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Preferred Apical Distribution of Glycosyl-Phosphatidylinositol (GPI) Anchored Proteins: A Highly Conserved Feature of the Polarized Epithelial Cell Phenotype

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Summary. We use a sensitive biotin polarity assay to survey the surface distribution of glycosyl-phosphatidylinositol (GPI) anchored proteins in five model epithelial cell lines derived from different species (dog, pig, man) and tissues, i.e., kidney (MDCK I, MDCK II, LLC-PK₁) and intestine (Caco-2 and SK-CO15). After biotinylation of apical or basolateral surfaces of confluent monolayers grown on polycarbonate filters, GPI-anchored proteins are identified by their shift from a Triton X-114 detergent-rich phase to a detergent-poor phase in the presence of phosphatidylinositol-specific phospholipase C. All GPI-anchored proteins detected (3–9 per cell type, at least 13 different proteins) are found to be apically polarized; no GPI-anchored protein is observed preferentially localized to the basal surface. One of the GPI-anchored proteins is identified as carcinoembryonic antigen (CEA). Survey of MDCK II-RCA^r, a mutant cell line with a pleiotropic defect in galactosylation of glycoproteins and glycolipids (that presumably affects GPI anchors) also reveals an apical polarization of all GPI-anchored proteins. In contrast, analysis of MDCK II-ConA^r (a mutant cell line with an unknown defect in glycosylation) revealed five GPI-anchored proteins, two of which appeared relatively unpolarized. Our results indicate that the polarized apical distribution of GPI-anchored proteins is highly conserved across species and tissue-type and may depend on glycosylation.

Key Words protein targeting · biotin labeling · epithelial polarity · glycolipids · glycosyl-phosphatidylinositol

Introduction

The conventional mechanism by which glycoproteins are anchored to the cell surface of mammalian cells involves a stretch of relatively hydrophobic amino acids which acts both to anchor the extracellular domain to the cell surface and as a stop transfer signal during translocation to the lumen of the ER (Blobel, 1980). An alternative mechanism for membrane protein anchoring involves the covalent linkage of the extracellular protein domain via a glycolipid, glycosyl-phosphatidylinositol (GPI) (Low et al., 1986; Cross, 1987; Low & Saltiel, 1988).

More specifically, the C-terminal amino acid is bound (via ethanolamine) to an oligosaccharide chain, which in turn is linked to the inositol ring of phosphatidylinositol (Ferguson & Williams, 1988). During synthesis, the precursor polypeptide containing a C-terminal hydrophobic sequence is cleaved and the glycolipid is added *en bloc* (<1 min after synthesis) in the endoplasmic reticulum (Bangs et al., 1985; Ferguson et al., 1986; Conzelmann, Spiazzi & Bron, 1987; He, Finne & Goridis, 1987). Although the basic structural features of this glycolipid have been identified, relatively little is known about the precise function of this new mode of protein anchoring.

Recent work has provided suggestive evidence that, after insertion in the membrane of the endoplasmic reticulum, integral membrane proteins are transported by default with the bulk membrane flow along the secretory pathway (Wieland et al., 1987; reviewed by Pfeiffer & Rothman, 1987). Proteins destined to remain in the endoplasmic reticulum or the Golgi apparatus are believed to possess specific structural information (“retention signals”) that, through interaction with putative organelle-specific receptors, allow for retention at these organelles (Machamer & Rose, 1987; Poruchynsky & Atkinson, 1988).

At the Golgi apparatus, however, sorting “decisions” are made that imply the existence of specific “organelle targeting” mechanisms, in addition to the retention systems discussed above. In one case, that of lysosomal hydrolases, a specific signal has been uncovered, mannose-6-phosphate (reviewed by Sly, 1982), that via a specific Golgi receptor, *targets* these proteins to the lysosomes. Plasma membrane proteins are believed to proceed by default to the cell surface. However, in epithelial cells some plasma membrane proteins are sorted in the Golgi apparatus into apically or basolaterally tar-

geted vesicles, which implies that at least one of these pathways to the cell surface is signal mediated (reviewed by Rodriguez-Boulan & Salas, 1989; Wandinger-Ness & Simons, 1989). In spite of several studies with chimeras of apical and basolateral proteins, the nature of the sorting information in these proteins remains unsolved.

Here, we present strong correlative evidence for a role of GPI-anchors in the sorting of attached proteins to the apical surface. We employ an improved-sensitivity version of a polarity assay for GPI-anchored proteins (Lisanti et al., 1988) that takes advantage of several conserved structural features of this mode of protein anchoring, to survey the surface distribution of these proteins in several model polarized epithelial cell lines. Preliminary observations with MDCK cells identified six GPI-anchored proteins, all of them preferentially distributed to the apical surface (Lisanti et al., 1988). The results reported here indicate that the polarized apical distribution of GPI-anchored proteins is highly conserved across species and tissue-type, and may be a generalized feature of the polarized epithelial cell phenotype.

Materials and Methods

MATERIALS/REAGENTS

Sulfosuccinimidobiotin (sulf-NHS-biotin) was purchased from Pierce (Rockford, IL). Phenyl-Sepharose, bovine serum albumin (BSA) and Triton X-114 (TX-114) were obtained from Sigma (St. Louis, MO). Phosphatidylinositol-specific phospholipase C (PI-PLC) [purified from *Bacillus thuringiensis*], was the generous gift of Martin Low (Columbia University College of Physicians and Surgeons, New York). Caco-2 was the gift of Dr. Alain Zweibaum (Unite de Recherches sur le Metabolisme et la Differentiation de Cellules en Culture, INSERM U178; Villejuif, France); SK-CO15 was the gift of Dr. Francisco Real (Instituto Municipal de Investigaciones Medicas; Barcelona, Spain). Streptavidin and molecular mass markers were from Bethesda Research Laboratories. Carcinoembryonic antigen (CEA) antibodies (polyclonal, rabbit anti-human) were obtained from Dako (Santa Barbara, CA). Goat anti-rabbit IgG, conjugated to alkaline phosphatase, and the appropriate substrates for color reaction were from Promega (Madison, WI).

CELLS AND CELL CULTURE

Madin-Darby Canine Kidney (MDCK), types I and II, MDCK II-RCA^r, MDCK II-ConA^r, as well as LLC-PK₁, were maintained in Dulbecco's modified Eagle's medium (DME) (Gibco, Grand Island, NY) supplemented with 10% (vol/vol) horse serum (HS) (Hyclone Laboratories, Logan, UT) and antibiotics (Rodriguez-Boulan, 1983). Caco-2 and SK-CO15 were maintained in DME supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Gibco) and nonessential amino acids.

Cells from a single confluent 75-cm² flask were trypsinized and transferred to six Transwell filter chambers (24.5 mm, 0.4 µm polycarbonate, tissue cultured treated) [Costar, Cambridge, MA] (van Meer et al., 1987). Media was changed every two days for renal cell lines and every day for intestinal cell lines. For the development of a tight, well-differentiated epithelial monolayer, approximately 7–9 and 11–14 days after plating were required for renal and intestinal cell lines, respectively.

In certain experiments, cells were serum starved 12–16 hr before processing in DME containing 0.2% bovine serum albumin. Filter-grown monolayers were assayed for impermeability to [³H]-inulin (NEN, 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 hr at 37°C) were discarded.

DOMAIN-SELECTIVE LABELING WITH SULFO-NHS-BIOTIN

Biotinylation was performed essentially as described previously (Lisanti et al., 1988; Sargiacomo et al., 1989). Filter-grown monolayers were washed (3×, 10 min) with ice-cold phosphate buffered saline containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS-C/M). Sulfo-NHS-biotin (0.5 mg/ml in PBS-C/M) was added either to the apical or basolateral compartment of the filter chamber. Compartments not receiving sulfo-NHS-biotin were filled with an equivalent volume of PBS-C/M alone. After 30 min of agitation at 4°C, filter chambers were incubated with ice-cold serum-free DME (1×, 10 min) and washed with ice-cold PBS-C/M (2×). Three filter chambers were used per experimental condition.

After extraction with Triton X-114 and analysis by SDS-PAGE and ¹²⁵I-streptavidin blotting, the overall amount of integral and peripheral proteins appeared greater on the basolateral side (for all seven cell lines studied) (*data not shown*; Lisanti et al., 1988; Le Bivic, Real & Rodriguez-Boulan, 1989; Sargiacomo et al., 1989), consistent with the notion that the basolateral domain usually exceeds the apical domain in surface area (von Bonsdorff, Fuller & Simons, 1985; Vega-Salas et al., 1987). Immunofluorescence on semi-thin 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). Furthermore, experiments employing a nonpolarized epithelial cell line suggest that labeling from the basolateral compartment with the small biotin analog is not significantly impaired by the polycarbonate filter (Lisanti et al., 1989).

PI-PLC AND PHENYL-SEPHAROSE TREATMENT

PI-PLC treatment was performed as described previously (Conzelmann et al., 1986; Lisanti et al., 1988), with the addition of phenyl-Sepharose treatment. Briefly, after extraction and phase separation with the detergent Triton X-114, detergent phases enriched in membrane (hydrophobic) forms of glycosyl-PI anchored proteins were treated with PI-PLC (6 U/ml). These conditions produced maximal release of PI-PLC sensitive GPI-anchored proteins (Lisanti et al., 1988). Detergent phases were additionally re-extracted (1×) with PI-PLC incubation buffer containing 0.25 M methyl- α -D-mannopyranoside before incubation with PI-PLC; methyl- α -D-mannopyranoside was also in-

cluded during PLC treatment. After PI-PLC treatment, resulting aqueous phases (containing soluble [hydrophilic] forms of glycosyl-PI anchored proteins) were re-extracted (2×) and phenyl-Sepharose (250 μ l of a 75% slurry) was added to each 1-ml sample. Prior to addition, phenyl-Sepharose was washed (4×) with Tris-buffered saline (10 mM Tris, pH 7.4/0.15 M NaCl/1 mM EDTA). After 16–20 hr rotating at 4°C, phenyl-Sepharose was removed by centrifugation (14,000 \times *g* for 1 min). The resulting supernatants were then quantitatively precipitated using sodium deoxycholate and trichloroacetic acid (Bensadoun & Weinstein, 1976). After solubilization in Laemmli sample buffer (50 μ l/sample), excess acid was neutralized with 1.5 M Tris, pH 7.3 (10 μ l/sample) and NH₄OH vapors. 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. TX-114 was precondensed according to Bordier (1981).

DETECTION OF BIOTINYLATED PROTEINS

Samples were electrophoresed under reducing conditions in SDS-polyacrylamide (10% slab gels) (Laemmli, 1970) and transferred to nitrocellulose (Towbin, Staehelin & Gordon, 1979). Nitrocellulose sheets were incubated with [¹²⁵I]-streptavidin under conditions that reduce nonspecific binding (Birk & Koepsell, 1987). Blots were blocked with 3% BSA/1% non-fat dry milk (Carnation) for 1 hr in PBS containing 0.5% (vol/vol) Tween 20, 10% (vol/vol) glycerol, 1 M glucose (TGG) at 25°C. After rinsing (2×) with PBS containing 0.5% Tween 20, [¹²⁵I]-streptavidin (0.5–1 \times 10⁶ cpm/ml in TGG containing 0.3% BSA) was allowed to bind for 2 hr. After washing (3×, 10 min each) with PBS containing 0.5% Tween 20, blots were dried and autoradiographed (2–48 hr at –70°C with an intensifying screen) on Kodak XAR-5 film. Autoradiographs were scanned using a GS-transmittance/reflectance densitometer (Hoefer, San Francisco, CA). The most prominent band in the lane corresponding to the apically biotinylated PI-PLC-treated sample was adjusted to 90% absorbance and compared with the untreated controls and corresponding basolaterally biotinylated samples. Scans of PI-PLC-treated samples and untreated controls were then automatically subtracted and the area of each peak determined using the GS-365 Data System/IBM PC version.

Iodinated streptavidin was prepared using chloramine T and Na ¹²⁵I (New England Nuclear, Chadds Ford, PA), yielding a specific activity of 5 μ Ci/ μ g. Molecular mass markers are as indicated (in kDa): myosin heavy chain, 200; phosphorylase *b*, 97.4; bovine serum albumin, 68; ovalbumin, 43; carbonic anhydrase, 29; *b*-lactoglobulin, 18.4; and lysozyme, 14.3.

CEA IMMUNOBLOTTING

After autoradiography, blots were rehydrated in Tris-buffered saline (10 mM Tris, pH 7.4/0.15 M NaCl) containing 0.05% Tween 20 (TBST) and blocked for 1 hr in TBST containing 3% BSA/1% non-fat dry milk. After rinsing (2×) with TBST, blots were incubated for 1 hr with a 1:1,000 dilution of rabbit anti-human CEA IgG (Dako, Santa Barbara, CA) in TBST containing 3% BSA/1% non-fat dry milk. Bound CEA IgG were visualized via incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG and the appropriate substrates as described by the manufacturer (Promega, Madison, WI).

Table 1. Polarity assay for GPI-anchored proteins (PI-PLC sensitive)

Steps	Rationale
1. Domain-selective labeling with sulfo-NHS-biotin (0.5 mg/ml; 30 min at 4°C)	Biotinylates primary amino groups of all cell surface components; membrane and tight junction impermeant
2. Extraction and phase separation with the detergent Triton X-114; collect detergent phases	Separates hydrophilic peripheral from hydrophobic integral membrane proteins; membrane (hydrophobic) forms of GPI-anchored proteins partition with the detergent phase
3. PI-PLC treatment (6 U/ml; 60 min at 37°C)	Induces a transition from the hydrophobic to the hydrophilic state due to specific cleavage of the GPI anchor and loss of the putative glycerolipid detergent binding domain
4. Triton X-114 phase separation; collect aqueous phase	Soluble (hydrophilic) forms of the GPI-anchored proteins are now found in the aqueous phase
5. Phenyl-Sepharose treatment (20 hr at 4°C); collect resulting supernatant	Removes residual hydrophobic glycoproteins present in the aqueous phase
6. [¹²⁵ I]-streptavidin blotting (1–2 \times 10 ⁶ cpm's/ml; 2 hr at 25°C)	Allows the visualization of biotinylated proteins by autoradiography

Results

POLARITY ASSAY FOR GPI-ANCHORED PROTEINS

GPI-anchored proteins of a given epithelial cell line were identified according to the criterion of sensitivity to PI-PLC. The experimental approach (Table 1) combined domain-selective labeling using a membrane/tight junction impermeant marker (sulfo-NHS-biotin), with TX-114 phase separation and PI-PLC release of GPI-anchored proteins from the detergent into the aqueous phase. In the original assay (Lisanti et al., 1988), this release overlapped with a background level of spontaneous protein partitioning into the aqueous phase which partially obscured the action of PI-PLC. This background release was attributed to the inefficiency inherent in TX-114 phase separation; the aqueous phase retains 0.7 mM TX-114, which is 3× above the critical micellar concentration of this detergent and is sufficient to solubilize certain membrane proteins of intermediate hydrophobicity (Pryde, 1986). Accordingly, we used a hydrophobic resin in an attempt to

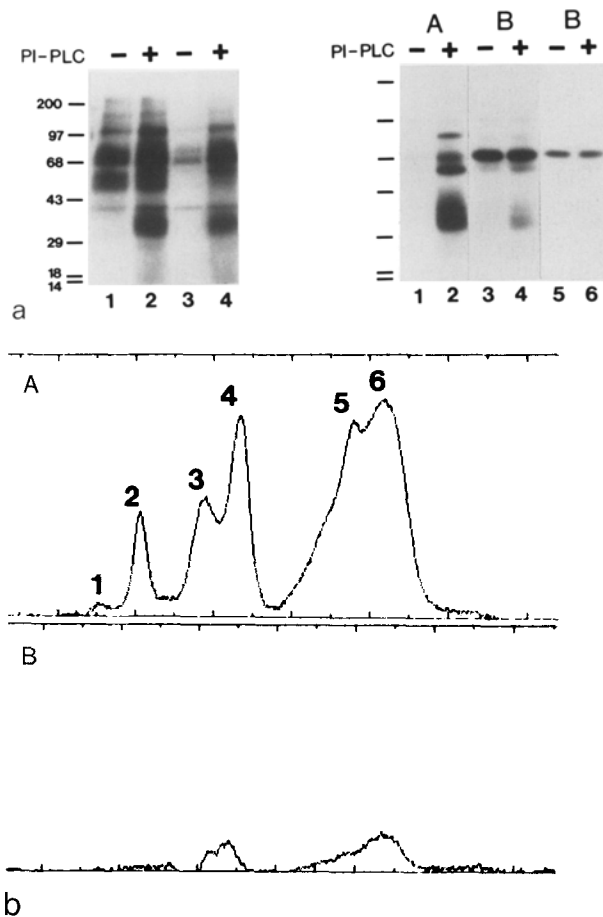


Fig. 1. Phenyl-Sepharose treatment drastically reduces spontaneous (PI-PLC independent) release without diminishing PI-PLC specific release. (a) Autoradiography. Left panel: Plastic-grown MDCK (type II) monolayers, two days confluent, were surface-labeled with sulfo-NHS-biotin. Right panel: Tight MDCK (type II) monolayers, 7–9 days confluent on polycarbonate-based filter chambers, were apically (A) or basolaterally (B) biotinylated. Following cell-surface labeling, monolayers were extracted with TX-114 and the resulting detergent phases incubated in the absence (lanes –) or presence (lanes +) of PI-PLC (6 U/ml). After PI-PLC treatment, the resulting aqueous phases were treated with phenyl-Sepharose (left panel, lanes 3 and 4; right panel, lanes 1–6) or left untreated (left panel, lanes 1 and 2). Six GPI-anchored proteins were visualized by [¹²⁵I]-streptavidin blotting/autoradiography. Note that phenyl-Sepharose treatment dramatically increases the sensitivity of detection of PI-PLC sensitive GPI-anchored proteins. No GPI-anchored proteins with a basolaterally polarized distribution were observed. In the right panel, lanes 3 and 4 are also shown at half the exposure time (as lanes 5 and 6) to partially correct for the larger surface area and protein mass of the basolateral cell surface. (b) Densitometric analysis. The above autoradiograph (Fig. 1a, right panel) was scanned and lanes corresponding to PI-PLC treated samples (+) and untreated controls (–) were automatically subtracted by the computer. (A) Apically biotinylated, lane 2 minus lane 1. (B) Basolaterally biotinylated, lane 4 minus lane 3. Peaks numbered 1–6 correspond to 110, 85, 70, 55, 38 and 35 kDa, respectively. The area of each peak was then determined and the percentage confined to the apical surface calculated (%Ap); values are listed in Table 2

remove both residual TX-114 and hydrophobic proteins without affecting the soluble (hydrophilic) set of GPI-anchored proteins released by PI-PLC.

Phenyl-Sepharose had been employed previously to distinguish between hydrophobic and hydrophilic forms of trehalase and decay accelerating factor (both GPI-anchored proteins) (Takesue et al., 1986; Davitz et al., 1987). Figure 1a (left panel) shows that addition of phenyl-Sepharose (step 5 in Table 1) drastically removed proteins that partition spontaneously into the aqueous phase without affecting PI-PLC specific release. Removal of spontaneously released proteins correlated well with the loss of detergent, suggesting that these proteins were kept in solution by TX-114 in the aqueous phase. This simple step greatly increased the sensitivity of detection of GPI-anchored proteins allowing a more stringent assessment of their polarized apical distribution.

Using this improved polarity assay, all GPI-anchored proteins detected in MDCK II cells were apically polarized. To quantitate the apical distribution of GPI-anchored proteins, scanning densitometry of autoradiographs was performed (Fig. 1b). The percentage of each GPI-anchored protein confined to the apical surface ranged from 86.7 to >99% (see Table 2). No GPI-anchored proteins with a preferred basolateral distribution were observed (Fig. 1a, right panel), consistent with our preliminary observations (Lisanti et al., 1988). Since this might be a property specific to MDCK II cells, we sought to generalize this observation using polarized epithelial cell lines of diverse species and tissue origin.

POLARIZED APICAL DISTRIBUTION OF GPI-ANCHORED PROTEINS IN RENAL CELL LINES

Several well-characterized epithelial cell lines retain the polarized phenotype (Gstraunthaler, 1988; Rodriguez-Boulan et al., 1989). MDCK cells (derived from canine kidney) are the best characterized in this regard; two strains (I and II) have been isolated. Both appear to originate from the distal tubule/collecting duct, based on hormone-responsiveness studies (ADH/aldosterone sensitivity) and lack the ability to develop a “brush border” (Handler, Perkins & Johnson, 1980; Ojakian, Romain & Herz, 1987). MDCK, type I, “high resistance strain” (transmonolayer electrical resistance [TER] >1,000 $\Omega \cdot \text{cm}^2$) comprises a basolateral surface with 7.6 \times the area of the apical surface; in contrast, MDCK, type II, “low resistance strain” (TER 100–200 $\Omega \cdot \text{cm}^2$) has a basolateral surface with 2.8–4.0 \times area of the apical surface (von Bonsdorff et al., 1985; Vega-Salas et al., 1987). LLC-PK₁, an epithelial cell line derived from porcine kidney, retains the

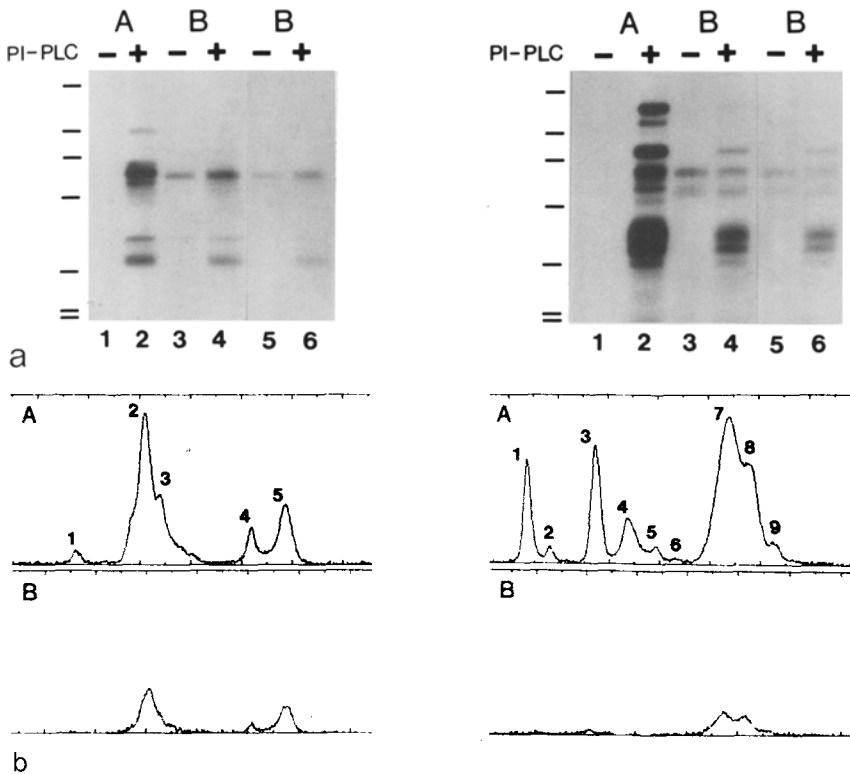


Fig. 2. Polarized apical distribution of GPI-anchored proteins in two renal epithelial cell lines. (a) Autoradiography. Tight, filter-grown monolayers [left panel, MDCK (type I); right panel, LLC-PK₁], 7–9 days confluent, were apically (A) or basolaterally (B) biotinylated. Following extraction with TX-114 and PI-PLC treatment, the resulting aqueous phases were incubated batchwise with phenyl-Sepharose. GPI-anchored proteins were visualized after transfer to nitrocellulose via [¹²⁵I]-streptavidin blotting/autoradiography. Analysis of MDCK (type I), revealed six GPI-anchored proteins of similar molecular weight to those detected for MDCK (type II), but in different relative proportions. Nine GPI-anchored proteins were detected using LLC-PK₁. No GPI-anchored (PI-PLC sensitive) proteins with a preferred basolateral distribution were observed. Lanes 3 and 4 are also shown at half the exposure time (as lanes 5 and 6) to partially correct for the larger surface area and protein mass of the basolateral surface. Molecular mass standards are as indicated in Fig. 1 (left panel). (b) Densitometric analysis. The above autoradiographs were scanned and lanes corresponding to PI-PLC treated samples (+) and untreated controls (–) were automatically subtracted. (A) Apically biotinylated, lane 2 minus lane 1. (B) basolaterally biotinylated, lane 4 minus lane 3. Peaks numbered 1–5 correspond to 110, 70, 55, 35 and 38 kDa, respectively [left, MDCK (type I)]. Similarly, peaks numbered 1–9 correspond to 180, 150, 85, 70, 55, 45, 38, 35 and 30 kDa, respectively (right, LLC-PK₁).

ability to form a relatively tight monolayer (TER 400 $\Omega \cdot \text{cm}^2$), with a B/A surface area ratio of 1–1.5. LLC-PK₁ cells appear to originate from the proximal tubule as indicated by a well-developed brush border, and the presence of glucose (Na-dependent) and phosphate transport systems (Hull, Cherry & Weaver, 1976; Rabito, Kreisberg & Wight, 1984).

All three cell lines met the criteria necessary for domain-selective labeling with sulfo-NHS-biotin, namely (i) growth and attachment to polycarbonate-based filter chambers and (ii) the generation of a tight monolayer as evidenced by impermeability to a small diffusible radioactive tracer ([³H]-inulin). Confluent monolayers were biotinylated from the apical or the basolateral side, extracted with TX-114 and treated with PI-PLC, according to the protocol described in Table 1. All three cell lines

showed a polarized apical distribution of GPI-anchored proteins. Analysis of MDCK, type I, revealed six GPI-anchored proteins of the same molecular weights previously detected for MDCK, type II, (110, 85, 70, 55, 38 and 35 kDa) (Fig. 2a, left panel) although in different relative proportions. Scanning densitometry (Fig. 2b, left panel) indicated that, as in MDCK II cells, GPI-anchored proteins were predominantly (71.7–>99%) apical (Table 2). No basolaterally polarized GPI-anchored proteins were observed; however, a larger percentage of these proteins was detected on the basolateral surface, probably due to the larger area of this surface domain in MDCK I cells. It should be noted that although possession of similar molecular weights is not definitive proof of the identity of apical and basolateral bands, if putative basolaterally

Table 2. Molecular weights and percentage apical polarity of GPI-anchored proteins detected in seven polarized epithelial cell lines

Renal cell lines					Intestinal cell lines	
MDCK II	MDCK I	LLC-PK ₁	MDCK-RCA'	MDCK-ConA'	Caco-2	SK-CO15
*180 (ND)	110 (ND)	180 (ND)	65 (91.8)	85 (ND)	200 (ND)	200 (ND)
*110 (ND)	*85 (ND)	150 (ND)	50 (82.6)	70 (ND)	95 (95.5)	95 (80.9)
85 (93.1)	70 (78.6)	85 (95.2)	35 (74.3)	55 (68.3)	70 (98.1)	70 (76.2)
70 (93.6)	55 (84.0)	70 (ND)	30 (73.6)	38 (91.9)	40 (98.7)	
55 (90.2)	38 (84.6)	55 (ND)		35 (54.6)	38 (96.3)	
38 (92.8)	35 (71.7)	45 (ND)				
35 (86.7)		38 (89.2)				
		35 (85.1)				
		30 (83.9)				

Confluent epithelial cell monolayers were biotinylated, extracted with TX-114 and treated with PI-PLC/phenyl-Sepharose sequentially. GPI-anchored proteins were visualized after transfer to nitrocellulose via [¹²⁵I]-streptavidin blotting/autoradiography. The molecular weight (M_r) of the GPI-anchored proteins detected are listed above with the percentage that appeared confined to the apical surface indicated in parentheses.

The most abundant GPI-anchored proteins showed basolateral counterparts of identical molecular weight and reduced intensity. Assuming that such apical and basolateral bands corresponded to the same protein, the percentage of a given protein that appeared on the apical surface was calculated using the formula, $[A \text{ (apical band)}/[A \text{ (apical band)} + A \text{ (basal band)}] \times 100$, where A represents the area of a given peak obtained by densitometric analysis of the autoradiograph. ND (not determined), denotes that a basolateral counterpart band was not observed, i.e., that greater than 99% was present on the apical surface. In addition, certain bands only appeared on overexposure of the autoradiograph and are denoted by an asterisk (*).

polarized GPI-anchored proteins existed we should have observed some basolaterally polarized bands with apical counterparts of lower intensity.

Analysis of LLC-PK₁ revealed nine GPI-anchored proteins of molecular weights 180, 150, 85, 70, 55, 45, 38, 35 and 30 kDa (Fig. 2a, right panel), with percentage apical polarity ranging from 83.9 to 99% (Table 2). As observed for MDCK cells, only the most abundant GPI-anchored proteins showed basolateral counterparts of identical molecular weight and reduced intensity (Fig. 2b, right panel), confirming the preferred apical distribution of this protein group in LLC-PK₁ cells.

POLARIZED APICAL DISTRIBUTION OF GPI-ANCHORED PROTEINS IN INTESTINAL CELL LINES

We next investigated the distribution of GPI-anchored proteins in two intestinal epithelial lines, Caco-2 and SK-CO15. Caco-2 is a well-characterized cell line, derived from a human colon carcinoma, which develops $200 \Omega \cdot \text{cm}^2$ (Grasset et al., 1984), displays an extensive brush border with ordered apical microvilli, and expresses a variety of microvillar hydrolases (sucrase-isomaltase, leucine aminopeptidase, alkaline phosphatase, etc.) typical of enterocytic differentiation (Fogh & Trempe,

1975; Pinto et al., 1983; Hauri et al., 1985; Rousset, 1986). We also studied a previously uncharacterized intestinal cell line, SK-CO15 (also derived from a human colon adenocarcinoma), which by various morphological and biochemical criteria displays a pattern of differentiation closer to colonic intestinal epithelia (Le Bivic et al., 1989). Both lines attached to polycarbonate-based filter chambers and appeared impermeable to [³H]-inulin (<1% transported in 2 hr at 37°C, which made domain-selective labeling with sulfo-NHS-biotin feasible (Sargiacomo et al., 1989).

Apically or basolaterally biotinylated monolayers were extracted with TX-114, and treated with PI-PLC and phenyl-Sepharose sequentially. As with the kidney cell lines examined, both demonstrated a polarized apical distribution of GPI-anchored proteins. Analysis of Caco-2 cells revealed five GPI-anchored proteins of molecular weights 200, 95, 70, 40 and 38 kDa (Fig. 3a, left panel). Similarly, SK-CO15 contained three GPI-anchored proteins of identical molecular weights (200, 95, and 70 kDa) to three Caco-2 proteins (Fig. 3b, left panel). Percentage apical polarity ranged from 95.5 to 99% for Caco-2 and from 76.2 to >99% for SK-CO15 (Fig. 3c; Table 2).

We suspected that the 200-kDa band might be carcinoembryonic antigen (CEA) since this protein

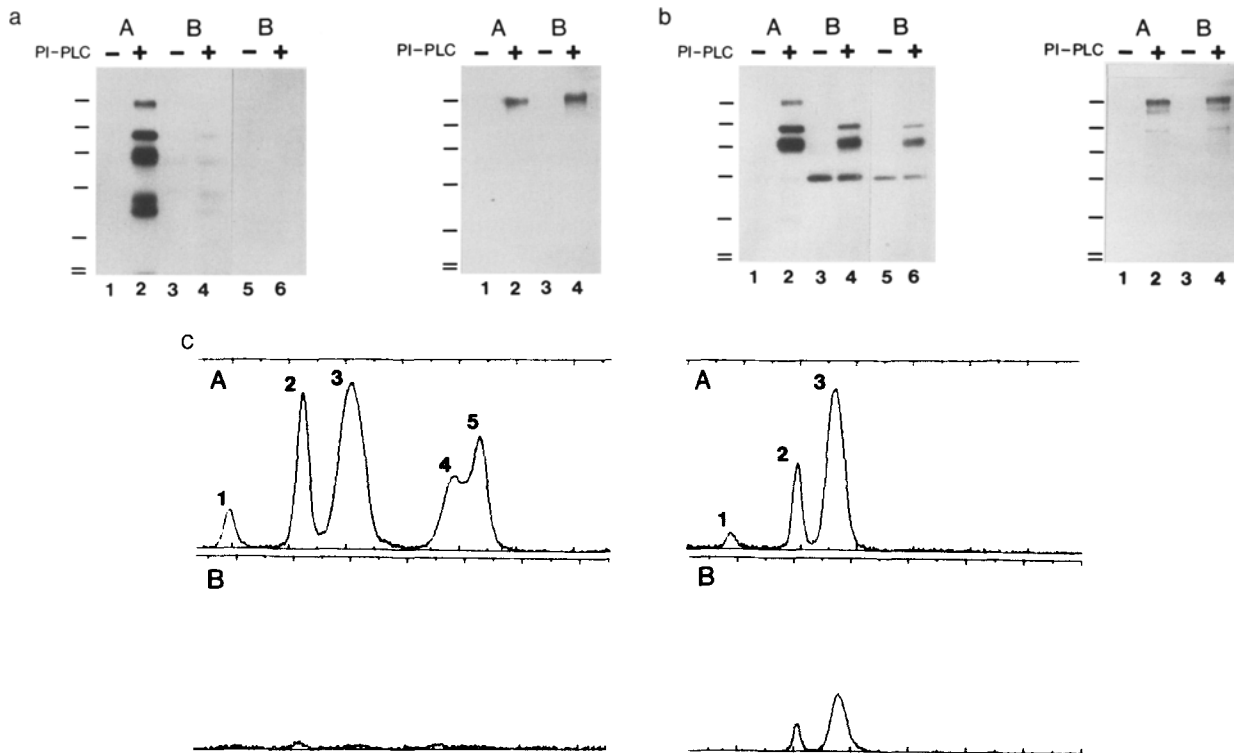


Fig. 3. Polarized apical distribution of GPI-anchored proteins in two human intestinal cell lines: identification of the 200-kDa GPI-anchored protein as CEA. (a and b) Autoradiography and Western blotting. Tight, filter-grown monolayers, (a) Caco-2; (b) SK-CO15, 11–14 days confluent, were apically (A) or basolaterally (B) biotinylated. Labeled monolayers were extracted with TX-114, and treated with PI-PLC and phenyl-Sepharose sequentially. Left panels: GPI-anchored proteins were visualized after transfer to nitrocellulose via [125 I]-streptavidin blotting/autoradiography. Analysis of Caco-2 cells revealed five GPI-anchored proteins with M_r of 200, 95, 70, 40 and 39 kDa. Similarly, SK-CO15 contained three GPI-anchored proteins of identical M_r (200, 95 and 70 kDa). Lanes 3 and 4 are also shown at half the exposure time (as lanes 5 and 6) to partially correct for the larger surface area of the basolateral surface. Right panels: After autoradiography, blots were rehydrated and coated with rabbit anti-human CEA IgG's and bound antibody visualized with the appropriate alkaline phosphatase-conjugated secondary antibody. A 200-kDa band, corresponding to the known molecular weight of CEA, was observed only in PI-PLC treated samples. A band of identical molecular weight was observed after PI-PLC treatment of apically biotinylated monolayers [(a) and (b) left panels, lane 2]. Molecular mass standards are as indicated in Fig. 1 (left panel). Note that Western blotting with anti-CEA antibodies cannot be domain selective; apically or basolaterally biotinylated samples are identical except for the selective presence of the biotin tag on apical or basolateral proteins. (c) Densitometric analysis. The above autoradiographs [(a) and (b), left panels] were scanned and lanes corresponding to PI-PLC treated samples (+) and untreated controls (-) automatically subtracted. (A) Apically biotinylated, lane 2 minus lane 1. (B) Basolaterally biotinylated, lane 4 minus lane 3. Peaks numbered 1–5 correspond to 200, 95, 70, 40 and 38 kDa, respectively (right, Caco-2; left, SK-CO15). Note that peaks numbered 4 and 5 are absent from SK-CO15

is present in an apically polarized distribution in well-differentiated adenocarcinoma of the colon (Hamada et al., 1985) and has recently been shown to possess a GPI anchor (Sack et al., 1988; Takami et al., 1988). To identify the 200-kDa band as authentic CEA, the Caco-2 and SK-CO15 blots (shown in the left panels of Fig. 3a and b) were incubated with rabbit anti-human CEA IgG's after autoradiography and bound IgG visualized with alkaline-phosphatase conjugated goat anti-rabbit IgG. CEA was released to the aqueous phase only in samples which had been treated with PI-PLC (Fig. 3a and b, right panels) but was biotinylated only when sulfo-NHS-biotin was added to the apical compartment of the filter chambers (Fig. 3a and b,

left panels, lane 2). Thus, CEA is present in an apically polarized distribution in these two human intestinal cell lines. The results described thus far indicate that the polarized apical distribution of GPI-anchored proteins is highly conserved across species and tissue type.

GLYCOSYLATION CAN AFFECT THE POLARITY OF GPI-ANCHORED PROTEINS

Various structural features of GPI appear to be highly conserved, including (i) linkage to the C-terminal amino acid to ethanolamine phosphate; (ii) the presence of phosphatidylinositol and (iii) a glycan bridge [(Man) $_3$ -GlcN] linking ethanolamine

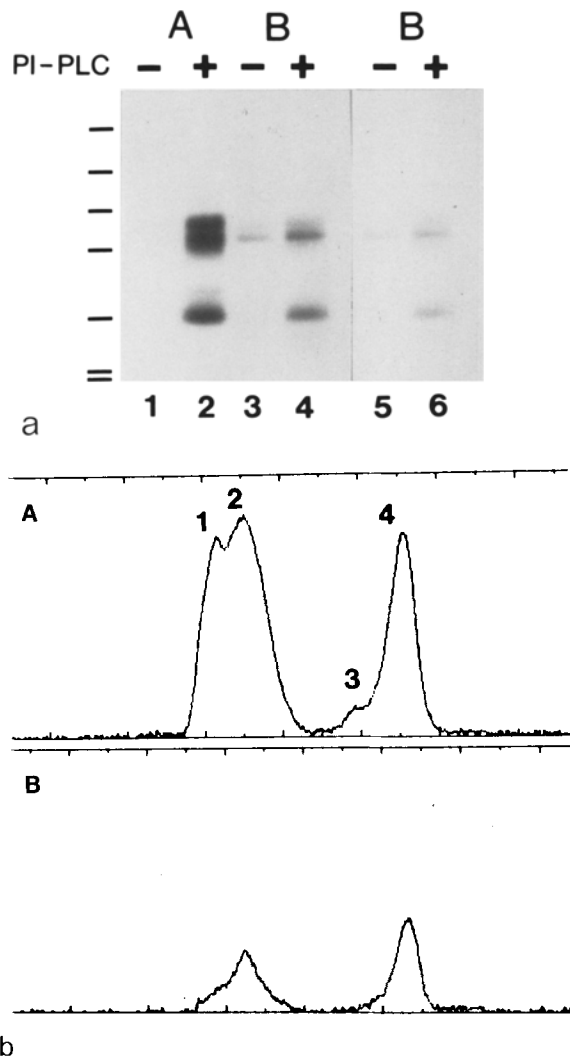


Fig. 4. Galactosylation is not required for the polarized apical distribution of GPI-anchored proteins in MDCK cells. (a) Autoradiography. Tight MDCK II-RCA^r monolayers, 7–9 days confluent, were apically (A) or basolaterally (B) biotinylated. Monolayers were extracted with TX-114, and treated with PI-PLC and phenyl-Sepharose sequentially. GPI-anchored proteins were visualized after transfer to nitrocellulose via [¹²⁵I]-streptavidin blotting/autoradiography. Four GPI-anchored proteins of apparently lower molecular weight (in comparison with MDCK II-WT, Fig. 1) were observed (65, 50, 35 and 30 kDa). Since the lectin-resistant mutant cell line, MDCK II-RCA^r, contains a pleiotropic defect in galactosylation (affecting both glycosphingolipid synthesis and protein glycosylation), our results suggest that galactosylation is not required for the polarized apical expression of GPI-anchored proteins. Lanes 3 and 4 are also shown at half the exposure time (as lanes 5 and 6) to partially correct for the larger surface area of the basolateral surface. Molecular mass standards are as indicated in Fig. 1 (left panel). (b) Densitometric analysis. The above autoradiograph was scanned and lanes corresponding to PI-PLC treated samples (+) and untreated controls (–) automatically subtracted. (A) Apically biotinylated, lane 2 minus lane 1. (B) Basolaterally biotinylated, lane 4 minus lane 3. Peaks numbered 1–4 correspond to 65, 50, 35 and 30 kDa, respectively

phosphate with phosphatidylinositol (Low & Saltiel, 1988; Ferguson & Williams, 1988). Since additional glycosylation (galactosylation and mannosylation) of the GPI-glycan moiety may vary (Ferguson et al., 1988; Homans et al., 1988), we investigated whether it plays a role in generating the polarized apical distribution of GPI-anchored proteins in epithelial cells. To test this hypothesis, we took advantage of a lectin-resistant mutant cell line, MDCK II-RCA^r, which contains a pleiotropic defect in galactosylation, while retaining the polarized epithelial cell phenotype (Meiss, Green & Rodriguez-Boulan, 1982; Brandli et al., 1988). The pleiotropic nature of the defect (affecting both N- and O-linked glycosylation, as well as glycosphingolipid synthesis) apparently results from defective uptake of UDP-galactose by the Golgi complex. Four GPI-anchored proteins were observed (Fig. 4a) with apparent molecular weights (65, 50, 35 and 30 kDa) lower than those of corresponding wild-type MDCK GPI-anchored proteins (consistent with the possession of truncated carbohydrate side chains). The percentage apical polarity ranged from 73.6 to 91.8 (Table 2), somewhat lower than in wild-type MDCK cells. All appeared in a polarized apical distribution, with only the most abundant GPI-anchored proteins showing basolateral counterparts of identical molecular weight and reduced intensity (Fig. 4b). Our results suggest that galactosylation is not required for the polarized apical expression of GPI-anchored proteins.

We next examined another lectin-resistant mutant cell line, MDCK II-ConA^r, which contains an unknown glycosylation defect. Comparison of VSV G-protein and influenza HA produced by infection of this cell line has suggested that this mutation affects only a subclass of core high-mannose oligosaccharides, possibly those not destined for processing into complex forms (Meiss et al., 1982). A similar differential effect is observed in processing of N-linked oligosaccharides by a class E Thy-1 negative lymphoma cell line (also derived by ConA^r, Chapman, Fujimoto & Kornfeld, 1980), which contains a pleiotropic defect in GPI-anchoring and secretes a hydrophilic form of Thy-1 (a GPI-anchored protein) into the culture media (Conzelmann et al., 1986).

Analysis of MDCK II-ConA^r cells revealed five GPI-anchored proteins of molecular weights almost identical to those observed in wild-type MDCK cells (85, 70, 55, 38, and 35 kDa; Fig. 5a). However, two bands (55 and 35 kDa) seemed relatively unpolarized with 31.7 to 45.4% appearing on the basolateral surface (Fig. 5b; Table 2). Other bands (85, 70 and 38 kDa) remained highly apically polarized as in wild-type cells (with 91.9 to >99% confined to the apical surface). These results indicate that a de-

fect in glycosylation can affect the polarity of certain GPI-anchored proteins. Such a change in polarity may be the direct result of an alteration in GPI-anchor biosynthesis/side chain addition or secondary to alterations in N-linked oligosaccharide processing. GPI-anchored proteins that remained highly polarized may be sorted based on information present in their extracellular domains, since redundant apical targeting information can be present in the ectodomain of a GPI-anchored protein (Lisanti et al., 1989).

Table 2 summarizes the apparent molecular weights and percentage apical polarity of the GPI-anchored proteins detected in the seven cell lines analyzed. A cumulative minimum of 13 different GPI-anchored proteins (based on distinct molecular weights) were detected and all appeared apically polarized. It should be noted that virtually identical results were obtained when monolayers (of all seven cell lines) were serum starved for 12–16 hr (*data not shown*). These results suggest that the observed polarized apical distribution of GPI-anchored proteins is independent of serum factors, which, either directly (e.g., proteases or PI-specific phospholipases) or indirectly (hormones, growth factors stimulating anchor degrading enzymes) might contribute to this polarity by acting preferentially on one epithelial surface.

Discussion

Central to the study of epithelial cell polarity is the question of how proteins are targeted and retained by specific plasma membrane domains. The results of this report describe a strong correlation between possession of a GPI-anchor and apical polarization of a protein in epithelial cells. To survey the surface distribution of GPI-anchored proteins in polarized epithelial cells, we employed a domain-selective labeling procedure and a sensitive assay that depends upon several conserved structural features of the GPI-membrane anchor (Lisanti et al., 1988). Domain-selective labeling with the cell surface/tight junction impermeant marker, sulfo-NHS-biotin, allows for the analysis of apical and basolateral membrane components without the need for complex subcellular fractionation and the burden of possible cross-contamination. Biotinylated GPI-anchored proteins can then be identified based on their sensitivity to cleavage of their membrane anchor by PI-PLC, which causes a transition from a hydrophobic membrane form to a hydrophilic soluble form, detectable by TX-114 phase separation (*see* Table 1). Unlike simple PI-PLC release from intact metabolically labeled cells, this assay system allows the de-

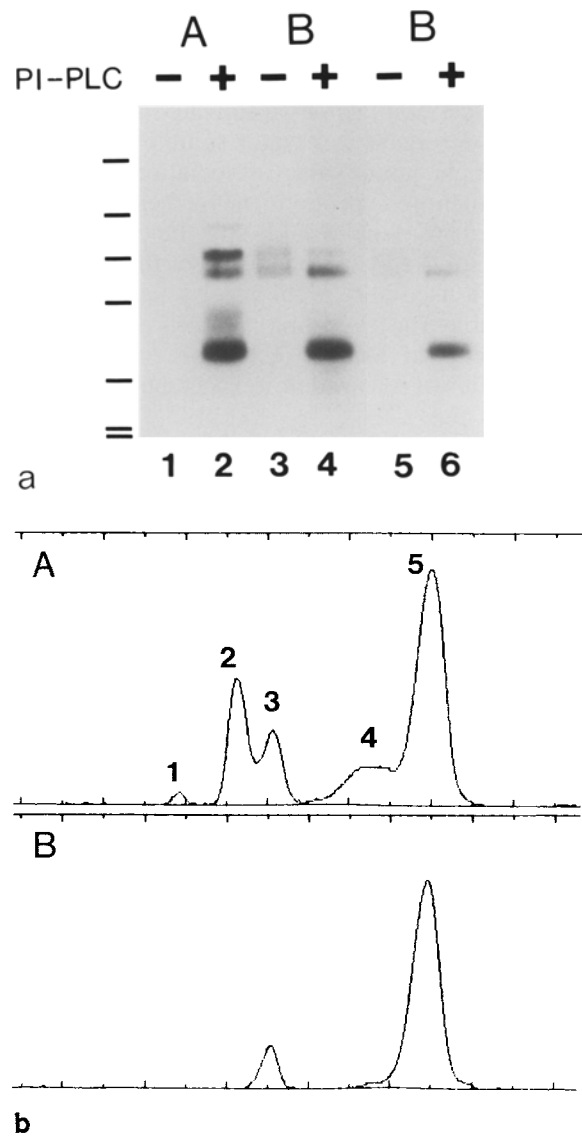


Fig. 5. A defect in glycosylation can affect the polarity of GPI-anchored proteins. (a) Autoradiography. Apically (A) or basolaterally (B) biotinylated monolayers of MDCK II-ConA^r. 7–9 days confluent, were extracted with TX-114 and subjected to PI-PLC and phenyl-Sepharose treatment. After transfer to nitrocellulose, GPI-anchored proteins were visualized via [¹²⁵I]-streptavidin blotting/autoradiography. Five GPI-anchored proteins of molecular weights 85, 70, 55, 38 and 35 kDa were observed. Two GPI-anchored proteins (55 and 35 kDa) appeared relatively unpolarized. Although the defect in these cells is unknown, it appears to involve mannosylation (Meiss et al., 1982). Lanes 3 and 4 are also shown at half the exposure time (as lanes 5 and 6) to partially correct for the larger surface area of the basolateral surface. Molecular mass standards are as indicated in Fig. 1 (left panel). (b) Densitometric analysis. The above autoradiograph was scanned and lanes corresponding to PI-PLC treated samples (+) and untreated controls (-) were automatically subtracted. (A) Apically biotinylated, lane 2 minus lane 1. (B) Basolaterally biotinylated, lane 4 minus lane 3. Peaks numbered 1–5 correspond to 85, 70, 55, 38, and 35 kDa, respectively

tection of only cell surface antigens containing an inositol phosphate bound, PI-PLC cleavable, hydrophobic domain. However, it cannot be excluded that the conversion of some biotinylated apical proteins from a detergent to a water soluble form may result from tight noncovalent association with another GPI-anchored protein (nevertheless, such GPI-anchored protein would still be apical, in agreement with the main conclusion of this report). The approach used here is the most sensitive technique available for revealing the set of GPI-anchored proteins of a given cell type; vanishingly small quantities of released proteins can be detected (as little as 1 pg).

Other methods of identification of GPI-anchored proteins, such as metabolic labeling with anchor-specific components ($[^3\text{H}]$ -ethanolamine or $[^3\text{H}]$ -inositol), cross reactivity with the trypanosome membrane anchor (cross-reactive domain [CRD] antibody) or PI-PLC treatment of intact cells metabolically labeled with $[^{35}\text{S}]$ -methionine, were pursued, but were unsuccessful (*data not shown*). Little or no signal was detected by immunoblotting with CRD after PI-PLC treatment or by metabolic labeling with anchor-specific components. In contrast, a high background (due to either cell lysis or secretion) was encountered during PI-PLC treatment of $[^{35}\text{S}]$ -methionine labeled cells. This was not surprising since these methods have been successful only when coupled with immunoprecipitation or an enzymatic assay to detect a specific candidate protein or when large quantities of purified protein have been available (as in CRD immunoblotting).

We utilized the stringent biotin/PI-PLC polarity assay to investigate the surface distribution of GPI-anchored proteins in five epithelial cell lines which have served as *in vitro* model systems for the study of epithelial cell polarity. This diverse group includes cells with canine, porcine and human origins which express certain features of renal (proximal tubule, distal tubule) or intestinal (enterocytic, colonic) epithelia *in vivo*. All GPI-anchored proteins detected in the five cell lines displayed a polarized apical distribution. No GPI-anchored proteins with a preferred basolateral distribution were observed. The detection of basolateral counterparts of apical GPI-anchored proteins was roughly related to the basolateral/apical surface area ratio, since it was more evident in MDCK I (B/A ratio = 7.6, von Bonsdorff et al., 1985) and SK-CO15 cells (which also have a well-developed basolateral surface, Le Bivic et al., 1989) than in MDCK II cells (B/A ratio 2.8–4) and minimal in cell lines with extensive brush-border development such as LLC-PK₁ (B/A ratio 1–1.5, Pfaller et al., 1988) and Caco-2 (Pinto et al., 1983; Hauri et al., 1985). These results are con-

sistent with our preliminary observations with the low resistance MDCK II subline (Lisanti et al., 1988) and with the apical polarity of several well-known endogenous and recombinantly expressed GPI-anchored proteins (Kollias et al., 1987; Low & Saltiel, 1988; Ferguson & Williams, 1988; Brown et al., 1989; Lisanti et al., 1989). In fact, some of these markers (renal dipeptidase [130 kDa], trehalase [96 kDa], 5' nucleotidase [73 kDa] and alkaline phosphatase [58–70 kDa]) are present in the cell lines we studied (Gstraunthaler, 1988) and have similar molecular weights to the GPI-anchored proteins we detected. The emerging picture is that the apical polarity of GPI-anchored proteins is observed in a variety of epithelial cells derived from different tissues and species and constitutes a highly conserved feature of the polarized epithelial cell phenotype.

Chemical analyses of the GPI anchors isolated from a variety of different GPI-anchored proteins have indicated that certain components of the core structure appear almost invariable (Low & Saltiel, 1988; Ferguson & Williams, 1988). However, considerable structural heterogeneity also exists, even within a single cell type. Elucidation of the complete structure of the GPI-anchors of two distinct glycoproteins, the *T. brucei* variable surface glycoprotein (VSG) and rat brain Thy-1, revealed extensive differences in side chain groups on the glycan moiety (Ferguson et al., 1988; Homans et al., 1988). More specifically, the Thy-1 GPI anchor contained extra ethanolamine phosphate, α -mannose and β -N-acetyl galactosamine residues, but lacked the 2-4 α -galactose residues normally observed for the GPI anchor of VSG. Synthesis of the GPI anchor appears to occur via glycosylation of phosphatidylinositol (by the sequential addition of glucosamine, mannose and ethanolamine-P) with the addition of other sugars occurring later, possibly after attachment to the C-terminal of the mature protein (Masterson et al., 1989). A putative precursor lipid, that may give rise to the GPI anchor, lacks galactose, suggesting that galactosylation occurs after synthesis (Krakow et al., 1986; Menon et al., 1988; Masterson et al., 1989) possibly at the level of the Golgi apparatus (Grab, Webster & Verjee, 1984). Thus, GPI anchors may have a common core structure in a variety of cell types, but variation can be expressed in side chain moieties without a loss of GPI anchoring. The preferred apical distribution of GPI-anchored proteins in the lectin-resistant mutant cell line, MDCK II-RCA', which contains a pleiotropic defect in galactosylation, suggests that galactose residues are not required to generate this polarity. (It seems unlikely that such a pleiotropic defect would spare GPI biosynthesis and processing.) In contrast, two of five GPI-anchored proteins in the

mutant cell line, MDCK II-ConA', appeared relatively unpolarized. Although the underlying defect is not known for this cell line, it appears to involve mannosylation (Meiss et al., 1982). This is especially interesting since another form of mannosylation, mannose-6-phosphate, acts as a targeting signal for lysosomal enzymes (Sly, 1982). Perhaps mannosylation of the side chain moiety of the GPI anchor contributes to efficient apical localization.

We can only speculate on the mechanism by which GPI-anchored proteins become apically distributed. Three possibilities may be envisioned. First, enzymatic modification at the basolateral surface might render them undetectable by the criterion of PI-PLC specific release. Such modifications might include cleavage of the GPI membrane anchor and subsequent secretion (Chan et al., 1988), or acylation of the inositol ring rendering GPI-anchored proteins insensitive to PI-PLC treatment (Roberts et al., 1988), thereby escaping detection by our assay system. Although not directly studied in this work, this possibility appears unlikely since application of alternative polarity assays (e.g., immunocytochemistry or immunoprecipitation of specific endogenous or transfected GPI-anchored proteins from monolayers biotinylated from the apical or the basolateral side) invariably detect the apical distribution of GPI-linked proteins (Lisanti et al., 1989). GPI-anchored decay accelerating factor (DAF, Davitz et al., 1987; Medof et al., 1987) introduced via transfection into MDCK cells is expressed on the apical surface while no DAF molecules are released to the basal medium, as measured by a sensitive RIA (Lisanti et al., 1989). Thus, all the available evidence taken together indicates that the apical polarity of GPI-anchored proteins is not artifactual, but a feature of the polarized epithelial cell phenotype.

A second possibility is that the targeting of plasma membrane proteins to the basolateral surface is mediated by signals in the transmembrane and/or cytoplasmic domains as shown for some Golgi proteins (Machamer & Rose, 1987); attachment to neutral GPI anchors results, in this view, in targeting to the apical membrane by a default pathway. Although this hypothesis is supported by recent evidence that VSV G-protein transmembrane and cytoplasmic domains may target an attached secretory protein to the basolateral surface (Brown & Rose, 1988), it is challenged by reports on the presence of sorting information in the ectodomain of apical proteins (Rodriguez-Boulán & Salas, 1989).

The third possibility invokes a more active role of GPI in apical sorting. Work by several laboratories (reviewed by van Meer, 1988; van Meer & Si-

mons, 1988) has demonstrated that several glycolipids are enriched two to 10-fold in the apical surface, presumably through sorting in the trans-Golgi network (TGN) (van Meer et al., 1987). This has led to the hypothesis that the sorting of glycolipids and apical proteins may be linked (van Meer & Simons, 1988; Wandinger-Ness & Simons, 1989). Since some glycolipids have a high tendency towards self-aggregation (Thompson & Tillack, 1985), apical proteins might be sorted through their affinity for glycolipid patches in the TGN, which would allow their incorporation into apical carrier vesicles. GPI-anchored proteins might be sorted through the affinity of GPI for the glycolipid patches (Rodriguez-Boulán & Nelson, 1989). Biophysical and biochemical dissection of the possible sorting role of GPI may provide useful insights on how the sorting of epithelial plasma membrane proteins is carried out.

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