9. To confluent 100 m*M* dishes, 5 ml of DMEM with 250 μCi of [^{35}S]cysteine per milliliter, with or without protamine sulfate (100 μg/ml), was added and allowed to incubate for 3 hours. Media were collected and centrifuged for 10 min at 4500*g*. Triton X-100 (1%), SDS (0.1%), sodium deoxycholate (0.5%), NaCl (200 m*M*), and phenylmethylsulfonyl fluoride (40 μg/ml) were added to the supernatant to the final concentrations indicated. Aliquots of 1 ml were used for immunoprecipitations. Antibody-antigen complexes were recovered with protein A Sepharose, and the beads were washed three times in 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1*M* NaCl, and 10 m*M* Hepes (*p*H 7.4). Three secondary washes were performed in a low-salt buffer (200 m*M* NaCl) with the same detergent concentrations, as indicated, before samples were loaded onto a 12% nonreduced SDS-polyacrylamide gel [U. K. Laemmli, *Nature* **227**, 680 (1970)]. After electrophoresis the gels were stained with Coomassie G250 and treated with Amplify (Amersham) before drying and autoradiography.
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Mechanism of Membrane Anchoring Affects Polarized Expression of Two Proteins in MDCK Cells

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The signals that direct membrane proteins to the apical or basolateral plasma membrane domains of polarized epithelial cells are not known. Several of the class of proteins anchored in the membrane by glycosyl-phosphatidylinositol (GPI) are expressed on the apical surface of such cells. However, it is not known whether the mechanism of membrane anchorage or the polypeptide sequence provides the sorting information. The conversion of the normally basolateral vesicular stomatitis virus glycoprotein (VSV G) to a GPI-anchored protein led to its apical expression. Conversely, replacement of the GPI anchor of placental alkaline phosphatase with the transmembrane and cytoplasmic domains of VSV G shifted its expression from the apical to the basolateral surface. Thus, the mechanism of membrane anchorage can determine the sorting of proteins to the apical or basolateral surface, and the GPI anchor itself may provide an apical transport signal.

CELLS IN EPITHELIAL TISSUE ARE joined by tight junctions (*1*). These junctions encircle each cell, dividing the surface into apical and basolateral domains. The two membrane regions contain different sets of proteins, which are not free to intermix. Madin-Darby canine kidney (MDCK) cells, which form polarized monolayers at confluency, are a useful model system for studying the sorting of membrane proteins [reviewed in (*1*)]. In MDCK cells, newly synthesized proteins destined for each surface are delivered there directly, without passage through the other membrane (*2*). Proteins are present in the same membrane compartment as far as the late Golgi (*3*) and are probably sorted from each other in the trans Golgi network (*4*). The location of sorting signals in membrane proteins is controversial. Several studies with truncated and hybrid proteins suggest a role for the extracellular domain in sorting. However, some work suggests that the cytoplasmic domain can also contain sorting signals [reviewed in (*5*)]. Interpretation of all these experiments is complicated by the fact that the default pathway for membrane proteins without sorting signals is not known.

A class of proteins, the members of which are anchored in the plasma membrane by a glycosyl-phosphatidylinositol (GPI) moiety instead of a classical proteinaceous transmembrane domain, has been described (*6*). These GPI-anchored proteins are synthesized with hydrophobic COOH-terminal domains that are removed almost immediately after translation. The new COOH-terminal amino acid is then covalently linked to presynthesized GPI (*7*).

The expression of several GPI-linked proteins in MDCK cells has been examined (*8*) by a procedure that should detect any such proteins on either the apical or basolateral surface. Though several GPI-anchored proteins were found on the apical surface, none could be detected on the basolateral membrane. Thus, it is possible that GPI itself might act as an apical sorting signal, although it cannot be the only signal for apical transport, as not all proteins on this surface

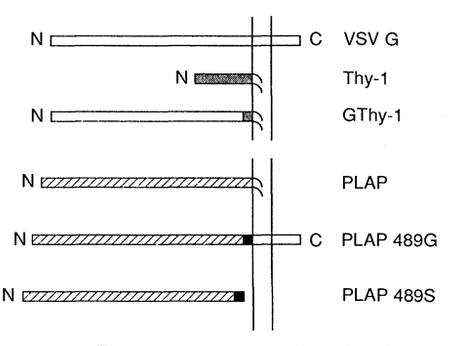

Fig. 1. Schematic diagram of the proteins used in this study. GThy-1 (*9*) contains amino acids 1 to 463 (the extracellular domain) of VSV G and amino acids 110 to 131 and the GPI moiety from Thy-1, assuming that cleavage and addition of GPI occur at Cys^{131} as in Thy-1. For expression in strain II MDCK cells, the GThy-1 cDNA was cloned into the unique Xho I site of pRSVΔSX (*11*). VSV G was expressed from pSV2*neo*-SVGΔSX (*11*). The hybrid protein PLAP 489G contains the transmembrane and cytoplasmic domains of VSV G attached to the extracellular domain of PLAP (*16*). PLAP 489S (*18*) is a truncated form of PLAP that terminates five amino acids COOH-terminal to the normal site of cleavage. It is secreted and does not acquire GPI; it is not known whether the five COOH-terminal amino acids are removed. All constructs were transfected as described (*11*). All plasmids except pSV2*neo*SVGΔSX were coexpressed with pSV2*neo* (*27*). G418-resistant clones were screened for high expression of transfected proteins by metabolic labeling and immunoprecipitation with antisera to VSV or PLAP. Open boxes, VSV G sequence; stippled boxes, Thy-1 sequence; hatched boxes, mature PLAP sequence; solid boxes, PLAP sequence normally removed after addition of GPI (five residues) and linker-encoded sequence (three residues) (*16*). GPI anchorage is symbolized by two short curved lines.

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contain the modification (*1*). Alternatively, sorting information might reside in the amino acid sequence of these proteins. A third possibility is that the lack of a peptide transmembrane span or cytoplasmic domain in GPI-linked proteins may lead to expression on the apical surface.

We used hybrid proteins to examine the effects of GPI and protein anchors on sorting. The first hybrid, GThy-1 (Fig. 1), will be described in detail elsewhere (*9*). The GThy-1 cDNA codes for the entire extracellular domain of vesicular stomatitis virus glycoprotein (VSV G) followed by the COOH-terminal 53 amino acids of the precursor of mouse Thy-1. Thy-1 is a GPI-anchored protein expressed on T lymphocytes. The COOH-terminal 31 amino acid residues of the Thy-1 precursor constitute the hydrophobic domain that is cleaved off after translation (*10*). A GPI anchor is then added to the new COOH-terminus. We expressed GThy-1 in HeLa cells and found that it forms trimers and is transported to the plasma membrane (*9*). It can be labeled with [^{3}H]ethanolamine and is sensitive to removal from the cell surface with phosphatidylinositol-specific phospholipase C (PI-PLC) (*Bacillus thuringiensis*), showing that it contains a GPI anchor. We assume that cleavage and addition of GPI occur at the same site as in Thy-1. Thus, mature GThy-1 presumably contains the entire extracellular domain of VSV G fused to the 22 COOH-terminal amino acids of mature Thy-1 linked to GPI.

MDCK cell lines expressing VSV G or GThy-1 were derived as described (Fig. 1) (*11*). We found that both proteins were transported efficiently to the plasma membrane and that GThy-1, but not VSV G, could be released from cells with PI-PLC (*12*). To study the polarity of surface expression of these proteins, we performed surface immune precipitation on monolayers (Fig. 2). Fifteen percent of the total cell-surface VSV G could be detected on the apical surfaces of duplicate monolayers, whereas 85% of the protein was recovered from the basolateral surface (Fig. 2A). This finding is consistent with previous studies demonstrating basolateral localization of this protein (*11, 13*). In contrast, we found 89% of GThy-1 on the apical surface of cells expressing this protein (Fig. 2B). Thus, addition of a small amount of Thy-1 protein sequence to the extracellular domain of VSV G and the substitution of a GPI anchor for the normal transmembrane span and cytoplasmic tail caused relocalization to the apical surface. Human Thy-1 is expressed apically in kidney tubules of transgenic mice (*14*). Our data indicate that an apical sorting signal from Thy-1 has been transferred to GThy-1. Alternatively, the loss of the transmembrane and cytoplasmic domains from VSV G might cause redirection to the apical surface.

An apical signal, if it existed, could either be contained in the GPI anchor or in the 22 membrane-proximal amino acid residues derived from Thy-1. We attempted to discriminate between these possibilities by using a GThy-1 protein from which most of the Thy-1 residues had been deleted. Unfortunately, this protein was not transported out of the endoplasmic reticulum.

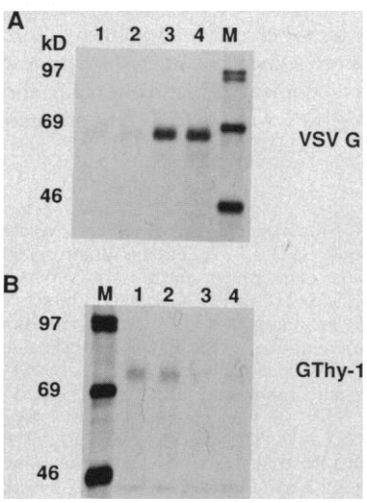

Fig. 2. Quantitation of cell-surface VSV G and GThy-1. MDCK (strain II) cell lines expressing VSV G or GThy-1 were grown on polycarbonate filters (0.4-μm pore size; Transwell, Costar). Filters were affixed to the bottoms of chambers suspended in wells, so that separate pools of media bathed apical and basolateral surfaces. Monolayers were judged to be polarized by two criteria: they maintained an electrical resistance greater than 100 ohms-cm^2 and they showed polarized uptake of [^{35}S]methionine (>10:1 basolateral:apical) (*28*). Medium was adjusted 12 to 14 hours before harvest to contain 10 m*M* sodium butyrate. Cells were labeled for 2 hours in carrier-free media containing 10 m*M* sodium butyrate and [^{35}S]methionine (200 μCi/ml) (Translabel, ICN). Monolayers were then incubated for 2 hours in ice-cold phosphate-buffered saline containing 1 m*M* each of $CaCl_2$ and $MgCl_2$ and 3% bovine serum albumin. Antiserum to VSV (10 μl/ml) was added to the buffer bathing the apical or basolateral surface as appropriate. After extensive washing to remove unbound antibodies, cells were lysed in detergent solution (*29*) containing aprotinin (100 U/ml). *Staphylococcus aureus* (Pansorbin, Calbiochem) was added to portions of lysates containing equal amounts of trichloroacetic acid–precipitable radioactivity. Antibody-antigen complexes were eluted and subjected to SDS–polyacrylamide gel electrophoresis and fluorography (*30*). Bands on autoradiograms were quantitated by densitometry. Quantitation presented in the text is the average of the duplicates shown. (**A**) Immunoprecipitates from cells expressing VSV G; (**B**) immunoprecipitates from cells expressing GThy-1. Lanes 1 and 2, antiserum added apically; lanes 3 and 4, antiserum added basolaterally. M, molecular size markers. Similar results were obtained in three similar experiments.

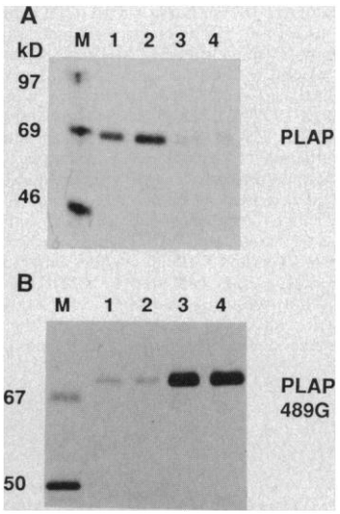

Fig. 3. Quantitation of cell-surface PLAP and PLAP 489G. Immunoprecipitates from lysates of cells expressing PLAP (**A**) or PLAP 489G (**B**) were prepared as described in Fig. 2. Antiserum to human PLAP (Dakopatts, Denmark) was substituted for antiserum to VSV. Lanes 1 and 2, antiserum added apically; lanes 3 and 4, antiserum added basolaterally. M, molecular size markers. Similar results were obtained in three similar experiments.

We also used a second hybrid protein of a design complementary to GThy-1. Placental alkaline phosphatase (PLAP) is a GPI-linked protein (*15*). A cDNA clone, pBC12-PLAP, has been shown to encode GPI-anchored PLAP when expressed in fibroblasts (*16*). We transfected MDCK cells with pBC12-PLAP and found that PLAP was expressed and could be removed from the surface by PI-PLC, indicating that GPI had been added to the protein (*12*). We found by surface immune precipitation that 92% of the total plasma membrane PLAP was expressed apically (Fig. 3A). We next examined sorting of PLAP 489G (*17*) (Fig. 1), which contains the entire amino acid sequence of mature PLAP but is anchored in the membrane by the transmembrane and cytoplasmic domains of VSV G, rather than by GPI. We expressed PLAP 489G in MDCK cells and found it primarily (91%) on the basolateral surface (Fig. 3B). We also examined a truncated, secreted form of PLAP [PLAP 489S (*18*) (Fig. 1)]. MDCK cells expressing PLAP 489S were labeled by means of a pulse-chase protocol. Immune precipitation was performed on apical and basolateral medium and on cell lysates (Fig. 4). Eighty-six percent of the total PLAP 489S was secreted; of this, 70% was apical. In other experiments with two independent lines, 61

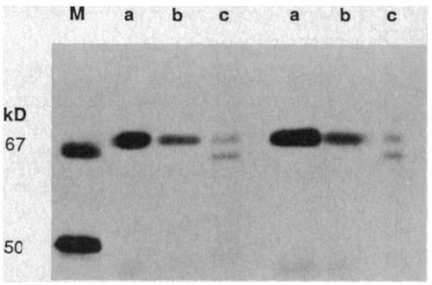

Fig. 4. Quantitation of secreted and cell-associated PLAP 489S. Duplicate filters were seeded with cells expressing PLAP 489S, which were grown and labeled essentially as described in Fig. 2 except that cells were expressed to a higher radioactive concentration of [^{35}S]methionine (400 μCi/ml) for a shorter time (15 min); media was then replaced with that containing 5 m*M* unlabeled methionine, and the cells were incubated for a further 2 hours. Immunoprecipitation with antiserum to human PLAP was performed on media bathing apical (a) and basolateral (b) surfaces and on cell lysates (c). Similar results were obtained in two experiments. M, molecualr size markers.

to 73% of the secreted PLAP 489S was recovered in the apical medium. This result suggests the possibility that the extracellular domain of PLAP contains apical sorting information. However, previous experiments in which secretion of truncated, soluble forms of membrane proteins were used to search for sorting signals, have yielded contradictory results (*19*). Additionally, the polarity of secretion of growth hormone, which appears to lack sorting signals when expressed transiently (*20*), is very heterogeneous among cloned lines of transfected cells. Many lines secrete this "unsorted" protein predominantly apically (*21*). Thus, the usefulness of secreted proteins in defining targeting signals is not clear. In any case, the polarity of PLAP expression (92% apical) is more pronounced than that of PLAP 489S (61 to 73% apical). This suggests that GPI attachment is required to assure complete apical polarity of the protein.

Focusing on the membrane-bound proteins we have studied, GPI anchoring of VSV G causes this normally basolateral protein to be expressed apically. In addition, removal of GPI from PLAP and substitution of a conventional protein anchor from VSV G redirects the protein from the apical to the basolateral surface. We conclude that the mechanism of membrane anchoring can affect the sorting of proteins to apical or basolateral membranes. This conclusion is consistent with recent data from another group (*21*) showing that both a secretory protein and a basolateral membrane protein are expressed on the apical surface after addition of sequences specifying GPI linkage.

That the fate of membrane proteins lacking sorting signals is not known prevents us from concluding whether the GPI anchor, the transmembrane anchor, or both are providing transport signals. It has been proposed (*22*) that proteins without specific sorting information may follow a "default" pathway. This kind of transport is analogous to the bulk flow postulated (*23*) to occur in nonpolarized cells in order to carry proteins from the Golgi apparatus to the surface without targeting signals. At least three possibilities can be imagined for protein targeting in polarized cells: (i) that apical transport occurs by default, and that basolateral transport requires specific signals; (ii) that basolateral transport occurs by default; or (iii) that there is no default path, and that specific transport to either surface requires a signal.

Our data suggest different conclusions when viewed from the perspective of each of these models. If apical transport occurs by default, then there is no need to postulate specific targeting signals in PLAP or GThy-1. We would conclude that VSV G and PLAP 489G are targeted basolaterally through recognition of signals in the transmembrane or cytoplasmic domains. However, it seems unlikely that apical transport is actually a default path. Proteins on this surface are generally specialized and rarely present on nonepithelial tissue (*1*). The basolateral surface, which is in contact with the circulation and thus in the same environment as other, nonpolarized cells, contains many proteins in common with other cells. As plasma membrane proteins in these cells may be transported by bulk flow from the Golgi apparatus to the plasma membrane (*23*), it would be surprising if the same proteins contained sorting information in polarized cells. Additionally, an interesting comparison can be made with another epithelial cell system. In hepatocytes, both apical and basolateral proteins are delivered initially to the basolateral surface (*24*). Proteins destined for the apical surface are then selectively removed. Apical delivery is clearly not the default pathway in these cells.

It seems likely that basolateral transport in MDCK cells occurs by default, or that transport to both membranes is mediated by specific signals and there is no default path. In either case, transport to the apical surface would require sorting information. Our data argue that GPI anchorage could provide such a signal, but the mechanism of this sorting process is unknown. Glycosphingolipids are highly enriched in the apical membrane of MDCK cells (*25*), and it has been proposed (*26*) that self-aggregation of these molecules may aid in their sorting. Although GPI contains the glycerolipid phosphatidylinositol instead of the ceramide moiety found in glycosphingolipids, the possibility that GPI-linked proteins are sorted by a related mechanism remains intriguing.

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