Correctly Sorted Molecules of a GPI-anchored Protein Are Clustered and Immobile When They Arrive at the Apical Surface of MDCK Cells

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Abstract. Glycosyl-phosphatidylinositol (GPI)-anchored proteins are sorted to the apical surface of many epithelial cell types. To better understand the mechanism for apical segregation of these proteins, we analyzed the lateral mobility and molecular associations of a model GPI-anchored protein, herpes simplex virus gD1 fused to human decay accelerating factor (gD1-DAF) (Lisanti, M. P., I. W. Caras, M. A. Davitz, and E. Rodriguez-Boulan. 1989. *J. Cell Biol.* 109:2145–2156) shortly after arrival and after long-term residence at the surface of confluent, polarized MDCK cells. FRAP measurements of lateral diffusion showed that the mobile fraction of newly arrived gD1-DAF molecules was much less than the mobile fraction of long-term resident molecules (40 vs. 80–90%). Fluorescence resonance energy transfer measurements showed that the newly arrived molecules were clustered, while resident molecules were not. Newly delivered gD1-DAF molecules were clustered but not immobilized in mutant, Concanavalin A–resistant MDCK cells that failed to sort gD1-DAF.

Our results indicate that GPI-anchored proteins in MDCK cells are clustered before delivery to the surface. However, clustering alone does not target molecules for apical delivery. The immobilization observed when gD1-DAF is correctly sorted suggests that the clusters must associate some component of the cell's cytoplasm.

THE segregation of proteins and lipids into polarized surface domains is key to the vectorial function of epithelial cells (Rodriguez-Boulan and Nelson, 1989; Simons and Fuller, 1985). Apical and basolateral proteins are sorted at two sites, the *trans*-Golgi network (TGN)[1] or the basolateral endosome, depending upon the protein and the epithelial cell type (Mostov et al., 1992; Rodriguez-Boulan and Powell, 1992). Sorting of apical and basolateral proteins in the TGN is thought to involve their incorporation into distinct sets of transport vesicles. Discrete cytoplasmic determinants which may or may not overlap with endocytic signals may direct basolateral proteins into basolateral vesicles, presumably through interaction with adaptin-like molecules (Casanova et al., 1991*a*; Brewer and Roth, 1991; Hunziker et al., 1991; Le Bivic et al., 1991).

Only one apical sorting signal has been identified, glycosyl-phosphatidylinositol (GPI). GPI-anchored proteins are polarized to the apical surface of MDCK cells (Lisanti et al., 1988), and other epithelial cell lines (Lisanti et al., 1990). Addition of GPI to the ectodomain of two basolateral proteins, vesicular stomatitis virus G protein and herpes simplex virus gD1 protein, redirects these proteins to the apical surface by altering their sorting at the level of the TGN (Brown et al., 1989; Lisanti et al., 1989).

GPI-anchored proteins are contained solely in the luminal leaflet of the TGN and transport vesicles; hence, they cannot interact directly with the vesicle-transporting machinery in the cytoplasm. Therefore, the sorting mechanism for these proteins is expected to involve luminal-side interactions with molecules of transport vesicles. Since glycosphingolipids tend to self-associate in model systems (Rock et al., 1990; Masserini et al., 1989; Pascher, 1976), it has been proposed that clustering of glycosphingolipids and of GPI-anchored proteins in the luminal leaflet of the TGN could partition these molecules for targeted delivery to the cell surface (Simons and van Meer, 1988; Lisanti and Rodriguez-Boulan, 1990). Sorting of molecular clusters of GPI-anchored proteins could still require transmembrane proteins, either trapped in the cluster, or sensing the aggregated state of membrane lipids; such transmembrane proteins could interact with cytoplasmic sorting machinery (Simons and Wandinger-Ness, 1990). Recent experiments showing changes in detergent solubility of some GPI-anchored proteins as they reach early Golgi compartments (Brown and Rose, 1992) support the hypothesis of GPI-anchored protein clustering. Furthermore, some GPI-anchored proteins have been shown to self-associate in a low pH- and ion-dependent manner (Fukuoka et al., 1992). Others form noncovalent complexes

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1. *Abbreviations used in this paper*: Con A^r, concanavalin A resistant; D_{lat}, lateral diffusion coefficient; DPPIV, dipeptidyl peptidase IV; FRET, fluorescence resonance energy transfer; gD1-DAF, herpes simplex virus gD1 fused to human decay accelerating factor; GPI, glycosyl-phosphatidyl inositol; HH, Hepes Hanks'; PIPLC, phosphatidylinositol-specific phospholipase C; R, mobile fraction; TGN, *trans*-Golgi network; TxR, Texas red.

with transmembrane proteins (Stefanova and Horejsi, 1991; Stefanova et al., 1991). However, the data do not provide any insight on whether clusters are related at all to apical sorting, or on possible mechanisms for sorting.

If GPI-anchored proteins are clustered in Golgi membranes and transport vesicles, then we expect to find molecules in close proximity to one another shortly after they are delivered to the cell surface. Diffusion of the clustered molecules is expected to be limited if the clusters contain transmembrane proteins which interact with sorting machinery in the cytoplasm. We have examined the lateral mobility and lateral organization of one apically sorted GPI-anchored protein, the Herpes simplex virus coat glycoprotein gD1 fused to the GPI attachment signal of human decay accelerating factor (gD1-DAF), expressed in polarized MDCK cells (Lisanti et al., 1989; Lisanti et al., 1990; Caras and Weddell, 1989) and in mutant MDCK cells which do not sort gD1-DAF.

Our results suggest that GPI-anchored proteins are clustered for packaging into transport vesicles. Sorting correlates with immobilization; this suggests recognition of the cluster by a sorting molecule, and association with fully polarized cytoplasmic sorting machinery.

Materials and Methods

IgG Digestion and Fab Conjugation

Rabbit polyclonal antibodies raised against gD1 were obtained from Dako Corp., Carpinteria, CA (Showalter et al., 1981). Mouse monoclonal antibodies to gp135 (Ojakian and Schwimmer, 1988) were the generous gift of Dr. G. Ojakian. MDCK cells expressing dipeptidyl peptidase IV (DPPIV) and rabbit polyclonal antibodies to DPPIV were the generous gift of Dr. A. Hubbard. Fab fragments from IgG molecules were prepared by papain digestion as described elsewhere (Porter, 1959). The Fab were purified on Sephadex G-100. Fluorescein conjugates of Fab molecules were prepared by 24-h incubation of the protein with a 50-fold molar excess of FITC at 4°C in 0.5 M sodium bicarbonate buffer, pH 9.5 (Wood et al., 1965). Texas red (TxR) conjugates were prepared similarly, except using a 90-fold molar excess TxR. Both fluorophores were obtained from Molecular Probes, Inc., (Eugene, OR). Conjugates were purified by passage over Sephadex G-50 obtained from Sigma Chemical Co. (St. Louis, MO), with the added purification of TxR conjugates by five successive passages over SM-2 beads (Bio-Rad Laboratories, Richmond, CA) (Spack et al., 1986). Dye/protein ratios were 1.4 for FITC-Fab and 3.7 for TxR-Fab.

Cell Culture

MDCK (type II) cells expressing gD1-DAF or rat DPPIV (obtained from Dr. A. Hubbard) were maintained in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Reheis Division of Armour, Phoenix, AZ) and Gibco nonessential amino acids at 37°C and 5% CO_2. Cells were grown on nitric acid-cleaned coverslips. 0.1 ml of cells was plated at a density of 10^5 cells/ml, grown to confluence, and in 10 mM sodium butyrate overnight, which increases transcriptional levels of gD1-DAF and DPPIV.

Unpolarized cells were plated at a low density (0.1 ml at a density of 10^3 cells/ml) and grown overnight in 10 mM sodium butyrate.

All buffers in which the cells were incubated (0.01 M Hepes, pH 7.35, and 0.1 M MES, pH 6.0) were supplemented Hanks' salts (100 mM NaCl, 5 mM KCl, 2 mM Na_2HPO_4, 4 μM $MgSO_4$, 1 mg/ml glucose, 12.5 mM $CaCl_2$). Hepes-Hanks' is hereafter referred to as HH.

Expression of gD1-DAF in Wild-type and Concanavalin A-resistant (Con A^r) MDCK Cells

Wild-type and Con A^r MDCK cells were cotransfected with cDNA coding for the ectodomain of the Herpes simplex virus glycoprotein gD1 linked to the 37 COOH-terminal amino acids of DAF (coding for GPI linkage) and the selectable marker pmV6(neo). The gD1-DAF cDNA was inserted in a nonselectable plasmid between an RSV promoter and an SV-40 polyadenylation signal (Caras et al., 1987). Clones expressing gD1-DAF were selected with 1 mg/ml G418.

Cell Labeling

Fab at 0.1 mg/ml was ultracentrifuged at 100,000 *g* for 1 h to remove any insoluble aggregates (Bierer et al., 1987). 25 μl of conjugate was applied to cells on a coverslip and incubated for 30 min at 4°C. When double labeling, equimolar concentrations of Fab conjugates or the FITC-Fab and unconjugated Fab were mixed after ultracentrifugation and used as described above. The cells were rinsed at least five times over a 20-min period at 4°C to remove any nonspecifically bound antibody.

Treatment of Cells with Phosphatidylinositol-specific Phospholipase C (PIPLC)

0.5 U (10 U/ml) of *Bacillus thurigiensis* PIPLC (Low and Saltiel, 1988; Ferguson and Williams, 1988), the generous gift of Dr. R. Pagano, was applied to each coverslip and incubated for 1 h at 20°C. Enzyme-treated cells did not label with FITC-Fab anti-gD1-DAF (data not shown). The cells were rinsed at least three times with HH, pH 7.35, and incubated at 37°C for 30 min. The cells were then labeled as described above. FRAP or fluorescence resonance energy transfer (FRET) measurements were taken immediately after staining, or following incubation at 37°C for another 30–90 min.

Changing Extracellular pH

Confluent monolayers of cells were incubated for 15 min at 37°C in HH, pH 7.35, or MES Hanks', pH 6.0. The cells were rinsed with HH, pH 7.35, immediately before labeling. The effect was not reversed for several hours; this allowed us to stain and perform FRAP and FRET measurements at pH 7.35.

Changing Intracellular pH by Treatment with Chloroquine

Confluent monolayers of cells were incubated in 10 mM chloroquine for 1 h at 37°C (Caplan et al., 1987). Alternatively, PIPLC was added to the same buffer to remove GPI-linked proteins at 20°C for 1 h. The cells were then rinsed with HH, pH 7.35, and incubated for 1 h at 37°C. Labeling, washing, and FPR or FRET measurements were performed as described below.

Measurement of Lateral Mobility by FRAP

Lateral mobility of membrane proteins was measured by FRAP (for review see Jacobson et al., 1983; Wolf, 1989). Cells are stained with a fluorophore-conjugated monovalent antibody (Fab). A small spot is bleached on the cell surface by focusing an intense laser beam on the cell, thus irreversibly bleaching a fraction of the fluorophores. The recovery of fluorescence results from the diffusion of fluorescent molecules into the spot, and is observed by a low intensity monitoring beam. Microvilli in the cells do not affect the measure of lateral mobility by FRAP (Wolf et al., 1982).

The general design of our apparatus has been described (Wolf, 1989). We used a bleach spot of 0.6 μm radius and power of densities of ~0.5 kW/cm^2 (probe) and 1 MW/cm^2 (bleach). Probe measurements were made four times a second. All measurements were performed at 37°C. Data were collected and analyzed on an IBM PC using customized software. Lateral diffusion coefficients and mobile fractions are calculated according to methods described elsewhere (Axelrod et al., 1976).

FRET Measurements

An excited-state fluorophore may lose energy and return to the ground state in four ways: (1) various processes which involve nonradiative loss of energy, (2) emission of a photon, (3) resonance transfer of energy to an acceptor molecule, or (4) chemical reaction while in the excited state. The third process is detected as quenching of donor fluorescence and enhancement of acceptor fluorescence. This process requires that the fluorescence donor and acceptor be separated by no more than 10's of Å. The fourth process, chemical reaction, is seen as bleaching of fluorescence with time. Energy transfer to an acceptor (process 3) lowers the net rate of excited-state chemical reactions (process 4). FRET can be detected in terms of a decreased rate of photochemically induced fading of the fluorescent donor

when the only additional decay process is by FRET (Jovin and Arndt-Jovin, 1989; Kubitscheck et al., 1991). This is readily measured in the microscope.

We used a modification of our FRAP apparatus for these measurements. The laser beam was expanded to ~40 μm in diameter. The fluorescence intensity was monitored continuously. The laser power density was ~1 KW/cm^2 and did not differ between samples. 15 areas, each covering ~four cells, were measured for each treatment and labeling pair. The fluorescence intensity was normalized for each measurement.

Results

We probed the organization of gDl-DAF in the apical membrane of MDCK cells using two biophysical techniques: FRAP and FRET. FRAP measures the lateral mobility of fluorescently labeled molecules in terms of a lateral diffusion coefficient (D_{lat}) and a mobile fraction (R) (Axelrod et al., 1976). D_{lat} is derived from the rate at which the intensity of fluorescence recovers after photobleaching. R is the fraction of the labeled molecules free to diffuse in the time scale of our measurement (minutes). R is derived from the extent of FRAP.

At 37°C D_{lat} of the resident (steady-state population) gDl-DAF molecules was typical of that measured for many GPI-anchored and transmembrane proteins, 2–4 × 10^{-10} cm^2 s^{-1} (Edidin, 1991; Zhang et al., 1991). R of resident gDl-DAF was high, 80–90% (Fig. 1 *A*).

We removed resident gDl-DAF molecules from the surface by treating the cells with PIPLC under conditions (20°C) which block delivery of transport vesicles containing newly synthesized gDl-DAF to the surface (Griffiths et al., 1989; Matlin and Simons, 1983). Cells treated with PIPLC (Lisanti et al., 1989; Low and Saltiel, 1988) for 1 h at 20°C were not labeled by FITC-Fab anti-gDl. gDl-DAF reappeared on the surface when the enzyme was washed away and the cells were shifted to 37°C (Fig. 1 *B*). After a 15-min recovery the cells were not detectably labeled by FITC-Fab, but by 30 min the fluorescence intensity of the surface gDl-DAF population was one fourth that of the steady-state population. While D_{lat} of this newly delivered population of gDl-DAF molecules was the same as that measured for resident molecules, R was significantly lower than that of the resident population (40%). Similar results were obtained for unpolarized cells (data not shown).

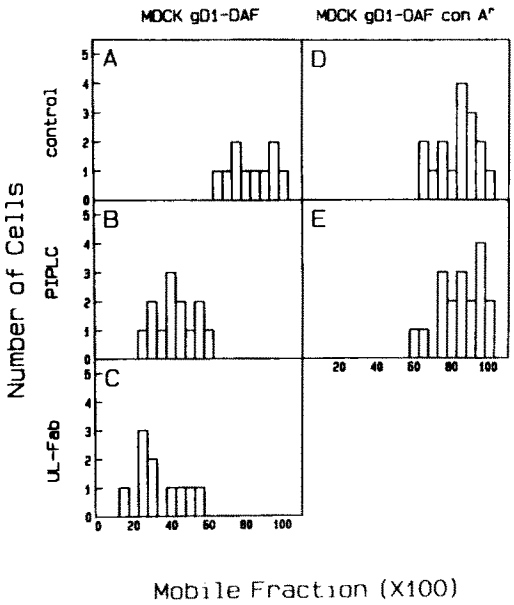

Figure 1. Mobility of gDl-DAF upon delivery to the surface of wild-type and Con A^r MDCK cells. Wild-type or Con A^r MDCK cells were grown to confluence on glass coverslips to be used for FRAP experiments. *A* depicts the mobile fraction for the steady-state population of gDl-DAF in wild-type cells. *B* depicts the mobile fraction of a newly delivered population of gDl-DAF isolated by PIPLC digestion at 20°C for 1 h, and followed by a recovery period of 30 min at 37°C. *C* demonstrates isolation of newly delivered protein by masking the steady-state population with unlabeled Fab at 20°C for 1 h, followed by a 30-min recovery period at 37°C. The mobile fraction of the steady-state population of gDl-DAF in Con A^r cells is depicted in *D*, and enzymatically isolated newly delivered gDl-DAF in *E*.

As a control for nonspecific effects of PIPLC treatment we compared D_{lat} and R of two apically expressed transmembrane proteins, endogenous gp135 (Ojakian and Schwimmer, 1988) and a rat hepatocyte protein DPPIV expressed in MDCK cells (Casanova et al., 1991*b*). These cells were treated with PIPLC using the protocol previously described. There was no effect of enzyme treatment on labeling, D_{lat} (3 × 10^{-10} for DPPIV and 1 × 10^{-9} for gp135) or R (80 and 43% for DPPIV and gp135, respectively) of these proteins.

Newly synthesized gDl-DAF could also be selectively labeled without using PIPLC. This was done by masking resident molecules with unlabeled Fab at 20°C for 1 h, shifting these cells to 37°C for 1 h, and then labeling with FITC-Fab. After this treatment, the R of labeled molecules was as low as that of molecules labeled after PIPLC treatment and regeneration of the surface gDl-DAF (Fig. 1 *C*).

The R of newly arrived molecules of gDl-DAF, labeled with FITC-Fab reached ~80% of control values within 2 h after PIPLC treatment, and did not increase beyond that when incubated for an additional 6 h (data not shown).

If immobilization is related to sorting, then we expect to see no change in R in cells which do not sort gDl-DAF properly. A Con A^r mutant MDCK cell line (Meiss et al., 1982) does not sort some endogenous GPI-anchored proteins (Lisanti et al., 1988). gDl-DAF is also not sorted efficiently in this cell line; approximately half of the total gDl-DAF is found in the basolateral domain and half in the apical domain (Fig. 2). Basolaterally sorted pool of gDl-DAF in both mutant and wild-type cells reproducibly migrates faster in SDS-PAGE, the reason for this is not yet clear. The R of resident gDl-DAF is as high in these cells as it is in wild-type cells. In contrast to the wild-type cells, the R of the newly delivered population of gDl-DAF (isolated by PIPLC treatment) is also high (Fig. 1, *D* and *E*).

The FRAP microscope was used as a microfluorimeter to directly examine the extent of self association, or clustering, of molecules of gDl-DAF in terms of FRET. As discussed in Materials and Methods, FRET can be detected in terms of a decreased rate of photochemically induced fading of the fluorescent donor when acceptor is present (Jovin and Arndt-Jovin, 1989; Kubitscheck et al., 1991). Excited state molecules can lose energy in many ways. The conditions we used compare decay from the excited state in the presence or the absence of the acceptor. Hence, any change in the photobleaching rate is the result of FRET. Energy transfer can only occur if the donor and acceptor molecules are sufficiently close, in this case 80 Å (Matko et al., 1992).

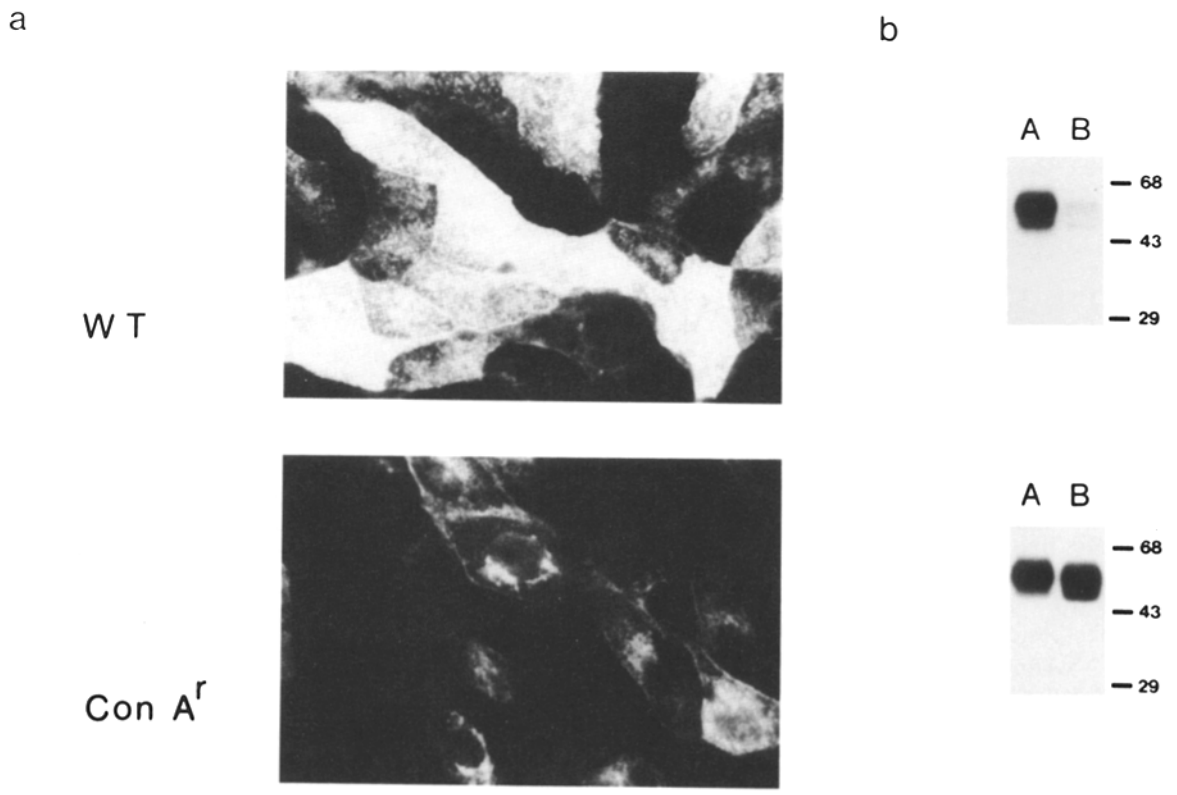

Figure 2. Expression of gD1-DAF in wild-type and Con A^r MDCK cells. Two clones of MDCK cells and Con A^r MDCK cells expressing gD1-DAF shown after treatment with 10 mM sodium butyrate. (*a*) Immunofluorescent detection of gD1-DAF in digitonin-permeabilized cells. Note the predominantly apical staining of wild-type cells, and the intracellular (Golgi-like staining) and lateral staining of the Con A^r cells. (*b*) Biotin polarity assay as described elsewhere (Lisanti et al., 1990). Monolayers of wild-type and Con A^r cells were grown to confluence on polycarbonate filters and biotinylated from the apical or from the basolateral side, extracted, and immunoprecipitated with gD1 antibodies. Biotinylated gD1-DAF was detected by SDS-PAGE and blotting with ^{125}I-streptavidin. Note the polarized apical distribution of gD1-DAF in wild-type cells and the unpolarized distribution in Con A^r MDCK cells.

FRET measurements of cells labeled with mixtures of FITC-Fab (donor), TxR-Fab, the fluorescent acceptor, and unlabeled Fab gave no evidence that resident populations of gD1-DAF molecules were clustered. The rate of photobleaching of donor-acceptor labeled cells was the same as donor-unlabeled labeled cells (Fig. 3 *A*). However, the newly delivered population of gD1-DAF did contain molecular clusters (Fig. 4 *B*). This is demonstrated by a decreased extent and rate of fading of donor fluorescence in the presence of TxR-Fab compared with that observed in its absence. The clusters disperse within 1 h (Fig. 3 *C*). gD1-DAF was similarly clustered on the apical surface of unpolarized MDCK cells (data not shown) and in Con A^r MDCK cells (Fig. 3, *D–F*). The fluorophore also seems to be in a different environment in Con A^r cells than in the wild-type cells since the fading of the donor-only sample is much higher than in the wild type. Exactly how the environment is different is unclear. Photobleaching is highly dependent on such factors as local oxygen concentration, pH, and the dielectric constant of the medium.

It has been proposed that sorting of apical GPI-anchored molecules occurs in an acidic compartment of the Golgi or TGN (Simons and van Meer, 1988; Simons and Wandinger-Ness, 1990; Lisanti and Rodriguez-Boulan, 1990). If this is so, and if acidity is important for clustering or immobilization, then we expect that chloroquine treatment of cells, which raises the pH of the Golgi compartments, will influence the mobility and/or organization of newly synthesized gD1-DAF. Almost all (90%) newly delivered gD1-DAF was mobile after chloroquine treatment, with no change in the diffusion coefficient. However, FRET measurement showed that the mobile molecules were still clustered (data not shown).

We attempted to mimic the environment of the TGN by incubating intact MDCK cells in pH 6.0 buffer (Griffiths and Simons, 1986). This treatment reduced R with no change in D_{lat} of gD1-DAF on wild-type cells, but had no effect in the mutant cell line (Fig. 4). gD1-DAF immobilized by brief treatment with pH 6.0 buffer remained immobile when cells were returned to pH 7.35 for 1 h (data not shown). We could not make interpretable FRET measurements on cells exposed to pH 6.0 buffer. Fluorescence on these cells consistently faded more rapidly in the presence of an acceptor than in the absence. Apparently the environment of the donor and acceptor has been changed by prior treatment with pH 6.0 buffer in such a way as to decrease FRET.

Discussion

Our experiments bear on models for the sorting of glycosphingolipids and GPI proteins in polarized epithelial cells (Simons and van Meer, 1988; Lisanti and Rodriguez-Boulan, 1990). According to these models, clusters interact indirectly, via an "apical sorting receptor," a putative trans-

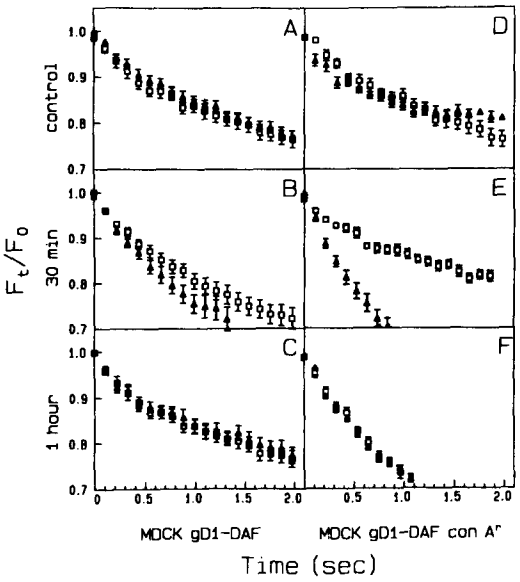

Figure 3. Clustering of gD1-DAF upon delivery to the surface of wild-type and Con A^r cells. Confluent monolayers of wild-type (*A–C*) or Con A^r (*D–F*) MDCK cells both expressing gD1-DAF were treated with PIPLC as described previously, and stained after 30 min at 37°C. FRET measurements were performed either immediately (*B* and *E*) or after an additional 30-min incubation at 37°C (*C* and *F*). These curves represent the average normalized fluorescent intensities and standard errors of 15 different regions of the monolayer. (▲) Cells labeled with FITC-Fab and unconjugated-Fab; (□) FITC-Fab– and TxR-Fab–labeled cells. Differences in the rate and extent of photobleaching can be noted. *A* and *D* represent untreated cells.

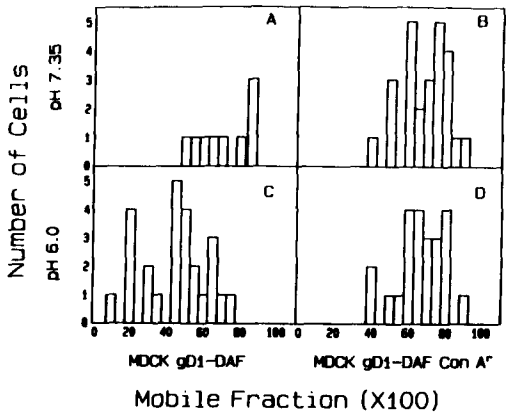

Figure 4. Mobile fraction of gD1-DAF after exposure to pH 6.0 in wild-type and Con A^r cells. Cell lines expressing gD1-DAF were grown to confluence, and treated with buffer at pH 7.35 (*A* and *B* for wild-type and Con A^r cell lines, respectively) or pH 6.0 (*C* and *D* for wild-type and Con A^r cell lines, respectively) for 15 min at 37°C. The cells were washed with pH 7.35 buffer and stained for gD1-DAF. Mobile fraction was obtained by FRAP measurements performed at 37°C.

membrane protein that recognizes sorting signals in the vesicle lumen (Simons and Wandinger-Ness, 1990). The models are supported by very recent results of Brown and Rose (1992) showing that GPI-anchored proteins become detergent insoluble as they undergo posttranslational modifications compatible with arrival into the early Golgi complex, and can be purified as an insoluble complex with glycosphingolipids. However, those experiments are performed on cell extracts and may be subject to numerous artifacts associated with detergent extraction.

Our experiments were performed on intact cells. Two distinct populations of gD1-DAF can be differentiated on the cell surfaces by exploiting the fact that the protein can be released from the diacylglycerol anchor by PIPLC digestion. One population consists of gD1-DAF molecules which have resided at the surface for more than a few hours. The second population of gD1-DAF molecules consists of molecules which have recently arrived at the cell surface. We could not detect resonance energy transfer between individual gD1-DAF molecules resident at the cell surface. By this criterion, the molecules are not clustered.

Newly arrived gD1-DAF molecules are clustered in wild-type polarized, wild-type nonpolarized, and polarized Con A^r cells. This indicates that clustering alone is not sufficient for apical targeting. The resident gD1-DAF monomeric molecules are all free to diffuse in the plane of the apical membrane as indicated by a nearly 100% R in FRAP measurements.

In contrast, more than half the population of newly delivered gD1-DAF is immobile on a time scale of minutes; i.e., $D_{lat} < 10^{-12}$ cm^2 sec^{-1}. This effect is not likely to be due to the size of the clusters alone. D_{lat} is proportional to ln (r) where r is the radius of the diffusing species (Saffman and Delbruck, 1975; Hughes et al., 1982). Hence, clusters would have to be of macroscopic size (mm) to be immobilized by size alone.

Sparse MDCK cells cluster gD1-DAF, and the molecules are immobile when delivered to the surface. Apically sorted molecules are always delivered to the free surface of the cells, even though cells are not fully polarized. Thus, apical targeting and immobilization appear to go together.

Newly delivered gD1-DAF clusters are fully mobile in Con A^r MDCK cells, cells which do not sort gD1-DAF to the apical surface. Hence, clustering by itself is not sufficient for apical sorting. Immobilization appears to be an essential correlate of proper sorting. The same distinction between clustering and immobilization is also observed for chloroquine-treated cells. Hence, the correct assembly of gD1-DAF clusters with a potential sorter molecule may require an acid environment.

The nature of the Con A^r mutation is at the moment unknown. Whatever the defect, it would appear that it affects a key sorting function in the TGN. It is unclear whether this altered function is a consequence of alterations in a passenger protein associated with the cluster of gD1-DAF, or in the sorter itself. This may be the direct consequence of altered glycosylation or an indirect effect of glycosylation on the folding of the molecule (Neefjes et al., 1988; Gallagher et al., 1988; Green et al., 1981).

The data presented in this paper suggest that delivery of GPI-anchored proteins to the surface is a two-step process. The first generates clusters of GPI-anchored proteins, probably including transmembrane proteins. The second step, which is fundamental to proper sorting, involves recognition of the cluster by a transmembrane protein that mediates interaction with the sorting machinery. This second interaction may depend on low pH (as indicated by its block in the presence of chloroquine, and induction on the cell surface in acidic conditions) suggesting that it takes place in the acidic TGN (Anderson and Pathak, 1985).

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