeiss confocal at ×100 magnification in the x–z plane.
The ARE is involved in apical membrane-specific sorting (6). The ARE of the host cell has also been implicated in the directional targeting of the virus in polarized epithelial cells. It was reported that the F glycoprotein contains an apical targeting signal (7), but our work demonstrates that this signal is not sufficient for the apical sorting and release of the whole virus. Although we have not yet tested the importance of the N or M proteins in the directional targeting and release of HRSV from polarized epithelial cells, we can conclude from the present study that the three glycoproteins—SH, G, and F—are not the major determinants for the apical maturation of HRSV. This was a surprising result in light of the observation of an intrinsic apical targeting signal in F glycoprotein (7). It is possible that many of the viral proteins have their own intrinsic apical targeting signals that allow them to localize to the apical membrane independently, at which point they can interact.

Marburg viruses that their viral glycoproteins localized to the opposite surface from viral budding in polarized epithelial cells (24, 39). Further evidence against the glycoprotein hypothesis came from studies, using whole VSV and influenza virus, which introduced mutations within the glycoprotein genes that altered their cellular localization but caused no effect on the specificity of directional release (2, 47). Similarly for HRSV, it was reported that the F glycoprotein contains an apical targeting signal (7), but our work demonstrates that this signal is not required for the apical sorting and release of the whole virus.

The host cell has been shown to have involvement in the apical targeting of HRSV. One example of this is through the interactions of HRSV with lipid rafts, which are characterized by Triton-insoluble membranes that localize to the apical surface of polarized epithelial cells (reviewed by Schuck and Simons [40]). Lipid rafts have been implicated in the assembly process of HRSV since viral particles were shown to contain lipid raft markers, and matrix, SH, G, and F proteins were each found to interact with lipid rafts (8–10, 18, 19, 25–27, 30, 37). The ARE of the host cell has also been implicated in the directional targeting of the virus in polarized epithelial cells (6). The ARE is involved in apical membrane-specific sorting of host proteins (28), and it has been shown that when this sorting process is interrupted, HRSV replication is decreased and the virus does not assemble or bud from the apical membrane (6).

Although the infected cell clearly has involvement in directional targeting of HRSV, viral protein(s) may also be responsible for interacting with these cellular pathways and thus in directing the virus to the apical membrane. Having shown that deletion of the HRSV glycoprotein genes does not affect apical maturation, the likely remaining candidates for the specific targeting of the virus are the structural proteins nucleocapsid (N) or matrix (M). Either of these proteins may have the ability to direct the assembly of the glycoproteins and the genome prior to viral release, despite not having a known apical targeting signal. It has been found for the measles, Sendai, and Marburg viruses that matrix protein is the directional determinant in the release of the virus from polarized epithelial cells (22, 29, 41). The matrix protein of HRSV has been shown to interact with the plasma membrane (25), as well as with G glycoprotein (15). Also, the HRSV G and F glycoproteins have been reported to interact and to form a complex at the cell surface (1, 23) although where and when these interactions take place is still unknown. They may occur along their trafficking route to the cell surface, where one protein such as N or M protein, may direct the others to the apical membrane, or they may all have their own intrinsic apical targeting signals that allow them to localize to the apical membrane independently, at which point they can interact.

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FIG. 8. Quantitative cell surface localization of HRSV proteins expressed by viruses having multiple glycoprotein deletions. Duplicate cultures of MDCK cells grown on filter inserts were infected with engineered viruses containing F or G glycoproteins at a multiplicity of 0.25. At 3 days postinfection the cells were fixed, blocked, and stained with primary and secondary antibodies either in the apical or basolateral chamber. The amounts of HRSV F glycoprotein (A) and G glycoprotein (B) localizing to the apical and basolateral membranes of infected cells were quantified by using cell ELISA as described in Materials and Methods. Error bars represent standard deviations from at least three experiments.