

Glycosylphosphatidylinositol-Anchored Proteins Are Preferentially Targeted to the Basolateral Surface in Fischer Rat Thyroid Epithelial Cells

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Abstract. Glycosylphosphatidylinositol (GPI) acts as an apical targeting signal in MDCK cells and other kidney and intestinal cell lines. In striking contrast with these model polarized cell lines, we show here that Fischer rat thyroid (FRT) epithelial cells do not display a preferential apical distribution of GPI-anchored proteins. Six out of nine detectable endogenous GPI-anchored proteins were localized on the basolateral surface, whereas two others were apical and one was not polarized. Transfection of several model GPI proteins, previously shown to be apically targeted in MDCK cells, also led to unexpected results. While the ectodomain of decay accelerating factor (DAF) was apically secreted, 50% of the native,

GPI-anchored form, of this protein was basolateral. Addition of a GPI anchor to the ectodomain of Herpes simplex gD-1, secreted without polarity, led to basolateral localization of the fusion protein, gD1-DAF. Targeting experiments demonstrated that gD1-DAF was delivered vectorially from the Golgi apparatus to the basolateral surface. These results indicate that FRT cells have fundamental differences with MDCK cells with regard to the mechanisms for sorting GPI-anchored proteins: GPI is not an apical signal but, rather, it behaves as a basolateral signal. The "mutant" behavior of FRT cells may provide clues to the nature of the mechanisms that sort GPI-anchored proteins in epithelial cells.

THE vectorial properties of epithelial cells derive from the polarized distribution of their plasma membrane proteins and lipids into characteristic apical and basolateral domains, separated by tight junctions (33, 38). It has become clear that this asymmetric molecular distribution depends on several mechanisms, such as intracellular sorting in the Golgi apparatus and vectorial surface delivery (21, 22, 30), domain-selective membrane recycling (8), and interaction with domain-specific membrane cytoskeletons (24, 25, 36). Two major biosynthetic pathways have been defined that carry epithelial plasma membrane proteins to their respective surface: a direct route from the TGN and transcytotic routes that bypass the Golgi apparatus (23, 32). Recent work has identified some protein and lipid signals that mediate intracellular sorting of proteins in the TGN and direct them along apical and basolateral routes (23, 34). Protein signals include poorly defined conformational features of the ectodomain of apical proteins (7, 35, 39) and distinct cytoplasmic determinants, related to coated pit internalization motifs, in several basolateral proteins (2, 5, 10, 12).

At present the best defined signal for apical delivery is a glycopospholipid, glycosylphosphatidylinositol (GPI),¹

the membrane anchor of a subset of plasma membrane proteins widely distributed in nature (13, 18). Although not exclusively found in epithelial cells, membrane anchoring via GPI has been correlated strictly with the apical localization of certain proteins in a variety of epithelial cells of diverse origin. These include cell lines such as the kidney derived MDCK I and II, LLC-PK1, the human intestinal carcinoma lines Caco-2 and SK-CO-15 and native epithelia such as hepatocytes (14, 15, 17). Not only endogenous GPI proteins are apical but also exogenous ones, expressed from their transfected cDNAs (4, 15, 19, 29, 44). Furthermore, recombinant DNA replacement of transmembrane and cytoplasmic domains of basolaterally targeted viral glycoproteins by GPI resulted in their rerouting to the apical surface (4, 15). In agreement with these results, transfection into MDCK cells of GPI- and transmembrane-anchored isoforms of the neural cell adhesion molecule (N-CAM) results in apical targeting of the former and basolateral segregation of the latter (28).

Recent work has demonstrated that epithelial cells display a striking variability regarding the polarized distribution of plasma membrane proteins and the intracellular sorting of these molecules en route to the cell surface (9, 23, 34). The Fischer rat thyroid (FRT) cell line is highly polarized and accurately sorts several exogenous and endogenous plasma membrane proteins (transferrin receptor, dipeptidylpeptidase IV, E-cadherin, Na,K-ATPase, influenza hemagglu-

1. *Abbreviations used in this paper:* DAF, decay accelerating factor; FRT, Fischer rat thyroid; GPI, glycosylphosphatidylinositol; HSV, Herpes simplex virus; met, methionine; PIPLC, PI-specific phospholipase C.

tinin, Vesicular Stomatitis Virus G protein) to the same domains as MDCK cells, using similar targeting pathways (26, 46–48). We report here that FRT cells differ fundamentally from MDCK and all other characterized epithelial cell lines in their handling of GPI-anchored proteins. Most of the endogenous GPI anchored proteins of this cell line are basolateral. Furthermore, two model GPI-anchored proteins that are apically localized when transfected in MDCK cells are basolateral or unpolarized in FRT cells. We conclude that GPI does not act as an apical sorting signal in FRT cells; rather, GPI acts as a basolateral signal or GPI-anchored proteins are transported basolaterally by default.

Materials and Methods

Reagents

Cell surface labeling reagents were from Pierce (Rockford, IL). PI-specific phospholipase C (PIPLC), purified from *Bacillus thuringiensis*, was the gift of Martin Low (Columbia University, New York) and contained no detectable protease activity. Rabbit antibodies (Ig fraction) against Herpes simplex virus (HSV) (used for immunoprecipitation) and gD-1 mAb (IgG_{2a}; used for immunofluorescence) were obtained as described (15).

Cells and Cell Culture

FRT cells were maintained in F12 Coon's modified medium supplemented with 5% FBS. MDCK cells, type II, were cultured in DME containing 10% of FBS. Cells from a single confluent 10-cm² flask were trypsinized and plated on six-well Transwell^R clusters (Costar Corp., Cambridge MA) at high density (~2,000,000 cells per filter). The intactness of the monolayer was monitored by measuring the transepithelial electrical resistance (TER).

Transfection and Selection of Clones

FRT cells were transfected as described previously (45), using a modification of the calcium phosphate precipitation procedure. Briefly, ~10 µg/dish of the nonselectable plasmid and 1 µg/dish of pMV6(neo) were added to subconfluent FRT cells growing in 10 cm dish in normal medium. After a 3-h incubation at 37°C, a 15% glycerol shock was applied; the cells were allowed to recover for 2–3 d, trypsinized, diluted 1:4, and plated in 10-cm dishes containing 500 µg/ml of G418. Resistant colonies were detected and collected after ~14 d.

Domain-selective Biotinylation

Filter-grown monolayers were washed (2×) with ice cold PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS-C/M) before addition of a biotin derivative (see below). After labeling, cell monolayers were washed with PBS-C/M (2×) and processed for immunoprecipitation.

Steady State. 1 ml of sulfo-NHS-LC-biotin (0.5 mg/ml in ice-cold PBS-C/M) was added to either the apical or the basolateral compartment; the other compartment received an equivalent volume of PBS-C/M (37). After 20 min at 4°C, the medium was removed and the labeling procedure repeated (2×). After labeling, excess reactive biotin was quenched by incubation with Hepes-buffered serum-free DME (10 min at 4°C).

Cell-surface Delivery. To follow the appearance of a newly synthesized GPI-anchored protein at the cell surface, we use a protocol previously described for MDCK cells (16). Briefly, cell surface free amino groups were quenched with 1 ml of sulfo-SHPP (0.5 mg/ml in ice-cold PBS-C/M) added to both the apical and the basolateral compartments. Approximately 90% of reactive cell surface amino groups are quenched after only a single labeling with sulfo-SHPP (40). After 15 min at 4°C, the solution was removed and the labeling procedure repeated (5×); residual reactive sulfo-SHPP was quenched after the final labeling by incubation with serum-free DME as described above for sulfo-NHS-biotin. The appearance of new free surface amino groups, after incubation at 37°C with pre-warmed normal medium, was monitored by transfer, after various times, to ice-cold PBS-C/M and domain selective labeling with sulfo-NHS-biotin (as described above).

To capture the first wave of newly synthesized transmembrane proteins arriving at the cell surface, 5–7-d-old filter-grown monolayers were labeled

for 1 h with [³⁵S]methionine/cysteine and then biotinylated with NHS-SS-Biotin on the apical or the basolateral membrane (48). The cells were extracted and biotinylated proteins were purified by successive precipitation with the appropriate antibody and streptavidin agarose and processed for SDS/PAGE and fluorography.

PIPLC Treatment

PIPLC treatment was performed as previously described (14). Briefly, after extraction and phase separation with TX-114, detergent phases enriched in membrane (hydrophobic) forms of GPI-anchored proteins were treated with PIPLC (6 U/ml). After PIPLC treatment and phase separation, resulting aqueous phases containing soluble (hydrophilic) forms of GPI-anchored proteins were purified by immunoprecipitation.

Metabolic Labeling

To measure the apical and basolateral secretion of secretory forms of HSV gD1 and DAF, confluent filter-grown FRT monolayers were incubated for 30 min with DME lacking methionine (met) and cysteine (cys) and then labeled overnight with ~100 µCi/ml of [³⁵S]met/cys. Apical and basal media were collected, centrifuged (14,000 g, 30 s) and supplemented with a cocktail of protease inhibitors (1). 200-µl aliquots were then diluted fivefold with TBS containing 1% Triton X-100 (TBST) and immunoprecipitated with either HSV or DAF IgGs, as described (15).

Detection of Biotinylated Proteins

After NaDodSO₄-PAGE (10% acrylamide) and transfer to nitrocellulose, biotinylated proteins were detected by blotting with ¹²⁵I-streptavidin (37). Autoradiographs were scanned using a Microtex scanner and analyzed with Image 1.41 software in a MacIntosh computer.

Fluorescence Microscopy

FRT cells expressing gD1, gD1-DAF, and DAF were trypsinized and transferred either to coverslips and grown for 2–3 d, or to polycarbonate filters for 4–5 d, washed with PBS, fixed and processed for immunofluorescence with gD-1 or DAF antibodies (15). Bound IgGs were visualized with FITC-conjugated secondary antibodies by using a Nikon fluorescence microscope coupled to a Molecular Dynamics Laser Scanning Confocal Microscope.

Results

Preferred Basolateral Distribution of Endogenous GPI-anchored Proteins

We analyzed the surface distribution of endogenous GPI-anchored proteins of FRT cells using a protocol developed earlier by Lisanti et al. (14) that combines domain-selective biotinylation with TX-114 phase separation and PIPLC treatment. Cells grown to confluency on filters for 5–7 d were labeled from either the apical or the basolateral side with sulfo NHS-LC-biotin. The proteins were then extracted in lysis buffer containing TX-114 and subjected to temperature-induced phase separation. The detergent phases obtained from either apically or basolaterally labeled filters were enriched in membrane forms of GPI-anchored proteins. They were subsequently treated with PIPLC, which released the protein moieties from their lipid anchors into the aqueous phase. Biotinylated proteins in this aqueous phase were resolved on SDS-PAGE, transferred to nitrocellulose, and then revealed by blotting with ¹²⁵I-streptavidin (Fig. 1). Comparison between the PIPLC-treated samples (+) and the untreated controls (–) surprisingly revealed 6 GPI-anchored proteins (58, 48, 45, 39, 25, and 16 kD) present almost exclusively in the basolateral membrane. A major GPI-protein (90 kD) was found to be unpolarized while two minor products (36 and 18 kD) were predominantly apical (Fig. 1). This

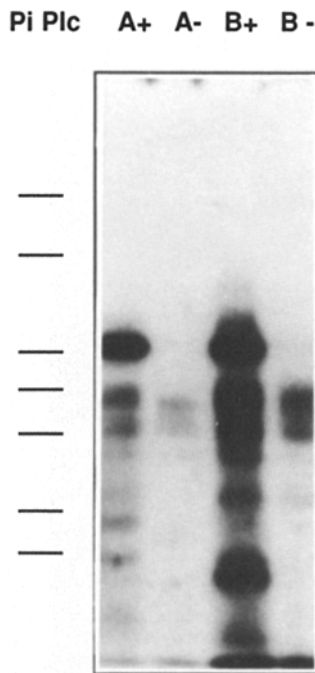


Figure 1. Polarized distribution of endogenous GPI-anchored proteins in FRT cells. Filter-grown FRT monolayers (5–7 d confluent) were biotinylated from the apical (lanes A) or basolateral (lanes B) sides. After extraction with Triton X-114 and phase separation, detergent phases were incubated in the presence (+) or absence (–) of PI-PLC (6 U/ml). After phase separation, biotinylated proteins in the aqueous phases were run on SDS-PAGE, transferred to nitrocellulose, and visualized with ^{125}I -labeled streptavidin. Molecular masses (top to bottom) are 116, 82, 64, 58, 36, and 26 kD.

striking result distinctly contrasted with all previous observations in different epithelial cell lines which always found an absolute correlation between the presence of a GPI moiety and apical protein localization (14, 15, 17).

Distribution of Transfected GPI-anchored Proteins in FRT Cells

To further understand the role of the GPI moiety as a sorting signal in FRT cells, we transfected these cells with cDNAs encoding three model proteins previously expressed in MDCK cells: the HSV native transmembrane glycoprotein gD1, the native GPI-protein decay accelerating factor (DAF) and a fusion protein between the ectodomain of gD1 and the GPI-attachment signal for DAF, gD1-DAF. Immunofluorescence images of uncloned populations of FRT cells transfected with gD1 (Fig. 2, A and B), DAF (Fig. 2, C and D) and gD1-DAF (Fig. 2, E and F) were obtained from intact cells (Fig. 2, A, C, and E) and from cells permeabilized with saponin (Fig. 2, B, D, and F). As previously shown in MDCK cells (15), FRT cells transfected with native gD1 displayed a typical, ring-like, basolateral staining as well as staining of intracellular vesicles (Fig. 2 B) but were devoid of apical staining (Fig. 2 A). Cells transfected with DAF showed, instead, both typical apical (punctate, microvillar) (Fig. 2 C) and basolateral staining patterns (Fig. 2 D), suggesting a nonpolarized distribution. Strikingly, gD1-DAF, which was localized preferentially to the apical membrane of MDCK cells (15), displayed a reversed distribution in transfected FRT cells, with a typical basolateral staining (Fig. 2, E and F).

Several clones positive for each protein were selected for resistance to G-418 and for expression of the protein by immunofluorescence. The cloned cells were seeded onto polycarbonate filter chambers to further analyze the polarity of gD1 and gD1-DAF and DAF by laser scanning confocal mi-

croscopy (LSCM). The same distribution described in the uncloned population grown on coverslips was found in the cloned cells grown on filters. In particular, we noted the exclusive distribution of the fusion protein gD1-DAF on the basolateral domain of transfected FRT cells (Fig. 3).

To quantify the distribution of the various transfected proteins in the cloned and uncloned population grown on filters for 5–7 d we used a domain selective biotinylation assay (Figs. 4 and 5). In agreement with the distribution seen by immunofluorescence we found that gD1 was basolaterally localized (Fig. 4), as previously shown in MDCK cells. In conformity with the LSCM results, the fusion protein gD1-DAF, which is localized exclusively on the apical membrane of transfected MDCK cells (15), was present only on the basolateral surface of FRT cells, as shown in Fig. 4. In both the uncloned population and in one selected clone, between 95 and 99% of the total surface protein was localized at steady state on the basolateral membrane of FRT cells. To verify that gD1-DAF was anchored to the membrane via a GPI moiety, apically and basolaterally biotinylated monolayers were extracted with Triton X-114, phase separated and treated with PIPLC followed by immunoprecipitation. GPI-anchored gD1-DAF appeared basolaterally polarized (Fig. 4, left) as a band of ~ 45 kD. This distribution of gD1-DAF was completely reversed with respect to that found for the same protein transfected in MDCK cells. The results of this experiment conclusively demonstrate that, in FRT cells, the addition of a GPI moiety to the ectodomain of a native basolateral protein does not reverse its polarity.

To further analyze the polarity of DAF we performed domain-selective biotinylation of the unselected population and of two different clones expressing DAF. We detected similar amounts of the protein (50:50%) in both the apical and the basolateral membrane domain (Fig. 5). Treatment of these clones with PIPLC indicated that only a small fraction of immunoprecipitable DAF ($\sim 1/10$) was susceptible to enzymatic cleavage; both PIPLC-sensitive and PIPLC-insensitive DAF were unpolarized (data not shown). These results are compatible with a fraction of DAF being anchored via a transmembrane domain (highly unlikely, see Discussion) or, alternatively, with the GPI of DAF rendered unsusceptible to PIPLC by acylation, as shown for the erythrocytes acetyl cholinesterase (31).

Polarity and Targeting Pathways in FRT Cells Expressing Exogenous GPI-anchored Proteins

To demonstrate that transfected FRT cells did not have altered polarity compared with the wild-type cells we performed control experiments to analyze the steady-state distribution of different apical and basolateral antigens in the transfected cells. By both immunofluorescence and domain-selective biotinylation we found DPPIV apically localized, while the 35–40 kD antigen, α and β subunits of Na,K-ATPase and transferrin receptor were basolaterally polarized (data not shown) as previously shown in wild-type cells (46, 48). Furthermore, we examined the plasma membrane delivery of newly synthesized DPPIV and Ag 35–40 kD (two model proteins previously used to characterize the targeting phenotype of FRT cells) (46) in FRT cells expressing gD1 or gD1-DAF in comparison to wild-type FRT cells using a biotin targeting assay (48) (Fig. 6 A and B). We found that

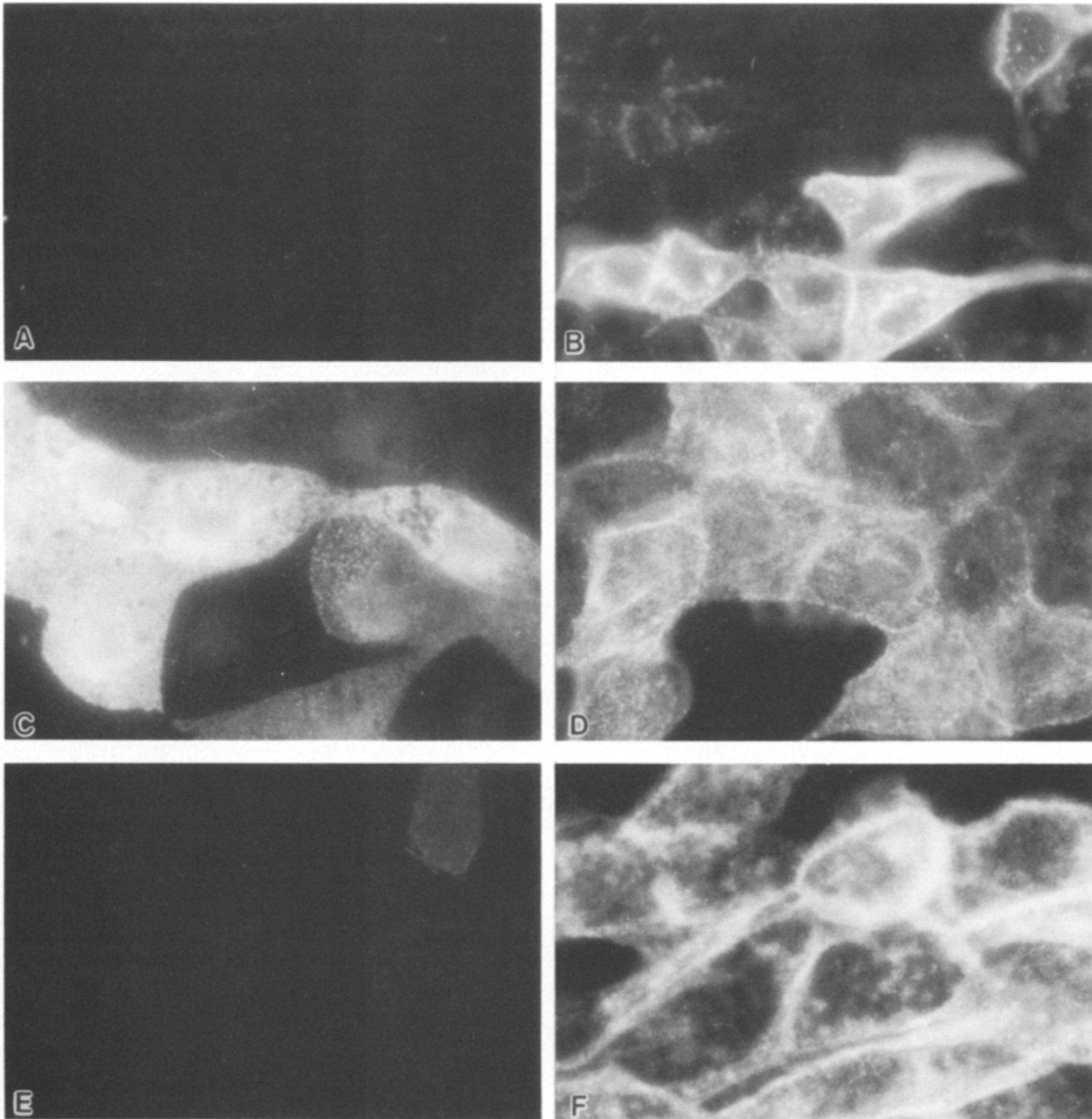


Figure 2. Immunofluorescent localization of native gD1 and (A and B), DAF (C and D) and gD1-DAF fusion protein (E and F) in transfected FRT cells. G418-resistant transfected FRT cells (uncloned cell populations) were grown at confluency on glass coverslips. Confluent monolayers were fixed and either permeabilized with saponin (B, D, and F) or left intact (A, C, and E) and then stained with HSV or DAF mAbs and revealed with an appropriate FITC-conjugated second antibody. Native gD1 appeared concentrated both intracellularly and at the basolateral surface (B), DAF was found both at the apical and the basolateral membranes (C and D) and gD1-DAF fusion protein appeared to be highly polarized on the basolateral membrane (F).

after 7 d of filter culture both the apical DPPIV (Fig. 6 A) and the basolateral 35–40-kD antigen (Fig. 6 B) were directly targeted to the appropriate domain and with the same efficiency both in the transfected and in the wild-type cells. These experiments clearly show that neither the plasma membrane polarity of apical and basolateral antigens nor the sorting capacity of FRT cells were affected by the transfection of the cDNAs encoding the exogenous proteins gD1 and gD1-DAF.

Surface Delivery of gD1-DAF

To determine whether the basolateral distribution of gD1-DAF in FRT cells was due to direct delivery from the Golgi apparatus or to transcytosis from a precursor apical pool, we performed a biotin surface targeting assay developed by Lisanti et al. (16). In this assay, free surface amino groups are quenched at 4°C with sulfo-SHPP (the water-soluble Bolton-Hunter reagent, which lacks a biotin moiety) and the

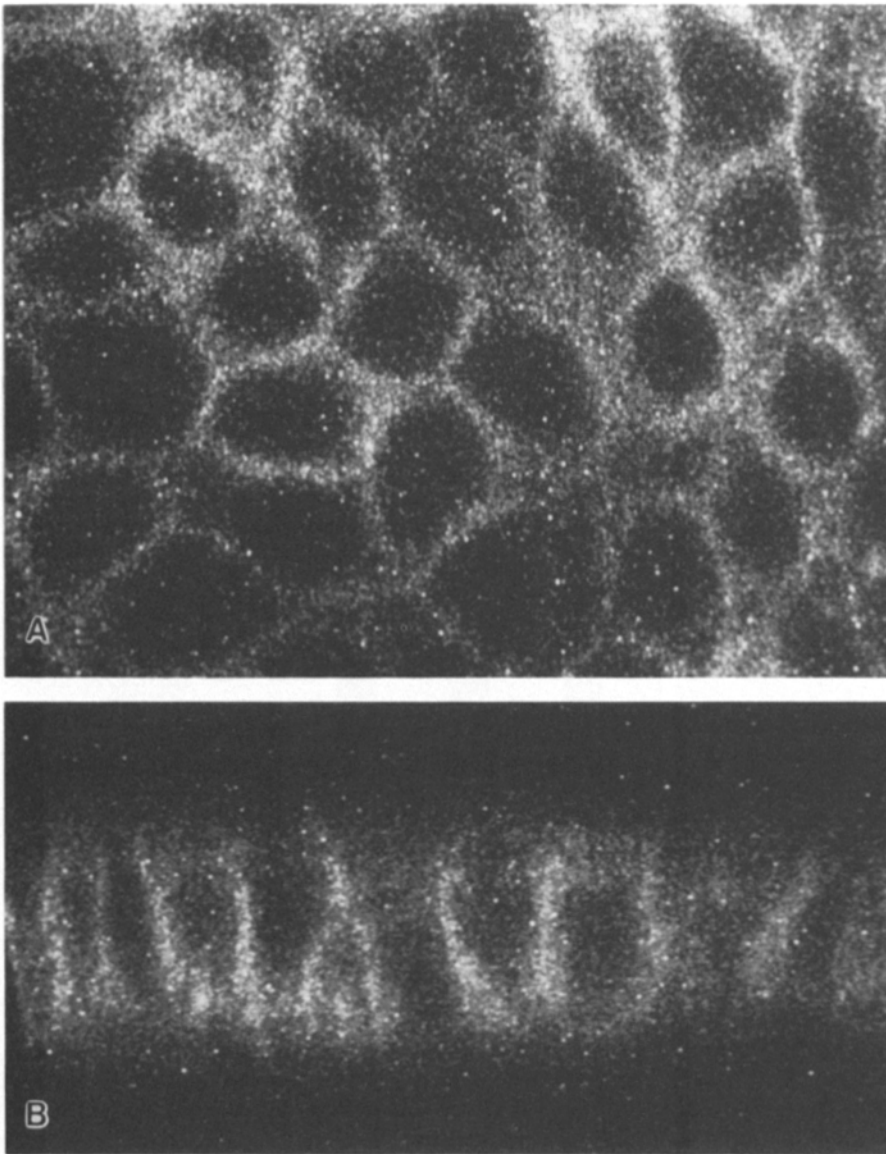


Figure 3. Immunofluorescent localization of gD1-DAF fusion protein in a selected clone of transfected FRT cells. A G418-resistant clone of transfected FRT cells was grown on filters for 5 d then fixed and processed for immunofluorescence. Immunostaining for gD1-DAF was revealed with an appropriate FITC-conjugated second antibody and viewed by laser scanning confocal microscopy on horizontal (A) and vertical (B) sections.

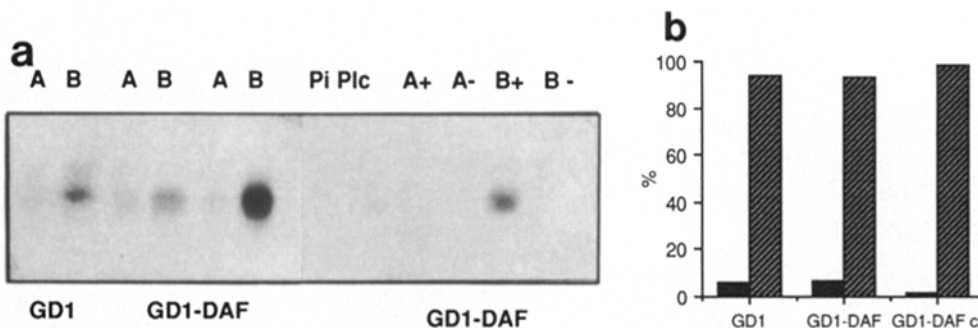


Figure 4. Basolateral localization of the native transmembrane HSV glycoprotein gD1 and the fusion protein gD1-DAF in transfected FRT cells. 5-7-d-old confluent filter-grown monolayers expressing gD1 or gD1-DAF were subjected to apical (A) or basolateral (B) biotinylation, extracted, and immunoprecipitated with anti HSV IgG. (A) Both gD1 (left two lanes) and

gD1-DAF (next four lanes representing the uncloned population and a selected clone of FRT cells) were found highly polarized at the basolateral surface. To confirm that the fusion protein gD1-DAF was GPI anchored, FRT expressing cells were extracted in Triton X-114 after biotinylation and PIPLC treated (+, 6 U/ml; -, untreated) and immunoprecipitated with anti HSV. As shown in the last four lanes on the right in one of the clones, gD1-DAF was highly sensitive to PIPLC treatment. Biotinylated proteins were detected by ¹²⁵I-streptavidin blotting/autoradiography. (B) Fluorograms of two independent gels were quantitated and the results were expressed as percent apical and percent basolateral surface expression of gD-1 and gD1-DAF in transfected FRT cells (uncloned population) or in a selected clone (FRT gD1-DAF cl).

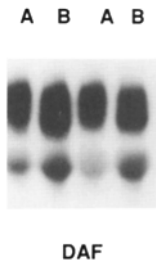


Figure 5. Unpolarized distribution of DAF in transfected FRT cells. Two selected clones expressing DAF were seeded on polycarbonate filters for 5–7 d and then biotinylated either from the apical (lanes *A*) or the basolateral (lanes *B*) surface, extracted and immunoprecipitated with anti-DAF IgG. Biotinylated proteins were visualized by ¹²⁵I-streptavidin blotting/autoradiography.

insertion of protected intracellular amino groups at the cell surface (after incubation at 37°C) can be detected by domain-selective biotinylation and ¹²⁵I-streptavidin blotting.

Using this assay system, the surface appearance of gD-1-DAF was time and temperature-dependent (undetectable at 4°C; rapid at 37°C) and basolaterally polarized (Fig. 7 *A*). Basolateral gD1-DAF was clearly visible as early as 30 min after transfer to 37°C, and continued to increase linearly at a much higher rate than the rate of apical appearance, reach-

ing a plateau between 120 and 150 min (Fig. 7, *A* and *B*). These results are consistent with intracellular sorting and vectorial basolateral delivery of gD1-DAF. Using the same assay, gD1-DAF was previously found to be apically targeted in MDCK cells (16).

Secretion of Anchor Minus gD1 and DAF

The basolateral distribution of gD-1-DAF can be accounted for by either a dominant basolateral signal present in the ectodomain of the native HSV gD-1 or by an altered signaling role of GPI in FRT cells (basolateral sorting signal?).

To discriminate between these possibilities we sought to determine whether the ectodomains of gD-1 and DAF contain sorting information. To this end, we expressed truncated (secreted) forms of these proteins by permanent transfection in FRT cells. cDNAs encoding residues 1–300 of the gD-1 ectodomain and a secretory form of DAF (Δ 1 DAF, lacking 17 carboxy-terminal amino acids required for GPI anchoring) were transfected into FRT cells. Interestingly, secretory

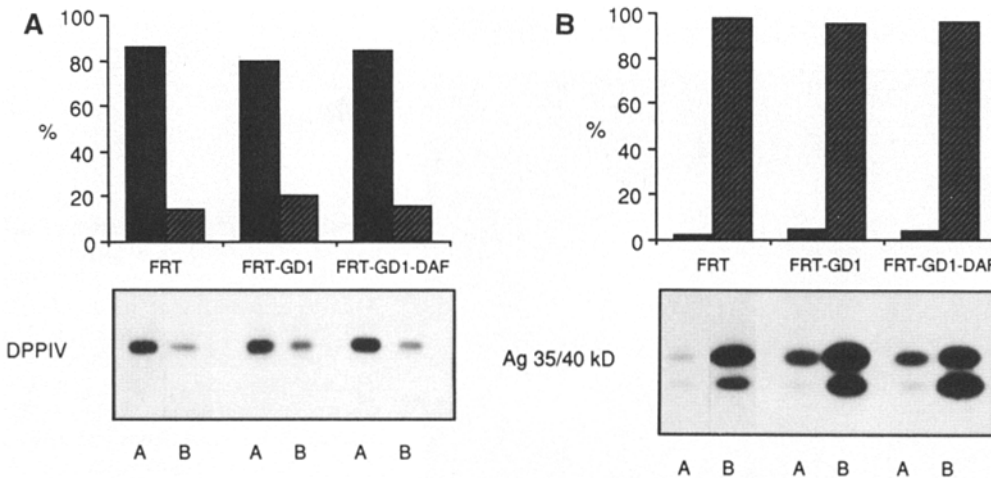


Figure 6. Polarized delivery of newly synthesized endogenous apical (DPPIV) (*A*) and basolateral (Ag 35–40 kD) (*B*) proteins in wild-type FRT cells and in transfected clones expressing gD1 and gD1-DAF. Wild-type and two selected clones of FRT cells expressing gD1 and gD1-DAF were grown on filters for 5–7 d and then pulsed for 1 h with [³⁵S]met/cys. Newly synthesized DPPIV and Ag 35–40 kD were detected at the cell surface by domain selective biotinylation, then immunoprecipitated with specific antibodies and reimmunoprecipitated with streptavidin beads and analyzed by SDS-PAGE and fluorography. Fluorograms of two independent experiments were quantitated and the results are expressed as percent of apical (lanes *A*) and basolateral (lanes *B*) surface expression of each antigen. (*A*) DPPIV was directly targeted to the apical surface in FRT cells (as previously shown) (46) as well as in transfected clones expressing gD1 or gD1-DAF. (*B*) Ag 35–40 kD was targeted directly to the basolateral surface of FRT cells (as previously shown) (46) as well as in transfected clones expressing gD1 or gD1-DAF.

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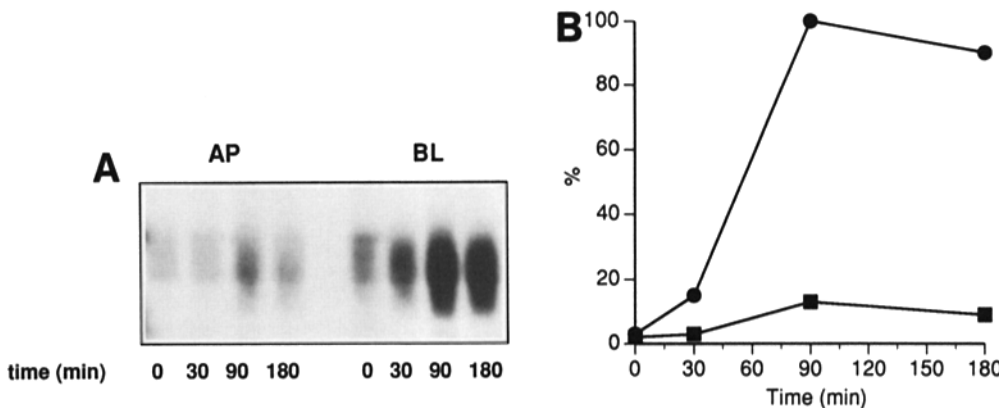


Figure 7. Polarized basolateral delivery of gD1-DAF in transfected FRT cells grown on filters for 5–7 d. (*A*) After surface amino groups were quenched with six consecutive rounds of sulfo-SHPP, cells were warmed at 37°C for various times and subjected to apical (*AP*) or basolateral (*BL*) biotinylation. The monolayers were then extracted and immunoprecipitated with anti-HSV IgG and run on SDS-PAGE. Biotinylated proteins were revealed with ¹²⁵I-strept-

avidin blotting/autoradiography. (*B*) Fluorograms of two independent experiments were quantitated and the results were expressed as percentage of the amount at the time of maximal expression at cell surface. (■) Apical; (●) basolateral.

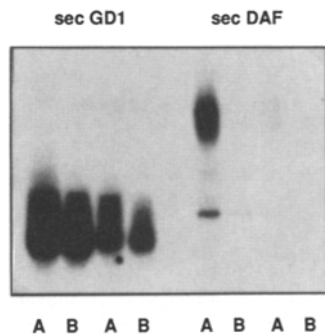


Figure 8. Secretion of anchor minus gD1 and DAF in transfected FRT cells. Filter-grown monolayers were metabolically labeled overnight. Apical (lanes A) and basolateral (lanes B) media were collected, centrifuged (16,000 g for 30 s) and exposed to protease inhibitors (15). Aliquots of media (500 and 200 μ l) were then diluted with TBST to 1 ml (final volume) and immunoprecipitated with either anti HSV or anti DAF IgG. Note that truncated gD1 is secreted in a nonpolarized fashion, whereas truncated DAF (Δ 1 DAF) secretion is primarily apical.

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DAF was released apically from FRT cells whereas the gD-1 ectodomain was released without polarity (Fig. 8). These results indicated the presence of an apical targeting signal in the DAF ectodomain, as previously shown in MDCK cells (15). They also showed that the basolateral distribution of gD-1-DAF cannot be accounted for by a dominant basolateral signal in the ectodomain of gD1.

Discussion

We describe here the unexpected localization of GPI-anchored proteins in the rat thyroid cell line FRT. Unlike MDCK cells and other cell lines characterized to date, GPI-anchored proteins do not appear to have a preferential apical localization in FRT cells. Most endogenous GPI-anchored proteins are basolateral in FRT cells (six clearly defined bands of 58, 48, 45, 39, 25, and 16 kD), whereas two minor bands (36 and 18 kD) are apical and one (90 kD) is symmetrically distributed. Furthermore, exogenous GPI-anchored proteins expressed from transfected cDNAs are also not restricted to the apical surface. Thus, anchorage via GPI does not ensure apical localization in FRT cells.

We have previously used the fusion protein gD-1-DAF as a model for our studies on the sorting of GPI-anchored proteins in epithelia. This protein was constructed by the addition of 37 COOH-terminal aminoacids of DAF to 300 aminoacids of the gD1 ectodomain; this is sufficient to produce an apically localized GPI-anchored fusion protein in MDCK cells. The apical localization of this protein is not determined by the presence of a cryptic apical targeting signal in the 37 amino acids donated by DAF since most of these amino acids (28 of them) are cleaved at the moment of GPI addition in the RER, and eight of the remaining nine amino acids can be removed with no effect on either GPI-addition or on the apical localization of an attached reporter protein, human growth hormone (19). We found that, upon transfection into FRT cells, gD1-DAF is sensitive to PIPLC treatment and is highly polarized to the basolateral surface (Fig. 4). Targeting experiments indicated that the basolateral distribution can be accounted for by polarized delivery from the Golgi apparatus to the cell surface. This mechanism can fully explain the observed surface distribution since it was previously shown that endocytosis and transcytosis of gD1-DAF are negligible (16). Since the secretory form of gD1, lacking transmembrane and cytoplasmic regions, is secreted

without polarity (Fig. 6), it is clear that it is the anchoring via GPI that directs gD1-DAF to the basolateral surface. This could occur via a default or a signal-mediated mechanism (see below).

In contrast to gD1-DAF, DAF, which was previously shown to be apical when expressed endogenously in two intestinal cell lines (Caco-2 and SK-CO-15) and exogenously in MDCK cells (15), was unpolarized in several clones of transfected FRT cells. A large fraction (\sim 90%) of DAF was found to be insensitive to PIPLC; this may reflect the presence of transmembrane isoforms of DAF (for which there is no precedent) or of an acylated form of GPI insensitive to PIPLC, as previously described for DAF itself and for acetyl cholinesterase in erythrocytes (31). Both the PIPLC insensitive and the PIPLC sensitive forms of DAF appeared to be unpolarized. The unpolarized distribution of DAF is especially surprising since, as previously shown in MDCK cells (15), a truncated secretory form of this protein is secreted apically (Fig. 8), indicating the presence of an apical signal in the ectodomain which is recognized by the FRT sorting machinery.

The results presented in this report generate interesting conclusions and raise new questions regarding the mechanisms of protein sorting in epithelial cells. First, they confirm the remarkable flexibility of the protein sorting machinery of epithelia (23, 34), since the same GPI-anchored proteins are sorted differently in different epithelial cell types. Recent work has shown that a typical apical protein, dipeptidyl peptidase IV, may reach the apical membrane via different routes in different epithelial cells, e.g., by transcytosis from the basolateral membrane in liver cells and directly from the TGN in MDCK and in FRT cells (6, 20, 46). Furthermore, in transgenic mice, the low density lipoprotein receptor is distributed basolaterally in liver and intestinal cells but apically in proximal tubular kidney cells (27). Finally, the viral glycoproteins of Semliki Forest and Sindbis viruses, which are distributed basolaterally in infected MDCK cells, are apically localized in FRT cells (47). Second, our data reopen the discussion on the mechanisms of basolateral protein delivery in epithelial cells. Recent work by several laboratories has shown that discrete cytoplasmic determinants, related to coated pit internalization signals, direct the Golgi sorting and surface delivery of certain basolateral proteins (2, 5, 10, 12). This led to the suggestion that basolateral delivery was signal mediated and would require cytosolic factors related to the family of adaptins (10, 12). The results presented here show for the first time that, at least in FRT cells, certain proteins may be targeted preferentially and with high efficiency to the basolateral membrane without any cytoplasmic domain. Since in these cells receptors with basolateral cytoplasmic determinants (such as transferrin receptor) (46) are also basolateral, our results imply that there exist at least two mechanisms for basolateral delivery in FRT cells. It is likely that two different types of basolaterally targeted vesicles are produced in the TGN which are transported independently to the basolateral membrane. A similar hypothesis was recently put forward for MDCK cells on the basis of the different sensitivity of the transport of secretory and membranous basolateral proteins to microtubule-disrupting agents (1). Alternatively, in FRT cells, certain GPI-anchored proteins, such as gD1-DAF, may associate with a transmembrane protein with

cytoplasmic basolateral determinants which mediates their transfer to the basolateral membrane. If this is the case, the association with the putative basolateral "receptor" may not be via the ectodomain of gDI-DAF since this does not seem to contain basolateral targeting signals (Fig. 8). This suggests either that GPI behaves as a basolateral targeting signal in FRT cells or that GPI-proteins follow a default basolateral pathway (38) in this cell line.

Third, our findings open a new avenue to explore the targeting role of GPI. Recent results by Brown and Rose in MDCK cells (3) have shown that GPI-anchored proteins associate with sphingoglycolipid-rich domains in "patches" insensitive to disruption by non-ionic detergents. Since certain sphingoglycolipids have been shown to be targeted preferentially to the apical membrane of MDCK and Caco2 cells (42, 43), this led to the suggestion that co-clustering with sphingoglycolipids might be essential for the incorporation of GPI-anchored proteins into apically targeted vesicles in the TGN (3, 38, 41). According to this hypothesis, the deficient apical targeting of GPI-proteins in FRT cells might be accounted for by their inability to associate with sphingoglycolipid-rich patches in the TGN that facilitate their incorporation into apically targeted vesicles or, alternatively, by defective sphingoglycolipid patching in FRT cells. Under these two scenarios GPI-anchored proteins would be transported to the basolateral membrane by a default mechanism. A third possible scenario is that patches of GPI-anchored proteins and sphingoglycolipids are preferentially targeted to the basolateral surface of FRT cells and therefore, GPI would act as a basolateral signal by promoting association with these patches. The FRT cell line provides an excellent tool to discriminate between these possibilities and to investigate the role of glycolipid clustering in the sorting of proteins and lipids in epithelial cells.

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