Transepithelial Transport of a Viral Membrane Glycoprotein Implanted into the Apical Plasma Membrane of Madin-Darby Canine Kidney Cells. II. Immunological Quantitation

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ABSTRACT The envelope of vesicular stomatitis virus was fused with the apical plasma membrane of Madin-Darby canine kidney cells by low pH treatment. The fate of the implanted G protein was then followed using a protein A-binding assay, which was designed to quantitate the amount of G protein in the apical and the basolateral membranes. The implanted G protein was rapidly internalized at 31°C, whereas at 10°C no uptake was observed. Already after 15 min at 31°C, a fraction of the G protein could be detected at the basolateral membrane. After 60 min 25-48% of the G protein was basolateral as measured by the protein A-binding assay. At the same time, 25-33% of the implanted G protein was detected at the apical membrane. Internalization of G protein was not affected by 20 mM ammonium chloride or by 10 μM monensin. However, the endocytosed G protein accumulated in intracellular vacuoles and redistribution back to the plasma membrane was inhibited. We conclude that the implanted G protein was rapidly internalized from the apical surface of Madin-Darby canine kidney cells and a major fraction was routed to the basolateral domain.

Enveloped RNA viruses have provided excellent tools to study the intracellular pathway of membrane proteins from their site of synthesis to the plasma membrane (see reference 8). Recently, the use of viruses and their envelope glycoproteins has been extended to study endocytosis of the cell surface (12, 30).

In the preceding paper we developed another approach to study the traffic to and from the cell surface in Madin-Darby canine kidney (MDCK) cells (17). In this case the proteins were not introduced into the plasma membrane from within after synthesis, but inserted there from the outside by low pH-induced fusion of the viral envelope with the cellular plasma membrane (16, 31, 32). MDCK cells are polarized epithelial cells, the plasma membrane of which is differentiated into two structurally and functionally different domains separated by tight junctions, namely the apical surface facing the growth medium and the basolateral surface facing the neighboring cells and the substratum (4, 11, 19, 20, 24). Normally during vesicular stomatis virus (VSV) infection of MDCK cells the G proteins are mainly transported to the basolateral plasma membrane (25). In the present study we implanted the G protein of VSV into the apical plasma membrane of these MDCK cells. Our previous morphological study (17) showed that the implanted G proteins are rapidly endocytosed and that some of them are distributed to the basolateral surface.

In the present study we used a protein A-binding assay to characterize the internalization and redistribution of the implanted G proteins in more detail.

MATERIALS AND METHODS

The cells, virus preparations, the implantation procedure of the G protein into the apical plasma membrane of MDCK cells, and the immunofluorescence staining technique are described in our previous study (17).

125I Protein A-binding Assay: The assay was adapted from that described for chicken embryo fibroblasts infected with Semliki Forest virus (7). To assay antigens in the apical cell surface, we fixed cultures directly before the 125I protein A-binding assay. To assay the whole cell surface, we made the basolateral antigens accessible for the reagents prior to fixation by washing briefly twice with 2 ml of PBS lacking Ca** and Mg** and incubating the cells for 5 min at 31°C with 5 mM EGTA (see also, in reference 17, Fig. 2). Both solutions were prewarmed to 31°C. Fixation was performed in the cold with 3% (wt/vol) formaldehyde, which was thereafter quenched with 50 mM NH4Cl at room temperature. All solutions used for EGTA-treated cells after the fixation step lacked Ca** and Mg**. Fixed cells were overlaid with 250 μl of antibody diluted in PBS containing 0.2% (wt/vol) of gelatin (PBS-gelatin) and incubated for 30 min at room temperature. After three washes with PBS-gelatin the cells were incubated with 250 μl of 125I protein A prepared in PBS-gelatin for 30 min at room temperature. The label was removed and the plates were washed.
RESULTS

$^{125}$I Protein A-binding Assay

To implant the envelope glycoprotein G of VSV into the apical surface of MDCK cells, 0.5–1.25 μg of VSV (protein) was added together with 125,000 cpm of $^{3}$H]uridine labeled VSV in binding medium (pH 6.3) to plates containing 2 × 10$^6$ MDCK cells, and incubated for 1 h in the cold. The unbound virions were washed away with binding medium. In the presence of calcium the tight junctions remain intact and only the apical surface is accessible to the virus (5, 14, 17). From each amount of virions added ~13% was bound to the cell surface (Fig. 1A). The rest of the plates were then put on a waterbath of 37°C and overlaid for 20 s with fusion medium (pH 5.4) prewarmed to 37°C to allow the envelope of the virions to fuse with the apical membrane of the cells. The monolayers were then treated with 0.5 mg/ml of trypsin for 90 min in the cold to release bound but unfused virions from the cell surface. For each amount of VSV added to the cells, 3% was fused with the cellular plasma membrane (Fig. 1A). To another set of cultures 0.5–1.25 μg of unlabeled VSV (protein) was added. After the binding and fusion steps the unfused virions were removed by a 5-min incubation with 20 mM EDTA in the cold (17). The cells were then fixed, treated with anti-VSV antibody, followed by $^{125}$I protein A, solubilized, and counted for cell-associated radioactivity. Fig. 1B shows that the assay was linear for various amounts of G protein at the apical surface.

Quantitation of G Protein at the Apical Surface

VSV (1 μg of protein) was bound and fused to 2 × 10$^6$ cells as above, followed by EDTA-treatment to release unfused virions. As previously shown, about 26,000 G proteins are implanted per cell under these conditions (3, 17). After implantation the cultures were incubated at 31°C and the G protein at the cell surface was quantitated by the $^{125}$I protein A-binding assay. The amount of G protein at the apical surface decreased rapidly (Fig. 2, solid circles). After 60 min the level of G protein had declined to ~25–33% (range of 10 experiments). The decrease of G protein at the surface was due to internalization rather than release into the medium. This was verified by following the appearance of acid-precipitable radioactivity into the medium during the incubation of cells to which $[^{35}]$S]methionine VSV was fused (see below). The amount of G protein implanted in the apical plasma membrane did not influence the extent of internalization within the range of 0.1 to 10 μg of VSV (protein) added per 2 × 10$^6$ cells (data not shown). No internalization of G protein could be detected at an incubation temperature of 10°C (Fig. 2, squares).

Quantitation of G Protein at the Basolateral Surface

Depletion of Ca$^{++}$ ions with EGTA treatment at 37°C opens up the tight junctions of MDCK cells, thus allowing access of the reagents to the basolateral surface (5, 14). Cells with 26,000 virions were added together with 125,000 cpm of $[^{3}]$H]uridine labeled VSV to monolayers of 2 × 10$^6$ MDCK cells at pH 6.3 and 0°C. After washing away the unbound virions the cell-associated radioactivity was determined. From these figures the amount of viral protein bound to the cells was calculated and plotted against the respective amount of virus added to the cell (O). The binding efficiency was a constant 13% of the added virus. In another set of cultures fusion was induced by pH 5.4 after the binding step and the unfused virions were removed by trypsin-treatment in the cold. The radioactivity was determined to calculate the cell-associated amount of viral protein (O). For each amount of virus added, 3% was fused with the apical membrane (see reference 17). (B) 0.5–1.25 μg of VSV (protein) was added per plate of 2 × 10$^6$ MDCK cells and after the binding and fusion steps the unfused virions were removed with a 5-min incubation of the cells with EDTA in the cold. The cells were then fixed and treated with anti-VSV antibody followed by $^{125}$I protein A per plate. The cell-associated radioactivity was determined and plotted against the respective amount of virus originally added to the monolayers for implantation.
G protein molecules inserted into the apical surface per cell (see above) were incubated at 31°C in the presence of 20μg cycloheximide/ml. 7 min before the indicated time points in Fig. 2.4 the cells were washed twice with PBS lacking Ca++ and Mg+++, and incubated with 2 mM EGTA at 31°C for 5 min. The amount of G protein detected after EGTA treatment at the cell surface with the 125I protein A-binding assay was larger than that detected on the apical surface only (Fig. 2A, open circles). The difference between the values obtained for the surface expression before and after EGTA treatment was taken to represent basolateral G protein (Fig. 2B). After 60 min at 31°C, the fraction of basolateral G protein varied between 25% and 48% in 10 experiments. If cycloheximide was omitted from the incubation medium the same results were obtained. The incubation time with EGTA appeared to be critical. A treatment of 2 to 5 min at 31°C was found to give maximal values for 125I protein A-binding. Incubation times >10 min gave lower values since the cells begin to round up after prolonged treatments with EGTA and came off the plates in subsequent manipulations (Table I).

An attempt was made to quantitate the amount of G protein in the basolateral plasma membrane directly by treating the cells with saturating amounts of anti-VSV antibody (0.83μg/ml) and unlabeled protein A (0.2μg/ml) for 15 min at 0°C before opening of the tight junctions with EGTA at 31°C to render the apical cell surface unreactive in the subsequent 125I protein A-binding assay (Fig. 3). It was not possible, however, to quench >50% of the 125I protein A-binding activity of the apical surface. Nevertheless, Fig. 3 shows that the amounts of basolateral G protein detected under the above conditions were reasonably similar to figures obtained without quenching of the apical surface with unlabeled protein A.

**Degradation of Internalized Viral Proteins**

VSV (1μg of protein) labeled with 300,000 cpm of [35S]-methionine was added to plates of 2 x 10^6 MDCK cells, followed by the binding and fusion steps and EDTA treatment to release unfused virions. The cultures were incubated at 31°C in the presence or the absence of 20 mM NH4Cl. At different time points acid-soluble and acid-precipitable radioactivity of the medium and the total cell-associated radioactivity were determined. Fig. 4 shows that after a lag period of 30 min acid-soluble radioactivity started to appear in the medium, indicating degradation of the viral proteins. Therefore, the rate of degradation was 15%/h of the total cell-associated viral radioactivity. Ammonium chloride (20 mM) in the incubation medium inhibited degradation. Within 15 min after the cultures had been shifted to 31°C there was an initial loss of ~15% of the cell-associated acid-precipitable radioactivity into the medium (Fig. 4). It most probably was derived from unfused virions left after EDTA treatment which were eluted from the cell surface by neutral pH at 31°C. No further loss of acid-precipitable material occurred.

**Implantation and Internalization of G Protein Do Not Induce Random Membrane Uptake**

**Fluid-phase Uptake:** To study whether the low pH-induced fusion of VSV with the cell surface or internalization of G protein affected general parameters of the cells, we first measured fluid phase uptake of MDCK cells using fluorescein-conjugated dextran (FITC-dextran) as a marker. Untreated control cultures and cultures with implanted G protein (26,000 molecules per apical cell surface) were incubated at 31°C with 1 ml of minimal essential medium containing 20 mg/ml of FITC-dextran. At different time points cells were harvested by scraping, lysed, and the cell-associated fluorescence intensity was determined. Fig. 5 shows that both sets of

![Figure 2](https://example.com/figure2.png)

**Effect of EGTA Treatment on MDCK Cells**

| Incubation time with EGTA | Bound 125I protein A |
|--------------------------|----------------------|
| min          | cpm        |
| 0            | 7,500      |
| 2            | 11,000 (100%) |
| 5            | 11,000 (100%) |
| 10           | 10,700 (97%)  |
| 15           | 10,000 (91%)  |
| 20           | 8,700 (78%)   |

26,000 G protein molecules were implanted into the apical surface of MDCK cells and the cultures were incubated at 31°C. After 45 min duplicate dishes were treated for different times with 5 mM EGTA at 31°C and the amount of G protein on the cell surface was estimated with the 125I protein A-binding assay using 42,000 cpm of the label per plate. Immediately after implantation 15,000 cpm of 125I protein A was bound to the cells.

![Figure 3](https://example.com/figure3.png)
plates displayed the same kinetics of uptake of FITC-dextran. Uptake was linear for at least 3 h. The rate of uptake was 4.5 nM/l/h/10^6 cells. Some of the cultures were assayed for apical G protein. The same kinetics of internalization of G protein were obtained in the absence or in the presence of FITC-dextran (Fig. 5).

**Ammonopeptidase:** Next we wanted to see whether internalization of G protein from the apical cell surface was paralleled by uptake of an apical membrane protein of the cell, ammonopeptidase. The 125I protein A–binding assay was used to follow the level of ammonopeptidase at the apical membrane during the time when G protein rapidly disappeared from the cell surface. After insertion of G protein into the apical surface as before, the cultures were incubated at 31°C in the presence of 20 μg/ml of cycloheximide. One set of plates was assayed for G protein and another for ammonopeptidase using the respective antibodies. The amount of G protein decreased 78% in 15 min (Fig. 6). However, the amount of ammonopeptidase barely changed in 15 min and decreased only 10% in 30 min. The same decrease of ammonopeptidase at the surface was obtained for cultures which had been incubated at 31°C in the presence of cycloheximide without G protein implantation. Thus, the slight decrease of ammonopeptidase at the apical surface was due to the drug or the shift from 37°C to 31°C and not to the virus or the low pH-treatment.

**Inhibition of Redistribution of G Protein**

The effect of ammonium chloride and monensin on G protein redistribution was tested since they have been reported to interrupt recycling of the cell surface receptors (2, 6, 9, 10, 28). When 20 mM ammonium chloride was added to the incubation medium, implanted G protein was internalized rapidly from the apical surface as in the control cultures. The level of apical G protein continued to decline and decreased in 60 min to 7% at the apical and to 5% at the basolateral surface (Fig. 7). In parallel controls, ~20% of the G protein could be detected at the apical and ~30% at the basolateral surface after a 60-min incubation. The effect of ammonium chloride on the level of G protein at the cell surface was concentration dependent. Similar effects were obtained with 20 and 10 mM concentrations, but a 2-mM concentration showed no effect within 60 min. Essentially the same results were obtained with 10 μM monensin instead of 20 mM ammonium chloride. The effect of ammonium chloride and monensin on the fate of the implanted G protein could be seen also by indirect immunofluorescent labeling. In the presence of the drugs G protein disappeared from the apical surface and accumulated in large intracellular vacuoles (Fig. 8). There was no detectable redistribution of G protein to the basolateral surface domain. Thus, G protein accumulated inside the cells in the presence of ammonium chloride or monensin. These results imply that most of the G protein was assayed before (closed symbols) or after EDTA treatment (open symbols) using 45,000 (closed symbols) or 51,000 cpm (open symbols) of 125I protein A per plate. After implantation of G protein before incubation at 31°C, 17,800 cpm of 125I protein A was bound to the cell surface (100%). The amount of cell-associated 125I protein A decreased only 10% in 30 min. The same decrease of ammonopeptidase at the apical surface was due to the drug or the shift from 37°C to 31°C and not to the virus or the low pH-treatment.

**Figure 4** Degradation of internalized VSV proteins. 300,000 cpm of 35S-methionine-labeled VSV and lug of unlabeled VSV (protein) were added per plate of MDCK cells. After the binding and fusion steps unfused virions were removed by EDTA-treatment and the cultures were incubated at 31°C in the presence of cycloheximide (closed symbols), or both cycloheximide and 20 mM ammonium chloride (open symbols). At different time points duplicate cultures were harvested and the acid-soluble (circles) and acid-precipitable radioactivity (triangles) of the medium, as well as the total cell-associated radioactivity (squares) were determined.

**Figure 5** Effect of internalization of implanted G protein on fluid phase uptake of MDCK cells. Untreated MDCK cells (squares) or cells to which G protein was implanted (see Fig. 2) (triangles and circles) were incubated in the presence (closed symbols) or absence (open circles) of 20 mg/ml of FITC dextran at 31°C. At different time points one set of cells (squares and triangles) were scraped off the plates, washed and lysed with PBS containing 0.1% SDS and the fluorescence intensity of the cell-associated FITC-dextran was determined using excitation and emission wavelengths of 490 and 520 nm, respectively. Another set of plates (circles) was fixed and the amount of G protein at the apical cell surface was determined with the 125I protein A–binding assay using 40,000 cpm of the label per plate.

**Figure 6** Level of ammonopeptidase at the apical surface during internalization of G protein. G protein was inserted into the apical surface of MDCK cells (see Fig. 2) and the cultures were incubated at 31°C in the presence of cycloheximide. The levels of G protein (△) and ammonopeptidase (○) at the apical surface of the cells were determined using the 125I protein A–binding assay. One set of cultures was not treated with virus, but incubated at 31°C in the presence of cycloheximide (△) and assayed for ammonopeptidase. To assay ammonopeptidase 20 μg/ml of antibody was used and the radiolabeled protein A was diluted with 10 ng/ml of unlabeled protein A. In the assay 15,400 cpm (△), 12,300 cpm (○), or 11,600 cpm (△) of 125I protein A was bound per plate out of 70,800 cpm (△) or 61,800 cpm (○, △) 125I protein A added per plate prior to incubation at 31°C. The amount (in %) of 125I protein A bound to the cell surface compared to time 0 are plotted against times of incubation.

**Figure 7** Inhibition of redistribution of internalized G protein to the cell surface by ammonium chloride (2%) protein A–binding assay). G protein was inserted into the apical surface of MDCK cells (see Fig. 2) and the cultures were incubated at 31°C in the presence of cycloheximide (open symbols) using 45,000 (closed symbols) or 51,000 cpm (open symbols) of 125I protein A per plate. After implantation of G protein before incubation at 31°C, 17,800 cpm of 125I protein A was bound to the cell surface (100%). The amount of cell-associated 125I protein A is plotted against the time of incubation.

**Figure 8** Degradation of internalized VSV proteins. 300,000 cpm of 35S-methionine-labeled VSV and lug of unlabeled VSV (protein) were added per plate of MDCK cells. After the binding and fusion steps unfused virions were removed by EDTA-treatment and the cultures were incubated at 31°C in the presence of cycloheximide (closed symbols), or both cycloheximide and 20 mM ammonium chloride (open symbols). At different time points duplicate cultures were harvested and the acid-soluble (circles) and acid-precipitable radioactivity (triangles) of the medium, as well as the total cell-associated radioactivity (squares) were determined.
Figure 8: Inhibition of redistribution of G protein to the cell surface by ammonium chloride (indirect immunofluorescent staining). After implantation of G protein to the apical surface of MDCK cells as in Fig. 2, the monolayers were directly fixed (a–c) or incubated at 31°C for 30 min (d–i) in the absence (d–f) or presence (g–i) of 20 mM ammonium chloride prior to fixation. In a, d, and g the cells were fixed and treated directly with anti-VSV antibody followed by rhodamine-conjugated anti-IgG antibody to stain the apical cell surface. In b, e, and h the tight junctions were opened by EGTA treatment at 31°C before fixation to gain access for the reagents also to the basolateral cell surface. In c, f, and i the cells were permeabilized with 0.1% Triton X-100 after fixation to visualize internalized antigens. For more detail see reference 17. Bar, 8 μM.

Molecules detected at the apical surface under normal incubation conditions did not represent a static and immobile pool, but were also capable of being endocytosed. In contrast to G protein, 20 mM ammonium chloride or 10 μM monensin had no effect on the level of aminopeptidase at the apical surface. In the presence of the drugs the same results were obtained as shown for control cultures in Fig. 6.

Discussion

In this study we used a protein A–binding assay to follow the internalization and the reappearance at the cell surface of G protein after implantation into the apical plasma membrane of MDCK cells. Apical proteins could be monitored with the assay after fixation of the cell monolayer. Only the apical surface domain is accessible to the antibodies because the cells in the monolayer are sealed together by tight junctions (4, 5, 14, 17). Proteins present in the apical and the basolateral surface domains could be monitored after opening the tight junctions by calcium-depletion before fixation. Whether all basolateral proteins became accessible to the reagents after the EGTA treatment is difficult to judge. It is possible that the basolateral values obtained with this assay are underestimated because of steric hindrance through cell-cell and cell-substratum interactions.

The protein A–binding assay showed that the implanted G
proteins were internalized rapidly from the apical surface. The half-life of the implanted G protein at the apical surface was <10 min at 31°C. At 10°C internalization was not observed. The process was thus similar with regard to kinetics and temperature-dependence to receptor-mediated endocytosis (see reference 26). The implantation procedure did not perturb the properties of the apical membrane as judged by two parameters. First, we found no change in the rate of fluid phase endocytosis after fusion of the virus with the plasma membrane. Second, we could not detect any appreciable loss of aminopeptidase from the apical membrane during the internalization of implanted G protein.

The morphological studies in the preceding paper (17) showed that the G protein was not only endocytosed after implantation, but a portion was redistributed to the basolateral surface. Some also seemed to be recycled to the apical surface. Here the redistribution of the endocytosed G protein to the basolateral surface could be quantitated using the protein A–binding assay. The rate of the appearance of G protein at the basolateral surface varied between experiments (cf. Figs 2 and 7), but 15 min after implantation some basolateral G protein was usually detectable. After 60 min the fraction of G protein at the basolateral surface was estimated to be 25-48% of the implanted proteins. The routing of G protein to the basolateral cell surface could be almost completely inhibited both by the carboxylic ionophore monensin which catalyzes the exchange of Na⁺ and H⁺ across biological membranes (22) and by ammonium chloride. The latter weak base accumulates in acidic compartments and increases their pH (18, 21).

Using these drugs the apical surface practically cleared of G protein and the protein accumulated in intracellular vacuoles. Previous studies have shown that the recycling of cell surface receptors can be inhibited to varying degrees by these drugs (2, 6, 9, 10, 28). Exactly how they exert their action is not known, but evidence is accumulating that endosomes might be the site at which these drugs affect recycling. Recent studies have shown that endosomes as well as lysosomes have an acidic pH (13, 18, 23, 29). An increase in the endosomal pH may prevent dissociation of ligand-receptor complexes and recycling to the cell surface. The immunoperoxidase labeling studies revealed most of the intracellular G proteins in endosomes, some in multivesicular bodies, and very rarely in secondary lysosomes in the first 10 min after implantation (17). Moreover, virtually no degradation of the viral polypeptide was observed within 30 min after implantation at 31°C. Thus, lysosomes may not be an obligatory intermediate on the transepithelial transport route. It seems more likely that the endosome is the organelle from which the internalized G protein is routed to the basolateral surface (cf. reference 1) and that it is here that monensin and NH₄Cl block the transepithelial transport of the G protein. Further studies are underway to characterize the organelles involved in the transepithelial route.

Our studies suggest that the apical and the basolateral surface domains are connected by an intracellular route in MDCK cells. An implication of these findings is that continuous sorting of membrane components would have to take place to maintain the unique composition of the apical and the basolateral surface domains. We are now studying the fate of the influenza virus hemagglutinin implanted by low pH-fusion into the apical plasma membrane. Hemagglutinin appears at the apical surface of MDCK cells after de novo synthesis (25). A future goal of this work will be to find out where in the cell apical and basolateral proteins are sorted from each other during endocytosis.

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