Transcytosis of the G Protein of Vesicular Stomatitis Virus after Implantation into the Apical Membrane of Madin-Darby Canine Kidney Cells.

II. Involvement of the Golgi Complex

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ABSTRACT In the preceding paper (Pesonen M., W. Ansorge, and K. Simons, 1984, J. Cell Biol., 99:796-802), we have shown that transcellular transport of the membrane glycoprotein G of vesicular stomatitis virus implanted into the apical membrane of Madin-Darby canine kidney cells is transcytosed through the endosomal compartment to the basolateral plasma membrane. To determine whether the Golgi complex was involved in this process, G protein lacking sialic acid or all of the terminal sugars was implanted into the apical membrane and allowed to move to the basolateral membrane. Using the criteria of endoglycosidase H sensitivity, binding to Ricinus communis agglutinin and two-dimensional gel electrophoresis, the sugars on the transcytosed G protein were found to be the same as in the starting material. The absence of any involvement of the Golgi complex in transcytosis was supported by subcellular fractionation studies in which transcytosing G protein was never found in fractions containing galactosyl transferase.

Implantation of the G protein of vesicular stomatitis virus (VSV) into the apical plasma membrane of Madin-Darby canine kidney (MDCK) cells by low pH-induced fusion (1) leads to transcytosis of G protein to the basolateral plasma membrane (1, 2). In the preceding paper (3) we have shown that implanted G protein is rapidly internalized into a nonlysosomal compartment with the characteristics of endosomes in other cells (4). We could exclude that the lysosomes were an important kinetic intermediate in the transcytotic route. At 21°C when traffic to the lysosomes was arrested, transcytosis of G protein was still taking place. At higher temperatures some G protein was routed to the lysosomes for degradation but this occurred after most of the G protein had already been transported to the basolateral membrane.

In this paper we have studied the possible role of the Golgi complex in the transcytosis of G proteins implanted into the apical membrane of MDCK cells. The G protein of VSV contains two oligosaccharide side-chains which each have a triantennary structure [NeuNAc-Gal-GlcNAc]-[Man]-GlcNAc-GlcNAc[Fuc]-peptide (5). The terminal glycosylation of the G protein has been shown to occur in the Golgi complex (6, 7). To study the involvement of the Golgi complex in the transcytosis of implanted G protein, we produced VSV particles in which the G protein was underglycosylated and investigated whether it became reglycosylated during transcytosis. To complement these studies subcellular fractionation was used to detect G protein in Golgi elements during transcytosis.

MATERIALS AND METHODS

The MDCK and baby hamster kidney (BHK) cells, the virus preparations, the implantation procedure of the G protein into the apical membrane of MDCK cells, the 125I-protein A-binding assay, and the method to study degradation of viral proteins are described in the preceding paper (3).

The Chinese hamster ovary (CHO) 15B cells were grown in minimum essential medium (MEM) with 5% fetal calf serum. Both nonlabeled VSV and VSV-labeled with [35S]methionine were produced in CHO 15B cells and purified as described for BHK cells (1).

Desialylated VSV was prepared by incubating the virus in 450 μl of minimum essential medium pH 6.3 with 0.05 U of neuraminidase (Clostridium
Cell Electrophoresis: Monolayers of cells were solubilized in PBS containing 2% SDS, 5 mM EDTA, 1 mM iodoacetamide, and 1 mM phenylmethylsulphonyl fluoride. Ice-cold trichloroacetic acid (TCA) was added to a final concentration of 10%, the samples were incubated on ice for 1 h and centrifuged for 3 min in a table top Eppendorf centrifuge. The TCA-precipitates and the virus preparations were subjected to one-dimensional gel electrophoresis as described in the preceding paper (3).

For two-dimensional gel electrophoresis virus preparations were subjected to phase separation using Triton X-114 (3), or cell monolayers were solubilized into PBS containing 0.75% of Triton X-114 instead of 2% SDS (see above) and then were subjected to phase separation. The detergent phases were dried under N₂ and resuspended in lysis buffer (8). The procedures for two-dimensional gel electrophoresis were essentially those described before (9) with some modifications. The first dimension separation was performed as acidic electrofocusing (IEF, 20 h x 1,200 V) in 230 x 2.1 mm 4% (wt/vol) polyacrylamide gels containing 2% ampholytes (1.6% pH 5.7, 0.4% pH 3.5-10). The second dimension was carried out in an SDS polyacrylamide gel as described (9). The slab measured 25 x 25 cm. The gels were processed for fluorography as described (10).

Endoglycosidase H-Digestions: Virus preparations or cell monolayers were solubilized in 250 μl of 0.2 M sodium citrate buffer, pH 5.5, containing 1% SDS, and incubated with 5 μl of endo-N-acetyl-d-galactosaminidase H (endoglycosidase H) ( Streptomyces plicatus; Miles Laboratories Inc., Elkhart, IN) for 16 h at 36°C in the presence of antipain (1 μg/ml; Sigma Chemical Co.), benzamidine (17.5 μM/gluc; Sigma Chemical Co.), pepstatin (1 μg/ml; Sigma Chemical Co.), aprotinin (10 μg/ml; Boehringer Mannheim Biochemica, Federal Republic of Germany) and phenylmethylsulfonyl fluoride (1 mM; SERVA, Heidelberg, Federal Republic of Germany). The reaction was stopped by the addition of 500 μl of ice cold 10% TCA, and the precipitates were collected on Whatman GF/C filters (Whatman Chemical Separation, Inc., Clifton, NJ), washed with 2% Triton X-114 instead of 2% SDS (see above) and dissolved in 500 μl of 3 N HCl. Finally the samples were loaded on a column of Sephadex G-25 (1 x 30 cm) and eluted with water. The fractions containing protein were collected on icecold trichloroacetic acid (TCA) was added to a final concentration of 10%, the samples were incubated on ice for 15 min. The precipitates were collected on Whatman GF/C filters (Whatman Chemical Separation, Inc., Clifton, NJ), washed with 5% TCA, air dried, and counted for radioactivity. Fetal (type IV, Sigma Chemical Co.) was desialylated by mild acid hydrolysis with 0.01 N H₂SO₄ for 60 min at 80°C, neutralized, and passed over a column of Sephadex G-25 (1 x 30 cm) and eluted with water. The fractions containing protein (assayed by the Bio-Rad kit, Bio-Rad Laboratories, Richmond, CA) were pooled, dried by lyophilization, and reconstituted with water to a concentration of 88 mg/ml.

Subcellular Fractionation of Infected Cells: One tray (17 x 17 cm) of MDCK cells was infected with 20 pfu/cell of VSV in minimum essential medium, pH 7.4, for 1 h at 37°C. The inoculum was replaced by fresh MEM, pH 7.4. After 3 more hours at 37°C the cells were shifted to 20°C for 90 min. Thereafter, the cells were harvested by scraping, homogenized, and subjected to density gradient centrifugation in 20% Percoll as described above. Galactosyltransferase and aminopeptidase were assayed as described in reference 3. G protein was assayed using a radioimmunoassay on microtiter plate wells as described in reference 3 for aminopeptidase by binding 100 μl of each fraction directly to the wells and using 0.28 μg/ml of anti-VSV antibody.

RESULTS

Transcytosis of Underglycosylated G Protein

Underglycosylated G proteins were used as probes to determine whether reglycosylation took place during transcytosis. Underglycosylated G protein was produced in two ways: VSV was grown either in mutants of CHO cells (CHO 15B), which lack N-acetylgalcosaminyltransferase I (13, 14), or in BHK cells that produce fully glycosylated G protein. The VSV particles were then digested with neuraminidase to remove terminal sialic residues. The glycans of G protein produced in CHO 15B cells are of the high-mannose type with the structure (Man₉)(GlcNAc)₂ (14), being thus sensitive to endoglycosidase H (17). The VSV particles with underglycosylated G protein were implanted into the apical plasma membrane of MDCK cells by low pHi-induced fusion of the viral envelope with the plasma membrane (1). Since both sialyltransferase and N-acetylgalcosaminyltransferase I are thought to be located in the Golgi complex (12, 15, 16), reglycosylation during transcytosis of underglycosylated G protein would indicate involvement of the Golgi complex in the transcellular route.

ENDOGLYCOSIDASE H-SENSITIVE G PROTEIN: [³⁵S]-methionine-labeled VSV was produced in CHO 15B cells. Fig. 1 shows that G protein of VSV₁₅₈ migrated slightly faster in gel electrophoresis than fully glycosylated G protein originating from virus grown in BHK cells (VSV₅₈). G protein of VSV₁₅₈ was endoglycosidase H-sensitive and G protein of VSV₅₈ was endoglycosidase H-resistant (Fig. 1). VSV₁₅₈ was then fused with the apical surface of MDCK cells. [³⁵S]methionine-labeled VSV₁₅₈ was incubated with MDCK cell monolayers at pH 6.3 for 1 h in the cold, and the cell-associated radioactivity was determined. 40% of the...
added virions were bound to the cell surface, while the binding efficiency of \( ^{35} \text{S} \) VSVBH was 12\% (1). Table I shows that most of the bound VSViSB could be removed from the cell surface with EDTA treatment in the cold. The fusion efficiency was 24\%, which was similar to that of VSVBH (Table I). Table II shows that implanted endoglycosidase H-sensitive G protein was internalized to an extent similar to the fully glycosylated G protein and that the two different G proteins were redistributed to the basolateral surface with the same efficiency. The rates of intracellular degradation of the viral proteins were also similar in both cases (Table II).

Since implanted endoglycosidase H-sensitive G protein was indeed transcytosed from the apical to the basolateral membrane domain, possible alterations in its glycans during transcytosis could be monitored. Cells containing \( ^{35} \text{S} \)-labeled implanted G protein were harvested immediately or incubated for 60 min at 31\°C. Half of the preparations were then digested with endoglycosidase H. Thereafter, the samples were subjected to TCA-precipitation followed by SDS gel electrophoresis. Fig. 2 shows that implanted G protein remained fully susceptible to endoglycosidase H after transcytosis for 60 min at 31\°C.

**Desialylated G protein:** \( ^{35} \text{S} \)-G protein isolated from desialylated virus particles (VSViSact) was subjected to two-dimensional gel electrophoresis to check whether most of the sialic acid residues had been removed by the neuraminidase treatment. G protein of VSVBH showed six spots (Fig. 3 A), probably representing different degrees of sialylation and sulfation of G protein (18). After neuraminidase digestion there were only two major and one minor spot on the basic side of the spectrum (Fig. 3 B). For VSViSact the binding and fusion efficiencies, as well as internalization, redistribution, and degradation of the G protein were similar to those of intact G protein (Tables I and II).

\[ \text{Virus} \quad \text{Virus not released by EDTA}^* \quad \text{EDTA treatment and Fusion efficiency}^* \]

<table>
<thead>
<tr>
<th>Virus</th>
<th>After low pH treatment</th>
<th>Fusion efficiency</th>
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<tbody>
<tr>
<td>VSViSB</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>VSViSact</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td>VSVBH</td>
<td>16</td>
<td>40</td>
</tr>
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1.3 x 10^6 cpm (1 \mu g virus protein) of \( ^{35} \text{S} \)-labeled virus grown in CHO 15B cells (VSViSact) or in BHK cells (VSVBH) or VSVBH digested with neuraminidase (VSVBH) were incubated with MDCK cell monolayers (2 x 10^6 cells) for 1 h at 0°C at pH 6.3 to allow binding of virions to the cell surface. Binding was 40, 19, and 12\% of the added VSViSB, VSViSact, VSVBH, respectively. The data are expressed as percentages of the total \( ^{35} \text{S} \)-radioactivity initially bound to the cells after 1 h at pH 6.3 and 0°C and represent the mean of two experiments.

* Parallel monolayers were treated after the binding step with EDTA in the cold to remove bound virions. The remaining cell-associated radioactivity is referred to as EDTA background.

**Internalization, Transcytosis, and Intracellular Degradation of Underglycosylated G Protein**

<table>
<thead>
<tr>
<th>Source of G protein</th>
<th>Apical G protein</th>
<th>Appearance of basolateral G protein</th>
<th>Intracellular degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VSViSB</td>
<td>38</td>
<td>26</td>
<td>12</td>
</tr>
<tr>
<td>VSViSact</td>
<td>30</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>VSVBH</td>
<td>30</td>
<td>28</td>
<td>15</td>
</tr>
</tbody>
</table>

1 \mu g of VSV (virus protein) grown in CHO 15B cells (VSViSact) or in BHK cells (VSVBH) or VSVBH digested with neuraminidase (VSVBH) were incubated with MDCK cell monolayers (2 x 10^6 cells) for 1 h at 0°C at pH 6.3. After washing the pH was dropped to 5.3 for 20 s at 37°C and the unfused viruses were removed by EDTA treatment at 0°C (1). The cultures were then incubated at 31°C for different periods of time. The amounts of apical and basolateral G protein were measured by an \( ^{125} \text{I} \)-protein A-binding assay (2). The values given are the means of two experiments.

* The apical G protein is expressed as the percentage of G protein detected at the apical surface 30 min at 31°C after implantation.

† The basolateral protein is the percentage of G protein transcytosed to the basolateral surface after 30 min at 31°C (see reference 3).

‡ VSViSact, VSViSact, and VSVBH were fused with MDCK cells exactly as described above, except that \( ^{35} \text{S} \)-labeled virus of VSViSact, VSViSact, or VSVBH type was added to each respective preparation before binding. During incubation at 31°C the TCA-soluble radioactivity appearing in the extracellular medium was monitored. For each virus, after a lag of 30 min, TCA-soluble \( ^{35} \text{S} \)-radioactivity started to accumulate at a linear rate into medium. The value (mean of two experiments) is the percentage of the total cell-associated radioactivity degraded after 90 min at 31°C (2).

**FIGURE 2** Endoglycosidase H-sensitivity of G protein of VSViSB after transcytosis in MDCK cells. \( ^{35} \text{S} \)-Methionine-labeled VSViSB was fused with the apical surface of MDCK cells and nonfused virions were removed by EDTA treatment using the same protocol as before (3). The cells were harvested for SDS gel electrophoresis immediately (A and B) or after a 60-min incubation at 37°C in the presence of 20 \mu g/ml of cycloheximide (C and D). Samples in B and D were digested with endoglycosidase H prior to gel electrophoresis. 18,000 cpm were loaded in each slot.

**TABLE I**

**Fusion Activity of Underglycosylated G Protein**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Fusion efficiency</th>
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<tbody>
<tr>
<td>VSViSB</td>
<td>24%</td>
</tr>
<tr>
<td>VSVBH</td>
<td>24%</td>
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</tbody>
</table>

**TABLE II**

**Internalization, Transcytosis, and Intracellular Degradation of Underglycosylated G Protein**

<table>
<thead>
<tr>
<th>Source of G protein</th>
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<tr>
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<td>28</td>
<td>15</td>
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**FIGURE 2**
FIGURE 3 Analysis of G protein by two-dimensional gel electrophoresis. The G protein was extracted from [35S]methionine-labeled VSV with Triton X-114, and subjected to two-dimensional isoelectric focusing-SDS PAGE and fluorography prior to (A) or after (B) neuraminidase digestion. The neuraminidase-digested 35S-VSV was fused with the apical surface of MDCK cells, which were incubated after EDTA-treatment for 60 min at 31°C (C) or for 90 min at 21°C (D) in the presence of 20 μg/ml of cycloheximide. The cells were solubilized into 0.75% Triton X-114, G protein was isolated by phase separation into the detergent, and the samples were subjected to two-dimensional gel electrophoresis as above. Separation from top to bottom is by apparent molecular weight and from left to right by isoelectric point with the acidic end at the right. The x marks the position of viral N protein which serves as an internal marker.

tored by affinity chromatography using RCA-Sepharose. This lectin binds terminal galactose residues (19). Fig. 4 shows that most G protein (72%) did not bind to the lectin before digestion, whereas after digestion the G protein was almost completely bound, and could be eluted from the column by displacement with 0.1 M galactose. Implantation of fully glycosylated 35S-G protein and subsequent incubation of the cells for either 60 min at 31°C or for 90 min at 21°C did not increase the fraction that was bound to the lectin, suggesting that sialic acid residues were not lost during transcytosis (Fig. 4A). Likewise, incubation of cells with implanted 35S-asialo G protein at either temperature did not decrease the fraction bound to the lectin, suggesting that addition of sialic acid residues did not take place either (Fig. 4B). However, since it is not known how many of the maximum number, i.e., six, of the sialic acid residues present in a fully glycosylated G protein are necessary for lectin-binding, we assayed the degree of sialylation of G protein using a more sensitive method, two-dimensional gel electrophoresis. Cultures with implanted 35S-asialo G protein were incubated for 60 min at 31°C or for 90 min at 21°C. The 35S-G protein was extracted into Triton X-114 (see reference 3) and the samples were then subjected to two-dimensional gel electrophoresis (Fig. 3, C and D). The same two major spots and the one minor spot of G protein were found as seen in two-dimensional gels of G protein obtained directly after neuraminidase treatment (Fig. 3B).

These results show that both desialylated and endoglycoside-H sensitive G protein were endocytosed and transcytosed in a similar fashion as fully glycosylated G protein. However, no evidence for reglycosylation during transcytosis could be found with the methods used.

IN VITRO SIALYLATION OF IMPLANTED DESIALYLATED G PROTEIN: A control experiment was performed in vitro to find out whether desialylated and low-pH treated G protein can be resialylated by the sialyltransferases present...
in the Golgi fraction of MDCK cells. 4 x 10^6 cpm of 35S-VSV<sub>brk</sub> was digested with neuraminidase and bound to the apical surface of a monolayer of 70 x 10^6 cells at pH 6.3 in the cold. Fusion was performed as before. The cells were solubilized in 10 ml of PBS containing 0.75% Triton X-114, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 1 mM iodoacetamide. The suspension was centrifuged for 5 min at 2,500 rpm in the cold to remove unsolubilized debris, incubated for 3 min at 30°C, and centrifuged for 3 min at 10,000 rpm at room temperature. The supernatant was discarded and the detergent phase containing the 35S-G protein was assayed for binding to RCA-Sepharose. Fig. 5A shows that in this experiment ~50% of the radioactivity eluted off the column as a broad peak with PBS. The rest could be displaced with 0.1 M galactose. A second cycle of Triton X-114 extraction did not alter the affinity chromatography profile. This preparation was used as substrate in the in vitro resialylation experiment.

Golgi-derived vesicles containing sialyltransferase were isolated by Percoll density gradient centrifugation. MDCK cells were homogenized using SEAT-buffer and the postnuclear supernatant was subjected to density gradient centrifugation in 20% Percoll. The 500-μl fractions were assayed for sialyltransferase activity after phase separation into Triton X-114. A profile similar to the galactosyltransferase activity shown in Fig. 6 (see below) was obtained. The four peak fractions were pooled, diluted 10-fold with PBS, and subjected to phase separation into Triton X-114. The detergent phase served as the source of sialyltransferase in the following experiment.

For in vitro resialylation of G protein, 20 μl of the 35S-asialo G protein preparation described above were incubated with 20 μl of the sialyltransferase preparation for 60 min at 37°C with 70 μl of reaction mixture containing CMP-sialic acid, β-mercaptoethanol, and TX-100 in 0.1 M cacycylate buffer, pH 5.8 (see Materials and Methods). After cooling on ice the reaction product was assayed for binding to RCA-Sepharose. The 35S-radioactivity was quantitatively excluded from the column, indicating extensive resialylation of 35S-G protein (Fig. 5B). In another experiment 90% of the radioactivity was excluded from and 10% bound to the column. If the sialyltransferase preparation was omitted from the reaction mixture, an affinity chromatography profile similar to that obtained before the incubation was obtained: 46% of the radioactivity was excluded from the column (Fig. 5B). We cannot from these experiments completely exclude degradation, but our results strongly suggest that the sialyltransferases of MDCK cells were able to resialylate implanted, low pH-treated 35S-asialo G protein. Thus, the asialo G protein should serve as a proper substrate for sialyltransferases during transcellular transport.

**Subcellular Fractionation**

MDCK cells were fractionated to find out whether implanted G proteins passed through the Golgi complex during transcytosis. 35S-G protein from VSV<sub>brk</sub> was implanted into the apical cell surface of MDCK cells as before and the cultures were harvested immediately or after an incubation time of 8, 20, or 40 min at 31°C. The cells were homogenized and subjected to density gradient centrifugation in 20% Percoll. Aminopeptidase and β-hexosaminidase co-migrated in the gradient at a density of 1.05–1.07 g/cm³. Galactosyltransferase was well separated from the latter, banding at ~1.035 g/cm³ (Fig. 6A). All the 35S-G protein was found at a density of 1.05–1.07 g/cm³ with the plasma membrane and the lysosome markers. Fig. 6 shows that at none of the studied time points could any 35S-G protein be found co-migrating with galactosyltransferase.

To control whether newly synthesized G protein could be found in galactosyltransferase-containing fractions banding at 1.035 g/cm³, MDCK cells were infected with VSV and the cell homogenate was subjected to similar Percoll density gradient centrifugation as shown in Fig. 6. Under these conditions G protein assayed by the 125I-protein A-binding assay on microtiter plates could be detected in the fractions containing galactosyltransferase activity banding at 1.035 g/cm³ (Fig. 7).

**DISCUSSION**

In this paper we have studied whether during transcytosis G protein traverses intracellular compartments containing N-acetyl-glucosaminyltransferase I, sialyltransferase, or galactosyltransferase activity. Galactosyltransferase has been localized by immunoelectronmicroscopic methods to the trans cisternae of the Golgi complex in HeLa cells (20). Similar studies have not been carried out as yet for sialyltransferase and N-acetylglicosaminyltransferase I, but cell fractionation and autoradiographic studies have demonstrated that both enzymes are part of the Golgi complex (15, 16). Using underglycosylated G proteins as probes we have found no evidence for resialylation or acquisition of resistance to endoglycosidase H during transcytosis. Neither could we demonstrate significant amounts of G protein in subcellular fractions containing galactosyl- or sialyltransferase activity after implantation and endocytosis into MDCK cells. These studies confirm our earlier results obtained by localization of endocytosed G protein using immunoelectron microscopy (1). Practically no G protein could be detected in Golgi cisternae. These results suggest that the Golgi complex is not part of the route that implanted G protein is following to reach the basolateral...
plasma membrane through the cell. However, from these experiments we cannot conclusively exclude the possibility that a very small fraction of G protein in fact traversed glycosyltransferase-containing compartments. Neither can we exclude that G protein passed through the reticular membrane elements associated with the trans face of the Golgi stack as envisaged by Willingham and Pastan (21). However, this postulated part of the Golgi system has to be more precisely defined before its role in endocytic processes such as transcytosis can be delineated.

Our results are in keeping with those of Abrahamson and Rodewald (22) who showed that ferritin-labeled IgG were found in endosomes and lysosomes during receptor-mediated transcellular transport from the apical to the basolateral plasma membrane in new born rat intestinal cells. They did not demonstrate IgG in the Golgi complex. Neither did Herzog (23) for thyroglobulin transcytosed from the apical to the basolateral plasma membrane in thyroid follicle cells. On the other hand, it has been reported that desialylated transferrin is partially resialylated during membrane recycling at the sinusoidal plasma membrane of rat hepatocytes (24). Bergerson and co-workers (25) have also reported uptake of insulin into Golgi-like elements from the sinusoidal membrane of rat liver cells. Similarly endocytosis into the Golgi complex of cationized ferritin, a nonspecific membrane marker, has been demonstrated in secretory cells after hormonal stimulation of secretion (see reference 15). Similar endocytic pathways into the Golgi complex might very well exist in MDCK cells, but they appear not to constitute a major route in transcytosis.

It is interesting that G protein with both desialylated and high-mannose glycans is transcytosed as efficiently as fully glycosylated G protein. It has previously been shown that nonglycosylated or underglycosylated G protein is correctly routed to the basolateral plasma membrane during VSV infection of MDCK cells (26, 27). Similar results have also been obtained for apical viral glycoproteins (26, 27). These studies indicate that protein-bound glycans are not involved as sorting signals. Little is presently known of the mechanism by which apical and basolateral proteins become sorted to their correct destinations in epithelial cells. Sorting must occur not only during exocytosis of newly synthesized membrane proteins, but also during transcytosis to prevent intermixing of recycling surface components from the two surface domains. Our results suggest that lysosomes and the Golgi complex are not involved in transcytosis. Further work will be required to find out whether the endocytic and the exocytic routes meet or whether sorting during exocytosis and endocytosis occur at separate sites.

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