

A Glycophospholipid Membrane Anchor Acts as an Apical Targeting Signal in Polarized Epithelial Cells

Michael P. Lisanti,* Ingrid W. Caras,‡ Michael A. Davitz,§ and Enrique Rodriguez-Boulan*

*Department of Cell Biology and Anatomy, Cornell University Medical College, New York 10021; ‡Genentech, Inc., South San Francisco, California 94080; and §Department of Pathology and Environmental Medicine, New York University School of Medicine, New York 10016

Abstract. Glycosyl-phosphatidylinositol- (GPI) anchored proteins contain a large extracellular protein domain that is linked to the membrane via a glycosylated form of phosphatidylinositol. We recently reported the polarized apical distribution of all endogenous GPI-anchored proteins in the MDCK cell line (Lisanti, M. P., M. Sargiacomo, L. Graeve, A. R. Saltiel, and E. Rodriguez-Boulan. 1988. *Proc. Natl. Acad. Sci. USA.* 85:9557-9561). To study the role of this mechanism of membrane anchoring in targeting to the apical cell surface, we use here decay-accelerating factor (DAF) as a model GPI-anchored protein. Endogenous DAF was localized on the apical surface of

two human intestinal cell lines (Caco-2 and SK-CO15). Recombinant DAF, expressed in MDCK cells, also assumed a polarized apical distribution. Transfer of the 37-amino acid DAF signal for GPI attachment to the ectodomain of herpes simplex glycoprotein D (a basolateral antigen) and to human growth hormone (a regulated secretory protein) by recombinant DNA methods resulted in delivery of the fusion proteins to the apical surface of transfected MDCK cells. These results are consistent with the notion that the GPI anchoring mechanism may convey apical targeting information.

AN alternative mechanism whereby membrane glycoproteins can be anchored to the cell surface has recently emerged. This mode of anchoring involves the covalent attachment (via ethanolamine) of a glycolipid, glycosyl-phosphatidylinositol (GPI),¹ to the COOH-terminal amino acid of the extracellular protein domain (Low et al., 1986; Cross, 1987; Low and Saltiel, 1988; Ferguson and Williams, 1988). A growing number of ~30 proteins is known to be anchored in this fashion. The best characterized members of this group include the variable surface glycoprotein of *Trypanosoma brucei*, acetyl-cholinesterase, Thy-1, N-CAM, placental alkaline-phosphatase, and decay-accelerating factor (DAF) (see Ferguson and Williams, 1988, for the most current list). Attachment of the GPI-anchor occurs 1-2 min after synthesis of the precursor protein in the endoplasmic reticulum (Bangs et al., 1985; Ferguson et al., 1986; He et al., 1987; Conzelmann et al., 1987), after removal of a COOH-terminal hydrophobic sequence, which appears to act as a signal for incorporation of the glycolipid (Caras et al., 1989).

Decay-accelerating factor (DAF), a GPI-anchored complement regulatory protein (Davitz et al., 1986; Medof et al., 1986), has been extensively used to analyze the signal for attachment of the GPI-membrane anchor. Initial studies indicated that this information is contained within the COOH-

terminal 37 amino acids encoded by DAF mRNA (Caras et al., 1987a,b). Addition of this sequence to a secreted protein resulted in GPI-anchoring and targeting of the fusion protein to the plasma membrane (Caras et al., 1987b). Recent work has further defined two regions within the COOH-terminal peptide: a COOH-terminal 17-amino acid hydrophobic portion and an adjacent 20-amino acid region. Deletion of the 17-amino acid hydrophobic peptide prevents GPI attachment, resulting in secretion of anchor minus DAF into the medium (Caras et al., 1989). The "signal" function of this peptide seems to depend on overall hydrophobicity and not on a specific sequence, because it can be replaced by either the signal sequence of human growth hormone (hGH) or by a random hydrophobic sequence (Caras and Weddell, 1989). Both the COOH-terminal 17-amino acid hydrophobic domain and the adjacent 20-amino acid region are required to direct GPI attachment, the latter possibly containing a necessary cleavage/attachment site for the anchor (Caras et al., 1989). The exact site of the cleavage of the DAF signal for GPI-attachment is not known. By analogy with other GPI-anchored proteins where the cleavage sites for removal of the hydrophobic COOH-terminal peptide have been determined, ~8-10 of the 37 COOH-terminal amino acids could remain after cleavage. Experiments with other GPI-anchored proteins, namely Qa-2 and placental alkaline phosphatase, have also localized the signal for GPI attachment to COOH-terminal hydrophobic regions (Waneck et al., 1988; Berger et al., 1988).

1. *Abbreviations used in this paper:* DAF, decay-accelerating factor; GPI, glycosyl-phosphatidylinositol; hGH, human growth hormone; TBST, Tris-buffered saline containing 1% Triton X-100; TGN, *trans*-Golgi network.

Although the general structural features of the GPI anchor and the sequences directing its attachment are well characterized, its function remains unknown. Given that lipids follow defined biogenic pathways in eukaryotic cells (Pagano and Sleight, 1985; van Meer et al., 1987; Koval and Pagano, 1989), covalent attachment of GPI to proteins may influence their sorting. Here, we have studied the role of GPI anchoring in targeting the attached protein to the apical surface of polarized epithelia by transferring the DAF signal for GPI attachment to the ectodomain of the Herpes glycoprotein gD-1 and to hGH. We then assessed their surface distribution in transfected MDCK cells. Our results are consistent with the notion that the GPI-membrane anchoring mechanism may contain apical targeting information.

Materials and Methods

Materials/Reagents

Phosphatidylinositol-specific phospholipase C (PI-PLC) (purified from *Bacillus thuringiensis*), was the generous gift of Dr. Martin Low (Columbia University College of Physicians and Surgeons, New York). Caco-2 was the gift of Dr. Alain Zweibaum (Unité de Recherches sur le Metabolisme et la Differentiation de Cellules en Culture, Institut Nationale de la Santé et de la Recherche Medicale U178, Villejuif, France); SK-CO15 was the gift of Dr. Francisco Real (Instituto Municipal de Investigaciones Medicas, Barcelona, Spain). Both rabbit polyclonal and mouse monoclonal antibodies directed against human DAF were as described previously (Davitz et al., 1986; Medof et al., 1986; 1987). Rabbit IgG directed against the Herpes simplex virus (HSV), type 1 (MacIntyre) were purchased from Dako Corp. (Santa Barbara, CA). Purified rabbit IgG against hGH was provided by the Medicinal Analytical Chemistry Department at Genentech, Inc. (South San Francisco, CA).

Constructs

A full-length DAF cDNA (GPI-anchored form) was inserted into a mammalian expression vector between an RSV promoter and an SV40 polyadenylation signal (Caras et al., 1987a). Similar constructs for the expression of DAF fusion proteins (gD-1-DAF and hGH-DAF) contained the last 37 COOH-terminal amino acids of DAF as described previously (Caras et al., 1987, 1989). Constructs containing fewer COOH-terminal amino acids of DAF, i.e. 17 or 27, failed to become GPI-anchored after expression in CHO or COS cells (Caras et al., 1989).

Cells and Cell Culture

MDCK cells, type II, were maintained in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% (vol/vol) horse serum (HS) (Hyclone Laboratories, Logan, UT) and antibiotics (Rodriguez-Boulant, 1983). Caco-2, SK-CO15, and Hela cells were maintained in DME supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco Laboratories) and nonessential amino acids.

Cells from a single confluent 75-cm² flask were trypsinized and transferred to six filter chambers (Transwell, 24.5 mm, 0.4- μ m pore polycarbonate, tissue-culture treated; Costar Corp., Cambridge, MA). Media were changed every 2 d for renal cell lines and everyday for intestinal cell lines. For the development of a tight, well-differentiated epithelial monolayer, ~5-7 and 11-14 d after plating were required for renal and intestinal cell lines, respectively. Filter-grown monolayers were assayed for impermeability to [³H]inulin (New England Nuclear, Chadds Ford, PA) to assess the integrity of the monolayer as described previously (Caplan et al., 1986). Monolayers with a permeability of >1% (after 2 h at 37°C) were discarded.

Transfection and Selection

MDCK cells were transfected as described previously using a modification of the calcium phosphate precipitation procedure (Graham and van der Eb, 1973). Briefly, ~10⁶ subconfluent MDCK cells were trypsinized, resuspended in 1 ml DME containing 10% FBS, and transferred to a 100-mm dish. Cells were overlaid with 10 μ g/dish of the nonselectable plasmid and

1 μ g/dish of pMV6(neo) and incubated for 6-9 h at 37°C. After a 15% glycerol shock, cultures were allowed to recover for 2 d in DME containing 10% FBS. Cells were trypsinized, diluted 1:8, and replated in 150-mm dishes in DME containing 10% HS and 500 μ g/ml G418 (Gibco Laboratories). After 10-14 d, resistant colonies appeared. Individual clones were obtained by limiting dilution. Briefly, resistant colonies were trypsinized, diluted to two to five cells/ml and plated in 96-well dishes (150 μ l/well). After 10-14 d, colonies were screened for expression of the transfected gene product. pMV6 tk(neo), conferring resistance to the antibiotic G-418 (Maddon et al., 1985), was a gift of Dr. Moses Chao (Cornell University Medical College).

Screening

After 14 d, 96-well plates were trypsinized (25 μ l trypsin-EDTA per well + 175 μ l DME/10% HS per well) and duplicate plates generated by plating 100 μ l of cell suspension per well in a second 96-well dish. Duplicate plates were then screened for the presence of the transfected gene product after a 12-16-h incubation in DME containing 10% HS and 10 mM Na butyrate to elevate expression (Gorman and Howard, 1983; Gottlieb et al., 1986). Positive clones were detected after fixation and permeabilization by incubation with the appropriate polyclonal rabbit antiserum (1:100 dilution in PBS C/M containing 0.2% BSA) followed by [¹²⁵I]-protein A binding, or in the case of native hGH and Δ 1 DAF, by immunoradiometric assay (IRMA) of the culture media. Truncated gD-1 expressing clones were identified by using mouse mAbs (IgG2a; Bioproducts for Science Inc., Indianapolis, IN), adsorbed to 96-well U-bottom plastic plates, to capture antigen from tissue-culture supernatants. Bound antigen was visualized by using rabbit anti-HSV IgG and the anti-rabbit ABC Vectastain kit (Vector Laboratories, Burlingame, CA).

Domain-selective Labeling with Sulfo-NHS-biotin

Biotinylation was performed as described previously (Lisanti et al., 1988; Sargiacomo et al., 1989). Note that immunofluorescence on semithin frozen sections and immunoprecipitation of apical and basolateral markers have demonstrated that biotinylation is confined to either the apical or basolateral cell surface and is uniform up to the level of the tight junction (Sargiacomo et al., 1989).

Phosphatidylinositol-phospholipase C (PI-PLC) Treatment

PI-PLC treatment was performed as described previously (Conzelmann et al., 1986; Lisanti et al., 1988). Briefly, after extraction and phase separation with the detergent Triton X-114, detergent phases enriched in membrane (hydrophobic) forms of GPI-anchored proteins were treated with PI-PLC (6 U/ml). After PI-PLC treatment, resulting aqueous phases (containing soluble [hydrophilic] forms of GPI-anchored proteins) were subjected to immunoprecipitation. All solutions used for cell extraction phase separation and PI-PLC treatment were ice cold and contained each of the following protease inhibitors at 10 μ g/ml: leupeptin, pepstatin A, and antipain. Three filter chambers were used per experimental condition. TX-114 was precondensed according to Bordier (1981).

Metabolic Labeling with [³⁵S]cysteine and Methionine

Filter-grown, confluent MDCK monolayers were washed twice with DME lacking methionine (met) and cysteine (cys) and either labeled overnight or pulsed with [³⁵S]cys and met (New England Nuclear). For steady-state labeling, filters were labeled with 100 μ Ci/ml of both [³⁵S]cys and met in DME containing one-tenth the normal concentration of met and cys/10 mM Hepes, pH 7.3/0.2% BSA for 12-16 h; radiolabel was added only to the basolateral compartment since uptake is most efficient from the basolateral side.

For pulse-chase experiments, filters were labeled with 1 mCi/ml of both [³⁵S]cys and met in DME (lacking met and cys)/10 mM Hepes, pH 7.3 for 10 min at 37°C and chased for various times (as indicated) in DME containing excess methionine and cysteine. Approximately 150 μ l of labeling medium containing radio-label was added to the basolateral side of each filter chamber (placed inverted in a humidified chamber) (Kondor-Koch et al., 1985; Urban et al., 1987). Filters were then transferred after the pulse to six-well dishes and 1 ml of medium was added to the apical and basolateral compartments of the filter chamber.

Immunoradiometric Assay

Briefly, samples and DAF standards were added (25 μ l/well) to 96-well U-bottomed plastic plates (Becton Dickinson & Co., Oxnard, CA) pre-coated with anti-DAF mAb IA10 (20 μ g/ml) (Medof et al., 1987). After 1 h at 25°C, plates were washed three times with PBS containing 0.05% Tween and incubated with ¹²⁵I-labeled anti-DAF mAb IH6 (25 μ l/well; ~150,000 cpm/well). After washing as described above, wells were counted and the amount of DAF present was calculated by comparison with a standard curve generated using known quantities of purified DAF. For quantitating the release of DAF in Caco-2, SK-CO-15, and transfected MDCK clones expressing DAF, filter-grown tight monolayers (5–7 d after plating) were incubated for 12–16 h with DME containing 10% serum (apical compartment [1 ml]; basal compartment [1 ml]). An aliquot was removed (100 μ l) and adjusted to 50 mM Hepes, pH 7.3, and 25 μ l used for IRMA as described above. hGH was assayed as described by the manufacturer (Diagnostic Products Corp., Los Angeles, CA).

Immunoprecipitation of DAF

Cell Lysates (Biotin Labeled). Biotinylated, filter-grown monolayers were excised and extracted for 45 minutes at 4°C with intermittent mixing in 1 ml of Tris-buffered saline (10 mM Tris, pH 8.0, 0.15 M NaCl) containing 1% TX-100 (TBST) and protease inhibitors. After centrifugation (14,000 *g* for 10 min), lysates were precleared with 40 μ l/ml of Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ).

PI-PLC-treated Samples (Biotin Labeled). Samples from the PI-PLC assay were divided in half after phenyl-Sepharose treatment and precipitated with TCA or immunoprecipitated. The half to be immunoprecipitated was diluted 1:1 with TBST and additional protease inhibitors were added.

After preclearing, DAF mAbs (IA10) coupled to Sepharose were added (10 μ l/sample) and incubated overnight at 4°C rotating (Davitz et al., 1986). Sepharose CL-4B (40 μ l/sample) was added as a carrier just before washing the immunoprecipitates. Immunoprecipitates were washed five to six times with TBST and eluted with Laemmli sample buffer (100 μ l/sample) and boiling (1–3 min).

Note that the protease inhibitors used were the same as in the PI-PLC assay and three filter chambers were used per experimental condition.

Immunoprecipitation of DAF Fusion Proteins Cell Lysates (Biotin Labeled). Biotinylated filter-grown monolayers were extracted for 45 min at 4°C with 1 ml of Tris-buffered saline (10 mM Tris, pH 7.4, 0.15 M NaCl) containing 1% TX-114 and protease inhibitors. After temperature-induced phase separation, detergent phases were collected and reextracted twice. After reextraction, detergent phases were diluted to 1 ml with the above TBS (lacking detergent) and subjected to preclearing with protein A-Sepharose.

PI-PLC-treated Samples (Biotin Labeled). Samples were processed as described under immunoprecipitation of DAF.

Rabbit polyclonal antibodies, anti-HSV-1 (6 μ l/sample) or anti-hGH (4 μ l/sample), were allowed to bind protein A-Sepharose (10 mg/sample) overnight at 4°C in TBST containing 0.2% BSA. After washing twice with TBST, the antibody-protein A-Sepharose complexes were added to the above described extracts. After an overnight incubation rotating at 4°C, immunoprecipitates were washed and eluted as described for immunoprecipitation of DAF. Three filter chambers were used per experimental condition.

Immunofluorescence

Cells grown on glass coverslips (2–3 d confluent) were incubated 12–16 h in DME containing 10% HS and 10 mM Na butyrate, washed three times with PBS C/M and processed for surface staining. Monolayers were incubated for 5 min in PBS C/M containing 0.2% BSA and allowed to bind anti-DAF mAb IA10, anti-HSV-1 or anti-hGH (diluted 1:100) for 90 min. After extensive washing, coverslips were fixed with 2% paraformaldehyde and excess aldehyde groups quenched with NH₄Cl. Bound antibodies were visualized using the appropriate rhodamine-conjugated IgG (Cappel Laboratories, Cooper Biomedical Inc., Malvern, PA) at a dilution of 1:100. Na butyrate treatment was omitted for Caco-2 and SK-CO15 cells.

Detection of Labeled Proteins

Electrophoresis and Electroblothing. Samples were subjected to SDS-PAGE (Laemmli, 1970) under reducing conditions (10% or 15% [for hGH] acrylamide gels) and transferred to nitrocellulose (Towbin et al., 1979). After incubation with [¹²⁵I]-streptavidin (1–2 \times 10⁶ cpm/ml) for 2 h, blots were washed and dried as described previously (Sargiacomo et al., 1989).

Biotinylated proteins were visualized by autoradiography on Kodak XAR-5 film (–80°C with an intensifying screen).

After autoradiography, blots were rehydrated and reblocked with BSA/non-fat dry milk, and biotinylated proteins were directly visualized by incubation with alkaline phosphatase-conjugated streptavidin (1:250 dilution; Zymed, San Francisco, CA) and the appropriate substrates as described by the manufacturer (Promega Biotec, Madison, WI). Biotinylated bands were excised from the nitrocellulose sheets and counted in a gamma counter to quantitate the polarized apical distribution of GPI-anchored proteins in the various cell types examined. A corresponding piece of nitrocellulose (unbiotinylated controls) was counted and the signal was subtracted to yield specific counts. A background level of ~100–200 cpm was normally observed. Alternatively, autoradiographs were scanned using a GS-300 transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). Relative molecular weight markers are as indicated and are expressed in kilodaltons: myosin heavy chain, 200; phosphorylase b, 97.4; BSA, 68; ovalbumin, 43; carbonic anhydrase, 29; b lactoglobulin, 18.4; and lysozyme, 14.3.

Electrophoresis and Na Salicylate Treatment. Metabolically labeled samples were subjected to SDS-PAGE (Laemmli, 1970) under reducing conditions (10% polyacrylamide). After fixation (40% methanol/10% acetic acid for 30–60 min) gels were rehydrated (10% glycerol for 30 min) and impregnated with Na salicylate (1 M solution for 30 min) (Chamberlain, 1979). Dried gels were subjected to autoradiography (12 h to 4 d at –80°C) with Kodak XAR-5 film.

Results

Polarized Apical Distribution of Endogenous DAF in Intestinal Epithelial Cell Lines

The presence of DAF has been reported in a variety of epithelial cell-types, including intestinal mucosa (Medof et al., 1987), but its polarity was not described. To investigate the surface distribution of DAF, we used two human intestinal cell lines that retain the polarized phenotype *in vitro*. Caco-2 cells develop transmonolayer electrical resistance (200 Ω ·cm²) (Grasset et al., 1984), exhibit an extensive brush border with ordered apical microvilli, and express a variety of microvillar hydrolases (sucrase isomaltase, leucine aminopeptidase, alkaline phosphatase, etc.) typical of enterocytic differentiation (Fogh and Trempe, 1975; Pinto et al. 1983; Hauri et al., 1985; Rousset, 1986). SK-CO15 is a previously uncharacterized intestinal cell line (also derived from human adenocarcinoma of the colon), which by various morphological and biochemical criteria, displays a pattern of differentiation closer to colonic intestinal epithelia (Le Bivic et al., 1989).

Both cell lines were initially screened by immunofluorescence to detect the expression of DAF. DAF was present on the apical surface (as determined by surface immunofluorescence of intact cells) and exhibited the typical punctate pattern (corresponding to apical microvilli) observed with other apical membrane proteins (Fig. 1, *a* and *b*; *left*). Immunofluorescence on detergent permeabilized cells suggested that very little DAF was present on the basolateral membrane (data not shown). However, to conclusively determine whether DAF was apically polarized, we used a biochemical polarity assay: domain-selective biotinylation coupled with immunoprecipitation (Sargiacomo et al., 1989). Confluent monolayers grown on polycarbonate filter chambers (Transwell; Costar Corp.), with a permeability to [³H]inulin <0.5% per h, were selectively labeled at the apical or basolateral cell surface with the membrane/tight junction impermeant marker, sulfo-NHS-biotin. After biotinylation the filters were excised, the cells were extracted, and DAF was immu-

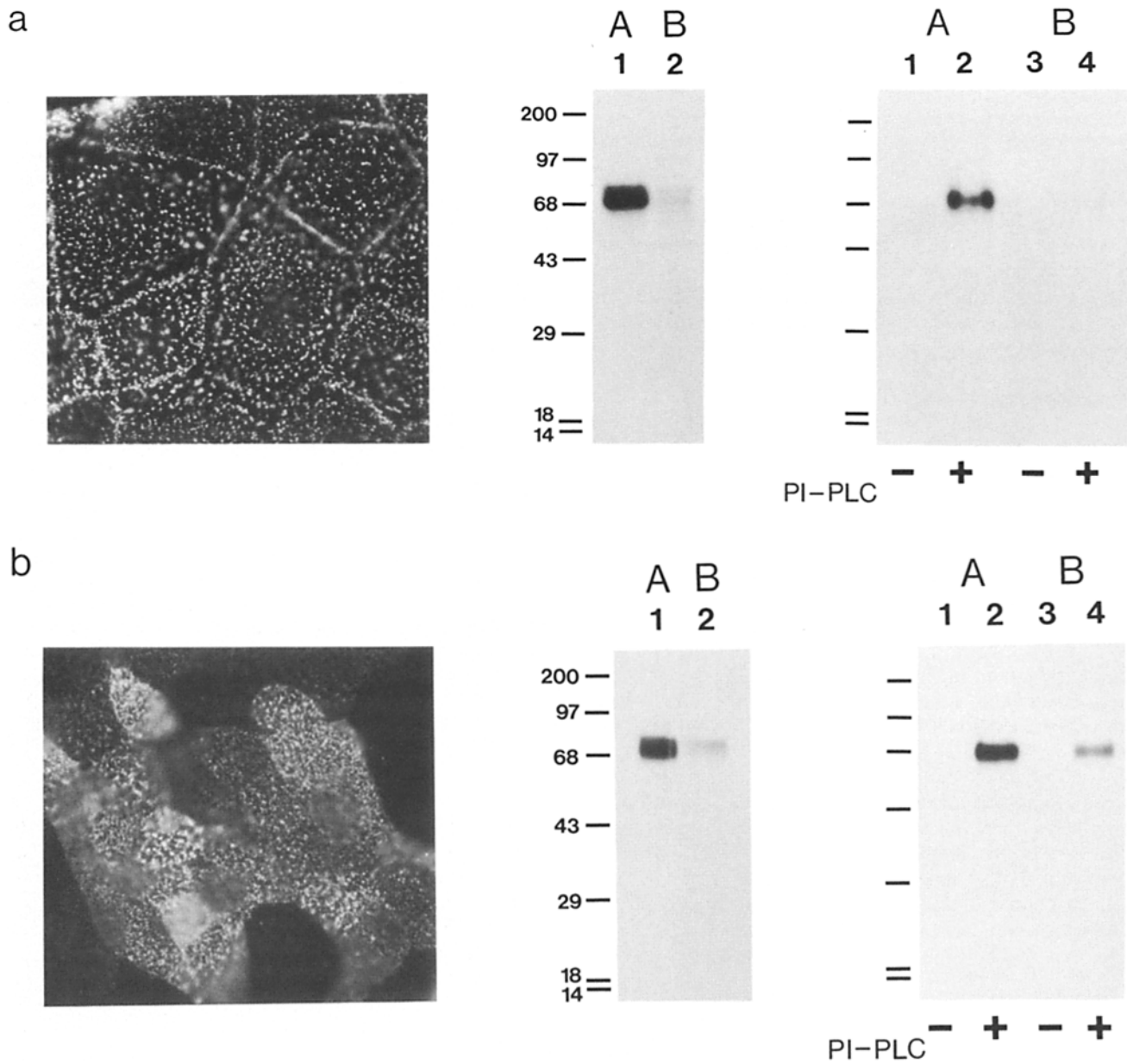


Figure 1. Polarized apical distribution of endogenous DAF in two human intestinal epithelial cell lines. (a) Caco-2; (b) SK-CO15. Intestinal cells were plated at high density onto glass coverslips or polycarbonate-based filter chambers. (Left) Coverslips, containing confluent monolayers, were incubated with a 1:100 dilution of anti-DAF mAb (IA10), postfixed with 2% paraformaldehyde (5 min at room temperature) and surface bound anti-DAF IgG visualized with rhodamine-conjugated goat anti-mouse IgG (Fab). (Middle) Filter-grown monolayers, 9–14 d confluent, were subjected to domain-selective labeling with sulfo-NHS-biotin either apically (A) or basolaterally (B). After solubilization with lysis buffer, DAF was immunoprecipitated with anti-DAF mAbs (IA10) coupled to Sepharose. [¹²⁵I]Streptavidin blotting, followed by autoradiography, was used to visualize biotinylated proteins. (Right) Filter-grown monolayer, 9–14 d confluent, were apically (A) or basolaterally (B) biotinylated. After lysis with the detergent TX-114 and PI-PLC treatment (–, untreated; +, 6 U/ml), the resulting aqueous phases were immunoprecipitated with anti-DAF mAbs coupled to Sepharose. Biotinylated proteins were visualized as described above. Note that endogenous DAF is GPI anchored and present in an apically polarized distribution in both cell lines tested. Molecular weight standards are indicated in kilodaltons (middle).

noprecipitated with a specific mAb. Biotinylated DAF was visualized by [¹²⁵I]streptavidin blotting, followed by autoradiography. In both cell lines, DAF was detected almost exclusively at the apical surface (Fig. 1, a and b; middle), clearly demonstrating its apical localization. DAF appeared as a doublet of 70 kD, as previously reported (Davitz et al., 1986).

To determine that the apically polarized DAF is GPI anchored, apically or basolaterally biotinylated monolayers were extracted with Triton X-114. The extracts were phase partitioned at 37°C (Bordier, 1981) and the detergent phases (containing GPI-anchored proteins) were subjected to PI-PLC treatment. DAF was immunoprecipitated after release into the aqueous phase by PI-PLC. A single band of 67 kD,

corresponding to the size of anchor-minus DAF (Davitz et al., 1986), was detected in the aqueous phase only after treatment with PI-PLC and was preferentially biotinylated from the apical side (Fig. 1, *a* and *b*; *right*).

To quantitate the polarized apical distribution of DAF, biotinylated bands were excised from the nitrocellulose after autoradiography (see Materials and Methods) and their radioactivity measured in a gamma counter. Approximately 90 and 80% of surface-biotinylated DAF was present on the apical domains of Caco-2 and SK-CO15, respectively. Because quantitative morphometric analyses of the surface areas occupied by the apical and basolateral membrane domains of these cell lines are not available, it is not possible to express the polarity of endogenous DAF on a per unit area basis.

Control experiments were performed to explore the possibility that the efficiency of labeling from the basolateral compartment was reduced by the presence of the filter. A non-polarized epithelial cell line, HeLa (derived from human cervical carcinoma), which expresses DAF (Medof et al., 1987) was chosen to evaluate this possibility. Confluent HeLa cell monolayers grown on Transwells (with a permeability to inulin of 10–16%) were biotinylated by adding sulfo-NHS-biotin to either the apical or basolateral compartment. After biotinylation, monolayers were extracted and immunoprecipitated with DAF mAbs. Alternatively, monolayers were extracted, treated with PI-PLC, and subjected to TCA or immunoprecipitation. In all cases, the levels of DAF that were detected were the same whether filter-grown monolayers were biotinylated from the apical or the basolateral compartment of the filter chamber (Fig. 2). These results indicate that the filter did not lower the efficiency of labeling from the basolateral compartment. In addition, the amounts of DAF recovered by direct immunoprecipitation or by TCA precipitation and immunoprecipitation after PI-PLC treatment were equivalent. Thus, it appears that our conditions for PI-PLC treatment quantitatively released the DAF present at the cell surface.

Polarized Apical Distribution of Transfected DAF in MDCK Cells

Having established the polarized apical distribution of endogenous DAF in two independent intestinal cell lines, we analyzed recombinant DAF expressed in MDCK cells. A cDNA encoding DAF (Caras et al., 1987a) was introduced into MDCK cells via cotransfection with a plasmid expressing neomycin resistance. After selection with the neomycin analogue G418, resistant cells were screened for the expression of DAF.

Positive clones were initially examined by immunofluorescence (in the absence of detergent) to qualitatively assess the polarized distribution of DAF. All eight positive clones expressed DAF on the apical surface, as shown by the typical microvillar (punctate) pattern (Fig. 3; *left*). Two clones were selected and seeded onto polycarbonate filter chambers to further assess the polarity of DAF using domain-selective labeling with sulfo-NHS-biotin. As with intestinal cells expressing endogenous DAF, MDCK cells expressed transfected DAF in an apically polarized distribution; two to three bands of 60–70 kD were specifically immunoprecipitated as compared with untransfected MDCK cell controls (Fig. 3;

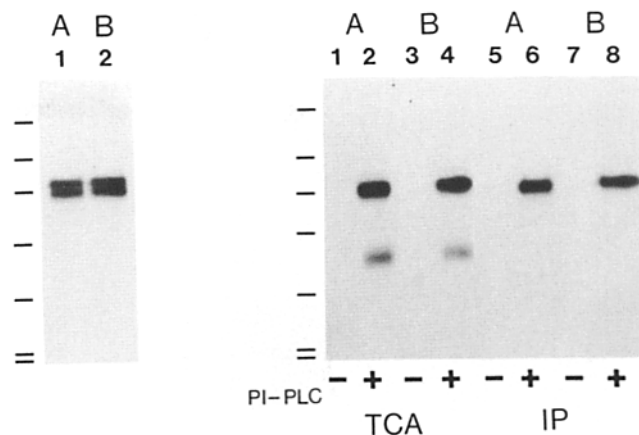


Figure 2. Identification of endogenous DAF in filter-grown HeLa cell monolayers. HeLa cells, 6–8 d confluent on polycarbonate-based filter chambers, were biotinylated by addition of sulfo-NHS-biotin to either the upper (*A*) or lower (*B*) compartment of the filter-chambers. (*Left*) After detergent solubilization, DAF was immunoprecipitated and visualized by [¹²⁵I]streptavidin blotting/autoradiography. (*Right*) Monolayers were extracted with TX-114 and subjected to PI-PLC treatment. (–, untreated; +, 6 U/ml). The resulting aqueous phases were then precipitated by the addition of Na deoxycholate and TCA (lanes 1–4) or immunoprecipitated (IP) with anti-DAF mAbs (lanes 5–8). Biotinylated proteins were visualized as described above. Because equal quantities of DAF were detected after biotinylation from either the upper or lower compartment of the filter chamber, the efficiency of labeling from the lower compartment is not significantly reduced by the presence of the filter. Molecular weight standards are as indicated in Fig. 1 (*middle*).

middle). Approximately 95% of surface-biotinylated DAF was present on the apical surface of transfected MDCK cells, yielding an A:B ratio of 68.4 per unit surface area. (This ratio was calculated based on morphometric analyses of the parental MDCK (type II) cell line (von Bonsdorff et al., 1985), from which DAF-expressing clones were derived).

To determine whether transfected DAF was anchored to the cell surface via GPI, biotinylated monolayers were extracted, treated with PI-PLC and DAF immunoprecipitated from the resulting aqueous phase. A major band of 65 kD was specifically immunoprecipitated only after treatment with PI-PLC and was present in an apically polarized distribution (Fig. 3; *right*). Thus, GPI-anchored DAF in MDCK was expressed with the same polarized distribution as endogenous DAF in two independent intestinal cell lines.

A possible cause of this polarized distribution of DAF was the action of an endogenous PI-PLC associated with the basolateral membrane. To evaluate whether DAF was being shed into the medium in a polarized fashion, we used a sensitive two-site IRMA that has been used extensively to measure nanogram quantities of DAF in a variety of body fluids (Medof et al., 1987). Analyses of the apical and basolateral media of filter-grown Caco-2, SK-CO15, and MDCK cells expressing transfected DAF failed to reveal the presence of DAF. These results suggest that the polarized apical distribution of DAF in these cell lines was not due to the activity of a basolaterally polarized GPI-anchor degrading enzyme, but was a consequence of the sorting processes carried out by the cells.

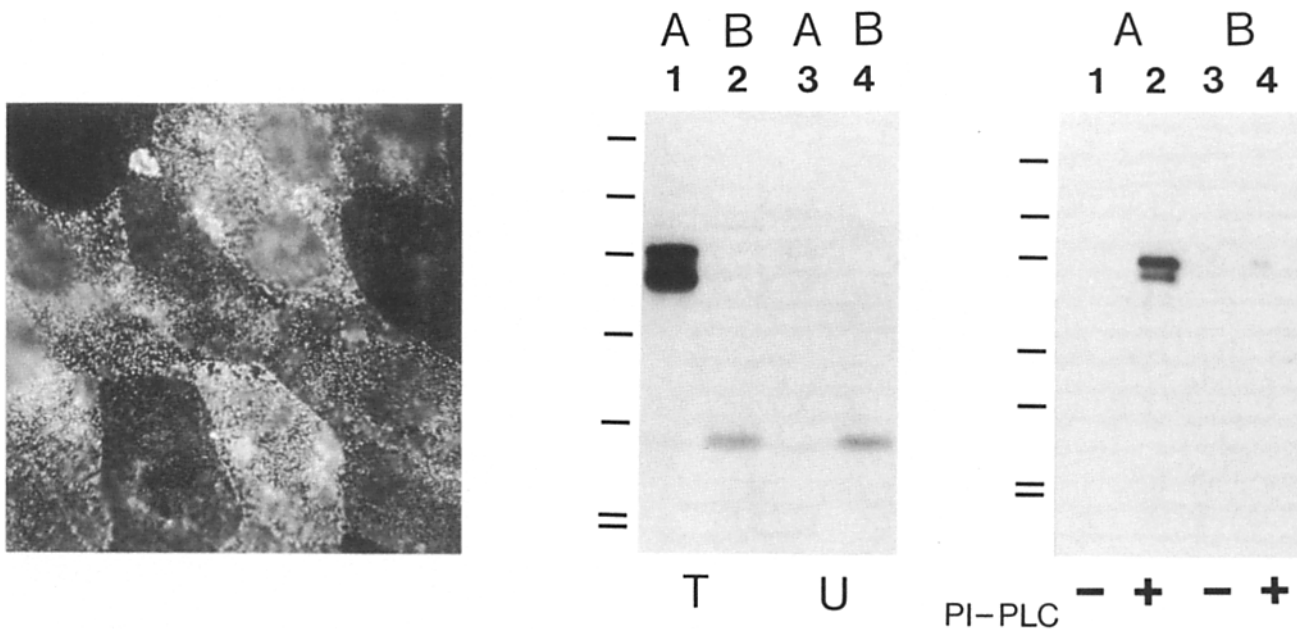


Figure 3. Polarized apical distribution of DAF in transfected MDCK cells. MDCK cells expressing DAF were seeded at high density onto glass coverslips or polycarbonate-based filter chambers. (Left) Coverslips, containing confluent monolayers, were incubated with anti-DAF IgG (1:100 dilution) and surface-bound anti-DAF IgG visualized with the appropriate secondary antibody coupled to rhodamine. (Middle) Filter-grown MDCK monolayers, 5–7 d confluent, were biotinylated either apically (A) or basolaterally (B) with sulfo-NHS-biotin. After cell lysis, anti-DAF mAbs coupled to Sepharose were added to extracts of transfected (T) MDCK cells expressing DAF or untransfected (U) MDCK cells. After immunoprecipitation, biotinylated proteins were visualized by [¹²⁵I]streptavidin blotting/autoradiography. (Right) Filter-grown transfected MDCK cells were subjected to domain-selective biotinylation, extraction with TX-114 and PI-PLC treatment (–, untreated; +, 6 U/ml). Resulting aqueous phases were immunoprecipitated with anti-DAF mAbs. Biotinylated proteins were visualized as described above. Note that transfected DAF is GPI anchored and present in an apically polarized distribution in MDCK cells. Molecular weight standards are as indicated in Fig. 1 (middle).

Apical Localization of DAF Fusion Proteins in MDCK Cells

gD-1-DAF Fusion Protein. To further study the role of GPI anchoring in targeting the attached protein to the apical surface, we used the DAF signal for GPI attachment fused to the ectodomain of a basolateral antigen, HSV-1 envelope glycoprotein D (gD-1). The fusion protein, containing 300 of the 340 residues thought to encode the extracellular domain of gD-1 (75% of gD-1) and the 37 COOH-terminal residues of DAF (10% of DAF) (Caras et al., 1987b), was expressed in MDCK cells by transfection of its cDNA. Immunofluorescence of positive clones indicated that a substantial proportion of gD-1-DAF was expressed on the apical surface (Fig. 4, a and b and Fig. 5, left). A cDNA encoding the native gD-1 glycoprotein was also transfected to evaluate the distribution of gD-1 in the absence of other viral components. Immunofluorescence localization of native gD-1 in transfected MDCK cells indicated that gD-1 was present primarily with nuclear/perinuclear localization, but also exhibited faint staining of the basolateral membranes (Fig. 4, c and d). This was not surprising, because members of the herpes virus family bud from the inner nuclear membrane, and only a fraction of their viral envelope glycoproteins apparently reaches the basolateral cell surface (Srinivas et al., 1986).

To determine the polarity of gD-1-DAF and native gD-1, high expressing clones were seeded on Transwells and subjected to domain selective labeling with sulfo-NHS-biotin (Fig. 5, middle). Apically or basolaterally biotinylated mono-

layers were extracted and immunoprecipitated with HSV-1 antibodies. gD-1-DAF appeared as a broad band with an M_r of 45–50 kD as reported previously (Caras et al., 1987b). Virtually all of the surface-biotinylated fusion protein was present on the apical surface. To verify that gD-1-DAF was anchored to the membrane via a GPI moiety, biotinylated monolayers (apically or basolaterally) were extracted and treated with PI-PLC followed by immunoprecipitation. GPI-anchored gD-1-DAF, corresponding to a M_r of ~45 kD, was also present in an apically polarized distribution (Fig. 5; right). The fusion protein appeared as a more compact/thinner band after PI-PLC treatment, as is observed with DAF (Davitz et al., 1986). Steady-state metabolic labeling detected a band of ~40 kD, in addition to the 45–50-kD band (Fig. 6 a, lane 2). Pulse chase experiments indicated that the 40 kDa species was rapidly converted (within minutes) to the 45–50-kD species, which was stable over at least 6 h of chase (Fig. 6 b; top); similar precursor-product relationships have been reported for gD-1-DAF in CHO cells (Caras et al., 1987b). Immunoprecipitation of the media after 6 h of chase did not detect any shedding of gD-1-DAF (Fig. 6 b; top).

In contrast to the fusion protein, native gD-1 could not be detected after cell-surface biotinylation (not shown), suggesting that the bulk of the protein was contained intracellularly. Steady-state metabolic labeling detected native gD-1 as two bands with M_r s of 60 and 70 kD (Fig. 6 a, lane 3). Pulse-chase experiments indicated that, in contrast to gD-1-DAF, native gD-1 was rapidly degraded (within 1–2 h) after processing from the 60- to the 70-kD form (Fig. 6 b; bot-

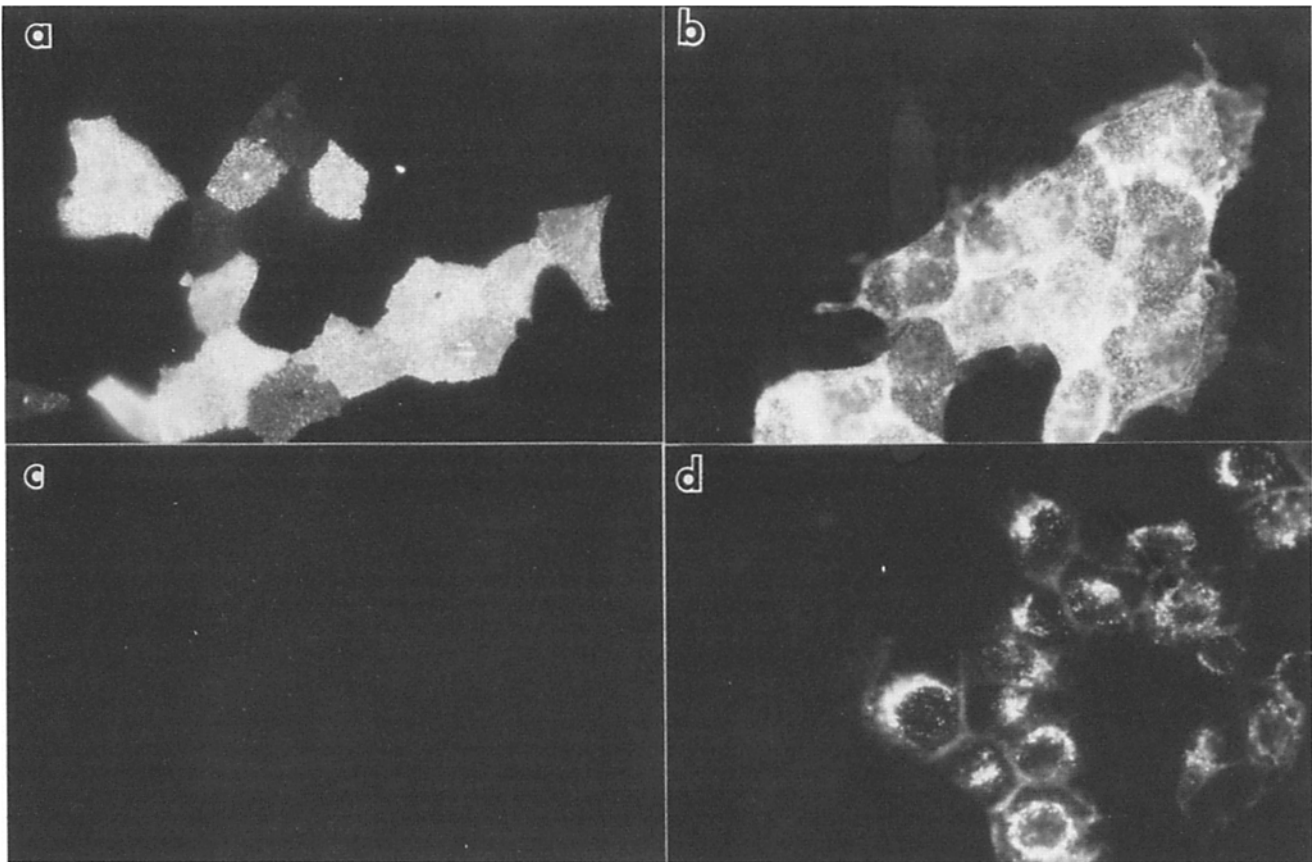


Figure 4. Immunofluorescent localization of gD-1-DAF fusion protein (*a* and *b*) and native gD-1 (*c* and *d*) in transfected MDCK cells. G418-resistant, transfected MDCK (uncloned cell populations) were seeded at high densities onto glass coverslips. Confluent monolayers were fixed and either permeabilized with saponin (*b* and *d*; to reveal intracellular and basolateral surface staining) or left intact (*a* and *c*; to visualize apical membrane staining). After incubation with anti-HSV rabbit IgG, bound antibody was detected with the appropriate rhodamine-conjugated second antibody. The gD-1-DAF fusion protein appeared in high concentration in the apical surface (note typical punctate pattern of microvilli), whereas native gD-1 was detected intracellularly (nuclear and perinuclear localization) and at the basolateral surface (faint intercellular rings).

tom), consistent with the low levels of basolateral surface fluorescence we observed (Fig. 4 *d*). Similar rapid turnover ($t_{1/2} \sim 1$ h) has been observed for another basolateral antigen, the VSV G protein, when expressed via transfection in MDCK cells (Compton et al., 1989).

Thus, replacement of the transmembrane and cytoplasmic domains of gD-1 with the DAF signal for GPI attachment drastically altered the fate of the fusion protein in MDCK cells. The GPI-anchored fusion protein was stable and specifically targeted to the apical cell surface with no release into the medium, whereas native gD-1 was synthesized but rapidly degraded intracellularly with only a minor fraction reaching the basolateral surface (as detected by immunofluorescence). Similarly, during viral infection of MDCK cells, native gD-1 is expressed primarily as a nuclear membrane antigen with a small fraction of the protein leaking to the basolateral cell surface (Srinivas et al., 1986), perhaps due to the high levels of expression characteristic of viral infection.

hGH-DAF Fusion Protein. GH, a regulated secretory protein, accumulates in secretory granules in its native environment (the anterior pituitary cell) and is released in response to specific stimuli (Niall et al., 1971). In contrast,

when transfected into epithelial cell lines, GH is released constitutively due to the absence of a regulated secretory pathway and is thought to be transported by "default" (without specific epithelial targeting information) to the cell surface (Kondor-Koch et al., 1985; Gottlieb et al., 1986; Rindler and Traber, 1988). In MDCK cells (derived from dog kidney) exogenous growth hormone is secreted "without polarity" and found in roughly equal quantities in the apical and basolateral medium (Kondor-Koch et al., 1985; Gottlieb et al., 1986). However, when expressed in Caco-2 cells, it is secreted in basolaterally polarized fashion, suggesting that the "default pathway" is basolaterally-directed in these cells (Rindler and Traber, 1988). Several other exogenous secretory proteins, both regulated (lysozyme, prochymosin) and constitutive (Ig- κ chain, liver $\alpha 2$ -microglobulin), were also reported to be secreted "without polarity" when expressed via transfection in MDCK cells (Kondor-Koch et al., 1985; Gottlieb et al., 1986).

To further explore the possibility that the GPI-anchor contains apical targeting information, we used the DAF signal for GPI attachment fused to a regulated secretory protein, GH. hGH containing the 37 COOH-terminal amino acids of DAF that signal GPI-attachment (Caras et al., 1989), was ex-

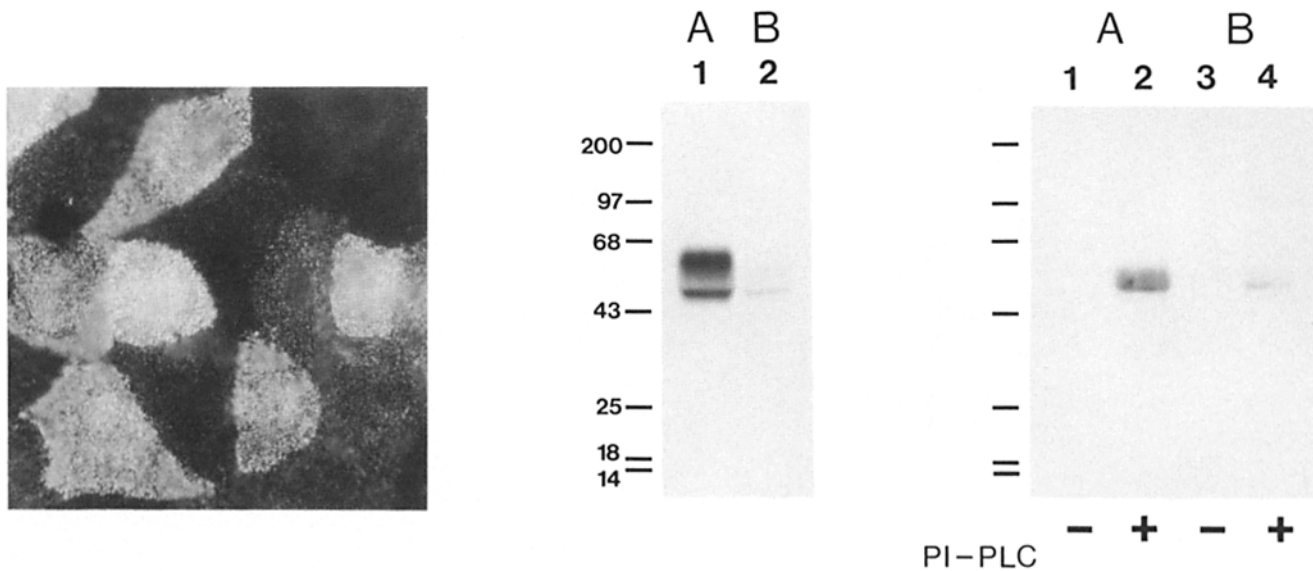


Figure 5. Apical localization of the gD-1-DAF fusion protein in transfected MDCK cells. Positive, high-expressing clones were seeded at high density onto coverslips or polycarbonate filter chambers. (Left) Surface immunofluorescence with anti-HSV-1 rabbit IgG (1:100 dilution). Bound IgG were visualized with rhodamine-conjugated goat anti-rabbit IgG (Fc). (Middle) Filter-grown monolayers expressing gD-1-DAF were subjected to domain-selective labeling with sulfo-NHS-biotin. Apically (A) or basolaterally (B) biotinylated monolayers were extracted and immunoprecipitated with anti-HSV rabbit IgG. (Right) Apically (A) or basolaterally (B) biotinylated monolayers were also extracted with TX-114 and treated with PI-PLC (-, untreated; +, 6 U/ml) to confirm that the fusion protein was GPI-anchored. Resulting aqueous phases were immunoprecipitated with anti-HSV IgG. Biotinylated proteins were visualized by [¹²⁵I]streptavidin blotting/auto-radiography. Molecular weight standards are indicated in kilodaltons (middle).

pressed via transfection in MDCK cells. High expressing clones displayed bright apical fluorescence (Fig. 7; left). Domain-selective biotinylation/[¹²⁵I]streptavidin blotting, coupled with immunoprecipitation using anti-hGH-IgG, confirmed the apical polarity of hGH-DAF (Fig. 7; middle). Greater than 95% was present in an apically polarized distribution; PI-PLC sensitive hGH-DAF was also present in an apically-polarized distribution, confirming that this fusion protein was GPI-anchored (Fig. 7; right).

Native hGH was also expressed in MDCK cells to confirm that it is secreted in a nonpolarized fashion. Substantial amounts of hGH were detected by RIA in both the apical and basolateral medium, however, the ratios varied from clone to clone and this variation did not appear to be dependent

upon the level of expression. Roughly, 45–80% of hGH appeared in the apical medium with 20–55% appearing in the basolateral medium (Table I). Such variation may be due in part to the fact that MDCK cell line is heterogeneous (Ojakian et al., 1987). Furthermore, transient expression or uncloned populations of permanently expressing cells were primarily used to evaluate the “bulk flow” markers previously (Gottlieb et al., 1986; Rindler and Traber, 1988). Nevertheless, our results demonstrate that a secretory protein can be directed to the apical surface by addition of the DAF signal for GPI attachment.

Polarized Secretion of Anchor-Minus gD-1 and DAF

To determine whether the ectodomains of gD-1 and DAF

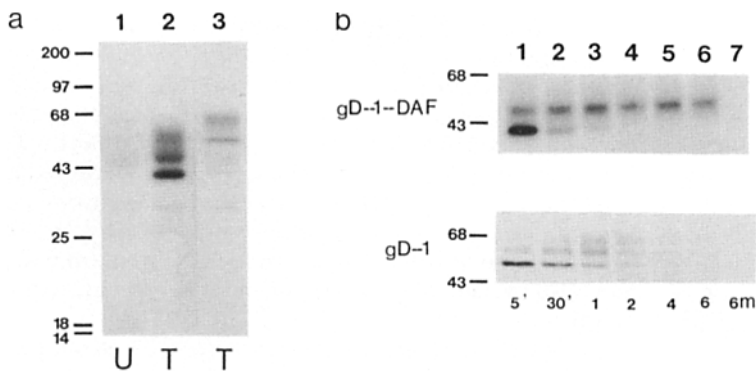


Figure 6. Metabolic labeling (a, steady-state; b, pulse-chase) of gD-1-DAF fusion protein and native gD-1 in transfected MDCK cells. (a) For steady-state labeling, cells were incubated overnight (12–16 h) with 100 μ Ci/ml of both [³⁵S]cys and met in DME containing one-tenth the normal concentration of cys and met. Cells were lysed and immunoprecipitated with anti-HSV-IgG: lane 1, untransfected (U) control; lane 2, gD-1-DAF-expressing cells; and lane 3, native gD-1-expressing cells. T denotes transfected, high-expressing clones. (b) gD-1-DAF- (top) and native gD-1- (bottom) expressing clones were pulsed with 1 mCi/ml of both [³⁵S]cys and met for 10 min and chased for various times (as indicated) into media containing excess met and cys. Cells were lysed and immunoprecipitated with anti-HSV IgG. In addition, after 6 hours of chase the media was immunoprecipitated (6 m) to detect spontaneous shedding or secretion. Note that gD-1-DAF is rapidly processed from a 40-kD to a 45–50-kD form that is stable, whereas native gD-1 is converted from a 60- to a 70-kD form and rapidly degraded (between 1 and 2 h of chase). Molecular weight standards are indicated and are expressed in kilodaltons.

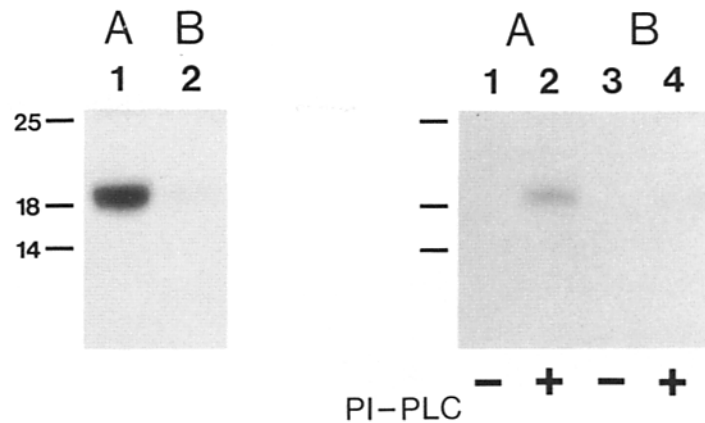


Figure 7. Apical localization of the hGH-DAF fusion protein. Transfected MDCK cells, expressing hGH-DAF, were plated at high density onto glass coverslips or polycarbonate filters. (Left) Anti-hGH rabbit IgG's (diluted 1:100) were added without detergent permeabilization to localize hGH-DAF expressed on the apical surface. Bound IgG were detected with the appropriate rhodamine-conjugated secondary antibody. (Middle) Confluent filter-grown monolayers were subjected to apical (A) or basolateral (B) biotinylation to detect hGH-DAF present on either cell surface. After biotinylation, monolayers were extracted and immunoprecipitated with anti-hGH rabbit IgG. (Right) Apically (A) or basolaterally (B) biotinylated monolayers were also extracted, PI-PLC-treated (-, untreated; +, 6 U/ml) and immunoprecipitated with anti-hGH to determine the polarity of GPI-anchored hGH-DAF. Biotinylated proteins were detected by [¹²⁵I]streptavidin blotting/autoradiography. Molecular weight standards are as indicated and are expressed in kilodaltons (middle).

possess sorting information, we expressed truncated forms of these proteins. cDNAs encoding residues 1-300 of the gD-1 ectodomain (a protein segment identical to that present in the gD-1-DAF fusion protein, Caras et al., 1987b) and a secretory form of DAF (Δ 1 DAF, lacking the last 17 COOH-terminal aminoacids required for GPI anchoring, Caras et al., 1989) were transfected into MDCK cells. For both proteins, a concentration-dependent effect was noticed. Clones expressing low levels of the transfected gene products exhibited polarized secretion, with truncated gD-1 secreted basolaterally (up to 90%) and Δ 1 DAF secreted apically (up to 90%) (Fig. 8). On the other hand, in higher expressing clones, both secretory proteins were released in a relatively unpolarized fashion, with roughly similar amounts present in the apical and basolateral media (data not shown).

These results suggest that sorting information is present within the ectodomains of gD-1 and DAF and that correct sorting of these secretory proteins is concentration-dependent and saturable. Because the ectodomain of gD-1 contains

basolateral sorting information, our results with gD-1-DAF indicate that GPI-anchoring can redirect a basolateral antigen to the apical surface. In addition, based on our results with anchor-minus DAF, redundant apical targeting information can be present in the ectodomain of GPI-anchored proteins. Apical secretion of an anchor-minus form of another GPI-anchored protein, placental alkaline phosphatase, has recently been observed (Brown, D., and J. Rose, personal communication).

Discussion

The sorting signals that direct the intracellular trafficking and vectorial delivery of membrane glycoproteins to either the apical or basolateral cell surface of polarized epithelia are currently an area of intense interest. Here, we used DAF and its signal for GPI attachment to investigate the role of the GPI-anchoring mechanism as a possible apical targeting signal. The results of this report indicate that DAF is expressed on the apical surface of two different intestinal cell lines. After transfection of its cDNA into MDCK cells, 95% of DAF was expressed on the apical surface, as detected with a biotin polarity assay. This indicates that the DAF signal(s) for apical localization are conserved between intestinal and renal epithelial cells. Presumably, the same distribution would be found in a variety of native epithelia where the presence of DAF has been documented previously, e.g., gastrointestinal mucosa, renal tubules, exocrine glands, and uterine mucosa (Medof et al., 1987). This is supported by the detection of a soluble form of DAF in urine, saliva, cerebrospinal fluid, and tears (Medof et al. 1987), which may be the result of phospholipase cleavage of apical DAF.

The apical localization of DAF is consistent with our previous observation that endogenous GPI-anchored proteins are apically polarized in MDCK cells (Lisanti et al., 1988). Because the only obvious common feature of this class of membrane proteins is the possession of a GPI-anchor, we

Table I. Secretion of Native hGH in Transfected MDCK Cells

Clones	[hGH] (ng/ml)		% Ap
	Ap	Bl	
A	370	255	59.2
B	42	34	58.0
C	90	30.5	74.2
D	250	66	79.1
E	22	25	46.8
F	170	60	73.9

Filter-grown monolayers (6 d confluent) of MDCK cells expressing native hGH were analyzed by a sensitive IRMA for hGH. The concentration of hGH found in both the apical (Ap) and the basolateral (Bl) medium (after 12-16 h of incubation) is shown for six independent clones (labeled A through F). Analysis of each clone was performed in duplicate. The % of total hGH that appeared in the apical compartment (% Ap) is listed at right.

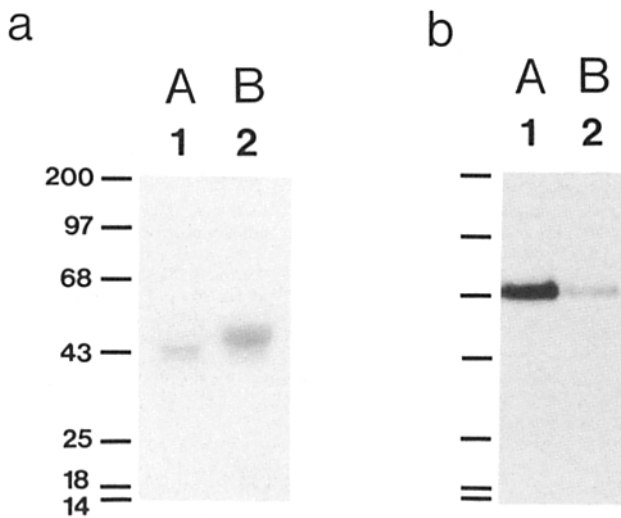


Figure 8. Polarized secretion of anchor-minus gD-1 and $\Delta 1$ DAF in transfected MDCK cells. (a) truncated gD-1; (b) $\Delta 1$ DAF. Filter-grown monolayers were metabolically labeled overnight (see Materials and Methods); sodium butyrate was employed to elevate expression levels. Apical (A) and basolateral (B) media were collected, centrifuged (14,000 g for 30 s) and protease inhibitors added. An aliquot of medium (200 μ l) was then diluted fivefold with TBST and immunoprecipitated with either anti-HSV or anti-DAF IgG. Note that truncated gD-1 is secreted in a predominantly basolateral fashion, whereas $\Delta 1$ DAF secretion is primarily apical. Molecular weight standards are as indicated and are expressed in kilodaltons.

speculated that this membrane anchoring mechanism might contribute the necessary information for targeting to the apical surface. To test this hypothesis further, we studied the distribution of two DAF fusion proteins (gD-1-DAF and hGH-DAF) after transfection of their cDNAs into MDCK cells. Both fusion proteins were linked to the membrane via GPI after transfection into MDCK cells (as shown by their sensitivity to PI-PLC).

During viral infection, gD-1, as well as other Herpes simplex viral glycoproteins, is primarily localized on the nuclear membrane and secondarily on the basolateral surface of MDBK cells (Srinivas et al., 1986), a cell line of bovine origin that supports viral budding with the same polarity as MDBK cells (Rodriguez-Boulant and Sabatini, 1978). In MDBK cells transfected with native gD-1 cDNA (this report), gD-1 was processed to a higher molecular mass form which was rapidly degraded with a half-life of ~ 1 h; only a small fraction reached the basolateral cell surface (as seen by immunofluorescence). In contrast, truncated gD-1 (lacking transmembrane and cytoplasmic domains) is stable and secreted basolaterally, suggesting that the ectodomain contains basolateral sorting information. However, when anchored via GPI, the ectodomain of gD-1 is targeted with high efficiency to the apical surface of MDCK cells. These results suggest that the GPI-anchoring mechanism can act as a dominant apical sorting signal because it overrides basolateral sorting information and redirects a basolateral secretory protein to the apical surface.

In our experiments, native hGH was secreted significantly from both apical and basolateral surfaces, but with a variable polarity ranging from 45 to 80% apical in different transfected MDCK clones. Similar variability has been observed

for MDCK transfectants expressing anchor-minus forms of both polymeric-Ig receptor (Mostov et al., 1987) and DAF (Lisanti et al., unpublished results). This variability may reflect variations in the default pathway between different MDCK clonal cell populations. Different expression methods and times of confluency of the monolayers when measurements were made may contribute additional variation between different laboratories. The fraction of hGH secreted to the apical medium by the most apically polarized hGH-secreting clone increases as a function of time of confluency from 60 (2 d) to 70 (4 d) and 75% (6–8 d) (Lisanti et al., unpublished results). Because endogenous apical secretory proteins (30–40-kD group) appear to be highly polarized (>85%) early after confluency (2–3 d, Kondor-Koch et al., 1985; Gottlieb et al., 1986), it seems that the MDCK secretory pathway may become progressively more polarized as the monolayer matures and behave distinctly from the putative signal-mediated apical secretory pathway.

Our results clearly show that two fusion proteins (gD-1-DAF and hGH-DAF), membrane-anchored via GPI encoded by the COOH-terminal 37-amino acid DAF sequence, are expressed with an apically polarized distribution on the surface of transfected MDCK cells. DAF itself, when expressed in MDCK cells from its transfected cDNA, is also highly polarized to the apical surface. The possibility that the apical polarity of these proteins results from following passively an apical default membrane pathway in MDCK cells is highly unlikely because (a) they have higher polarity levels (>90%) than secreted hGH (45–80%), and (b) transfected DAF expressed in MDCK cells is as highly apically polarized as in intestinal cells (Caco-2 and SK-CO15), where the default secretory pathway is basolateral (Rindler and Tauber, 1988; LeBivic, Rodriguez-Boulant et al., manuscript in preparation) and where (in Caco-2) transfected growth hormone is secreted basolaterally.

Our experiments with endogenous (Lisanti et al., 1988; this work) and transfected GPI-anchored proteins suggest that the GPI-membrane anchoring mechanism may convey apical targeting information. However, we cannot eliminate the possibility that a short DAF sequence remaining after GPI-attachment (8–10 amino acids, Caras, I., unpublished observations) may contribute to the apical targeting of DAF and the DAF-fusion proteins we studied. This sequence, which contains a putative cleavage/attachment site, cannot be reduced significantly without preventing GPI-anchoring (Caras et al., 1989). It is highly unlikely that this short sequence is responsible for apical targeting because (a) no obvious similarity exists between these 8–10 amino acids and corresponding regions of other apically polarized GPI-anchored proteins (Ferguson and Williams, 1988); (b) replacement of this sequence by a completely different upstream DAF sequence also results in GPI anchoring (Caras et al., 1989) and targeting of DAF and hGH-DAF to the apical surface of transfected MDCK cells (Lisanti et al., unpublished observations); and (c) the ectodomain of VSV G protein (a basolateral antigen) is targeted to the apical surface when linked to the Thy-1 signal for GPI-attachment (which leaves a completely different sequence remaining in the fusion protein; D. Brown and J. Rose, personal communication). On the other hand, the GPI moiety appears as a more likely candidate to mediate apical targeting because it is the only element clearly common to all apical GPI-anchored

proteins and possesses several highly conserved structural features.

Recent work has shown that several glycolipids are preferentially localized on the apical surface of epithelial cells, where they may constitute virtually 100% of the exoplasmic leaflet of the lipid bilayer (van Meer, 1988). Sorting of these glycosphingolipids appears to take place intracellularly in the *trans*-Golgi network (TGN) (van Meer et al., 1987), the putative sorting site for apical and basolateral membrane proteins in MDCK cells (Griffiths and Simons, 1986; Rodriguez-Boulan and Salas, 1989). This has led to the hypothesis that sorting of apical proteins and glycolipids may be linked (van Meer and Simons, 1988; Wandinger-Ness and Simons, 1989). Our results with GPI-anchored proteins provide support for this hypothesis (Rodriguez-Boulan and Nelson, 1989). Because certain glycolipids have a strong tendency towards self-aggregation (Thompson and Tillack, 1985), non-GPI-anchored proteins might be sorted through partition into glycolipid clusters in the TGN (van Meer and Simons, 1988). Coclustering of glycolipids and GPI-anchored proteins may promote their incorporation into apically targeted post-TGN vesicles. This hypothesis is amenable to experimental testing.

We thank Dr. Martin Low for his generous gift of PI-PLC; Dr. Alain Zweibaum (Villejuif, France) for Caco-2 cells and Drs. Francisco Real and Lloyd Old for SK-CO15 cells; Dr. Andre Le Bivic for kindly helping with Caco-2 and SK-CO15 cell cultures; Dr. Lutz Graeve for assistance with transfections and selection of MDCK clones; Dr. Erik Falck-Pedersen and JogiRaju Tantravahi for HeLa cells; Ms. Lori Van Houten for photographic work; and Ms. Francine Sanchez and Michelle Garcia for typing the manuscript.

M. P. Lisanti was supported by a Medical Scientist Training grant from the Cornell University M.D.-Ph.D. program; I. W. Caras was supported by Genentech, Inc.; M. A. Davitz, is a Lucille P. Markey (LPM) Scholar and was supported in part by a grant from the LPM Charitable Trust; and E. Rodriguez-Boulan by grants from the National Institutes of Health (GM-34107 and HL-37675) and the American Heart Association (grant in aid from New York Branch and Established Investigator Award).

Received for publication 16 June 1989 and in revised form 12 July 1989.

Note added in proof: We have recently observed that the apical polarity of endogenous GPI-anchored proteins is highly conserved across species and tissue type and is partially disrupted in a lectin-resistant MDCK mutant cell line. (Lisanti, M. P., A. LeBivic, A. R. Saltiel, and E. Rodriguez-Boulan. 1989. Preferred apical distribution of GPI-anchored proteins: a highly conserved feature of the polarized epithelial cell phenotype. *J. Membr. Biol.* In press.)

References

Bangs, J. D., D. Hereld, J. L. Krakow, G. W. Hart, and P. T. Englund. 1985. Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein. *Proc. Natl. Acad. Sci. USA.* 82:3207-3211.

Berger, J., A. D. Howard, L. Brink, L. Gerber, J. Hauber, and B. R. Cullen. 1988. COOH-terminal requirements for the correct processing of a phosphatidylinositol-glycan-anchored membrane protein. *J. Biol. Chem.* 263:10016-10021.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604-1607.

Caplan, M. J., H. C. Anderson, G. E. Palade, and J. D. Jamieson. 1986. Intracellular sorting and polarized cell surface delivery of (Na⁺, K⁺) ATPase, an endogenous component of MDCK cell basolateral plasma membranes. *Cell.* 46:623-631.

Caras, I. W., M. A. Davitz, L. Rhee, G. Weddell, D. W. Martin, and V. Nussen-zweig. 1987a. Cloning of decay-accelerating suggests novel use of splicing to generate two proteins. *Nature (Lond.)* 325:545-548.

Caras, I. W., and G. N. Weddell. 1989. Signal peptide for protein secretion directing glycosphingolipid membrane anchor attachment. *Science (Wash. DC).* 243:1196-1198.

Caras, I. W., G. N. Weddell, M. A. Davitz, V. Nussen-zweig, and D. W. Martin. 1987b. Signal for attachment of a phospholipid membrane anchor in decay accelerating factor. *Science (Wash. DC).* 238:1280-1283.

Caras, I. W., G. N. Weddell, and S. R. Williams. 1989. Analysis of the signal for attachment of a glycosphingolipid membrane anchor. *J. Cell Biol.* 108:1387-1396.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with a water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132-135.

Compton, T., I. E. Ianov, T. Gottlieb, M. Rindler, M. Adesnik, and D. Sabatini. 1989. A sorting signal for the basolateral delivery of the VSV G protein lies in its luminal domain: analysis of the targeting of VSV G-influenza HA chimeras. *Proc. Natl. Acad. Sci. USA.* 86:4112-4116.

Conzelmann, A., A. Spiazzi, R. Hyman, and C. Bron. 1986. Anchoring of membrane proteins via phosphatidylinositol is deficient in two classes of Thy-1 negative mutant lymphoma cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3291-3296.

Conzelmann, A., A. Spiazzi, and C. Bron. 1987. Glycolipid anchors are attached to Thy-1 glycoprotein rapidly after translation. *Biochem. J.* 246:605-610.

Cross, G. A. M. 1987. Eukaryotic protein modification and membrane attachment via phosphatidylinositol. *Cell.* 48:179-181.

Davitz, M. A., M. G. Low, and V. Nussen-zweig. 1986. Release of decay-accelerating factor (DAF) from the cell membrane by phosphatidylinositol-specific phospholipase C (PI-PLC). *J. Exp. Med.* 163:1150-1161.

Ferguson, M. A. J., M. Duszenko, G. S. Lamont, P. Overath, and G. A. M. Cross. 1986. Biosynthesis of Trypanosoma brucei variant surface glycoproteins. *J. Biol. Chem.* 261:356-362.

Ferguson, M. A. J., and A. F. Williams. 1988. Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. *Annu. Rev. Biochem.* 57:285-320.

Fogh, J., and G. Trempe. 1975. New human tumor cell lines. In *Human Tumor Cells In Vitro*. J. Fogh, editor. Plenum Publishing Corp., New York. 115-140.

Gorman, C. M., and B. H. Howard. 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* 11:7631-7648.

Gottlieb, T. A., G. Beaudry, L. Rizzolo, A. Colman, M. Rindler, M. Adesnik, and D. D. Sabatini. 1986. Secretion of endogenous and exogenous proteins from polarized MDCK cell monolayers. *Proc. Natl. Acad. Sci. USA.* 83:2100-2104.

Grasset, E., M. Pinto, E. Dussaulx, A. Zweibaum, and J. F. Desjeux. 1984. Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. *Am. J. Physiol.* 247:C260-C267.

Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456-467.

Griffiths, G., and K. Simons. 1986. The *trans* Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438-443.

Hauri, H. P., E. E. Sterchi, D. Bienz, J. A. M. Fransen, and A. Marxen. 1985. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. *J. Cell Biol.* 101:838-851.

He, H., J. Finne, and C. Goriadis. 1987. Biosynthesis, membrane association, and release of N-CAM-120, a phosphatidylinositol-linked form of the neural cell adhesion molecule. *J. Cell Biol.* 105:2489-2500.

Kondor-Koch, C., R. Bravo, S. D. Fuller, D. Cutler, and H. Garoff. 1985. Exocytic pathways exist to both the apical and the basolateral cell surface of the polarized epithelial cell MDCK. *Cell.* 43:297-306.

Koval, M., and R. E. Pagano. 1989. Lipid recycling between the plasma membrane and intracellular compartments. *J. Cell Biol.* 108:2169-2181.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature (Lond.)* 227:680-685.

LeBivic, A., F. Real, and E. Rodriguez-Boulan. 1989. Vectorial targeting of apical and basolateral plasma membrane proteins in a human intestinal epithelial cell line. *Proc. Natl. Acad. Sci. USA.* In press.

Lisanti, M. P., M. Sargiacomo, L. Graeve, A. R. Saltiel, and E. Rodriguez-Boulan. 1988. Polarized apical distribution of glycosyl-phosphatidylinositol anchored proteins in a renal epithelial cell line. *Proc. Natl. Acad. Sci. USA.* 85:9557-9561.

Low, M. G., M. A. J. Ferguson, A. H. Futterman, and I. Silman. 1986. Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins. *Trends Biochem. Sci.* 11:212-214.

Low, M., and A. R. Saltiel. 1988. Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science (Wash. DC).* 239:268-275.

Maddon, P. J., D. R. Littman, M. Godfrey, D. E. Maddon, L. Chess, and R. Axel. 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell.* 42:93-104.

Medof, M. E., E. I. Walter, W. L. Roberts, R. Haas, and T. L. Rosenberry. 1986. Decay accelerating factor of complement is anchored to cells by a C-terminal glycolipid. *Biochemistry.* 25:6740-6747.

Medof, M. E., E. I. Walter, J. L. Rutgers, D. M. Knowles, and V. Nussen-zweig. 1987. Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. *J. Exp. Med.* 165:848-864.

Mostov, K. E., P. Breitfeld, and J. Harris. 1987. An anchor-minus form of the

- polymeric immunoglobulin receptor is secreted predominantly apically in Madin-Darby canine kidney cells. *J. Cell Biol.* 105:2031-2036.
- Niall, H. D., M. L. Hogan, R. Sayer, I. Y. Rosenblum, and F. C. Greenwood. 1971. Sequences of pituitary and placental lactogenic and growth hormones: evolution from a primordial peptide by gene reduplication. *Proc. Natl. Acad. Sci. USA.* 68:866-869.
- Ojakian, G. K., R. E. Romain, and R. E. Herz. 1987. A distal nephron glycoprotein that has different cell surface distributions in MDCK sublines. *Am. J. Physiol.* 253:C433-C443.
- Pagano, R. E., and R. G. Sleight. 1985. Defining lipid transport pathways in animal cells. *Science (Wash. DC).* 229:1051-1057.
- Pinto, M., S. Robine-Leon, M. D. Appay, M. Keding, N. Triadou, E. Dusaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell.* 47:323-330.
- Rindler, M. J., and M. G. Traber. 1988. A specific sorting signal is not required for the polarized secretion of newly synthesized proteins from cultured intestinal epithelial cells. *J. Cell Biol.* 107:471-479.
- Rodriguez-Boulan, E. 1983. Polarized budding of viruses from epithelial cells. *Methods Enzymol.* 98:486-501.
- Rodriguez-Boulan, E., and W. J. Nelson. 1989. Morphogenesis of the epithelial cell phenotype. *Science (Wash. DC).* 245:718-725.
- Rodriguez-Boulan, E., and D. Sabatini. 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. *Proc. Natl. Acad. Sci. USA.* 75:5071-5075.
- Rodriguez-Boulan, E., and P. J. I. Salas. 1989. External and internal signals for surface molecular polarization of epithelial cells. *Annu. Rev. Physiol.* 51:741-754.
- Rousset, M. 1986. The human colon carcinoma cell lines HT-29 and Caco-2: Two in vitro models for the study of intestinal differentiation. *Biochimie (Paris).* 68:1035-1040.
- Sargiacomo, M., M. P. Lisanti, L. Graeve, A. LeBivic, and E. Rodriguez-Boulan. 1989. Integral and peripheral protein composition of the apical and basolateral membrane domains in MDCK cells. *J. Membr. Biol.* 107:277-286.
- Srinivas, R. V., N. Balachandran, F. V. Alonso-Caplen, and R. W. Compans. 1986. Expression of Herpes simplex virus glycoproteins in polarized epithelial cells. *J. Virol.* 58:689-693.
- Thompson, T. E., and T. W. Tillack. 1985. Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells. *Annu. Rev. Biophys. Chem.* 14:361-386.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Urban, J., K. Parczyk, A. Leutz, M. Kayne, and C. Kondor-Koch. 1987. Constitutive apical secretion of an 80-kD sulfated glycoprotein complex in the polarized epithelial Madin-Darby canine kidney cell line. *J. Cell Biol.* 105:2735-2743.
- van Meer, G., E. H. K. Stelzer, R. W. Wijnaendts-van Resandt, and K. Simons. 1987. Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J. Cell Biol.* 105:1623-1635.
- van Meer, G., and K. Simons. 1988. Lipid polarity and sorting in epithelial cells. *J. Cell Biochem.* 36:51-58.
- van Meer, G. 1988. How epithelia grease their microvilli. *Trends Biochem. Sci.* 13:242-243.
- von Bonsdorff, C.-H., S. D. Fuller, and K. Simons. 1985. Apical and basolateral endocytosis in MDCK cells grown on nitrocellulose filters. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2781-2792.
- Wandiger-Ness, A., and K. Simons. 1989. The polarized transport of surface proteins and lipids in epithelial cells. In *Intracellular Trafficking of Proteins*. J. Hanover and C. Steer, editors. Cambridge University Press, New York. In press.
- Waneck, G. L., D. H. Sherman, P. W. Kinkade, M. G. Low, and R. A. Flavell. 1988. Molecular mapping of signals in the Qa-2 antigen required for attachment of the phosphatidylinositol membrane anchor. *Proc. Natl. Acad. Sci. USA.* 98:577-581.