Transcytosis of the G Protein of Vesicular Stomatitis Virus after Implantation into the Apical Plasma Membrane of Madin-Darby Canine Kidney Cells. I. Involvement of Endosomes and Lysosomes

MARJA PESONEN, WILHELM ANSORGE, and KAI SIMONS

European Molecular Biology Laboratory, D-6900 Heidelberg, Federal Republic of Germany. Dr. Pesonen's present address is Recombinant DNA Laboratory, University of Helsinki, SF-00380 Helsinki 38, Finland.

ABSTRACT The G protein of vesicular stomatitis virus, implanted into the apical plasma membrane of Madin-Darby canine kidney cells, is rapidly transcytosed to the basolateral membrane. In this and the accompanying paper (Pesonen, M., R. Bravo, and K. Simons, 1984, *J. Cell Biol.* 99:803–809.) we have studied the intracellular route by which the G protein traverses during transcytosis. Using Percoll density gradient centrifugation and free flow electrophoresis we could demonstrate that the G protein is endocytosed into a nonlysosomal compartment with a density of ~1.05 g/cm^3, which has many of the characteristics of endosomes. Transcytosis to the basolateral membrane appeared to occur from this compartment. No direct evidence for the involvement of lysosomes in the transcytotic route could be obtained. No G protein was detected in the lysosomes when transcytosis of G protein was occurring. Moreover, at 21°C when passage of G protein to the lysosomes was shown to be arrested, transcytosis of G protein could still be demonstrated.

One hallmark of the epithelial cell surface is its polarity (1). The plasma membrane of each cell is divided into two domains, one apical and the other basolateral, which are separated from each other by a junctional complex encircling the apex of the cell (2). Little is known of how cell surface polarity in epithelial cells is generated and maintained. One promising experimental system to study these questions is the Madin-Darby canine kidney (MDCK)[1] line which grows as a polarized epithelium in culture (3–5). Not only have the apical and the basolateral plasma membrane domains been shown to exhibit different lipid and protein compositions as in other epithelial cells (6–8), but the MDCK cells can be infected with enveloped viruses that distribute the newly synthesized viral membrane glycoproteins into one or the other surface domain (9, 10). The G protein of vesicular stomatitis virus (VSV), for instance, is inserted mainly into the basolateral plasma membrane. The viral glycoproteins can thus be used to study how apical and basolateral proteins are routed to their correct destinations in the cell.

Using the VSV G protein we have recently demonstrated that the apical and the basolateral plasma membrane domains in MDCK cells are connected by an intracellular (transcytotic) route (11, 12), examples of which have been found in many other epithelial cells (13–17). We implanted the VSV G protein into the apical membrane, where the G protein is not normally found during viral infection and found that it was endocytosed and that the bulk was then redistributed to the basolateral membrane. Implantation was brought about by fusing the envelope of VSV with the apical plasma membrane of MDCK cells at low pH (18, 19). In this and the accompanying paper (20) we have used implanted G protein as a probe to characterize the transcytotic route in more detail.

[1] *Abbreviations used in this paper:* MDCK, Madin-Darby canine kidney cells; MEM, minimum essential medium; SEAT, 0.25 M sucrose/1 mM acetic acid/10 mM triethanolamine); TCA, trichloroacetic acid; VSV, vesicular stomatitis virus.

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MATERIALS AND METHODS

Growth of Cells and Virus: MDCK cells were grown as described (11) on plastic dishes (3-cm diam) or trays (17 × 17 cm) containing 2×10^6 or 70×10^6 cells, respectively. Confluent 2-d-old cultures were used. Unlabeled VSV (Indiana serotype) or [^{35}S]methionine-labeled VSV were grown in baby hamster kidney cells and purified by sucrose density gradient centrifugation in the presence of EDTA as described (11).

Implantation of VSV G Protein into the Cellular Plasma Membrane: VSV was bound to the apical cell surface of MDCK cell monolayers by incubation in minimum essential medium (MEM), pH 6.3, in the cold for 1 h. The unbound virions were removed and the cells were flooded for 20 s with prewarmed (37°C) MEM, pH 5.3, to fuse the virion envelope with the cellular plasma membrane (11, 12). The fusion medium was then replaced with ice-cold phosphate-buffered saline, pH 7.4 (PBS). The cultures were washed twice with cold PBS lacking Ca^{++} and Mg^{++}, incubated for 5 min with 20 mM EDTA in the same buffer on ice to remove unfused virions (11), and then returned to PBS. Incubation at different temperatures was performed in MEM, pH 7.4, usually containing 20 μg cycloheximide per ml (12).

Homogenization of the Cells: The cells were scraped off the trays into 10 ml of ice-cold PBS, sedimented, resuspended in SEAT-buffer (0.25 M sucrose/1 mM EDTA/10 mM acetic acid/10 mM triethanolamine, adjusted to pH 7.4), and lysed by 50 passes through the blue tip of a 1-ml Eppendorf pipette (21). Cells, nuclei, and debris were removed by successive 20-min centrifugations at 2,500 rpm and 3,000 rpm in a Sorvall SS34 rotor at 4°C. Recovery of ^{35}S-G protein in the postnuclear supernatant in 10 experiments was 25–50%. Recovery of total protein was in the same range. The postnuclear supernatant was used directly for Percoll density gradient centrifugation.

Percoll Density Gradient Centrifugation: The MDCK cell postnuclear supernatants were mixed with Percoll stock suspension (9 parts Percoll + 1 part 10-fold concentrated SEAT-buffer) and SEAT-buffer to yield 24 ml of 30% (wt/wt) Percoll suspension and centrifuged for 4 h at 16,000 rpm in a Beckman Ti70 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The density of the 0.8 ml fractions was determined by refractometry.

Free-flow Electrophoresis: Free-flow electrophoresis was performed in SEAT-buffer in an Elphor VapII apparatus (Bender and Hobein GmbH, Munich, Federal Republic of Germany) at 5°C, 200 mA, and 116 V/cm (21, 22). The fraction size was 3–4 ml and the flow rate about 575 ml/h. Recovery of both viral radioactivity and β-hexosaminidase activity was ~40%. Samples pooled from Percoll gradients were subjected to a spin for 20 min at 10,000 rpm to remove some of the Percoll prior to electrophoresis. No β-hexosaminidase activity or ^{35}S-radioactivity was lost during this spin.

Marker Assays: From the free-flow electrophoresis fractions β-*N*-acetylhexosaminidase (EC 3.2.1.20) and succinate-3-(4-iodophenyl)-2-(4-nitrophenyl-5-phenyl-2H-tetrazolium chloride (INT) reductase (EC 1.3.99.1) were assayed as described (21) and the total protein content was determined using the Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA), standardized with IgG. Aminopeptidase and Na^+/K^+ ATPase were concentrated by phase separation into Triton X-114 as described by Bordier (23) from the free-flow electrophoresis fractions. The detergent phase (<50 μl) was diluted with 50 μl of PBS and transferred into wells of microtiter plates pretreated with polylysine (1 mg/ml) for 20 min. The antigen was bound to the wells overnight and then fixed by adding 100 μl of 8% formaldehyde to each well for 20 min. After washes with distilled water 200 μl of 50 mM NH_4Cl was added for 20 min, followed by washes with distilled water and addition of 200 μl of Hb-PBS (20 mg/ml of hemoglobin in PBS, containing 0.02% of azide). After 1 h the Hb-PBS was removed and 200 μl of the respective antiserum (against aminopeptidase or Na^+/K^+ ATPase, dilution 1:100) was added in Hb-PBS for 16 h, followed by washes with distilled water and addition of ^{125}I-protein. The antibodies were gifts from Daniel Louvard, Institut Pasteur, Paris. After an incubation for 16 h the unbound protein A was removed, and the wells were washed and cut off with a hot wire for counting. All incubations were at room temperature. The Percoll density gradient fractions were assayed for aminopeptidase and Na^+/K^+ ATPase as above, except that the fractions were bound to the microtiter plate wells directly without phase separation into Triton X-114. Uridine diphosphate-galactosyl-glycoprotein galactosyltransferase (EC 2.4.1.38) was concentrated from both the free-flow electrophoresis and the Percoll density gradient fractions by phase separation into Triton X-114, and then assayed (24). The detergent phase (10–30 μl) was incubated with 55 μl of reaction mixture at 37°C for 1 h. The reaction mixture contained 50 mM HEPES, 40 mM β-mercaptoethanol, 0.4% Triton X-100, 40 mM $MnCl_2$, 17 mg/ml of ovomucoid, 1 mM uridine diphosphate [^{3}H]galactose, 2 mM ATP and 5 mg/ml of BSA. The reaction was stopped with 1 ml of ice-cold 10% trichloroacetic acid (TCA). After incubation for 15 min on ice the precipitates were collected on Whatman GF/C filters (Whatman Chemical Separation, Inc., Clifton, NJ), washed with cold 5% TCA, dried, and counted for radioactivity.

^{125}I protein A-binding Assay for Cell Surface G Protein: The assay and its calibration have been described (12). Briefly, G protein on the apical surface was assayed by fixing the cells, incubating them first with anti-VSV antibody, and then with ^{125}I-protein A followed by counting of cell-associated radioactivity. G protein on the entire cell surface was assayed by opening up the tight junctions with a 5-min incubation at 31°C with 5 mM EGTA prior to fixation and assay. The values for the basolateral surface were obtained by subtracting the apical values from those for the entire cell surface.

Degradation Studies: Intracellular degradation of [^{35}S]methionine-labeled viral proteins was followed by monitoring accumulation of TCA-soluble radioactivity into the growth medium as described (25).

Gel Electrophoresis: Polyacrylamide gels were prepared with an acrylamide gradient of 10–15% (wt/vol) containing 8 M urea, using the buffer system of Laemmli (26). Samples for gel electrophoresis were prepared as described (27). After running, gels were fixed in 45% (vol/vol) methanol and 7% (vol/vol) acetic acid, treated for fluorography using Enhance, vacuum dried, and exposed to XOMat-R film (Kodak, Rochester, New York) at −80°C.

RESULTS

A Nonlysosomal Intracellular Compartment Is Involved in Transcellular Transport of G Protein

We have previously shown that G protein of VSV, after implantation into the apical surface of MDCK cells, is internalized with a half time of ~7.5 min at 31°C, and redistributed to the basolateral cell surface (12). After 1 h ~25–48% of the implanted G protein was found on the basolateral cell surface using the ^{125}I protein A-binding assay and ~25–33% on the apical plasma membrane domain. The recovery of total cell surface G protein after 1 h at 31°C was 55–90%, the mean of 17 experiments was 70%. To study the intracellular organelles into which G protein was internalized, the following experiment was performed. About 35×10^6 cpm of [^{35}S]methionine VSV (35 μg of viral protein) was added to monolayers of 70×10^6 MDCK cells in MEM, pH 6.3, and implanted as described in Material and Methods. About 25,000 G protein molecules were implanted per cell under these conditions (11). This protocol was used in all the subsequent experiments. The cultures were either scraped off the dish directly for homogenization or they were incubated in MEM, pH 7.4, at 31°C for 7.5 or 15 min to allow internalization to occur prior to homogenization. After removal of cell debris and nuclei the homogenates were subjected to centrifugation in self-forming Percoll density gradients. The gradient fractions were assayed for ^{35}S-labeled G protein, the lysosomal marker β-hexosaminidase, the apical plasma membrane marker aminopeptidase (7), and the basolateral plasma membrane marker Na^+/K^+ ATPase (7). To localize the ^{35}S-G protein in the gradient fractions, the amphipathic G protein had to be separated from the other labeled viral proteins which had also been introduced into the cells by the implantation procedure. This was done by phase separation into the detergent Triton X-114 (23). Fig. 1 *B* shows that nearly all of ^{35}S-G protein and a very small amount of N protein of the virion, representing together ~21% of the total viral ^{35}S-radioactivity, were recovered in the detergent phase. The internal viral proteins were left behind in the aqueous phase. Similar results were obtained using cell fractions containing the viral proteins.

Immediately after implantation into the apical cell surface ^{35}S-G protein was found by Percoll density gradient centrifugation at a density of 1.070 g/cm^3, this peak having a shoulder at ~1.055 g/cm^3. (Fig. 2 *A*). The G protein overlapped with the aminopeptidase and the Na^+/K^+ ATPase activity. β-Hexosaminidase equilibrated at higher density than aminopeptidase and Na^+/K^+ ATPase. The peak of the implanted G protein always had a higher density than that of the peak of

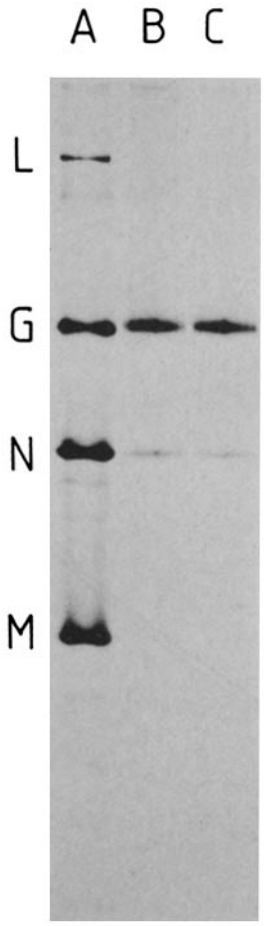

FIGURE 1 Isolation of G protein by detergent phase separation. 70,000 cpm of [^{35}S]methionine-labeled VSV was prepared for SDS gel electrophoresis directly (*A*) or after one (*B*) or two (C) cycles of phase separation into Triton X-114 (see reference 23), which was carried out as follows. 250 μl of 0.75% Triton X-114 in PBS was added to the virus samples of 5 μl and the mixtures were incubated for 3 min at 0 and 30°C successively. After centrifugation for 3 min at 300 *g* at room temperature the aqueous phase was discarded and the detergent phase was dried under nitrogen prior to preparation for SDS gel electrophoresis. Exposure time of autoradiogram was 44 h. *L*, *G*, *N*, and *M* are the structural proteins of VSV.

the aminopeptidase activity. The reason for this difference is not known but it could be due to inclusion of the dense viral nucleocapsids in plasma membrane vesicles formed after homogenization of apical membranes with implanted viral membranes. The internal proteins of the virus were found associated with the G protein-containing fractions in Fig. 2*A*. When free-flow electrophoresis was used as an analytical tool, newly implanted G protein co-migrated with the plasma membrane markers (see below). It should be noted that some of the plasma membrane markers in Fig. 2*A* are probably localized in intracellular compartments as well.

After incubation for 7.5 min at 31°C, G protein still equilibrated mainly at 1.07 g/cm^3 but some of it had now moved to a compartment of lower density (Fig. 2*B*). After 15 min at 31°C more than half of [^{35}S] G protein equilibrated around 1.05 g/cm^3 (Fig. 2*C*). Recovery of [^{35}S] protein applied to the gradients in Fig. 2, *A–C* was 90, 95, and 92%, respectively. Thus, during internalization from the apical cell surface ^{35}S-G protein moved into a compartment that contained little β-hexosaminidase and banded at 1.05 g/cm^3.

Transcytosis of G protein at Different Temperatures

Previous studies in other cells have shown that traffic of endocytosed ligands into lysosomes is temperature dependent (28–30). At ~20°C, endocytosis and membrane recycling continue, but entry into lysosomes is arrested. In the following experiments we therefore examined the temperature dependence of internalization, transcytosis, and intracellular degradation of G protein. About 25,000 G proteins were implanted as before. The cultures were incubated at different temperatures, and at different time points duplicate cultures were removed for fixation and assayed for ^{125}I-protein A-binding to measure the amount of G protein in the apical cell surface. Basolateral G protein was assayed in parallel duplicate cultures that were treated for 5 min with EGTA to open up the tight junctions prior to fixation. Fig. 3*A* shows that no internalization of G protein from the apical surface could be detected up to 2.5 h at 4°C or 10°C. At 15°C slow internalization occurred. At 21°C the half time of internalization was ~14 min, at 24°C, ~8 min, and at 31°C, ~7 min. At 15°C no basolateral G protein could be detected (Fig. 3*B*). At 21°C G protein started to appear in the basolateral surface after a lag of 30 min. For degradation studies 5.6 × 10^5 cpm of [^{35}S]-methionine-labeled VSV together with 1 μg of cold virus

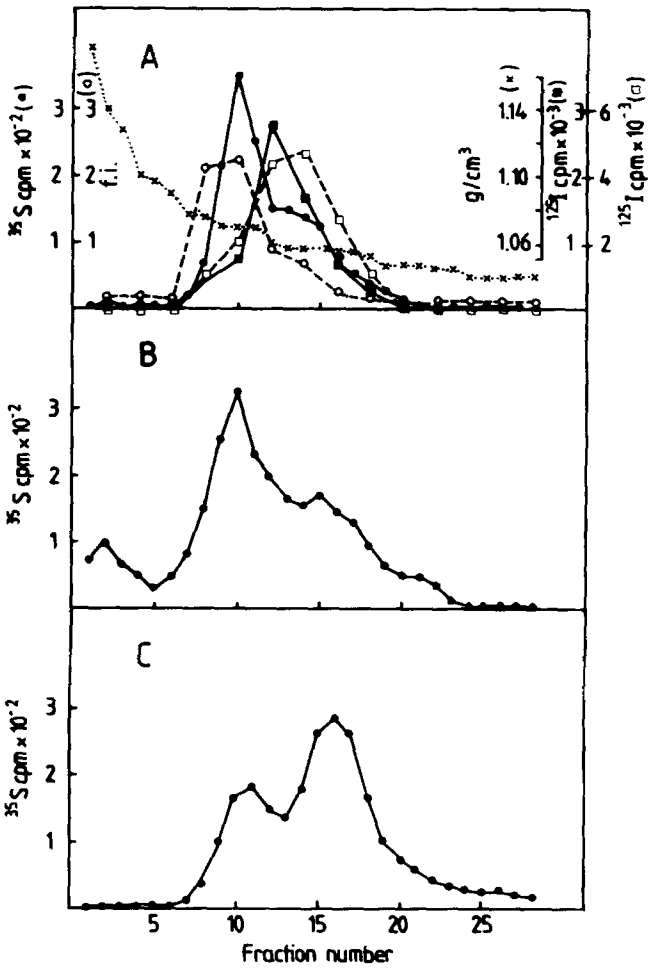

FIGURE 2 Percoll density gradient centrifugation of postnuclear supernate from MDCK cells with implanted ^{35}S-G proteins. ^{35}S-G protein was implanted into the apical surface of MDCK cells as described in the text. The cultures were homogenized immediately (*A*) or after incubation for 7.5 (*B*) or 15 min (C) at 31°C, and subjected to centrifugation in self-forming Percoll density gradients. Fractions of 800 μl were collected. 100-μl samples of the fractions were diluted 10-fold with PBS and subjected to phase separation with Triton X-114. The detergent phase containing the ^{35}S-G protein was counted for radioactivity. (●). 10 μl of every second fraction was used for the β-hexosaminidase assay (○); *f.i.* is fluorescence intensity in arbitrary units. 50 μl out of two successive fractions were combined into wells of microtiter plates for radioimmunoassay to detect aminopeptidase (■) or Na^+/K^+ ATPase (□). The density of the Percoll fractions was determined by refractometry (×).

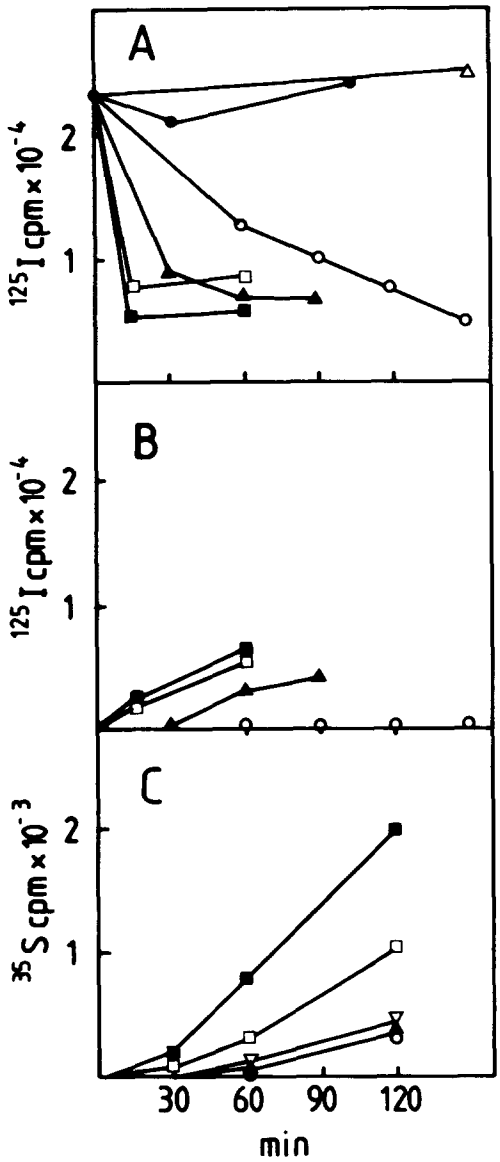

FIGURE 3 Temperature dependency of internalization, redistribution, and degradation of implanted G protein. Nonlabeled VSV (1 μg virus protein/plate) (*A* and *B*) or ^{35}S-labeled VSV (1 μg virus protein/plate) (*C*) was fused into the apical surface of MDCK cells (3-cm dishes) and the cultures were incubated in the presence of 20 μg/ml of cycloheximide at 31 (■), at 24 (□), 21 (▲), 15 (○), 10 (●), and 4°C (△) for the indicated periods of time. In *A* duplicate cultures were fixed and assayed for apical G protein by radioimmunoassay. In *B* the tight junctions of parallel monolayers were opened prior to fixation and radioimmunoassay to assay the entire cell surface for G protein. The values in *B* represent the level of basolateral G protein, obtained by subtraction of the apical values. The radioimmunoassays were performed using 50,000 cpm of ^{125}I-protein A per plate. In *C* the TCA-soluble radioactivity of the medium was determined for duplicate cultures at each time point. One set of cultures was incubated in the presence of 20 mM NH_4Cl at 31°C (▽).

(protein) was added per plate. After implantation the cultures were incubated at the different temperatures as before (Fig. 3*C*). One additional set of cultures was incubated at 31°C in the presence of 20 mM NH_4Cl. At 31°C degradation, as measured by appearance of TCA-soluble ^{35}S-radioactivity in the medium, started after a lag of about 30 min amounting thereafter to 14%/h of the total cell-associated radioactivity (12). At 21°C and 15°C degradation was negligible during the first hour as was also true at 31°C in the presence of NH_4Cl. At 24°C intermediate values were obtained.

To confirm that the soluble ^{35}S-radioactivity appearing in the medium originated at least partially from lysosomal degradation of G protein, the following experiment was performed. Cultures with implanted ^{35}S-G protein were again incubated at different temperatures for different periods of time. The cells were then solubilized with PBS containing 5 mM EDTA, 0.75% Triton X-114, 1 mM iodoacetamide, and 1 mM phenylmethylsulphonyl fluoride (PMSF) at 4°C. Cell debris was removed by centrifugation at 2,000 rpm for 5 min at 4°C. Thereafter ^{35}S-G protein was extracted into Triton X-114 for counting. At 31°C the amount of ^{35}S-radioactivity in the detergent phase started to decrease after a lag of 30 min; degradation amounting to ~18%/h. At 21°C no decrease of ^{35}S-radioactivity could be observed within 180 min. 20 mM NH_4Cl at 31°C also inhibited the decrease of ^{35}S-radioactivity in the detergent phase (data not shown).

These experiments show that internalization, transcytosis, and intracellular degradation of G protein are temperature-dependent events but each has a different threshold temperature. Internalization stopped at 10°C, transcytosis at 15°C, and degradation at 21°C (Fig. 4). The absence of degradation at 21°C suggests that delivery of G protein into the lysosomal compartment is inhibited since the lysosomal enzymes are known to be active at this temperature (28), a finding that we have confirmed for MDCK cells (data not shown).

Transport of G Proteins into Lysosomes

The involvement of lysosomes in the transcytotic route was studied more directly by isolating the lysosomes using a combination of Percoll density gradient centrifugation and free-flow electrophoresis (21). Fig. 5*A* shows the results of a free-flow electrophoresis run of the postnuclear supernatant obtained from control MDCK cells. The enzyme profile is similar to that obtained earlier for other cells (21, 22). The main peak of a lysosomal marker, β-hexosaminidase, was well separated from the plasma membrane marker aminopeptidase, the basolateral plasma membrane marker Na^+/K^+ ATPase, the Golgi complex marker galactosyltransferase, and the mitochondrial marker succinate-INT-reductase. Directly after implantation the ^{35}S-G protein was found in fractions

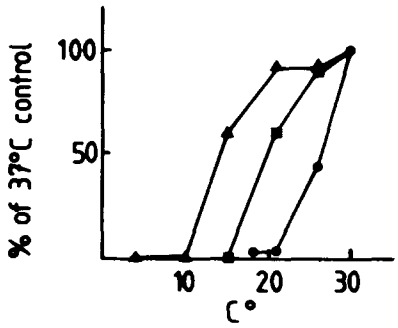

FIGURE 4 Summary of temperature dependency of internalization, transcytosis, and degradation of G protein. The relative levels of G protein on the apical surface (see Fig. 3*A*) and the basolateral surface (see Fig. 3*B*) after incubation for 60 min, and the relative amounts of TCA-soluble ^{35}S-radioactivity in the culture media after incubations of 60 min (Fig. 3*C*) are plotted against incubation temperature to indicate temperature-dependency of internalization (▲), transcytosis (■), and degradation (●) of G protein.

50–62, as was the aminopeptidase activity (Fig. 5 *B*). All the other enzymer markers migrated in the same positions as in the control experiment (see Fig. 5 *A*).

In the next experiments, MDCK cells with implanted ^{35}S-G protein (0.5–1.6 × 10^6 cpm per 70 × 10^6 cells) were incubated at 31°C for 15 or 60 min, or at 21°C for 40 min. The cells were then homogenized and the postnuclear supernatant was first subjected to Percoll density gradient centrifugation. The fractions containing both the lysosomal and the plasma membrane markers, corresponding to fractions 8–12 in Fig. 2 *C*, were pooled and subjected to free-flow electrophoresis to separate the lysosomes from the plasma membrane. Percoll density gradient centrifugation was necessary to remove the nonlysosomal 1.05 g/cm^3 compartment, which comigrated partly with the lysosomes in free-flow electrophoresis. The free-flow electrophoresis profiles showed that the ^{35}S-radioactivity was recovered in a single narrow peak coinciding with the plasma membrane markers, 15 min after implantation at 31°C as well as after 40 min at 21°C. No radioactivity could be detected in the β-hexosaminidase containing fractions (Fig. 6, *A* and *B*). However, if the cultures were incubated for 60 min at 31°C when degradation of the viral proteins could be observed (see Fig. 3 *C*), 8% of the ^{35}S-radioactivity was found in β-hexosaminidase-containing fractions, the rest migrating with the plasma membrane markers. Fractions 41 and 51–53 were subjected to phase separation using Triton X-114. In each case, ~50% of the radioactivity was recovered in the detergent phase. We conclude that under conditions where redistribution of ^{35}S-G protein to the basolateral surface was underway (15 min at 31°C or 40 min at 21°C), no G protein could be detected in lysosomes.

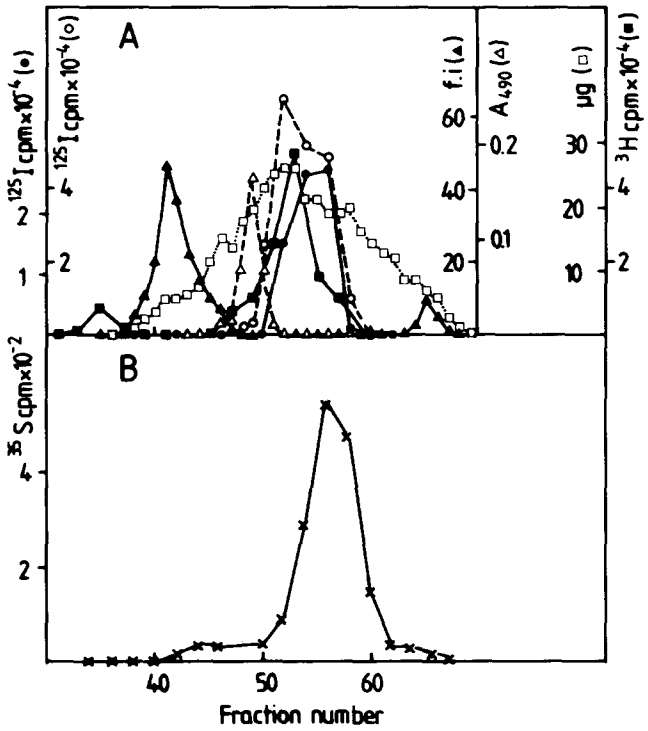

FIGURE 5 Free flow electrophoresis of MDCK cell homogenates. In *A*, 140 × 10^6 MDCK cells were subjected to free-flow electrophoresis after homogenization in SEAT-buffer as described in Material and Methods and 90 3 ml-fractions were collected. Each fraction was assayed for total protein content (□), β-hexosaminidase (▲), and succinate-INT-reductase (△). Galactosyltransferase (■) was assayed from every second fraction after phase separation into Triton X-114. Every second fraction was subjected to phase separation into Triton X-114 followed by binding of the detergent phase to microtiter plate wells and subsequent radioimmunoassay for aminopeptidase (●) or Na^+/K^+ ATPase (○). In *B*, ^{35}S-labeled VSV (32 µg of virion protein) was added to monolayers of 70 × 10^6 MDCK cells for binding at pH 6.3 in the cold, and subsequent fusion with the cells by low pH treatment. The cultures were harvested immediately. The cells were then homogenized and the postnuclear supernatant subjected to free-flow electrophoresis. The fractions were assayed for subcellular markers as before. The Triton X-114 phase of two successive fractions were combined and ^{35}S-G protein was measured by counting for radioactivity.

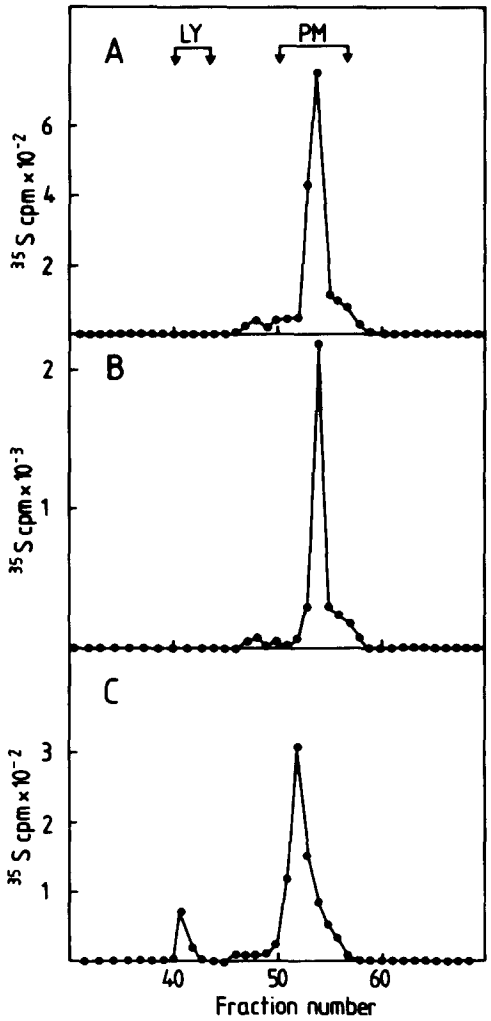

FIGURE 6 Free flow electrophoresis of the fractions containing lysosome and plasma membrane markers isolated by Percoll density gradient centrifugation. ^{35}S-labeled VSV (35 µg of virion protein) (*A–C*) was added to monolayers of 70 × 10^6 MDCK cells for binding and fusion. The cells were incubated for 15 min at 31°C (*A*), 40 min at 21°C (*B*), or 60 min at 31°C (*C*). Thereafter the cells were homogenized and the postnuclear supernatants subjected to Percoll density gradient centrifugation (Fig. 2). The fractions containing ^{35}S-G protein together with aminopeptidase and β-hexosaminidase (corresponding to fractions 8–12 in Fig. 2C) were pooled and subjected to free-flow electrophoresis. Out of the 3-ml-fractions, 2 ml were counted for ^{35}S-radioactivity: β-hexosaminidase (*LY*) and aminopeptidase (*PM*) were assayed as shown in Fig. 5*A*.

DISCUSSION

Most previous studies using the glycoproteins of enveloped viruses as probes to investigate intracellular traffic have been

done by infecting cells with viruses to study the transport of the newly-synthesized proteins to the plasma membrane. In this paper we have used a different approach. Instead of infecting cells with the virus, the viral envelope was fused with the plasma membrane. VSV was bound to the plasma membrane at 0°C and the fusion reaction was triggered by shifting the pH to 5.3 for 20 s at 37°C (11, 12). In this way, the membrane glycoprotein G of VSV was implanted efficiently into the plasma membrane of the cell and its subsequent fate could conveniently be followed.

The assumption underlying this approach is that the membrane recycling routes from the cell surface are not disturbed by implantation of the glycoprotein and that the intracellular routes followed by the implanted glycoproteins represent constitutive pathways characteristic of the cell. At least the first of these requirements appeared to be met since the fluid phase uptake rate into the cells and the level of aminopeptidase on the apical surface were not changed after implantation (12). The implanted G protein was rapidly endocytosed and a major fraction of it was redistributed to the basolateral plasma membrane (12).

In the present study we have used the implanted G protein as a probe to study the intracellular route by which the apical and basolateral membranes are connected in MDCK cells. We have found that the implanted G protein moved rapidly into a subcellular compartment that equilibrated at a density around 1.05 g/cm^3 in Percoll density gradients. The plasma membrane markers equilibrated at ~1.07 g/cm^3 and the bulk of the lysosomal marker β-hexosaminidase at ~1.08 g/cm^3. Previous immunoperoxidase studies at the electron microscope level have shown that most of the endocytosed G protein is localized in irregularly shaped smooth-surfaced vacuoles with an electron-lucent interior apparently devoid of lysosomal enzyme activities (11).

Using Percoll density gradient fractionation we could not separate the lysosomal enzyme marker from the plasma membrane markers. We therefore had to use a combination of Percoll density gradient fractionation and free-flow electrophoresis to determine whether implanted G protein entered into the lysosomal compartment. Our previous morphological studies indicated that only a small fraction of G protein could be localized to secondary lysosomes (11). The fractionation results confirmed these findings. During the time when redistribution into the basolateral membrane was occurring (15 min after implantation) no lysosomal G protein could be detected. Only later (60 min after implantation), after redistribution to the basolateral surface had already taken place, could some G protein be found in the lysosomal fraction (Fig. 6 *C*). Our evidence thus suggests that the bulk of endocytosed G protein was transported via a light fraction largely devoid of lysosomal enzyme activity. However, since the recovery of ^{35}S-G protein in the postnuclear supernatant was not complete (see Material and Methods), these experiments are not conclusive.

Further evidence suggesting that the lysosomes could be bypassed during transcytosis was obtained by studying the temperature dependence of endocytosis, transcytosis, and degradation of G protein. The rates of these processes had different temperature thresholds. At 10°C, internalization of G protein was inhibited. At 15°C, transcytosis stopped but uptake of G protein still proceeded, albeit at a slow rate. At 21°C, proteolytic degradation of G protein was almost totally inhibited but endocytosis from the apical membrane and redistribution of G protein to the basolateral membrane were still taking place. Previous studies in other cells have shown that receptor-mediated endocytosis continues at 16–21°C but traffic of ligands into lysosomes is arrested (28, 29). Free flow electrophoresis confirmed that no lysosomal G protein could be detected in lysosomes at 21°C. Thus, our studies gave no direct evidence for the involvement of the lysosomes in the transcellular route.

The subcellular compartment equilibrating at a density of 1.05 g/cm^3 into which G protein is endocytosed shares characteristics with endosomes in other cells (30). They have similar morphology, their buoyant density is usually lower than that of lysosomes, and they contain little or no lysosomal hydrolases (29–34). We will therefore henceforth refer to the subcellular compartment into which the G protein is endocytosed in MDCK cells as endosomes. The endosomal compartment in other cells is known to receive receptor-bound ligands from the cell surface for delivery to lysosomes (30–32). The available evidence suggests that many cell surface receptors that dissociate from their ligands at the low endosomal pH (29, 33, 35, 36) are recycled back to the cell surface before reaching the lysosomes (37–41). We have not yet demonstrated that the endosomal compartment in MDCK cells has an acidic pH. However, we have shown earlier that the transcytosis of endocytosed G protein is inhibited by monensin and NH_4Cl (12), both known to dissipate proton gradients and to inhibit receptor recycling in other cells (42–46).

These studies demonstrate that endosomes not only play an important role in membrane recycling, but in transcytosis in MDCK cells as well. Previous studies of ligands undergoing transcytosis through other epithelial cells have also implied that endosomes are involved in the process. Abrahamson and Rodewald (47) have shown that IgG molecules undergoing transcytosis from the apical surface to the basolateral plasma membrane of neonatal rat intestinal cells passed through intracellular structures with the morphological characteristics of endosomes. Some IgG could also be detected in lysosomes but whether these molecules were destined for degradation or transcytosis could not be ascertained by their methods. Similar results have recently been reported by Herzog (17) for TSH-stimulated transcytosis of thyroglobulin in thyroid follicle cells.

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