Nonpolarized Distribution of Glycosylphosphatidylinositols in the Plasma Membrane of Polarized Madin-Darby Canine Kidney Cells*

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Glycosylphosphatidylinositols (GPIs) are ubiquitous in eukaryotes and serve to anchor a variety of proteins to the exoplasmic leaflet of cellular membranes. GPIs are synthesized in the endoplasmic reticulum (ER), in excess of the amount needed for protein modification. The fate of the excess GPIs is unknown, but they may be retained in the ER, transported to other membranes, and/or metabolized. In relation to this problem, we were interested in determining whether GPIs were transported to the plasma membrane and whether, like GPI-anchored proteins, their presence was confined to the apical plasma membrane domain in polarized epithelial cells. Polarized Madin-Darby canine kidney epithelial cell monolayers were incubated with [3H]mannose or [3H]ethanolamine to label GPIs and then infected with enveloped viruses. We used influenza virus (flu) and vesicular stomatitis virus (VSV) for these experiments as these viruses are assembled at the cell surface and acquire their envelope lipids from the plasma membrane. Furthermore, flu and VSV bud specifically from the apical and basolateral plasma membrane domains, respectively. Flu and VSV were isolated from the apical and basolateral media, respectively, and subjected to lipid analysis. Radiolabeled GPIs were found in both viruses. Moreover, the membrane concentration of GPIs (i.e. GPI radioactivity normalized to membrane mass) in the two viruses was essentially the same. These observations suggest that (i) non-protein-linked GPIs are located at the plasma membrane; (ii) since GPIs are synthesized in the ER, they must be transported from the ER to the plasma membrane; and (iii) transport of non-protein-linked GPIs is not influenced by the sorting processes that target GPI-anchored proteins exclusively to the apical plasma membrane.

Glycosylphosphatidylinositols (GPIs) are a diverse family of eukaryotic glycolipids containing elements of the sequence ethanolamine-P-6Manα1–2Manα1–6Manα1–4Glcnα1–6myoinositol-1-P-lipid (1, 2). The core GPI structure is assembled in the endoplasmic reticulum (ER) (3) via sequential transfer of components to phosphatidylinositol (1, 2). Current models suggest that the biosynthetic pathway is localized to the cytoplasmic face of the ER (3–5) and that an ethanolamine-containing GPI structure flips across the ER bilayer for transfer to proteins bearing an appropriate carboxyl-terminal signal sequence (6).

GPIs are made in excess of the amount needed for protein modification, and eukaryotic cells contain significant pools of non-protein-linked GPIs (10^5–10^7 molecules/cell depending on GPI structure and cell type) in addition to GPI structures (anchors) covalently linked to protein. Evidence from different experimental systems suggests that a fraction of the non-protein-linked GPIs may exit the ER and relocate to other organelles, including the plasma membrane (PM) (7–9). Indeed, some members of a family of GPI-related glycolipids (glycoinositol phospholipids) in Leishmania parasites are found at the cell surface, where they are accessible to antibodies and where they can be modified by exogenously added glycosyltransferases (1, 10).

In renal and intestinal epithelial cell lines such as Madin-Darby canine kidney (MDCK) and Caco-2, GPI-anchored proteins are expressed exclusively at the apical PM domain (11–14). It has been proposed that GPI anchors are dominant apical sorting signals for GPI-anchored proteins, acting through biophysical interactions with glycosphingolipid (GSL) clusters in the trans-Golgi network of epithelial cells (11, 15, 16). In contrast, very little is known about the subcellular distribution and transport of non-protein-linked GPIs. We have studied the composition and distribution of non-protein-linked GPIs in MDCK cells to investigate if the GPI structure per se contains intracellular sorting information, reflected in a polarized distribution of non-protein-linked GPIs on the epithelial cell surface. By analysis of enveloped RNA viruses that bud from the two cell-surface domains of MDCK cells, we show that GPIs are indeed present in the PM, but that they do not display a polarized distribution between the apical and basolateral domains. In the framework of models for epithelial cell polarity (15–18), intracellular lipid transport (17, 19), and GPI biosynthesis (3–5), we suggest that non-protein-linked GPIs may be located in the cytoplasmic leaflet of the PM lipid bilayer.

**EXPERIMENTAL PROCEDURES**

Materials—[1-^3H]Ethan-1-ol-2-amino hydrochloride (25 Ci/mmol) was purchased from Amersham Corp. [d-(4,5-^3H)]Galactose (49 Ci/mmol) was purchased from Amersham Corp. [d-(4,5-^3H)]Galactose (49 Ci/mmol)

Darby canine kidney; GSL, glycosphingolipid; flu, influenza virus; VSV, vesicular stomatitis virus; PE, phosphatidylethanolamine; DAF, decay-accelerating factor; PAGE, polyacrylamide gel electrophoresis; G_3α_3, NeuAc(b2–8)NeuAc(a2–3)Gal(b1–4)Glc(p1–1)ceramide; G_3α_3, NeuAc(b2–3)Gal(b1–4)Glc(p1–1)ceramide.
RESULTS

GPIs in MDCK Cells—MDCK cells were metabolically labeled with [2-3H]mannose in the presence of tunicamycin. When glycolipids were extracted from the labeled cells and analyzed by TLC, 8–11 labeled products were resolved (Fig. 1A). The four most hydrophilic products were identified as GPIs by their sensitivity to GPI-specific phospholipase D (Fig. 1B) and nitrous acid (data not shown) (24). For the purposes of this paper, the structures will be designated GPI-1, GPI-2 (a mixture of two poorly resolved GPIs), and GPI-3, in order of increasing hydrophobicity (Fig. 1A). Cocrchromatography with mammalian GPI standards indicated that the more hydrophilic component of the GPI-2 doublet was a GPI structure containing 3 phosphoethanolamine residues and that GPI-3 contains 2 phosphoethanolamine residues (22, 23). This assignment was supported by the observation that both GPI-2 and GPI-3 could be metabolically radiolabeled via [3H]ethanolamine (data not shown). GPI-1 may be a lyso form of one of the components of GPI-2 or may contain an additional mannose residue. Yields of radiolabeled GPI-1 varied between experiments, and in some cases, GPI-1 was not detected. Also in some experiments, an additional GPI structure migrating between GPI-2 and GPI-3 was identified (see Fig. 3, A and B); this may be the lipid identified previously as H7 (22).

The remaining [3H]mannose-labeled products (running in
the upper half of the chromatogram in Fig. 1A) were not sensitive to various tunicamycin pretreatments and were GPI-specific phospholipase D-, nitrous acid-, and mild acid-resistant, but mild base-sensitive. Their identity is currently unknown, but based on their response to the various treatments, it is clear that they are not dolichol-P-monosaccharides, dolichol-PP-glicosaccharides, or GPIs. Work is in progress to identify the nature of these lipids.

Following published work on domain-specific biotinylation of cell-surface proteins in MDCK and other polarized cells (11), we investigated whether ethanolamine-containing GPIs could be derivatized at the cell surface by membrane-impermeant amine-reactive probes. Consistent with previous observations on GPI modification under approximately physiological conditions (3, 4), these attempts were unsuccessful (data not shown). We therefore decided to use enveloped RNA viruses to investigate the presence of GPIs in the plasma membrane. These viruses obtain their envelope lipids by budding from select membranes in their host cells; flu and VSV bud from the PM, whereas flu-infected cells samples, "Experimental Procedures." The extract was analyzed by TLC directly (A) or after treatment with rabbit serum, a source of GPI-specific phospholipase D (GPI-PLD) (B). The chromatogram was developed in chloroform/methanol/water (10:10:3, v/v/v) and visualized using a radioactivity scanner. The origin (o) and solvent front (f) are indicated, as are the migration positions of the GPI standards H8 (containing 3 phosphoethanolamine residues as shown; see Refs. 22 and 23) and H7 (with 2 phosphoethanolamine residues; the middle mannose is not substituted). The designations GPI-1, GPI-2 (H8 substituted), GPI-3 (H7) are used throughout this paper. EtN, ethanolamine.

Fig. 1. Analysis of GPIs in MDCK cells. MDCK cells were radio-labeled with [3H]mannose, and glycolipids were extracted as described under "Experimental Procedures." The extract was analyzed by TLC directly (A) or after treatment with rabbit serum, a source of GPI-specific phospholipase D (GPI-PLD) (B). The chromatogram was developed in chloroform/methanol/water (10:10:3, v/v/v) and visualized using a radioactivity scanner. The origin (o) and solvent front (f) are indicated, as are the migration positions of the GPI standards H8 (containing 3 phosphoethanolamine residues as shown; see Refs. 22 and 23) and H7 (with 2 phosphoethanolamine residues; the middle mannose is not substituted). The designations GPI-1, GPI-2 (H8 + another poorly resolved GPI), and GPI-3 (H7) are used throughout this paper. EtN, ethanolamine.

Fig. 2. Polarized budding of flu and VSV from the apical and basolateral surface domains of MDCK cells. MDCK cells were seeded on 75-mm Transwell filters, incubated with [3H]galactose to label GSLs, and infected with flu (A), VSV (B), or no virus (C). Released viruses in the apical (●) or basolateral (○) media were purified and analyzed by centrifugation in 7–52% linear sucrose gradients (A–C). Gradient fractions (0.5 ml) were collected from the top of the gradients and taken for liquid scintillation counting (A–C, radioactivity). The sucrose density in each fraction was determined by measuring the refractive index (C, sucrose density). As a control, MDCK cells were infected with flu or VSV for 4 h as described under "Experimental Procedures," followed by metabolic labeling for 4 h at 37 °C with 150 μCi/ml [35S]methionine. Released viruses were analyzed as described above, and 1.0-ml fractions were monitored for the marker proteins influenza HA2 and VSV-G by immunoprecipitation, followed by SDS-PAGE and densitometry (C, relative intensity percent).
were obtained on analysis of viruses derived from infected \(^{3}H\)ethanolamine-labeled cells (data not shown). The data indicate that free GPIs are present in the PM of MDCK cells. The \(^{3}H\)mannose-derived radioactivity recovered in lipid extracts of the viruses was 3–4% of that recovered from the infected cell sample, suggesting that at least this fraction of cellular GPIs is located at the PM. For a more accurate calculation of the distribution of GPIs between the PM versus intracellular membranes and between the apical versus basolateral surface domain, it was necessary to obtain estimates of GPI concentration in the viral membranes. This was accomplished by normalizing GPI radioactivity to the amount of PE in each virus sample. PE is an appropriate marker for membrane bulk as (i) it is equally distributed across the membranes of the various compartments in the exocytic and endocytic pathways (17), and (ii) it is uniformly distributed between the apical and basolateral surface domains of polarized MDCK cells. This second point derives from the observation that PE is enriched in the nonpolarized cytoplasmic leaflet of the PM lipid bilayer (17), which in polarized epithelial cells is continuous between the apical and basolateral domains (17, 18, 21). Therefore, normalization of GPI radioactivity to PE also serves to correct for the differences in budding efficiency of flu and VSV in individual experiments. PE was measured by phosphorus analysis; typically <5% of total cellular PE was recovered in the purified viruses (see legend to Table I).

The \(^{3}H\)mannose-labeled GPI/PE ratio (cpm/nmol) in purified viruses, representing the plasma membrane, was 0–30% lower than in cells (Table I, “Enrichment”), indicating that the free GPIs are probably not preferentially enriched in the plasma membrane. Moreover, comparison of the GPI/PE ratio in flu versus VSV showed that the GPIs were similarly concentrated in both viruses (polarity values of ~1) and hence did not display a polarized distribution in the MDCK cell plasma membrane (Table I, “Polarity”).

We also investigated whether GPI-anchored proteins could be recovered in enveloped viruses in a manner reflecting their polarized cell-surface distribution. Although cellular glycoproteins are generally not assembled into enveloped viruses (28), GPI-anchored proteins may be incorporated via the same mechanisms used to recruit plasma membrane lipids into viral envelopes. Indeed, Calafat et al. (28) reported the presence of a GPI-anchored protein, Thy-1 (29), in VSV and murine leukemia virus virions recovered from infected thymoma cells. Since Thy-1 is not found in epithelial cells (30) and as we wished to maximize our chances of detecting GPI-anchored proteins in viruses, we chose to analyze a stably transfected MDCK cell clone expressing high levels of a chimeric GPI-anchored protein, gD1-DAF (14). This cell line has been previously characterized and is known to express gD1-DAF exclusively at the apical cell-surface domain (14). SDS-PAGE and Western blotting analysis of purified virus virions recovered from infected thymoma cells. Since gD1-DAF is not found in epithelial cells (30) and as we wished to maximize our chances of detecting GPI-anchored proteins in viruses, we chose to analyze a stably transfected MDCK cell clone expressing high levels of a chimeric GPI-anchored protein, gD1-DAF (14). This cell line has been previously characterized and is known to express gD1-DAF exclusively at the apical cell-surface domain (14). SDS-PAGE and Western blotting of solubilized MDCK (gD1-DAF) cells clearly showed a band corresponding to the 40-kDa gD1-DAF precursor and a smear (50–55 kDa) corresponding to mature forms of the chimera (data not shown). These bands were not detected in wild-type cells. The gD1-DAF-expressing MDCK cells were then infected with virus (flu or VSV) under conditions chosen to maximize virus yield. Western blotting analysis of purified virus samples recovered from the infected cultures detected none of the gD1-DAF forms, indicating that gD1-DAF is not incorporated into the viral envelopes. Thus, the viral assay is not useful in probing the plasma membrane distribution of GPI-anchored proteins in general. These results contrast with those of Calafat et al. (28) described above. One explanation for these apparently conflicting observations is that relatively small GPI-anchored proteins such as Thy-1 (~24 kDa) may
Table I

| Sample | GPI/PE | Enrichment (virus/cells) | Polarity (flu/VSV) |
|--------|--------|--------------------------|-------------------|
| Flu    | 37.9   | 0.86                     | 1.16              |
| Flu-cells | 43.9   | 1.00                     |                   |
| VSV    | 32.7   | 0.88                     | 1.09              |
| VSV-cells | 32.4   | 1.00                     |                   |
| GPI-2  |        |                          |                   |
| Flu    | 132.9  | 0.86                     | 1.09              |
| Flu-cells | 155.0  | 1.00                     |                   |
| VSV    | 121.7  | 0.89                     |                   |
| VSV-cells | 136.6  | 1.00                     |                   |
| GPI-3  |        |                          |                   |
| Flu    | 55.4   | 0.72                     | 0.97              |
| Flu-cells | 76.6   | 0.80                     |                   |
| VSV    | 57.3   | 0.80                     |                   |
| VSV-cells | 71.8   | 1.00                     |                   |

*Flu and VSV represent purified virus samples. Flu-cells and VSV-cells represent infected cell samples after washing to remove viruses in the surrounding medium.*

Behave essentially as large glycolipids and escape the potential steric constraints or ectodomain interactions that result in the elimination of gD1-DAF (−50–55 kDa) from virus budding sites (28).

**Discussion**

We used enveloped RNA viruses to obtain purified samples of the compositionally distinct PM domains of polarized MDCK cell monayers in order to assay for the presence of non-protein-linked GPIs at the cell surface. The observation that particular classes of enveloped viruses bud from specific cellular membranes has been exploited to obtain very pure membrane samples of subcellular compartments (21, 31, 32). In this way, flu and VSV, which bud from the apical and basolateral PM domains, respectively, have been used to study the phospholipid and GSL composition of the PM domains, respectively, have been used to study the phospholipid and GSL composition of the PM domains in [32P]phosphatidylserine and in [3H]mannose for 8 h at 37 °C in the presence of 50 μg/ml tunicamycin and infected with virus as described under “Experimental Procedures”. Radiolabeled GPIs in flu (apical) and VSV (basolateral) were extracted, analyzed, and quantified using a TLC linear analyzer. PE was quantified by phosphorus analysis. The amounts of GPI in intact cells were normalized to the amount of PE and are presented as the ratio GPI/PE (cpm/nmol). The apical to basolateral polarity is calculated by taking the ratio GPI/PE in flu over the ratio GPI/PE in VSV: PE in flu-infected cells, 175 ± 36 nmol, and in flu, 58 ± 0.7 nmol (3.5 ± 1.0% of total PE); total PE in VSV-infected cells, 155 ± 11 nmol, and in VSV, 5.1 ± 1.9 nmol (3.8 ± 0.5% of total PE). Data are presented as the mean of two measurements.

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**Synthesis in the ER.** Consistent with this observation, previous work has shown that GPIs may be found in lipid extracts of rat liver PM (12) and that members of a family of GPI-related glycolipids (glycosphingolipid phospholipids) are expressed on the surface of the protozoan parasite Leishmania major (10). Since GPIs are synthesized in the cytoplasmic leaflet of the ER (3–5), they may be transported to the PM via monomeric diffusion or protein-mediated exchange through the cytosol or via a vesicular transfer mechanism. All three transport mechanisms would result in expression of GPIs at the cytoplasmic face of the PM (see below), unless transbilayer movement (flip-flop) occurs. Since differences in lipid composition between the apical and basolateral PM domains are confined to the exoplasmic leaflet of the membrane bilayer (17, 18), the nonpolarized distribution of GPIs at the PM (Table I) suggests that GPIs may indeed be located in the cytoplasmic leaflet of the PM. We were unable to confirm this directly by examining the orientation of the various GPI structures in the MDCK cell PM or in purified viruses. The probes (phosphatidylinositol-specific phospholipase C and concanavalin A) that have been used successfully in the analysis of transmembrane orientation of protozoan and mammalian GPIs lacking inositol acyl and side chain phosphoethanolamine groups (3, 4) do not hydrolyze or recognize the mammalian GPIs that we describe. Therefore, it cannot be ruled out that some or all of the nonprotein-linked GPIs in the PM are expressed at the cell exterior, albeit without polarity. This would involve flipping of GPIs into the exoplasmic membrane leaflet of an intracellular organelle such as the ER, followed by vesicular transport without sorting at the trans-Golgi network. The results and hypotheses presented in this paper demonstrate the contrast between the distribution and transport of free GPIs and GPI-anchored proteins. The latter are confined to the exoplasmic leaflet of cellular membranes and are presumably sorted in the trans-Golgi network for selective delivery to the apical PM domain via a vesicular mechanism (11, 16). Although we were unable to use our viral assay to confirm the cell-surface polarity of GPI-anchored proteins, the exclusive localization of these proteins to the apical plasma membrane domain in MDCK cells has been extensively documented using immunofluorescence (14), domain-selective biotinylation (12, 14), domain-selective radioiodination (30), and cell-surface immunoprecipitation (13).

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