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Replacement of the cytoplasmic domain alters sorting of a viral glycoprotein in polarized cells

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ABSTRACT **The envelope glycoprotein (G protein) of vesicular stomatitis virus (VSV) is transported to the basolateral plasma membrane of polarized epithelial cells, whereas the hemagglutinin glycoprotein (HA protein) of influenza virus is transported to the apical plasma membrane. To determine if the cytoplasmic domain of VSV G protein might be important in directing G protein to the basolateral membrane, we derived polarized Madin–Darby canine kidney cell lines expressing G protein or G protein with its normal cytoplasmic domain replaced with the cytoplasmic domain from an influenza HA protein (GHA protein). Indirect immunofluorescence microscopy showed that G protein was present primarily on basolateral surfaces, whereas the GHA protein was present on the apical and basolateral membranes. These results suggest that the cytoplasmic domain can be an important determinant directing polarized expression of an integral membrane protein.**

Epithelial cells, such as Madin–Darby canine kidney (MDCK) cells, develop differentiated apical and basolateral plasma membrane domains with distinct protein and lipid compositions (1). Polarized budding of certain viruses from the apical or basolateral membranes of these cells is a manifestation of this differentiation. For example, vesicular stomatitis virus (VSV) buds from the basolateral surface of these cells, whereas influenza virus buds from the apical surface. This polarized budding is preceded by accumulation of the viral glycoproteins in the appropriate apical or basolateral plasma membrane where budding occurs (2, 3).

It has been reported that cDNAs encoding the envelope glycoprotein (G protein) of VSV or the hemagglutinin glycoprotein (HA protein) of influenza virus can be expressed transiently in polarized cells either from simian virus 40 (SV40) vectors or in SV40 or vaccinia virus vector-infected cells. The G and HA proteins are targeted correctly to the basolateral or apical plasma membrane domains of these cells (4–6). Thus, specific features of these glycoproteins must direct segregation to the appropriate plasma membrane domain. It is known also that the pathway of biogenesis of G and HA proteins proceeds from the rough endoplasmic reticulum through the Golgi complex to the plasma membrane and that both proteins are found in the same Golgi cisternae of MDCK cells doubly infected with VSV and influenza virus. Therefore, sorting of these proteins to their respective plasma membrane domains must occur during or following exit from the Golgi complex (7, 8). Also, in fully polarized cells, transport of these proteins proceeds from the Golgi complex directly to the appropriate membrane (9–12).

In the work reported here we began an analysis of the signals that direct proteins to specific plasma membrane domains in MDCK cells. We have shown previously that cDNA clones encoding VSV G protein with its normal 29 amino acid cytoplasmic domain replaced with the 10 amino acid cytoplasmic domain of an influenza virus HA protein or the 3 amino acid cytoplasmic domain of the IgM heavy chain (μ_m) are transported to the plasma membrane of transfected, nonpolarized COS-1 cells (13). In this study we expressed these cDNAs in epithelial cells to determine if the cytoplasmic domain of G protein has a role in targeting G protein to the basolateral membrane. Rather than using transient expression systems (which give only a very low percentage of MDCK cells expressing the protein) or the viral-based expression systems (in which cells are rapidly depolarized), we devised methods that allowed us to obtain stable MDCK cell lines expressing sufficient quantities of the wild-type or hybrid glycoproteins for immunolocalization studies.

MATERIALS AND METHODS

Construction of Expression Plasmids. Plasmids for selection of cell lines expressing the wild-type or mutant G proteins were constructed as shown in Fig. 1. Since aberrant splicing of the mRNA encoding G protein was apparently observed in the presence of the SV40 small tumor antigen intron (16), we deleted it from pSV2TG by first linearizing the plasmid with *Bgl* II and then trimming with BAL-31 exonuclease. *Bgl* II linkers (CAGATCTG) were inserted at the site of the deletion. DNA sequence analysis showed that nucleotides 4409–4691 (including the small tumor antigen intron; ref. 28) had been deleted in a plasmid designated pSV2TGΔS. This plasmid was cleaved with *Hin*dIII and *Bgl* II, and the BAL-31-trimmed TG gene was removed. The plasmid was religated in the presence of *Xho* I linkers (CCTCGAGG), introducing a unique cloning site. The *Bam*HI fragment containing the SV40 early promoter and transcription termination signals was purified from pSV2ΔSX and inserted into the unique *Bam*HI site in pSV2neo (ref. 15; provided by P. Southern, Scripps Clinic and Research Foundation, La Jolla, CA). Wild-type or mutant G genes were ligated into the *Xho* I site of pSV2neoSV and the orientation of the inserted genes was confirmed by mapping with restriction enzymes. An additional plasmid, pRSVG, for expression of the G gene under control of the Rous sarcoma virus (RSV) long terminal repeat was obtained from H.-P. Moore (University of California, San Francisco). The 900-base-pair (bp) *Bgl* II to *Bam*HI fragment in pRSVG (17) was replaced with the analogous 627-bp fragment from pSV2GΔS that lacked the SV40 small tumor antigen intron to generate the plasmid pRSVGΔS.

Cell Lines and Viruses. The parental MDCK cells (strain II) obtained from P. Maher and S. J. Singer (University of

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Abbreviations: SV40, simian virus 40; MDCK, Madin–Darby canine kidney; VSV, vesicular stomatitis virus; HA protein, hemagglutinin glycoprotein of influenza virus; G protein, envelope glycoprotein of VSV; RSV, Rous sarcoma virus.

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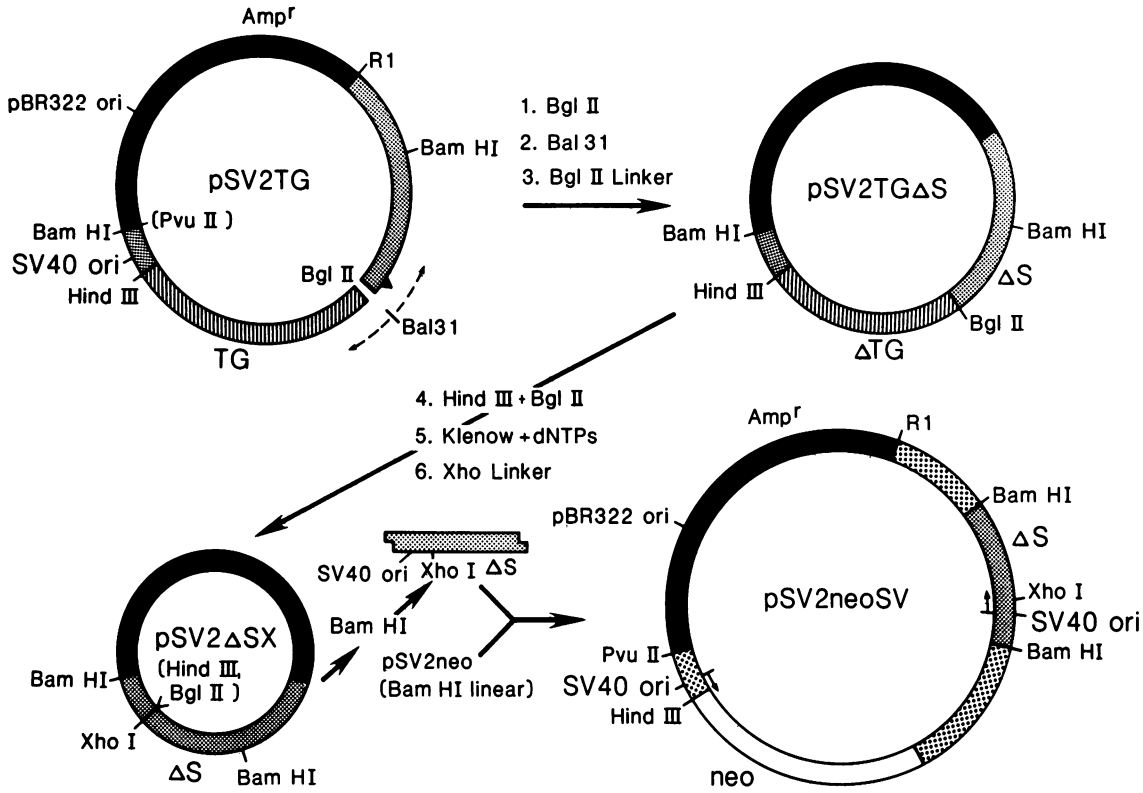

FIG. 1. Construction of the expression vector. Solid bars are pBR322 segments containing the origin of replication and the ampicillinase gene. The hatched segment in pSV2TG (14) indicates a truncated cDNA segment encoding the VSV G protein. Stipled bars indicate fragments of SV40 DNA that control expression of the cDNAs in eukaryotic cells. The open bar in pSV2neoSV indicates the gene encoding resistance to G418 (15).

California, San Diego) and G418-resistant MDCK cell lines selected in this study were maintained in Dulbecco–Vogt's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM nonessential amino acids. Twelve to 18 hr before transfection, $\approx 2.5 \times 10^6$ MDCK cells were plated on 10-cm tissue culture dishes. Transfection of 50–70 μg of plasmid DNA coprecipitated with calcium phosphate was performed as described except that the postadsorption incubation was increased to 9 hr (18). The medium was removed and 3.75 ml of 15% glycerol in Hepes-buffered saline (19) was added for 3.5 min at room temperature. In some transfections we extended the glycerol shock to 5 min or used a 10% dimethyl sulfoxide shock for 10 min, but we found no significant differences in transfection frequencies. In other experiments addition of 100 μg of chloroquine per ml to the postadsorption medium increased the transfection frequency from 3×10^{-5} to 1.2×10^{-4}. The cells were washed three times with DMEM and incubated in maintenance medium for an additional 36–48 hr before subculturing in medium containing 400 μg of antibiotic per ml ("active" G418, GIBCO) (15). The medium was changed every 4 days. G418-resistant colonies of cells were isolated 10–14 days after transfection and screened for expression of G protein by ELISA (as described below) or by immunofluorescence microscopy and immunoprecipitation as described for transfected COS-1 cells (14). Cell lines expressing wild-type or mutant G genes were recloned by limiting dilution.

ELISA. Clones of G418-resistant cell lines were cultured in duplicate or triplicate wells of 96-well tissue culture plates. Cells were induced with 10 mM sodium butyrate 14–16 hr before the assay. Sodium butyrate increases expression of genes under control of the SV40 early-region or RSV promoters (20). Monolayers were washed with Dulbecco's phosphate-buffered saline (DPBS) and then fixed with 3% paraformaldehyde in DPBS for 1 hr. Cells were washed with DPBS containing 10 mM glycine (DPBS/glycine; ref. 14) or 50 mM ammonium chloride (12) and then permeabilized with 1% Nonidet P-40 (Sigma) in the same buffer for 5 min. After washing, free aldehydes and protein-binding sites were blocked by incubation in DPBS/glycine containing 5% fetal calf serum (DPBS/fetal calf serum). Fifty microliters of a 1:300 dilution of ascites fluid containing a monoclonal antibody directed against G protein (I1; ref. 21) was added to each well and incubated 2 hr at room temperature. The plates were washed and 50 μl of a 1:750 dilution of peroxidase-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) was added to each well and incubated 1 hr at room temperature. The reaction was developed using 1 mM 2,2-azino-di-3-ethylbenzthiazoline sulfonate (Boehringer Mannheim)/0.1% H_2O_2 in citrate buffer (pH 4.2). The optical density of each well was measured at 415 nm.

RESULTS

Selection of MDCK Cell Lines Expressing Wild-Type or Mutant G Proteins. To obtain MDCK cell lines expressing wild-type or hybrid G proteins we first constructed the cDNA expression vector pSV2neoSV (Fig. 1) containing the dominant selectable gene *neo* (15), which confers G418 resistance to animal cells. The vector also contains a unique *Xho* I restriction site for insertion and expression of cDNA clones. The *neo* gene transcription and the cDNA transcription are driven by separate SV40 early-region promoters. Because we had previously encountered problems apparently resulting from splicing of sequences within the G mRNA to the 3' splice site in the SV40 small intron (16), this intron was eliminated from the vector.

Construction of cDNAs encoding G proteins with the normal cytoplasmic domain of 29 amino acids substituted by the 10 amino acid cytoplasmic domain of influenza virus HA

or the 3 amino acid cytoplasmic domain of μ_m heavy chain has been described (13). These were generated by an oligonucleotide-directed mutagenesis procedure in which nucleotides encoding the cytoplasmic domain of G protein were replaced with nucleotides encoding the cytoplasmic domains of the indicated proteins. The amino acid sequences of the normal and introduced cytoplasmic domains are shown in Fig. 2. The cDNAs encoding the G, GHA, or Gμ proteins were inserted at the single *Xho* I site of pSV2neoSV, and the resulting plasmids were designated pSV2neoSVG, pSV2neoSVGHA, and pSV2neoSVGμ. Another plasmid containing the VSV G cDNA under control of the RSV promoter (pRSVGΔS) was also constructed and was cotransfected into MDCK cells with pSV2neo. Colonies of G418-resistant cells obtained after transfection with each of these DNAs were screened for expression of G-related proteins by ELISA. The assay was performed after induction of the cells with sodium butyrate. Sodium butyrate increases the levels of proteins expressed under control of the SV40 early and RSV promoters as well as the levels of some cellular proteins (22). To quantify the total G-related protein expression, the ELISA was carried out on paraformaldehyde-fixed, permeabilized cells using a primary monoclonal antibody that recognizes an epitope shared among the wild-type and hybrid proteins (23).

The data from analysis of 335 clones transfected with pSV2neoSVGHA are given in Table 1. The majority of the cell lines (76%) expressed protein levels that fell in the lowest 17% of the assay range. These levels were found to be insufficient for reproducible detection of the protein by indirect immunofluorescence microscopy. Cell lines expressing the very highest levels of protein were chosen from the top 10–15% of the clones as determined by ELISA and screened further by immunofluorescence microscopy and radiolabeling followed by immunoprecipitation. It was necessary to screen about 300 cell lines to obtain several that were suitable for further analysis.

Analysis of Expression and Transport in MDCK Cell Lines. To determine if MDCK cell lines expressing high levels of G, GHA, or Gμ proteins encoded proteins of the appropriate apparent molecular weights, we labeled representative cell lines metabolically with [^{35}S]methionine.

Immunoprecipitates of cell lysates were then analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (NaDodSO$_4$/PAGE) (Fig. 3). Cell lines that had been transfected with the vector DNA alone (V3 and V7) were included as controls. G4 and R-G2 expressed G protein under control of the SV40

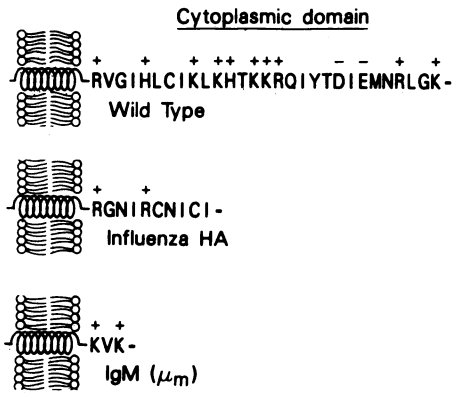

FIG. 2. Amino acid sequences of cytoplasmic domains in G, GHA, and Gμ proteins. DNAs encoding hybrid glycoproteins consisting of the extracellular and transmembrane domains of G protein linked to the cytoplasmic domains of the indicated proteins were generated as described previously and have been expressed in COS-1 cells and characterized (13). The sequences are shown in the single-letter code with net positive (+) or negative (−) charges indicated.

Table 1. Levels of GHA protein expression in G418-resistant MDCK cell lines

OD_{415}	Number	% of total
<0.10	155	46.2
0.10–0.15	100	29.9
0.15–0.20	38	11.3
0.20–0.30	24	7.2
0.30–0.40	9	2.7
0.40–0.50	3	0.9
0.50–0.60	3	0.9
0.70–0.80	1	0.3
>0.80	2	0.6

Results from an ELISA of 335 G418-resistant MDCK cell clones transfected with pSV2neoSVGHA DNA are shown. A background level optical density for control cells at 415 nm (OD_{415}) was subtracted in each case.

early promoter or RSV long terminal repeat, respectively. GHA7 and GHA10 expressed the GHA protein, and Gμ2 and Gμ7 expressed the Gμ protein under control of the SV40 early promoter. The G protein expressed by G4 and R-G2 comigrated with the authentic G protein from VSV-infected MDCK cells. The increased electrophoretic mobilities of GHA and Gμ proteins were those predicted from the DNA sequence. The localization of wild-type and hybrid proteins expressed in MDCK cells was assessed by direct or indirect immunofluorescence microscopy. Cells were grown on glass coverslips for 2–3 days after reaching confluence. At this time each cell line formed domes, suggesting that the cells were polarized (24). In the two cell lines that expressed G protein, the protein was only detectable by immunofluorescent labeling when the antigen was made accessible to antibodies either by permeabilization with detergent (Fig. 4*B*) or by treatment

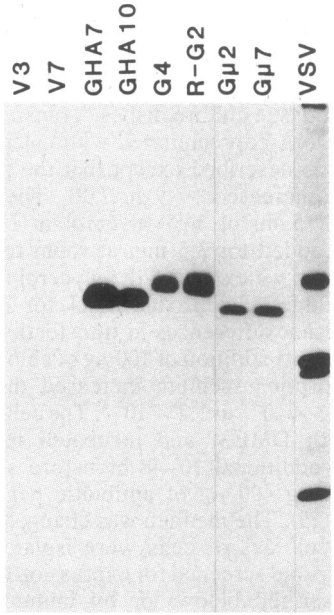

FIG. 3. Relative electrophoretic mobilities of G and G-related proteins. G418-resistant MDCK cell lines were derived from transfections with vectors encoding the G protein (G4, R-G2), GHA protein (GHA7, GHA10), or Gμ protein (Gμ2, Gμ7) or from transfections with vector alone (V3, V7). A 35-mm tissue culture dish of each cell line was labeled with 30 μCi (1 Ci = 37 GBq) of [^{35}S]methionine for 15 min after prior induction with 10 mM sodium butyrate for 16 hr. Proteins were immunoprecipitated from cell lysates and subjected to NaDodSO$_4$/PAGE. The lane labeled VSV contains the viral proteins from [^{35}S]methionine-labeled VSV-infected MDCK cells.

with 2 mM EGTA (Fig. 4*C*). The labeling pattern observed was typical of the basolateral pattern of G protein seen in VSV-infected cells or in cells expressing the G protein from cloned cDNA (2, 5, 11, 25). Although the majority of the cells showed the typical basolateral pattern of G protein expression, in some experiments we also observed a small fraction of the cells with G protein on the apical surface, suggesting that these cells were not polarized.

In nine cell lines expressing sufficient quantities of GHA protein for detection by immunofluorescence, we observed a strikingly different pattern of localization compared to G protein. The GHA protein was readily detectable without prior permeabilization or EGTA treatment of the cells. The typical patterns observed for nonpermeabilized or permeabilized cells are shown in Fig. 4 *D* and *E*. A punctate labeling pattern of the GHA protein was observed on the apical surface in both cases and presumably corresponded to GHA protein in microvilli. Even when these cells were left confluent for >3 days, they did not develop the basolateral distribution seen for G protein.

Although the GHA protein was clearly present on the apical surface, preliminary antibody-binding experiments performed on filter-grown cells suggested that GHA might also be present on basolateral membranes. Basolateral labeling of this protein might have been obscured by the intense apical labeling in the immunofluorescence experiments. To test this possibility, GHA protein on the apical surface was bound to an excess of guinea pig anti-VSV serum. The cells were then permeabilized with detergent and incubated with rabbit anti-VSV and fluorescein-conjugated anti-rabbit antibodies. As shown in Fig. 4*F*, the GHA protein was detected on the basolateral plasma membrane when the apical labeling of GHA on the apical surface was blocked.

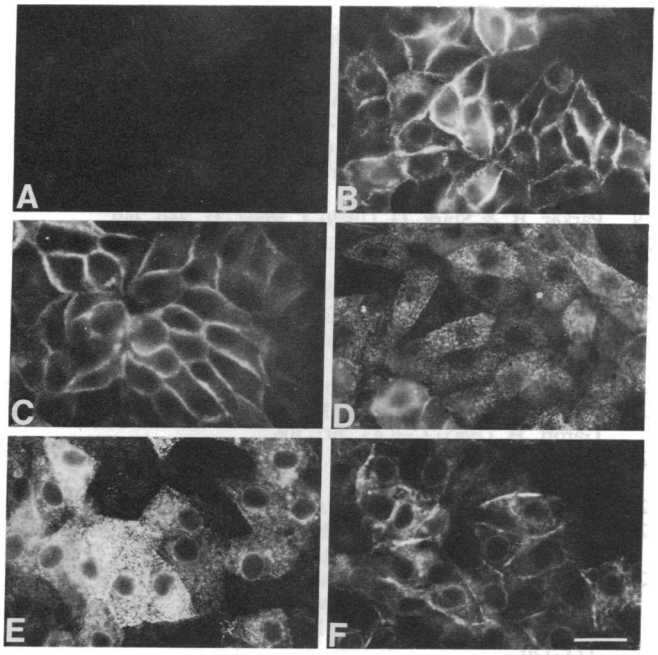

FIG. 4. Immunofluorescence microscopy. MDCK cell lines V3 (*A*), R-G2 (*B*), G4 (*C*), and GHA7 (*D–F*) were cultured on glass coverslips for 2 days after reaching confluence and were induced with sodium butyrate prior to preparation for immunofluorescence microscopy. Cells were fixed with paraformaldehyde prior to permeabilization with detergent (*A*, *B*, and *E*) or were fixed but not permeabilized (*D*). G4 cells (*C*) were incubated in 2 mM EGTA for 2 min to open tight junctions prior to fixation but were not permeabilized with detergent. Cells were labeled with a monoclonal antibody directed against a determinant in the extracellular domain of G protein (I1; ref. 23); this was followed by incubation in a 1:300 dilution of affinity-purified rhodamine-conjugated rabbit anti-mouse IgG. GHA cells in *F* were fixed and incubated with an excess guinea pig anti-VSV antibody (1:50 dilution) to block subsequent binding of antibodies to GHA. Cells were then permeabilized with detergent and labeled with rabbit anti-VSV and an affinity-purified fluorescence isothiocyanate-conjugated goat anti-rabbit IgG. The secondary antibody was preabsorbed on a column containing mouse immunoglobulins. Photographic exposures for the control (*A*) and experimental (*B*) cells were identical. (Bar = 10 μm.)

A possible explanation for the altered surface expression of GHA compared to G protein was that the cell lines expressing the GHA protein were not polarized. This explanation was ruled out in the following experiments. We examined the localization of normal G protein in the GHA line after infection of the cells with VSV (Fig. 5*A*). The viral-encoded G protein was present in a basolateral pattern, indicating that the GHA lines were polarized. It was possible to do this experiment in GHA10 cells that were not induced with sodium butyrate because they do not express levels of the GHA protein detectable by immunofluorescence microscopy or ELISA. To ensure that the butyrate-induced GHA cells were also polarized, we localized the Na^+,K^+-ATPase, a cellular protein present in the basolateral membranes of MDCK cells (26). As shown in Fig. 5*B*, the butyrate-induced cells expressing the GHA protein maintained a normal polarized (basolateral) pattern of Na^+,K^+-ATPase.

Several MDCK cell lines expressing the Gμ protein were also examined by indirect immunofluorescence. Although the ELISAs performed on permeabilized cells indicated that the levels of Gμ protein were comparable to those observed in cells expressing G or GHA proteins, we were unable to detect the Gμ protein in either an apical or basolateral pattern. Instead, the distribution suggested that the majority of Gμ protein was localized primarily in intracellular membranes of MDCK cells (data not shown).

DISCUSSION

The mechanisms that govern the transport of specific proteins to the apical or basolateral plasma membrane domains of polarized epithelial cells are not understood in molecular detail. To approach the question of which domains of the proteins might be recognized during the sorting process, we compared the localization of the VSV G protein to an altered G protein (GHA) with its entire cytoplasmic domain replaced by the cytoplasmic domain from an influenza virus HA protein. The G protein is normally localized on the basolateral membrane of MDCK cells, whereas the HA protein is

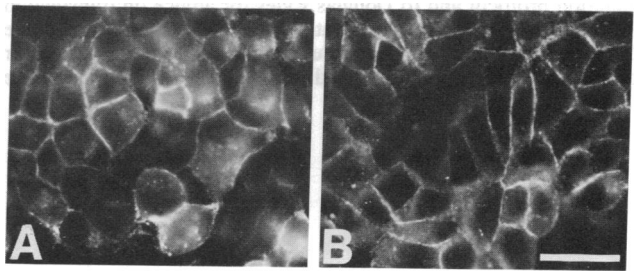

FIG. 5. Polarized expression of G protein or Na^+,K^+-ATPase in GHA-expressing cells. (*A*) GHA10 cells were infected with VSV for 1 hr at 37°C and then were shifted to 30°C for 3.5 hr. The cells were fixed, permeabilized, and labeled with I1 monoclonal antibody and rhodamine-conjugated anti-mouse IgG as described in the legend of Fig. 4. (*B*) GHA7 cells were induced with sodium butyrate for 16 hr; this was followed by fixation and permeabilization with detergent. Cells were then incubated with a 1:100 dilution of rabbit anti Na^+,K^+-ATPase serum provided by Jack Kyte (University of California, San Diego); this was followed by incubation in a 1:50 dilution of the fluorescein isothiocyanate-conjugated goat anti-rabbit antibody described in the legend of Fig. 4. (Bar = 10 μm.)

normally localized on the apical membrane. This has been demonstrated in virus-infected cells and in transient expression systems in which cDNA clones were expressed in polarized cells (2, 4–6, 11, 25).

In this study we constructed a vector suitable for deriving stable MDCK cell lines expressing G protein or altered G proteins from cDNA. In addition, we developed methods for obtaining cell lines expressing levels of protein high enough for analysis of their cell-surface localization by immunofluorescence microscopy. Our analyses showed that the VSV G protein was found predominantly on the basolateral plasma membrane, whereas the GHA protein was found on the apical and basolateral plasma membranes. MDCK lines expressing G protein with the cytoplasmic domain of an IgM (μ_m) molecule were also examined, but this protein was not detected on either cell surface. Gμ protein is known to be transported inefficiently to the surface of nonpolarized COS-1 cells (13); thus the absence of detectable surface labeling of this protein might be a consequence of this slow transport and the more rapid turnover of proteins expressed from transfected genes in MDCK cells compared to COS-1 cells (unpublished observations).

GHA protein either has lost its signal for sorting to the basolateral membrane or has gained a new signal that allows sorting of a fraction of the protein to the apical membrane. It was reported recently that a form of the HA protein lacking the transmembrane and cytoplasmic domains is secreted primarily from the apical membrane of polarized cells (27). Thus, the cytoplasmic domain of HA does not appear to be essential for HA transport to the apical surface. This result suggests that signals for sorting to the apical membrane of polarized cells could reside in the extracellular domain of the HA molecule alone. If we assume that the cytoplasmic domain of HA is not an apical sorting signal, then it seems likely that the altered sorting of the GHA protein is a consequence of the loss of the wild-type cytoplasmic domain from G protein. This normal cytoplasmic domain might therefore contain a positive sorting signal responsible for the localization of G protein in the basolateral membrane. Alternative models could invoke effects of the HA cytoplasmic domain on the conformation of the transmembrane or extracellular domains of the G protein. Proof that the cytoplasmic domain of G protein is acting as an independent element signaling basolateral transport might be obtained by demonstrating that it can direct nonsegregated secretory proteins specifically to the basolateral membrane of MDCK cell lines.

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