

Intracellular Sorting and Basolateral Appearance of the G Protein of Vesicular Stomatitis Virus in Madin-Darby Canine Kidney Cells

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ABSTRACT The polarity of the surface distribution of viral glycoproteins during virus infection has been studied in the Madin-Darby canine kidney epithelial cell line on nitrocellulose filters. Using a surface radioimmunoassay on Madin-Darby canine kidney strain I cells that had been infected with vesicular stomatitis virus or with avian influenza fowl plague virus, we found that the surface G protein was 97% basolateral, whereas the fowl plague virus hemagglutinin was 88% apical. Newly synthesized, pulse-labeled vesicular stomatitis virus appeared first on the basolateral plasma membrane as measured by an immunoprecipitation assay in which the anti-G protein antibody was applied to the monolayer either from the apical or the basolateral side. Labeled G protein could be accumulated inside the cell at a late stage of transport by decreasing the temperature to 20°C during the chase. Reversal to 37°C led to its rapid and synchronous transport to the basolateral surface at an initial rate 61-fold greater than that of transport to the apical side. These results demonstrate that the newly synthesized G protein is transported directly to the basolateral membrane and does not pass over the apical membrane en route. Since a previous study of the surface appearance of influenza virus hemagglutinins showed that the newly synthesized hemagglutinins were inserted directly from an intracellular site into the apical membrane (Matlin, K., and K. Simons, 1984, *J. Cell Biol.*, 99:2131-2139), we conclude that the divergence of the transport pathway for the apical and basolateral viral glycoproteins has to occur intracellularly, i.e., before reaching the cell surface.

The mechanism that generates surface polarity in epithelial cells remains unknown. One promising experimental system for the study of epithelial polarity is the Madin-Darby canine kidney (MDCK)¹ cell line which grows as a polarized epithelium in culture (2, 6, 12, 15). For example, the apical and the basolateral plasma membrane domains have been shown to exhibit distinct lipid and protein compositions (7, 21, 22, 32), as in other epithelial cells (see reference 26), and MDCK cells infected with enveloped viruses (28, 29) distribute the newly synthesized viral membrane glycoproteins into one or the other surface domain (27). Specifically, the G protein of

vesicular stomatitis virus (VSV) is inserted mainly into the basolateral plasma membrane, whereas the hemagglutinin of influenza virus is routed primarily to the apical membrane. Viral glycoproteins can thus be used to study how apical and basolateral proteins are transported to their correct destinations in the cell.

One problem in the use of viruses for studying polarity arises from the cytopathic effects of virus infection. Rodriguez-Boulan and Pendergast reported that the tight junctions of many VSV-infected cells have begun to open by the time virus budding started (27). In recent immunocytochemical studies of the distribution of viral membrane glycoproteins in infected MDCK cells grown on plastic, Rindler et al. found significant amounts of G protein on the apical plasma membrane (24, 25) in contrast to the electron microscopic observation that virus assembly seemed to occur predominantly at

¹ *Abbreviations used in this paper:* endo H, endoglycosidase H; FPV, fowl plague virus; HF, Hanks' balanced salt solution supplemented with 10% fetal calf serum; MDCK, Madin-Darby canine kidney; VSV, vesicular stomatitis virus.

the basolateral plasma membrane. They suggested that other viral or cellular components were responsible for the localization of budding to the appropriate domain (24).

The studies on virus assembly referred to above were performed with MDCK cells grown on solid supports. We have recently shown that the cytopathic effects of VSV infection could be considerably delayed in MDCK cells grown on nitrocellulose filters (3). The permeable support allowed the cells to feed through their basal surfaces as they would in an epithelium *in vivo* (5). Under these growth conditions, the MDCK cells formed epithelial monolayers that appeared more polarized than did monolayers on glass or plastic (1, 3). In filter-grown cells, the budding of VSV occurred exclusively from the basolateral side for as long as 10 h after infection (3). Moreover, immunofluorescent studies demonstrated that the G protein was nearly exclusively basolateral; in only 2% of the infected cells was G protein also seen apically.

These improvements in the growth conditions have made it possible to study the biogenesis of G protein in MDCK cells in more detail. In this paper, we have investigated whether the newly synthesized G proteins are transported directly to the basolateral surface, or are first sorted after their emergence at the cell surface. In the latter case, the G proteins would appear first on the apical surface en route to the basolateral surface or, alternatively, could be inserted randomly into both surface domains with subsequent correction through membrane recycling. Our results demonstrate that the newly synthesized G proteins are routed directly from an intracellular site to the basolateral surface.

MATERIALS AND METHODS

Cell Culture: MDCK strain I cells (23) were cloned from a low passage of MDCK cells and grown in plastic flasks as described previously (3). For experiments, cells released from plastic flasks with trypsin-EDTA were seeded onto 3- μ m-pore size nitrocellulose filters (20-mm diam. surface area 3.14 cm²; Thomapor AE97, Reichert, Heidelberg, FRG), clamped into mini-Marbrook chambers (Hendley Engineering, London, UK) as described previously (3). Six or seven such chambers were incubated in 70 ml medium for 4½ d at 37°C in 5% CO₂ without a medium change before use.

Monolayer Electrical Resistance: Electrical resistance of the monolayers, a measure of cell differentiation and monolayer integrity (2, 8), was measured using a device similar to that described by Perkins and Handler (16) in Hanks' balanced salt solution containing Mg⁺⁺ and Ca⁺⁺ supplemented with 10% fetal calf serum (HF).

Viral Infection: Filter-grown cultures of MDCK I were infected with VSV of the Indiana serotype in the following way. The chambers were placed upside down (basolateral side up) inside a humid chamber at 37°C. Virus suspended in 50 μ l (40 pfu/cell, 8 \times 10⁷ pfu/filter) serum-free culture medium was administered through the filter to the basolateral side of the cell monolayer (3). The chambers were covered with cups to maintain humidity and were incubated at 37°C for 1 h. The chambers were then transferred to cups containing 20 ml cell culture medium supplemented with 10% fetal calf serum and incubated basolateral side down at 37°C for an additional 2½–3½ h. Filter-grown MDCK I cells were infected with influenza fowl plague virus (FPV) as described previously (3).

Radioactive Labeling: The techniques developed for radioactive labeling, chasing of pulse-labeled protein, and antibody labeling of live cells (see below) were designed to minimize stress to the monolayer that would lead to leakage of antibody from one side to the other.

VSV-infected cultures were gently washed twice and incubated for 10 min at 37°C in cups containing 20 ml methionine-free culture medium. Methionine incorporation into protein by filter-grown MDCK I cells is dramatically higher when the label is administered from the basolateral side than from the apical side (1). As for virus infection, the chambers were placed basolateral side up in a humid chamber, and the cultures were labeled for 10 min at 37°C with 50 μ Ci [³⁵S]methionine (1,200 Ci/mmol; Amersham International, Amersham, UK) in a volume of 50 μ l methionine-free culture medium containing 0.1% bovine serum albumin (BSA).

At the end of the labeling period, the filters were transferred, apical side up, into cups (in water baths at the desired temperature) containing 20 ml culture medium supplemented to ten times the normal concentration of methionine and 10% fetal calf serum. Changes of chase temperature were accomplished by transferring the chambers from one cup to another at the new temperature. At the end of a chase period, filters were transferred to cups containing 20 ml ice-cold HF and held there until all chambers were ready for antibody labeling.

Immunological Analysis of Radioactively Labeled VSV G Protein: The appearance of pulse-labeled VSV G protein on the apical and basolateral cell surfaces of filter-grown cells was quantified using an adaptation of the immunoprecipitation method of Ploegh et al. (18). Endoglycosidase H-sensitivity of intracellular G protein was monitored on the same cells. The cells were infected with VSV and labeled with [³⁵S]methionine as before, and the VSV G protein on the cell surfaces was labeled for 1 h at 4°C with 50 μ l affinity purified anti-VSV G protein (40 μ g/ml) applied either apically or basolaterally in HF. At the end of the incubation period, excess fluid was removed by aspiration, 500 μ l HF was added to the treated side of the filter and immediately aspirated, and the assembled chambers were washed twice by submersion in large volumes of HF. The filters were then removed from the chamber, rinsed once in HF, and placed into dishes, apical (cell) sides up. The filters were washed once with 2 ml HF for 5 min at 4°C with gentle shaking, and then washed once similarly with PBS containing 0.1% BSA.

Next, 500 μ l (50 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.1% BSA, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) was added. The cells were scraped from the filters into the lysis buffer with the aid of a metal spatula, passed through a 22 G syringe needle into 1.5-ml Eppendorf centrifuge tubes, and centrifuged at 4°C for 5 min at 10,000 g. The supernatant fractions were transferred to fresh tubes, frozen in liquid N₂, and stored at -80°C.

To minimize nonspecific binding, fixed *Staphylococcus* bacteria (Pansorbin, Calbiochem, Giessen, FRG) were pretreated with a lysate of uninfected MDCK cells prepared by adding 0.5 ml of lysis buffer per 75-cm² bottle. The fixed *Staphylococcus* bacteria were washed twice in lysis buffer, resuspended in MDCK cell lysate, and incubated on ice for at least 1 h. Finally, the bacteria were centrifuged and resuspended to 10% wt/vol in fresh lysate.

The supernatant fractions of the [³⁵S]methionine-labeled cell lysates were thawed and centrifuged for 5 min at 10,000 g. 20 μ l of the washed *Staphylococcus* bacteria suspension was added to each supernatant and after vortex mixing, the tubes were incubated on ice for 5 min. The samples were centrifuged at room temperature for 1 min to give pellet and supernatant fractions. The bacterial pellet, containing the VSV G protein from the surface, was washed six times at 4°C with 0.5 ml volumes, twice with wash buffer (50 mM Tris-HCl, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 1 mg/ml BSA), twice with wash buffer containing 0.5 M NaCl, once with wash buffer minus BSA, and finally once with water. All centrifugations were for 1 min at room temperature. Pellets were resuspended using a 1-ml Gilson Pipetman (Gilson, Villier-le-Bel, France). The final washed pellets were taken up into 25 μ l vol of pH 8.8 gel sample buffer and stored frozen at -20°C. The supernatant fractions from the *Staphylococcus* treatment, containing intracellular VSV G protein that had not reacted with anti-G protein applied to the cell surface, were transferred to new tubes, centrifuged for 5 min at 4°C, and again transferred to new tubes. 2 μ l of anti-G protein was added to 200- μ l portions of each of the centrifuged supernatant fractions. The samples were incubated overnight with rotation at 4°C. Preabsorbed *Staphylococcus* bacteria were then added to each sample, and the samples were incubated and washed as described above. The washed bacterial pellets were suspended in 200 μ l of autoclaved 0.2 M citrate buffer solution at pH 5.5 with 1 mM PMSF and 0.1% SDS. Each fraction was split into two 100- μ l aliquots, to one of which was added 2.5 μ l (25 mU/ml) endoglycosidase H (Endo H) (Miles Research Products). The samples were incubated at 37°C for 10 h with constant rotation. An unchased sample, in which all labeled G protein would have been expected to be Endo H sensitive, was used to confirm that the enzymatic conversion was complete. Equal volumes of 40% TCA were added, and the samples were incubated overnight at 4°C. The tubes were centrifuged for 10 min at 10,000 g, the supernatant fractions were discarded, the tubes were again briefly centrifuged, and residual supernatant fluid removed. Finally, the pellets were resuspended in 25 μ l 1 M Tris base-containing sample buffer.

Gel Electrophoresis: Samples were prepared as described by Matlin and Simons (9) with minor modifications noted. Polyacrylamide gels (10%) were prepared as described (9). After running, the gels were fixed in 45% (vol/vol) methanol and 7% (vol/vol) acetic acid for 1 h, treated for fluorography with En³Hance (Amersham), vacuum dried, and exposed to Kodak XO Mat-AR film.

Analysis of Fluorogram Bands: Bands representing labeled VSV G protein were cut out of fluorograms and placed in 1.5-ml Eppendorf tubes. After incubating the film strips in 400 μ l 2 N NaOH for 30 min at room temperature, the silver grains were suspended by vortexing as described by

Suissa (30). The optical densities of the resultant suspensions were read in microtiter plates at 690 nm using a Uniscan spectrophotometer (Labsystems, Helsinki, Finland).

Surface Radioimmunoassay and Immunofluorescence: The ^{125}I -protein A-binding assay was adapted from that described for MDCK cells grown on plastic (17) and from the immunofluorescence assay previously used to stain either the apical or the basolateral side of MDCK cells grown on filters (3). Filters of MDCK cells infected with either FPV or VSV were removed from the mini-Marbrook chambers and fixed in 3% (wt/vol) paraformaldehyde at 4°C. The washed filters were then quenched with 50 mM NH_4Cl in phosphate-buffered saline (PBS) for 40 min. The filters were washed twice with PBS and twice with PBS-0.2% gelatin, by shaking the filter gently in the wash medium for 10 min. The filters were then treated with antibodies (35 μl) either from the apical or from the basolateral (filter) side on Parafilm at 37°C for 30 min. Nearly saturating concentrations of antibody were used, 2 $\mu\text{g}/\text{ml}$ for the affinity purified anti-G protein (11) and 2.1 $\mu\text{g}/\text{ml}$ for the affinity purified antihemagglutinin (9). The filters were then washed twice with PBS-gelatin, twice with PBS, and once again with PBS-gelatin. Then 35 μl ^{125}I -protein A (45 $\mu\text{g}/\text{ml}$ PBS-gelatin) was added to either the apical or the basolateral side of the filter as before for 20 min at 37°C. The filters were then washed five times with PBS-gelatin, and the center of the filter was punched out (discarding the outer 2 mm of the filter), and counted in a gamma scintillation counter (Nuclear Industries, Chicago, IL). The application of unlabeled protein A solution to the opposite side of the filter had no effect on the labeling with ^{125}I -protein A, indicating that there was little or no leakage through the cell monolayer under the conditions used for the assay.

For immunofluorescence studies, filter-grown cells were infected for 5 h with VSV or with FPV, and frozen sections were cut and stained with the appropriate antibodies as described by Tokuyasu (31). The cells were stained with Hoechst dye 33258 before the monolayer was sectioned (3).

RESULTS

Immunofluorescence Staining of VSV- and FPV-infected MDCK Cells and Quantitation of Viral Glycoprotein Surface Distribution

The clone of MDCK strain I cells used in these experiments has been described before (1, 3). Within 4 d of growth on 3- μm -pore size nitrocellulose filters, these cells form very tight monolayers with transepithelial resistances $>1,000 \text{ ohm} \cdot \text{cm}^2$. Figure 1A shows an immunofluorescence experiment of MDCK cells infected for 5 h with VSV. A frozen section cut perpendicular to the monolayer was labeled with anti-G protein and stained with rhodamine-labeled anti-IgG. The lateral and basal boundaries of the cells were clearly stained, whereas no staining was seen at the apical membrane. In Figure 1C, a section of FPV-infected MDCK cells is shown for comparison. Now the apical border of each cell was strongly labeled with anti-FPV hemagglutinin, but no labeling of the basolateral membrane could be discerned. The labeling above the nucleus and beneath the apical membrane was presumably derived from staining of the Golgi complex.

The accumulation of viral glycoprotein on the basolateral and apical surfaces of infected MDCK cells grown on filters after 5 h of infection was quantified by the surface radioimmunoassay (Table I). Of the G protein that had reached the surface, 96.6% was on the basolateral side and only 3.4% was on the apical side. In contrast, in cells infected with FPV, 88.1% of the surface FPV hemagglutinin was on the apical side and 11.9% on the basolateral side. These results convincingly demonstrated that under these conditions, VSV G protein is expressed on the basolateral cell surface in a strongly polar fashion.

Appearance of VSV G Protein on the Surface of MDCK at 37°C

To study the appearance of newly synthesized G protein on the cell surface, filter-grown MDCK cells were infected for

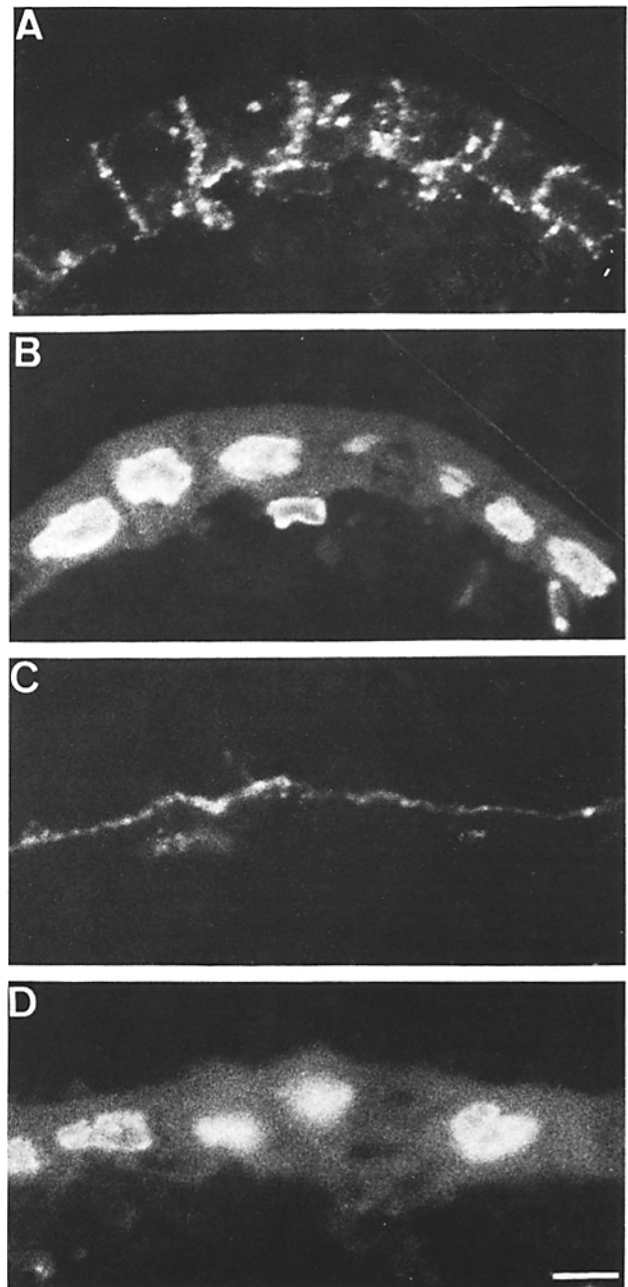


FIGURE 1 Immunofluorescence of frozen sections of infected MDCK I cells. MDCK cell monolayers were grown on 3- μm nitrocellulose filters for 4 d and infected with either VSV from the basolateral side (A and B) or with FPV from the apical side (C and D). The nuclei of the cells were stained with Hoechst dye 33258 (B and D). Frozen sections were cut perpendicular to the filter and stained (Materials and Methods) with anti-G protein (A) or anti-hemagglutinin (C). (A) The anti-G protein has labeled the basolateral cell surfaces; (B) the anti-hemagglutinin has labeled the apical surfaces; (C and D) Hoechst fluorescence from the corresponding fields. Bar, 10 μm .

5½ h with VSV, and then pulse-chase experiments were carried out as described in Materials and Methods (Fig. 2). At the end of the pulse, G protein was completely Endo H-sensitive, whereas after 20 min of chase, conversion to the Endo H-resistant form was essentially complete. In a parallel experiment, the electrical resistance was measured before the start of virus infection and at the end of the chase period. The

TABLE I. Surface Distribution of VSV G Protein and of FPV Hemagglutinin in Infected MDCK Cells*

	Apical membrane		Basolateral membrane	
	net cpm	%	net cpm	%
G protein*	261(8)	3.4	7325(9)	96.6
Hemagglutinin [§]	14856(6)	88.1	1806(7)	11.9

* MDCK cells were grown for 4 d on 3- μ m nitrocellulose filters, infected for 5 h with either VSV or FPV, and fixed with 3% paraformaldehyde. The apical and basolateral distributions were measured using a ¹²⁵I-protein A binding assay (Materials and Methods) after applying the appropriate antibody to either the apical or basolateral side of the monolayers. The number of filters used for each determination is shown in parenthesis.

[†] VSV-infected cells. The net cpm bound have been determined by subtracting "background" binding values from the apical (479 \pm 153 cpm) and basolateral (9630 \pm 1759 cpm) values. The background values (218 \pm 49 cpm and 2305 \pm 739 cpm for the apical and basolateral sides, respectively) were obtained by assaying ¹²⁵I-protein A binding to infected cultures to which 20 μ g/ml cycloheximide was added 1 h after virus infection to inhibit protein synthesis.

[§] FPV-infected cells. The net cpm bound have been determined by subtracting "background" binding values from the apical (14865 \pm 1755 cpm) and basolateral (3272 \pm 708 cpm) values. The background values (1518 \pm 223 cpm and 1466 \pm 313 cpm for the apical and basolateral sides, respectively) were obtained by assaying ¹²⁵I-protein A binding to infected cultures to which 2 μ g/ml actinomycin D was added during the 5 h of infection to inhibit RNA synthesis.

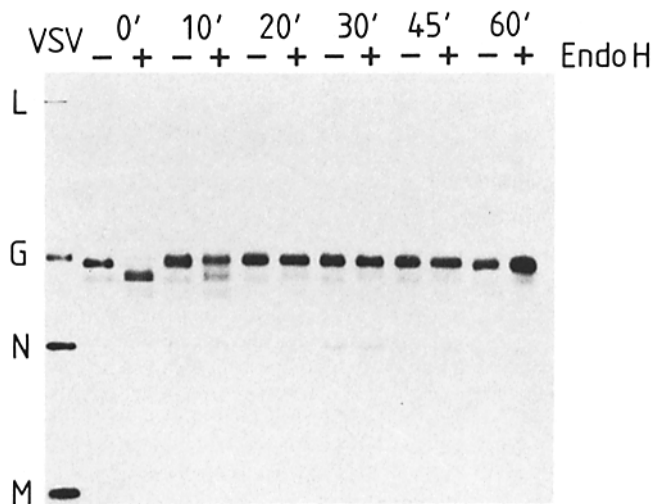


FIGURE 2 Conversion of VSV G protein from an Endo H-sensitive to a resistant form after pulse-labeling. MDCK I cells grown on nitrocellulose filters were infected with VSV for 5.5 h, pulse-labeled for 10 min with [³⁵S]methionine, and chased for 0–60 min at 37°C. Filters were sampled at each time point. One aliquot of each cell lysate was treated with endoglycosidase H (+) and another was not (–). The G proteins were analyzed by fluorography after immunoprecipitation and SDS gel electrophoresis. For details, see Materials and Methods. To the left are the structural proteins of purified VSV (L, G, N, and M) as molecular weight markers.

mean transepithelial resistance was 3014 \pm 386 ohm·cm² before infection, and 1133 \pm 232 ohm·cm² after the chase. This high transepithelial resistance implies that the infected monolayers remained tight throughout the experimental manipulations.

Surface appearance of the G protein was found to be polar. Most of the G protein emerged on the basolateral side where the G protein started to accumulate 20 min after the chase had begun (Figs. 3 and 4); only a small amount of G protein was detected when anti-G protein antibody was applied from the apical side.

Appearance of VSV G Protein on the MDCK Cell Surface after Blocking Intracellular Transport at 20°C

It has been shown previously that incubation at 20°C slows the transport of FPV hemagglutinin (9) and of VSV G protein (4) to the surface of MDCK cells. Since the FPV hemagglutinin acquired Endo H-resistance and the VSV G protein became sialated at this temperature (4), viral glycoprotein must accumulate inside the cell at a late stage in intracellular transport. We found this to be the case also for the transport of G protein in filter-grown MDCK cells. VSV-infected cells were labeled for 10 min at 37°C with [³⁵S]methionine and then

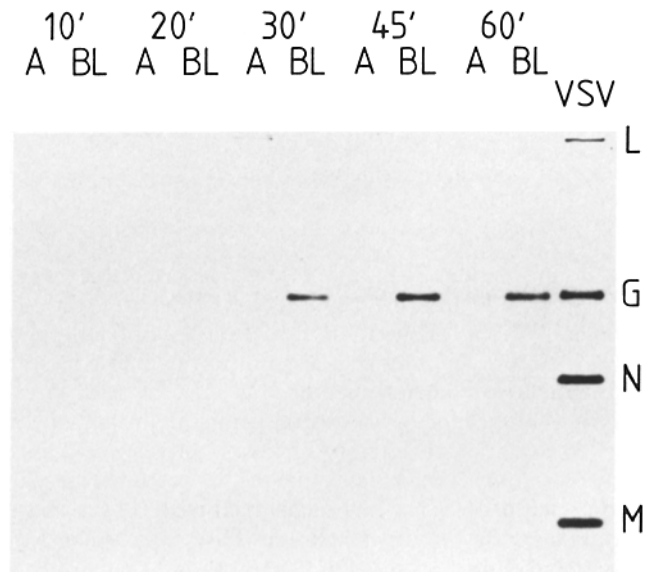


FIGURE 3 Transport of G protein to the apical and to the basolateral cell surface at 37°C. MDCK cells were infected with VSV for 5.5 h, pulse-labeled and chased as in Fig. 2. Duplicate filters were sampled at each time point. Anti-G protein antibody was applied to the apical side of one filter (A) of each pair and to the basolateral side of the other (BL). The amount of G protein immunoprecipitated was analyzed by fluorography after SDS gel electrophoresis. Markers are as in Fig. 2.

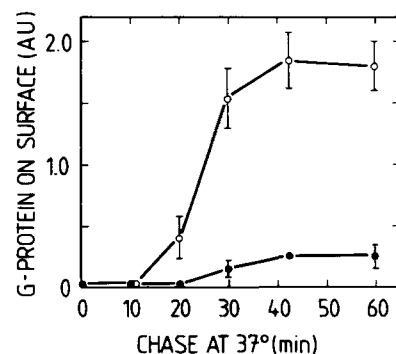


FIGURE 4 Quantitative analysis of the appearance of G protein on the basolateral and apical cell surface at 37°C. The transport of G protein to the cell surface was analyzed by fluorography after apical or basolateral immunoprecipitation and SDS gel electrophoresis (see Fig. 3). The amount immunoprecipitated was quantified by elution of fluorogram bands and optical measurements. The figure represents a composite of four different experiments. ●, apical G protein; ○, basolateral G protein. The bars represent standard errors of the means; where no bars are drawn, the errors fell within the size of the symbol. AU, arbitrary units.

incubated at 20°C in chase medium for times ranging from 30–180 min. In cells chased at 37°C, G protein became Endo H-resistant after 20 min. In contrast, 120 min was required for cells incubated at 20° (Fig. 5). The G protein is therefore slower down in its passage through the Golgi complex at 20°C as compared with 37°C. As seen in Fig. 6, most of the labeled G protein stayed inside the cell during the 3-h chase at 20°C. The small fraction of G protein that came to the cell surface under these conditions was largely basolateral (Fig. 6A).

By shifting the temperature from 20° to 37°C, rapid and synchronous externalization of the intracellular G protein could be achieved (Fig. 6A). When the temperature was shifted up after 2 h at 20°C, labeled G protein began to appear exclusively on the basolateral surface after 5 min. A more detailed analysis showed that the rate of appearance on the basolateral surface was 61-fold greater than on the apical plasma membrane (Fig. 6B). We conclude that newly synthesized G protein was inserted directly into the basolateral cell surface after blockage inside the cell at 20°C.

DISCUSSION

Matlin and Simons (10) have recently reported that newly synthesized influenza virus hemagglutinin is delivered directly from an intracellular site to the apical surface in filter-grown MDCK cells in amounts that account for its final polarized distribution. Although influenza virus-infected cells do express a small fraction of the hemagglutinin on the basolateral side (3), two approaches based on trypsin cleavage and antibody accessibility demonstrated that the basolateral hemagglutinin is not a necessary intermediate en route to the apical membrane (10). Similar conclusions have been reached by Misek et al. (14).

This investigation reports complementary studies on the biogenesis of a basolateral protein, the VSV G protein, in MDCK cells. It is clear from our results that the previous studies on MDCK cells grown on plastic, which showed that as early as 4 h after infection a significant and increasing fraction of the G protein was apical (24), must be a consequence of growth on plastic and the cytopathic effects of the VSV infection. The problem is exacerbated by the fact that VSV infects filter-grown MDCK cells only from the basolateral side because all the receptors for VSV are basolateral (3). VSV infection of cells grown on a solid support selects cells that are expressing apical VSV receptors since the basolateral

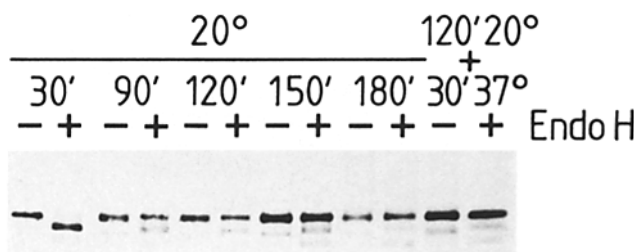


FIGURE 5 Conversion of G protein from an Endo H-sensitive to a resistant form at different chase temperatures. MDCK cells were infected with VSV, pulse-labeled for 10 min with [³⁵S]methionine, and chased at 20°C. The samples to the right were chased for 2 h at 20°C followed by 30 min at 37°C. Filters were sampled at each time point. One aliquot of the cell lysate was digested with Endoglycosidase H (+) and another was not (–). The G proteins were analyzed by fluorography after immunoprecipitation and SDS gel electrophoresis.

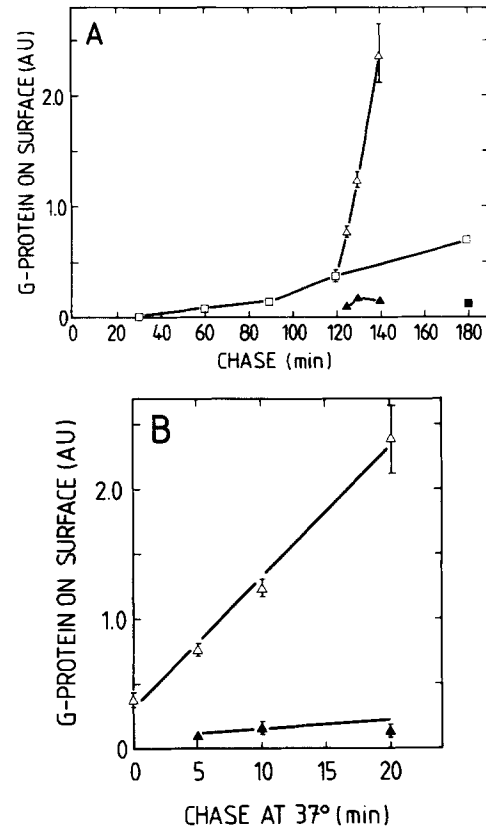


FIGURE 6 Quantitative analysis of the appearance of G protein on the basolateral and apical surfaces at 20°C and after reversal to 37°C. (A) MDCK cells were infected with VSV, pulse-labeled for 10 min with [³⁵S]methionine, and chased at 20°C for 0–180 min. Some filters were transferred after 120 min from 20°C to 37°C. The appearance of G protein on the apical and basolateral sides was quantified by immunoprecipitation and fluorography as in Fig. 4. □, basolateral G protein after chase at 20°C; ■, apical G protein after 180-min chase at 20°C; appearance at basolateral (Δ) and apical (▲) surface after a 120-min chase at 20°C followed by reversal to 37°C. (B) The data have been replotted to show more exactly the appearance of pulse-labeled G protein to the basolateral and apical cell surfaces after a chase at 20°C for 120 min and reversal to 37°C. The lines show the linear regression lines calculated from 0 to 20 min for the basolateral appearance (Δ, *n* = 20, standard error of the slope = 0.009 U/min) and for apical appearance from 5 to 20 min (▲, *n* = 21, standard error of the slope = 0.003 U/min). The ratio of the slopes of basolateral to apical appearance is 61.1:1. AU, arbitrary units. The bars represent standard errors of the means. At least five filters were used for each point.

side is not accessible. These will be the fraction of the population that are not fully polarized. In the present studies, when MDCK I cells grown on nitrocellulose filters were infected with VSV from the basolateral side, 96.6% of the G protein accumulated on the surface was on the basolateral side 5 h after infection, and only 3.4% was on the apical side. The initial surface appearance of newly synthesized G protein was also highly polarized to the basolateral side. Blocking the intracellular transport of G protein by incubation at 20°C and synchronizing its surface appearance by a shift to 37°C made this polarity very clear. Under these conditions, the rate of appearance on the basolateral surface was 61-fold greater than that on the apical surface. No evidence for the involvement of the apical membrane during the routing of G protein to the basolateral surface was obtained. Thus, proposals that the

protein would be first inserted either into the apical domain or into both surface domains (24), and only later achieve the proper basolateral localization, can be dismissed. Together, our studies with the newly synthesized FPV hemagglutinin and VSV G protein demonstrate that sorting to the apical and basolateral surface domains must be an intracellular event.

In this investigation, we also quantified the amount of virus glycoprotein on the two plasma membrane domains to obtain a measure for the steady-state distribution of the proteins on the polarized cell surface. We found that ~12% of the surface hemagglutinin was basolateral 5 h after FPV infection, whereas ~3% of the G protein was on the apical side after VSV infection. It should be emphasized that the values for these minority fractions are likely to be overestimates of the amounts in fully polarized MDCK cells for two reasons. First, the monolayer contains a small number of nonpolar cells (<2%) (3). After 4 d of growth, a few cells in the monolayer are still undergoing mitosis (mitotic index, 0.25%), and cells are continuously being pushed out of the monolayer into the medium, and probably account for some of the nonpolar cells. Second, minor leakages of antibody during the assay through the MDCK cell monolayer at sites locally damaged by experimental manipulations would inevitably lead to overestimates of the proportion of misdirected protein populations. Nevertheless, our studies show that it is possible to satisfactorily quantify the surface distribution of defined apical and basolateral proteins in an epithelial cell and that this distribution is very polar.

Our demonstration of more basolateral hemagglutinin than apical G is not limited to viral proteins. Endogenous apical proteins, i.e., a 114,000-D monoclonal antibody-specified protein (1) and leucine aminopeptidase (Roman, L., and K. Simons, unpublished observations), also have similar minority components detectable by immunofluorescence in filter-grown MDCK cells. Basolateral proteins also appear to be more stringently sorted than are apical proteins in some other epithelial cells (1, 13, 19, 20). Therefore, the routing of more hemagglutinin than G protein to the opposite side might be due to mechanisms intrinsic to the sorting process that generate and maintain the polarized distribution, and not due to the deleterious effects of virus infection. On the other hand, the relative surface areas of the apical and basolateral membrane should also be considered. Recent morphometric determinations of MDCK I cells growing on 0.45- μm^2 filters have indicated a value of 214 μm^2 for the apical surface area and an average value 7.6-fold larger for the basolateral surface area (Bonsdorff, C.-H., S. Fuller, and K. Simons, manuscript submitted for publication). Taking these differences into account, the relative surface densities of the minority G and hemagglutinin protein populations on the apical and basolateral surfaces, respectively, are not so different. However, since the mechanisms responsible for sorting and routing the surface proteins to their correct destinations are not known, we cannot yet evaluate which of the two parameters, amount or surface density of the "missorted" component, more closely reflects the mechanisms involved.

The exact site of sorting for apical and basolateral proteins is not yet known, but current results implicate a late Golgi compartment. Fuller et al. (4) have recently shown that in MDCK cells doubly infected with FPV and VSV, the viral glycoproteins remain together late in the pathway. The basolaterally directed VSV G protein was found to lose most of its sialic acid due to cleavage by the apically directed FPV

neuraminidase. The results were obtained early in double infection before loss of cell polarity had begun, and the data indicate that neuraminidase must still be in physical contact with the G protein during the terminal steps (i.e., post-sialation) of processing in the Golgi complex. Rindler et al. (24) also infected MDCK cells grown on plastic with both VSV and influenza viruses. Using immunoelectron microscopy, they found that the influenza hemagglutinin and VSV G protein were present in the same Golgi cisternae. Although they reasonably concluded that these proteins must therefore be sorted from each other during or after passage through the Golgi, this interpretation should be tempered by their observation that the cells were somewhat apolar with respect to G protein surface appearance. Further studies on the ultrastructural level are now necessary to pinpoint the site of sorting using MDCK strain I cells grown on nitrocellulose filters where viral glycoprotein sorting proceeds with a high degree of accuracy and reproducibility.

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