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

I. Morphological Evidence

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ABSTRACT The G protein of vesicular stomatitis virus was implanted in the apical plasma membrane of Madin-Darby canine kidney cells by low pH-dependent fusion of the viral envelope with the cellular membrane. The amount of fusion as determined by removal of unfused virions, either by tryptic digestion or by EDTA treatment at 0°C, was 22–24% of the cell-bound virus radioactivity. Upon incubation of cells after implantation, the amount of G protein as detected by immunofluorescence diminished on the apical membrane and appeared within 30 min on the basolateral membrane. At the same time some G protein fluorescence was also seen in intracellular vacuoles. The observations by immunofluorescence were confirmed and extended by electron microscopy. Using immunoperoxidase localization, G protein was seen to move into irregularly shaped vacuoles (endosomes) and multivesicular bodies and to appear on the basolateral plasma membrane. These results suggest that the apical and basolateral domains of Madin-Darby canine kidney cells are connected by an intracellular route.

In most cells the plasma membrane is continuously endocytosed (36) and the loss of surface membrane must be balanced by retrieval of membrane from inside the cell. Membrane recycling poses a special problem in epithelial cells because the plasma membrane is polarized; it is divided into apical and basolateral domains with distinct protein and lipid compositions (35). If the plasma membrane is continuously recycling, an important question is how the apical and basolateral domains preserve their unique compositions. If the apical and basolateral recycling routes are separated, randomization of the cell surface would of course be prevented. If, however, they connect at some point in the cell, continuous sorting of apical and basolateral proteins would have to take place.

In an attempt to map the membrane traffic routes to and from the cell surface in epithelial cells, we are using the Madin-Darby canine kidney (MDCK) cell line as our experimental system (7, 16). These cells display both structural and functional polarity when grown in culture (24, 32). The microvillar apical domain is freely accessible to the medium. The basolateral membrane attaches the cell to the culture dish and mediates contact with the neighboring cells. Each surface domain possesses a characteristic set of proteins. Aminopeptidase, for example, is found in the apical membrane, while the Na⁺K⁺ATPase has a basolateral location (17, 31). The two surface domains are separated from one another by tight junctions encircling the apex of the cell (21). Rodriguez-Boulan and colleagues (33, 34) have shown that in MDCK cells infected with enveloped viruses the newly synthesized viral membrane glycoproteins are inserted into one or the other surface domain. The influenza virus glycoproteins are distributed mainly to the apical domain while the vesicular stomatitis virus (VSV) G protein is inserted primarily in the basolateral domain. Because the viral glycoproteins are processed by cellular enzymes and transported through the cell to the plasma membrane via intracellular organelles, it seems likely that the processes leading to their polar distribution will
provide clues to the sorting mechanism and recycling pathways for normal plasma membrane constituents.

To investigate membrane recycling routes in epithelial cells, we implanted the basolateral VSV G protein into the apical membrane of the MDCK cells by low pH-induced fusion (22, 47), and followed its fate in the cell during subsequent incubations to find out whether it can be routed to the basolateral membrane. In this paper, we give morphological evidence that implanted G protein is rapidly endocytosed and that some of it is distributed to the basolateral plasma membrane.

In the following paper (28), the redistribution phenomenon is quantitated by an immunoradiometric assay and the redistribution process is described in more detail.

MATERIALS AND METHODS

Cell Cultures: MDCK cells were grown as described (22) in Earle's minimal essential medium supplemented with 5% fetal calf serum, 10 mM HEPES (pH 7.3), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (0.025 μg/ml). Cells were plated at 7 × 10^4 cells per 35-mm dish (with or without a glass coverslip) 2 d before the experiment and were confluent when used.

Virus: Subconfluent Falcon flasks (75 cm²; Falcon Labware, Oxnard, CA) of baby hamster kidney (BHK) cells were infected for 20 h at 37°C with 0.01 plaque-forming unit (pfu)/cell of VSV, Indiana serotype. The growth medium was aspirated, and centrifuged at 8,000 g for 20 min in a Beckman SW 27 rotor (Beckman Instruments, Inc.) for 90 min at 25,000 rpm and 0°C. The viral virus band was collected from the interface between the two sucrose concentrations, divided into aliquots, and stored at -80°C. The virus concentration of different batches of virus was 0.3–0.5 mg/ml.

Virus labeled with [35S]methionine (2 μCi/flask) or with [3H]uridine (2 μCi/flask) was prepared in BHK cells as described before (14, 22) and purified as described above.

Antibodies: Antibodies against VSV G proteins were produced in rabbits as described (22). The antiserum was further purified by affinity chromatography. VSV (5 mg protein) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and the affinity column was used to purify the anti-G protein antibodies as previously described (3). Elution of the antibodies was performed according to Ternynck and Avrameas (38).

Binding Assay and EDTA Treatment: Binding of [35S]methionine-labeled VSV to MDCK cells was measured using the procedure described previously (3). Implantation of the VSV G protein was determined after adsorption of the virus at 0°C and pH 6.3. The EDTA treatment to remove cell-bound virus was done by washing the cells after virus adsorption twice with 2 ml of cold phosphate-buffered saline, Ca- and Mg-free (PBS(-)), pH 7.4, and then with 20 mM EDTA in PBS(-), pH 7.4 for 5 min at 0°C. The monolayer was then washed twice with 2 ml PBS(-) before measuring the cell-bound radioactivity.

Fusion Assay: To measure fusion, virus labeled with either [35S]methionine or [3H]uridine was adsorbed to MDCK cells (35-mm dish) at pH 6.3 for 1 h at 37°C. The medium was removed from the dish, and the dish was then transferred to a waterbath at 37°C and flooded with warm acidic medium (pH 5.4) for 30 s. The dish was then cooled on ice, and the low pH medium was aspirated. Trypsin treatment was done as previously described (22). In most cases, EDTA washing at 0°C was substituted for trypsin treatment and was performed as described above. To test whether cell permeability was affected by fusing VSV membranes with the apical surface of MDCK cells, we measured transpatent blue uptake of the cells after fusion and incubation at 31°C of VSV membranes (1 μg protein of VSV added per 2.2 × 10⁶ cells). No cells were permeable to the dye after 15-min incubation.

Immunofluorescence: Immunofluorescence was performed by the indirect technique as described earlier (22). The first antibody was affinity-purified rabbit anti-G protein antibody (0.8 μg/ml). The second antibody was goat anti-rabbit IgG conjugated to rhodamine (7). To MDCK cells grown on coverslips in 35-mm plastic dishes, 2 μg of VSV were adsorbed at pH 6.3, fixed at pH 5.4, and treated with EDTA at 0°C. The dishes were then washed with PBS containing 0.09 mM Ca and 0.05 mM Mg (PBS(-)) at 0°C. For incubation the dishes were filled with medium at pH 7.4 (22) containing 20 μg/ml cycloheximide (17) and incubated for various times at 31°C in a 5% CO₂ atmosphere. To visualize the G protein on the apical surface, the cells were fixed with 3% (wt/vol) formaldehyde following by antibody staining. To visualize intracellular G proteins, we permeabilized the cells with 0.1% Triton X-100 after fixation (17). The basolateral surface was made accessible to antibodies by a treatment for 2–5 min at 37°C with 2 mM EGTA in PBS(-) (34). After fixation all subsequent washes of EGTA-treated samples were done with PBS(-). Coverslips were mounted in Moviol 4-88 (Hoechst, Frankfurt, Federal Republic of Germany) viewed through a Zeiss FRG photomicroscope III equipped with a Planapo 63 oil immersion objective.

Electron Microscopy: For ultrastructural localization of G protein by electron microscopy, the low pH-dependent fusion, EDTA treatment, and incubation were performed as described above, except that the incubations were done at 37°C and were terminated by the addition of fixative. In addition, some samples warmed at 37°C for 5 min or more were treated with 2 mM EDTA in PBS before fixation as described above to improve penetration of antibodies to the basolateral domain.

Cells for morphological analysis alone were fixed in 2% formaldehyde (wt/vol), 2% glutaraldehyde for 2 h at room temperature, postfixed in osmium tetroxide, and then stained with 0.5% uranyl acetate in an acid Michaels buffer (2). Fixation and processing procedures and cytochemical methods to detect lysosomal enzymes have been described in detail by Bainton and Farquhar (2), with the exception of the tritrophasmatid procedure which was performed according to Oliver (27).

Immunoperoxidase labeling was performed essentially as described by Ohtsuki et al. (26), Tougaard et al. (40), and Louvard et al. (18), but with the following modifications. The cells were fixed with 2% (wt/vol) formaldehyde and 0.05% (wt/vol) glutaraldehyde in PBS diluted to a total molarity of 100 mM NaCl for 30 min at room temperature. They were then washed three times with PBS containing 50 mM NaHCO₃ followed by PBS containing 0.05% (wt/vol) saponin and 0.2% (wt/vol) gelatin for 30 min at room temperature. Subsequently, the cells were treated with 1:100 dilution of affinity-purified rabbit anti-G protein antibody in PBS containing 0.05% (wt/vol) saponin for 30 min at room temperature. They were then washed three times with PBS containing 0.05% (wt/vol) saponin, treated with Fab fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase (Institut Pasteur, Paris) in the same buffer used for the first antibody. After three more washes with PBS, the cells were fixed with 1% (wt/vol) glutaraldehyde in 100 mM cacodylate buffer (pH 7.2). After three washes with the same buffer, they were left overnight at 4°C in sodium cacodylate buffer containing 5% sucrose and incubated in the medium devised by Graham and Karnovsky (11) containing 1 mg/ml diaminobenzidine and 0.01% (vol/vol) H₂O₂ for 1 h at room temperature in the dark. Next, they were washed with 0.1 M sodium cacodylate buffer with 5% sucrose and postfixed in reduced osmium as modified by Herzog and Miller (13). Briefly, the cells were covered with 1% unbuffered OsO₄ for 2 min, followed by 15 min in reduced osmium, produced by adding 5 mg of potassium permanganate to 1 ml of 1% osmium tetroxide. After removal of the OsO₄, the cells were rinsed for 2 min again in 1% unbuffered OsO₄ and dehydrated in graded ethanol. At this point, the cells were scored off into thin sections, and propylene oxide was squished directly onto the cells, making them float off the plastic dish. These small pieces of tissue were centrifuged in 0.4-m1 tubes at 10,000 g in a microfuge (Beckman Instruments, Palo Alto, CA). The pellets were gently cut out of the tubes and subsequently embedded in Epon. As a control for endogenous peroxidase, MDCK cells were fixed in 2% formaldehyde-0.05% glutaraldehyde before or after acid-induced fusion, and washed and incubated in diaminobenzidine-H₂O₂ as described above. Very little endogenous peroxidase was detected; when found, it was limited to rare Golgi cisternae and vacuoles. In other controls, one of the steps for demonstration of G protein was modified as follows: (a) virus was adsorbed but not subjected to acid treatment; (b) anti-G protein antibody was omitted, and replaced by anti-fowl plague virus antibody, and (c) the addition of virus was omitted. All three treatments produced a slight background on the apical domain significantly less than that seen after specific staining for implanted G protein.

RESULTS

Implantation of the VSV G protein by Fusion

Previously we have established that VSV binds optimally to the apical surface of MDCK cells at pH 6.3 and at 0°C does not enter the cells (22). The basolateral surface is not accessible to added viruses because the tight junctions do not allow passage of macromolecules between the cells (21). When the cells are overlaid with warm medium at pH 5.4 the viral envelope fuses with the apical plasma membrane (22, 47).
After fusion the G proteins are oriented in the plasma membrane with their glycosylated portions towards the outside (46). The nucleocapsid, consisting of the viral RNA and associated proteins, is introduced into the cytoplasm. To measure the efficiency of fusion, we made use of the previous observation that trypsin treatment at 0°C removes bound but not fused virions from the cell surface (22). By using VSV labeled in its RNA with [3H]uridine the degree of fusion could be measured as the amount of cell-associated radioactivity remaining after trypsin treatment. When 1 μg of VSV labeled with [3H]uridine was incubated for 1 h at 0°C with 2 × 10⁶ MDCK cells, 12% of the radioactivity was bound. After fusion at pH 5.4 and 37°C for 20 s, 28% of the bound radioactivity was resistant to trypsin. In controls, which were not subjected to low pH treatment, only 6% was resistant. Thus, ~22% of the virions originally bound to the cell surface had fused. In addition to removing bound virions from the cell surface, trypsin treatment can degrade some of the G protein in the plasma membrane (25). Thus, we could not use trypsin to remove unfused viruses that, if left on the cell surface, would be endocytosed and interfere with studies of the fate of implanted G protein in the cell. It was therefore necessary to devise a nondestructive way of eliminating unfused viral particles from the cell surface. Fig. 1 illustrates that treatment with EDTA in the cold effectively elutes bound virions from the cells. For 0.1–1.25 μg of [35S]methionine-labeled VSV added per 2 × 10⁶ cells, the fraction bound was a constant 12%. Treatment with 20 mM EDTA for 5 min at 0°C removed most of the radioactivity bound to the cell surface; ~16% was left. Prolonging the EDTA treatment to 15 min did not increase the amount released.

If 1 μg of radioactive VSV was added per 2 × 10⁶ cells, bound and fused at pH 5.4 as before, and then subjected to EDTA treatment, 40% of the cell-bound radioactivity remained. Since, in controls not treated at low pH, 16% remained cell-associated after EDTA treatment, we estimated that 24% of the added virus was fused. This figure compares favorably with the value of 22% fusion measured by the trypsin assay.

Redistribution of Implanted G Protein: Observations by Immunofluorescence

After fusion and EDTA washing to remove unfused viruses, the cells with implanted G protein were incubated at 31°C and the location of G protein was examined by immunofluorescence using specific anti-G protein antibodies. To visualize G protein in different parts of the cell, we employed three different treatments (Fig. 2). (a) To observe the apical surface added to MDCK cells (22) or to BHK cells (12), and probably represents viruses bound or fused mainly to microvilli on the cell.

![Figure 1](image1.png) **Figure 1** Binding and release of VSV from the cell surface. [35S]methionine labeled VSV (260,000 cpm) together with 0.25, 0.5, 0.75, 1.0, or 1.25 μg of unlabeled VSV (protein) was added per plate (2.2 × 10⁶ cells) in 200 μl of MEM, pH 6.3, and incubated for 1 h at 0°C. The unbound virions were washed off with cold binding medium and the cell-associated radioactivity of duplicate plates was determined before (○) or after (□) incubation with 20 mM EDTA for 5 min at 0°C. The amount of EDTA-resistant viral protein is plotted vs. the amount of viral protein added per plate.

![Figure 2](image2.png) **Figure 2** Schematic diagram illustrating the accessibility of cellular compartments to apically applied probes after various treatments. The large arrows indicate the tight junctions. The small circles represent G-protein-specific antibodies. (a) Only the apical surface is accessible. (b) Both the basolateral and apical domains can be reached by antibodies since the junctions are leaky (small arrows). (c) All compartments are accessible, because the plasma membrane has been permeabilized with detergent.

alone, the cells were fixed with formaldehyde, and solutions containing calcium and magnesium were used in the subsequent steps to keep the tight junctions intact. (b) To make the basolateral surface accessible to antibodies, the cells were treated with 2 mM EGTA at 37°C for 5 min (34) and divalent cations were omitted. The EGTA treatment of 37°C makes the tight junctions leaky (21). (c) To make cytoplasmic structures as well as both surface domains accessible to antibodies, the cells were permeabilized with Triton X-100 after fixation. By changing the focal plane and by comparison of the various labeling patterns, we could determine whether G protein was on the apical surface, the basolateral surface, or inside the cell.

Figs. 3 and 4 illustrate the patterns seen after fusion of 2 μg VSV with 2 × 10⁶ cells. Immediately after fusion (Fig. 3, a and a') the fluorescence appeared mainly as dots at the apical surface. This is the pattern generally seen when viruses are added to MDCK cells (22) or to BHK cells (12), and probably represents viruses bound or fused mainly to microvilli on the
Figure 4 Redistribution of VSV G proteins after implantation into MDCK cells. Samples from the same experiment shown in Fig. 3 were incubated at 31°C after implantation and EDTA treatment at 0°C and then processed for differential immunofluorescence staining as described in Fig. 2 and the text. (a-c) No incubation. (d-f) 15-min incubation. (g-i and g'-i'). 30-min incubation. In a, d, and g Ca and Mg were included in all solutions to keep tight junctions closed during fixation and staining. In b, e, and h the cells were treated with 2 mM EDTA in PBS for 2 min at 37°C before fixation, and Ca and Mg were omitted from all subsequent wash solutions to prevent the tight junctions from becoming impermeable. In c, f, and i the cells were treated with 0.1% (wt/vol) Triton X-100 (TX100) after fixation to permeabilize them before antibody staining (see text for more details). g'-i' are the Nomarski images of the fields shown in g-i. Arrowheads in h and h' mark the lateral borders of some cells. Bar, 8 μm. x 700.

Cell surface. When the cells were treated with 20 mM EDTA for 5 min at 0°C to remove unfused virions, the fluorescence pattern changed as expected to fewer dots (Fig. 3, b and b'). After the EDTA treatment, the cells were incubated in medium at 31°C for different periods. Cycloheximide was included to inhibit viral protein synthesis from infection. Before incubation at 31°C, no fluorescence was visible on the basolateral surface or inside the cell, since the staining patterns...
after all three treatments were similar and could only be seen on the apical surface (Fig. 4a, b, and c).

After a 15-min incubation, labeling of the apical surface dramatically decreased (Fig. 4d). Labeling of the basolateral surface could not be seen in most cells (Fig. 4e), but fluorescent vacuoles inside the cell were clearly visible (Fig. 4f). Basolateral staining could sometimes be seen after only a 15-min incubation, but was most frequently observed after ~30 min of incubation (arrows in Fig. 4h and h'). At this time some fluorescence also seemed to reappear on the apical surface of some cells (Fig. 4g). The latter consisted of a finely granular staining unlike the dots seen at the onset of incubation. Intracellularly, at this time, distinct vacuoles were labeled (Fig. 4, i and i'). The basolateral labeling pattern was more difficult to discern after Triton X-100 treatment. However, if more VSV (>3 µg per 2 × 10^6 cells) was used, basolateral G protein could be detected using this method (not shown). The results of this experiment suggest that the implanted G protein was rapidly internalized at 31°C; one fraction then appeared on the basolateral surface and another fraction was returned to the apical surface. Some G protein also remained inside the cell. Similar results were obtained by incubating the cells at 37°C after implantation, but the internalization and redistribution were more rapid.

Redistribution of Implanted G Protein: Electron Microscopic Observations

To confirm our immunofluorescence observations and to obtain more detailed information on the structures involved in the redistribution process, we localized the G protein by electron microscopy using immunoperoxidase staining. The cells used for the implantation studies were grown for 48 h in culture. At this stage, the MDCK cells formed confluent monolayers, with apical and basolateral domains connected by tight junctions (Fig. 5a). Intracellularly, the Golgi complex, typically located near the nucleus, and multivesicular bodies and secondary lysosomes filled with heterogeneous material (Fig. 5b) were prominent organelles. That these latter vacuoles were indeed lysosomal in nature was established by exploring the localization of three lysosomal enzymes: tri-methylphosphatase (Fig. 5c), arylsulfatase (Fig. 5d), and acid phosphatase (not shown). In general, the large vacuoles containing heterogeneous material stained heavily for hydrolytic enzymes, whereas in comparison the multivesicular-bodies and Golgi cisternae contained little or no reaction product.

After the implantation of G protein in the apical surface and incubation of the cells, we followed the localization of G protein in MDCK cells. To detect surface and intracellular G protein, we fixed cells with formaldehyde-glutaraldehyde, permeabilized to antibodies by treatment with saponin, and stained first with specific anti-G protein antibodies and then with peroxidase-conjugated Fab fragments directed against the first antibody. Observations were made during three time intervals: (a) after the first 20 s, when the viruses fused with the cells at pH 5.4 and 37°C; (b) 2–5 min after fusion, when the cells had been returned to growth medium at 37°C; and (c) later periods 10–40 min after fusion. Samples to be examined by electron microscopy were incubated at 37°C instead of 31°C as in the immunofluorescence experiments documented above. The two types of experiments can therefore be compared only qualitatively.

ACID-INDUCED FUSION AT THE APICAL SURFACE: The reaction product of oxidized diaminobenzidine consists of a dense, black, flocculent precipitate. After implantation of G protein by low pH-dependent fusion, the apical surface including microvilli and coated pits was stained, while the junctional complex throughout its entire length and the basolateral surface were unstained (Fig. 6a and b).

Often, microvilli could be seen which were more heavily stained than the rest of the membrane, and these may correspond to the dots seen in the immunofluorescence experiments. Occasional large intracellular vacuoles with reaction product on their membrane were also encountered (Fig. 6b). These presumptively originate from the endocytosis of implanted G protein during the 20-s warming period.

2–5 MIN AFTER FUSION: By 2 min after fusion, as depicted in Fig. 7, the amount of stain on the apical surface had decreased, and the heaviest amount of stain appeared to be in large vacuoles with clear matrices (arrows) varying in size and shape. A striking feature of these was their great diversity of size and contour. Their irregular shape could be seen in fortuitous serial sections, as illustrated in Fig. 8a and b. These vacuoles may be swollen as an artifact of the fixation and incubation procedure. This interpretation is based upon the morphology of similar vacuoles (see inset in Fig. 7) found in the same experimental material fixed with higher concentrations of fixative and not incubated for antigen localization. Besides the clear vacuoles, the other organelle which consistently stained was the multivesicular body (Figs. 7 and 8c–e). The amount of reaction product in multivesicular bodies seemed less than that observed in the clear vacuoles, and the distribution within the organelle itself varied. While the small vesicles in the matrix were always positive (Fig. 8c–e), the membrane of the multivesicular body was not so heavily stained. Invaginations of the labeled membrane suggested that the small vesicles pinch off from the outer aspect of the vacuolar membrane into the matrix (8). The only other positive organelles detected were secondary lysosomes but these were rarely stained. The basolateral aspect of the plasma membrane was negative in most cells examined at this time point, although rarely light stain was seen on the basolateral surface after only a 2-min incubation. No reaction product was observed in elements of the Golgi complex, or in the endoplasmic reticulum and mitochondria.

10–40 MIN AFTER FUSION: By 10–15 min (See Fig. 9, a and b), reaction product was detected on the basolateral membrane. Usually, the staining of the basolateral membrane could be seen in pockets where the membranes from two cells were separated from each other (Fig. 9a). Stain was also evident on the apical domain (Fig. 9a and b). The intracellular organelle most frequently labeled during this period was the multivesicular body, whereas positive clear vacuoles were rare. At early times, clear vacuoles outnumbered multivesicular bodies, whereas after 40 min the reverse was true. Reaction product in secondary lysosomes at this time was infrequent. These observations suggest that the implanted G protein is rapidly endocytosed and then distributed to the basolateral surface by an intracellular pathway involving the large, clear vacuoles and multivesicular bodies.

DISCUSSION

Our results demonstrate that the viral envelope glycoprotein G is a useful probe of endocytic pathways after its implantation in the plasma membrane by low pH-dependent fusion.
Our results show that G protein implanted into the apical membrane of MDCK cells is endocytosed and part of it is distributed to the basolateral surface. These findings strongly suggest that the apical and basolateral plasma membrane domains of MDCK cells are connected by an intracellular route.
Implantation

Previous studies have shown that VSV possesses an acidic pH-induced membrane fusion activity (22, 47). In this paper, we have taken advantage of this to introduce the glycoprotein G uniquely into one plasma membrane domain of MDCK cells. When 1 μg of viral protein was added to 2 × 10⁶ cells, 12% of the virions bound to the cell surface and 24% of the bound virions fused to the plasma membrane after 20 s at pH 5.4. This corresponds to about 26,000 G proteins (assuming...
FIGURE 7 MDCK cells after incubation in MEM culture medium, pH 7.4 for 2 min after fusion at pH 5.4 to illustrate the large number of stained intracellular vacuoles (arrows) found in both apical and basal cytoplasm. Occasional multivesicular bodies (mvb) also contain stain. The basal (B) and lateral (L) membranes are not stained. The usual morphologic appearance of mitochondria (m) is suboptimal due to the fixation procedure used. Bar 0.4 μm. x 20,000. (Inset) A vacuole from a cell treated in a similar manner experimentally but fixed for morphology alone (see Materials and Methods) and not subsequently processed for antigen localization. Note that this vacuole (double arrows) is not swollen as those seen in the rest of the figure (single arrows). Bar, 0.4 μm. x 40,000.

500 G proteins per virion; 6) implanted per cell. Most of the cell-bound virions which did not fuse could be removed by a short EDTA treatment at 0°C. This procedure did not disrupt cell contacts since the opening of tight junctions in MDCK cells by chelation of calcium ions occurs very slowly at low temperature (21). Binding of VSV to the cell surface thus seems to be mediated by divalent cations and is in this respect similar to the association of a number of physiological ligands with cells (15).

Optimal results in the experiments described here were obtained with virus which was not pelleted by centrifugation during purification. Moreover, EDTA had to be included in
FIGURE 8 (a and b) Fortuitous serial sections of cells that were fixed 2 min after warming to 37°C following acid-induced fusion. Note the immunolabel is present in intracellular vacuoles of different sizes and shapes (arrows). Also note the absence of stain on the lateral (L) and basal (B) membranes as well as in a secondary lysosome (sl). The only other intracellular organelle that was stained, was the multivesicular body as seen in c–e. Note that the vesicles in the matrix of the multivesicular bodies appear to be reactive. Bars: a and b, 0.4 µm; c–e, 0.3 µm. (a) × 20,000; (b) × 17,000; (c) × 56,000; (d) × 30,000; and (e) × 33,000.

Before these precautions were taken, different amounts of G protein remained at the apical cell surface after implantation and incubation. These variations could be due to differences in the release of the viral nucleocapsid from the envelope after fusion. Similar problems have been encountered with plasma membrane glycoproteins reconstituted into lipid vesicles and introduced into the cell membrane by polyethylene glycol.

the sucrose gradient to avoid aggregation of the virus (4).
mediated fusion (5).

Low pH-induced fusion is considerably more efficient than other fusion methods. Sendai virus mediated fusion or polyethylene glycol induced fusion have been used to implant membrane glycoproteins into cell membranes, but the time needed for fusion to occur is at least 5 min at 37°C (5, 30). With such long incubation times, it is difficult to distinguish between fusion at the cell surface and internalization of membrane vesicles by endocytosis. Moreover, both Sendai virus and polyethylene glycol may induce cell lysis (19, 29). After VSV fusion, we observed no permeabilization of the cells; antibodies applied to the apical surface did not penetrate inside the cell in the absence of detergent treatment (Fig. 4).

Endocytosis

The implanted G protein rapidly disappeared from the apical cell surface. Our evidence shows that it was endocytosed. Within 15 min after the fusion, G protein was detected in intracellular vacuoles by immunofluorescence. A small amount of intact G protein was lost in the medium after implantation (see following paper, reference 28). Endocytosis of implanted G protein was confirmed by electron microscopy. At early times after fusion, peroxidase reaction product was seen along the surface of smooth surfaced, irregularly shaped vesicles which we refer to as endosomes (20), and in multivesicular bodies. Cytochemical reactions suggest that endosomes and most multivesicular bodies in MDCK cells lack lysosomal hydrolases. This observation is consistent with the finding that degradation of implanted G protein into acid-soluble radioactivity in the medium is minimal during the duration of the experiment (see reference 28). The fact that many multivesicular bodies lack lysosomal hydrolases has been emphasized in a recent review by Goldfischer (10).

Others have also observed the endocytosis of probes and ligands into vacuoles morphologically similar to those described here (12, 45, 48). In particular Van Deurs et al. (42) reported that cationized ferritin initially entered electronlucent endocytic vacuoles and "light" multivesicular bodies containing some vesicular profiles, and only later could been seen in "dark" multivesicular bodies and dense bodies. The latter two structures, but not the former, were shown to be positive for acid phosphatase. Abrahamson and Rodewald (1) observed, in their study of receptor-mediated transport of IgG across the newborn rat enterocyte, that ferritin-conjugated IgG and horseradish peroxidase were taken up together into endocytic vesicles of irregular profile and into apical vacuoles, both devoid of acid phosphatase and aryl sulfatase. Both probes later appeared in dense bodies positive for these enzymes.

Redistribution

One most interesting observation was that some of the implanted G protein was found to rapidly redistribute within

FIGURE 9 Portions of MDCK cells to illustrate the redistribution of the apically fused G protein to the basolateral surface. After 10-min incubation (a) antigen staining can be seen in an expanded area (arrows) of the basolateral membrane. The exact mechanism of delivery to the lateral surface has not been seen. b illustrates the basolateral redistribution after 15 min at 37°C after fusion. Note the density in the basolateral region as compared with other membranes (double arrows). The apical membrane also has considerable staining. Bars, 1.0 μm. (a) × 9,900; (b) × 16,300.
the cell. Within 30 min at 31°C, G protein was seen on the basolateral domain by immunofluorescence. The immunofluorescence pattern was similar to that observed during infection of MDCK cells by VSV when the newly synthesized G protein is inserted into the basolateral domain (34) and also to the pattern seen in MDCK cells when Na⁺/K⁺-ATPase is labeled with specific antibodies (17). Localization of G protein to the basolateral domain was confirmed by electron microscopy after immunoperoxidase detection of G protein in cells permeabilized with saponin.

Our data strongly suggest that it is the endocytosed G protein that is routed to the basolateral surface, although it cannot be entirely excluded that G protein moves laterally through the tight junctions from the apical to the basolateral domain. By immunofluorescence, G protein appeared to move sequentially from the apical surface to the inside of the cells and only then to the basolateral surface. Also in electron micrographs, G protein was more readily detected on the basolateral domain at later times than at earlier times when it was easily seen in intracellular vacuoles.

Our data do not allow us to determine precisely the vesicular pathway traversing the cell from the apical to the basolateral domains. Most likely the endosomes are on this pathway. This interpretation, however, does not concur with that of van Deurs et al. (42). In their study of the transepithelial movement of cationized ferritin in the choriocarcinoma line, they concluded that transport through endocytic vacuoles and multivesicular bodies led consequentially to degradation in the dense lysosomes, while transfer to the basolateral domain went via a separate pathway. On the other hand, Abrahamsson and Rodewald (1), studying the transepithelial transport of IgG, suggested that transport to both the lysosomes and the basolateral domain begins in the endosomal compartment. Such an interpretation demands a new event in the endosomes in which some molecules are distributed to the plasma membrane and others are degraded in the lysosomes. A crucial factor in the selective mechanism may be the acidic pH of endosomes (9, 41) which recent work suggests is important for membrane recycling (23, 37, 43, 44).

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