Cell Surface Display and Intracellular Trafficking of Free Glycosylphosphatidylinositols in Mammalian Cells*

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In addition to serving as membrane anchors for cell surface proteins, glycosylphosphatidylinositols (GPIs) can be found abundantly as free glycolipids in mammalian cells. In this study we analyze the subcellular distribution and intracellular transport of metabolically radiolabeled GPIs in three different cell lines. We use a variety of membrane isolation techniques (subcellular fractionation, plasma membrane vesiculation to isolate pure plasma membrane fractions, and enveloped viruses to sample cellular membranes) to provide direct evidence that free GPIs are not confined to their site of synthesis, the endoplasmic reticulum, but can redistribute to populate other subcellular organelles. Over short labeling periods (2.5 h), radiolabeled GPIs were found at similar concentration in all subcellular fractions with the exception of a mitochondria-enriched fraction where GPI concentration was low. Pulse-chase experiments over extended chase periods showed that although the total amount of cellular radiolabeled GPIs decreased, the plasma membrane complement of labeled GPIs increased. GPIs at the plasma membrane were found to populate primarily the exoplasmic leaflet as detected using periodate oxidation of the cell surface. Transport of GPIs to the cell surface was inhibited by Brefeldin A and blocked at 15 °C, suggesting that GPIs are transported to the plasma membrane via a vesicular mechanism. The rate of transport of radiolabeled GPIs to the cell surface was found to be comparable with the rate of secretion of newly synthesized soluble proteins destined for the extracellular space.

Glycosylphosphatidylinositols (GPIs)$^1$ are a class of glycolipids present in all eukaryotic cells (1–3). Although these glycolipids were originally discovered covalently linked to eukaryotic cell surface glycoproteins, many cells contain large pools of non-protein-linked or “free” GPIs. In mammalian cells, estimates of the pool size of mature free GPIs (potential protein anchor precursors) range from $10^5$ to $10^7$ molecules/cell (4, 5). The potential functions of free GPIs are a matter for speculation, although numerous proposals exist that place these molecules in signal transduction pathways (reviewed in Refs. 6 and 7). Indeed, recent data indicate that free GPIs are essential for growth in the protozoan parasite *Leishmania mexicana*, although their precise mode of action remains to be determined (8). To gain insight into the biological significance of free GPI molecules, it is important to define their subcellular distribution and understand the pathways by which they are biosynthesized, turned over, and transported among various cellular membranes.

The sequence of reactions involved in GPI assembly is relatively well defined, and genetic approaches have identified a number of gene products that are required for the assembly process (9–11). However, GPI assembly is spatially and topologically complex (12). Synthesis of the first GPI intermediate, GlcNAc-PI, occurs uniformly throughout the endoplasmic reticulum (ER), whereas post-initiation GPI biosynthetic reactions are concentrated in a mitochondria-associated ER domain (13). Furthermore, GPI biosynthesis is initiated on the cytoplasmic face of the ER (14–20), but the end products of the assembly pathway must be present in the luminal leaflet where attachment to proteins occurs (21). Although the precise membrane topology of GPI assembly remains to be determined, analyses of biosynthesis requirements and transbilayer distribution of GPIs suggest that mannosylated GPI structures, including the anchor precursors, can undergo transbilayer movement (8, 14, 15, 18).

Early work by McConville and Bacic (22, 23) showed that glycoinositol phospholipids in *Leishmania* parasites could be detected by monoclonal antibodies or by sodium borotritide reduction following periodate or galactose oxidase-mediated oxidation of the cell surface. Subsequently, free mammalian GPIs were shown to be constituents of the plasma membrane-derived lipid envelope of influenza virus and vesicular stomatitis virus (VSV) (5) and were detected at the cell surface using amine-reactive membrane-impermeant reagents (24). GPIs were also identified by chemical radiolabeling in a plasma membrane fraction from rat liver (25). These studies provided the first indications that GPIs are exported from their site of synthesis. However, more detailed analyses are clearly required to obtain a quantitative picture of the subcellular distribution of GPIs and an understanding of GPI transport mechanisms.

In this study we use metabolic radiolabeling in conjunction with membrane isolation techniques to provide direct evidence that free GPIs, following their synthesis in the ER, redistribute...
to all major subcellular organelles, including the Golgi and plasma membrane. We show that although the membrane concentration of metabolically radiolabeled GIs under short term continuous labeling conditions (2.5 h) is roughly equal in all fractions derived from a post-mitochondrial supernatant, GIs are slowly concentrated in the exoplasmic leaflet of the plasma membrane over extended chase periods. Our analyses show that cell surface display of GIs requires vesicular transport through the Golgi apparatus and occurs at a rate similar to that with which proteins are secreted into the extracellular medium. The accumulation of GIs at the plasma membrane occurs in concert with catabolic/turnover processes that reduce the total cellular content of radiolabeled GIs.

**EXPERIMENTAL PROCEDURES**

**Materials**—n-[2-3H]Mannose (20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). t-[3-3H]Serine was from Amersham Pharmacia Biotech. Protease inhibitor mixture, DNase I, and tunicamycin were obtained from Calbiochem-Behring Corp. (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and penicillin/streptomycin were from Life Technologies, Inc. Glucose-free RPMI 1640 medium was purchased from Speciality Media, Inc. (NJ). VSV strain was obtained from Dr. Peter Butterkofer (University of Bern, Switzerland). Human Thy-1 antibodies were from Dr. Christopher Nichitita (Duke University Medical Center). Antibodies against the yeast mitochondrial proteins Hsp60 and Hsp70 were provided by Dr. Elisabeth Craig (University of Wisconsin-Madison). Rabbit polyclonal anti-GRP78 (BiP) antibodies were purchased from Affinity Bioreagents, Inc. (Golden, CO). Horseradish peroxidase-conjugated anti-rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL Western blotting detection system for chemiluminescent detection of Western blots was obtained from Amersham Pharmacia Biotech. Glass-backed silica 60 thin layer plates were from Merck.

**Cell Culture**—Cell lines were obtained from ATCC (Manassas, VA). The Thy-1 mouse thymoma cell line BW3147.3 was maintained in suspension culture in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO2 at 37 °C. MDCK and HeLa cell lines were cultured as cell monolayers under the conditions described for thymoma cells.

**Preparation of Subcellular Fractions from Metabolically Labeled Thymoma Cells**—Thymoma cells (5 × 106) were collected by centrifugation, washed twice with PBS, and resuspended in low glucose (0.1 mg/ml) RPMI 1640 medium (106 cells/ml) supplemented with 10% dialyzed fetal bovine serum. The cells were preincubated with 10 μg/ml tunicamycin for 30 min at 37 °C and labeled for 2.5 h with n-[2-3H]mannose (5–10 μCi/ml). After labeling, the cells were washed twice with PBS and resuspended in 5 ml of buffer A (0.25 mM sucrose, 10 mM Hepes/NaOH, pH 7.5, 1 mM EDTA) supplemented with protease inhibitor mixture. Cells were homogenized using a cavitator bomb (Kontes Glass Company, Vineland, NJ), and the resulting homogenate was treated with DNase I (0.5 mg/ml) for 30 min on ice. The homogenate was clarified by centrifugation at 1,000 × g for 10 min at 4 °C to remove large debris and nuclei. The resulting supernatant was centrifuged at 10,000 × g for 10 min at 4 °C to yield pellet (S10) and pellet (P10) fractions. S10 and P10 were analyzed directly or subjected to further fractionation as described below.

S10 was fractionated as described previously (14) and illustrated in Fig. 2A. Briefly, S10 corresponding to 5 × 106 cell equivalents was layered on top of a discontinuous sucrose gradient containing 1.5 ml of 35% sucrose, 1 ml of 30% sucrose, and 0.8 ml of 20% sucrose (all prepared in 10 mM Hepes/NaOH, pH 7.5, 1 mM DTT) supplemented with protease inhibitor mixture. Cells were homogenized using a cavitator bomb (Kontes Glass Company, Vineland, NJ), and the resulting homogenate was treated with DNase I (0.5 mg/ml) for 30 min on ice. The homogenate was clarified by centrifugation at 2,000 × g for 10 min at 4 °C to yield supernatant (S10) and pellet (P10) fractions. P10 and S10 were analyzed directly or subjected to further fractionation as described below.

P10 (10–15 mg/ml protein) was washed twice with buffer A and then resuspended in buffer A for enzyme activity measurement and lipid extraction. For further fractionation, P10 was resuspended in isolation buffer (250 mM mannitol, 25 mM Hepes/NaOH, pH 7.5, 0.5 mM EDTA) and resolved into mitochondria-associated membranes (MAM) and mitochondria (Mito) following previously established procedures (13, 26–28) (Fig. 3A). Briefly, P10 was layered on top of a 20-ml solution of 30% (v/v) Percoll (prepared in 225 mM mannitol, 25 mM Hepes/NaOH, pH 7.5, 1 mM EDTA) in a centrifuge tube. Following 30 min of centrifugation at 95,000 × g, a light diffuse upper band and a denser lower band were collected manually and diluted ~5-fold with buffer A. The diluted upper band was centrifuged for 30 min at 10,000 × g to yield pellet and supernatant fractions. The pellet derived from the dense lower band was resuspended in buffer A; based on marker-enzyme analysis (Fig. 3B) it was determined to be enriched in mitochondria and termed Mito. The supernatant was combined with the diluted diffuse upper band and centrifuged at 100,000 × g for 90 min at 4 °C to yield pellet MAM.

**Characterization of Subcellular Fractions**—The protein content of each fraction was determined using the Micro BCA Protein Assay Reagent (Pierce). Half of each fraction was taken for lipid extraction to determine phospholipid and GPI content. Labeled lipids were extracted and analyzed as described below. Total phospholipid content was determined following hydrolysis and phosphate measurement (29). To determine the lipid profile of each fraction, bulk lipids were extracted by the method of Bligh and Dyer (30). Lipids were analyzed by one-dimensional TLC using chloroform/methanol/acidic acid/formic acid/water (35:15:6:2:1, v/v/v/v/v), followed by hexane/diethylether/acetic acid (80:20:1, v/v/v) (31). To visualize the lipids, plates were aired after spraying with 5% CuSO4, 8% phosphoric acid. The resulting bands were identified by comparison with standards and were quantitated by digital densitometry using NIH Image.

Fractions were also characterized by measuring organelle-specific marker enzyme activities or by determining the amount of marker proteins via Western blot analysis. The following marker enzyme activities were assayed using procedures described by Storrie and Maden (32) unless otherwise specified: alkaline phosphodiesterase I (plasma membrane), α-mannosidase and β-galactosidase transferase (Golgi), succinate dehydrogenase (mitochondria; assayed as described in Ref. 33), and NADPH-cytochrome c reductase and dolicholphosphomannose synthase (ER markers; assayed as described in Ref. 14, 28, and 34). The ER proteins ribophorin I and BiP (GRP78) and the mitochondrial proteins Hsp60 and Hsp70 were quantitated using standard Western blotting procedures. After probing with primary antibodies, blots were developed using peroxidase-conjugated secondary antibodies and the ECL Western blotting detection system following the protocol provided by the manufacturer. Band intensities were determined by densitometry (at least two different amounts of protein (typically 1 and 2.5 μg) were analyzed, and several different film exposures were obtained to verify linearity of the film response for densitometric analysis).

**GPI Extraction and Analysis**—GPIs were extracted in chloroform/methanol/water (10:10:3, v/v/v) as described previously (5). The extract was desalted by n-butyl alcohol-water partitioning and analyzed by TLC (silica 60; chloroform/methanol/water, 10:10:3, v/v/v). TLC plates were scanned for radioactivity with a linear scanner (Berthold LB2842), and GPI species, identified by TLC mobility and diagnostic treatment with specific enzyme activities (glycosyl-GPI synthase MAM (Golgi), and analyzed as described above.

**GPI Extraction and Purification**—MDCK cell monolayers were grown to 80–90% confluence (approximately 107 cells/plate). Monolayers were washed several times with PBS followed by the addition of 7 ml of labeling medium consisting of low glucose (0.1 mg/ml) RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum. GIs were metabolically radiolabeled by incubating the cells with 20 μCi/ml n-[2-3H]mannose for 6 h. At the end of the labeling incubation period, the cells were washed twice with PBS and infected with vaccinia virus (VV) or VSV as described previously (5, 35). Cells were collected by scraping with 3% CuSO4, 8% phosphoric acid. The resulting bands were identified by comparison with standards and were quantitated by digital densitometry using NIH Image.

**Cells and Viruses**—Cells were infected with 5 plaque-forming units/cell of VV (IHD-J) strain for 24 h. The two intracellular forms of VV were purified essentially as described previously (35). Briefly, scraped and pelleted cells

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were ruptured in 10 mM Tris/HCl, pH 9.0, using a Dounce homogenizer with a large clearance. After centrifugation at 1,000 × g for 10 min at 4 °C to remove a large portion of cellular membranes, the resulting post-nuclear supernatant was sonicated in a water bath sonicator for 5 min at room temperature. The sonicated supernatant was layered onto a continuous sucrose gradient (25–50% sucrose in 150 mM NaCl, pH 9.0) buffer and centrifuged at 20,000 rpm (20 min) in an SW 28 rotor to collect IEV and IMV particles. The purity of the IMV and IEV fractions was assessed as described (35).

VSV infection was carried out as described previously (5). Cells were infected with 10 plaque-forming units/cell of VSV (San Juan strain) for 8 h. Following infection, the culture medium was collected, cleared of lysed cell debris by low speed centrifugation, and loaded onto a 0.25 mM sucrose cushion prepared in TE buffer (1 mM Tris/HCl, pH 7.4, 1 mM EDTA). Pellets recovered after centrifugation at 25,000 rpm for 3 h in an SW 28 rotor were resuspended in TE buffer, loaded onto a linear sucrose (7–32%, w/v) gradient, and centrifuged at 100,000 × g for 1 h in an SW 41 rotor. The VSV band was collected, diluted 4–fold with TE buffer, and recovered by centrifugation at 30,000 rpm for 2.2 h in an SW 41 rotor.

Vesiculation of the Plasma Membrane: Preparation of Plasma Membrane Blebs—MDCK and HeLa cell monolayers (grown to confluence in 150-mm diameter culture plates; ~10^7 cells/plate, ~2 × 10^6 cells/experiment) were washed several times with PBS followed by the addition of 7 ml of the labeling medium described above. The cells were then preincubated with 15 μg/ml (MDCK cells) or 6 μg/ml (HeLa cells) tunicamycin (added from a 10 mg/ml stock in Me2SO) for 30 min at 37 °C and labeled for 2.5 h with [2-3H]mannose (10 μCi/ml). For pulse-chase experiments metabolically labeled cell monolayers were washed twice with PBS before adding 10–15 ml of DMEM supplemented with 10% fetal bovine serum. Incubation was continued for different amounts of time as indicated in each experiment. In some experiments the cells were oxidized using 10 mM NaIO4 as described below before blebbing of the PM.

Vesiculation, or blebbing, of the plasma membrane of MDCK and HeLa cells was performed essentially as described by Scott (36). Briefly, metabolically radiolabeled cells were washed twice with 10 mM Hapes, 150 mM NaCl, pH 7.4. Vesiculation buffer (10 mM Hapes, 150 mM NaCl containing 2 mM CaCl2, 2 mM DTT, and 25 mM formaldehyde) (7 ml) was added to each plate, and vesiculation was allowed to occur for 2 h at 37 °C. The culture supernatant was then collected from the plates and pooled. The pooled supernatants were centrifuged at 1000 × g for 7 min to pellet any cells that may have dislodged from the plates. The plasma membrane preparation was then centrifuged at 30,000 × g (Beckman Ti 50.2 rotor, 1 h) to pellet the membranes, and the resulting pellet was resuspended in 10 mM Hapes, 150 mM NaCl, pH 7.4. Samples were taken directly for lipid analyses or were dialyzed for 24–30 h at 4 °C against 10 mM Hapes, 150 mM NaCl to remove residual vesiculation buffer components prior to protein and marker enzyme measurements. In parallel, an additional plate of metabolically labeled MDCK cells was treated with vesiculation buffer without CaCl2, DTT, and formaldehyde. Following incubation the cells were scraped and washed with 10 mM Hapes, 150 mM NaCl, and aliquots were used for lipid and marker enzyme analyses. The purity of the plasma membrane fraction was assessed by measuring the content of organelle-specific markers compared with that of the mock-treated cells. The amount of the ER marker ribophorin I and the mitochondrial marker Hsp70 was determined by Western blot analysis as described above. 5'-Nucleotidase activity (PM marker) was assayed as described previously (37).

Oxidation of GPIs by Sodium Metaperiodate—Metabolically labeled MDCK and HeLa cells, washed twice with ice-cold PBS (pH 7.5), were incubated on ice in the dark for 30 min in either PBS (control) or PBS containing 10 mM NaIO4. The reactions were quenched by washing the cells four times with ice-cold PBS containing 150 mM glycerol followed by two washes with PBS. In control experiments to determine the efficiency of oxidation as well as the efficiency of the quenching procedure, a fixed number of cells were metabolically labeled and induced to bleb, BFA (2.5 μg/ml) was included in the vesiculation buffer. [3H] Serine-labeled Protein Secretion Assay—HeLa cells, grown to confluence on 3-cm dishes, were preincubated with 750 μl of serine-free RPMI 1640 with or without BFA as described above. [3H] Serine (50 μCi) was then added, and the cells were incubated for 0–10 h at 37 °C. Following infection [3H] Serine-labeled proteins was assayed by processing the culture medium from a dish of cells as follows. A single dish was washed for each time point. The medium (150-μl aliquot) was centrifuged at maximum speed in a microfuge for 7 min to clear any cell debris and then treated with a mixture of chloroform and methanol to precipitate proteins according to Wessel and Flugge (38). The protein pellet so obtained was washed, then dried under a stream of nitrogen, and resuspended in SDS-PAGE sample buffer. A fraction of the sample was analyzed by SDS-PAGE (12.5% acrylamide gel) and fluorography (film was exposed for 3 weeks). The [3H] Serine-labeled proteins visualized on the film were quantitated by densitometric analysis.

RESULTS

Distribution of Free GPIs in P10 and S10 Subcellular Fractions—Previous work suggested that non-protein-linked (free) GPIs (henceforth referred to simply as GPIs) in mammalian cells are not restricted to their site of synthesis in the ER and can be transported to the plasma membrane (5, 18, 24, 25). To examine these results more closely, we used subcellular fractionation procedures to measure the distribution of metabolically labeled GPIs in different cellular organelles of thymoma cells. We first confirmed that when thymoma cells were incubated with [2-3H]mannose in the presence of tunicamycin, the main radiolabeled lipids recovered in organic solvent extracts were the well-characterized GPI molecules termed H7 and H8 (39). The structure of H8 is shown in Fig. 1A; H7 is identical to H8 except that it lacks the ethanolamine-phosphate group linked to the middle mannose residue. A representative thin layer chromatogram of a lipid extract from [2-3H]mannose-labeled thymoma cells is shown in Fig. 1B. The recovery of radiolabeled lipids was unchanged when labeling was carried out in the presence of the protein synthesis inhibitor cycloheximide (data not shown), indicating that the lipids are not derived via catabolism of GPI-anchored proteins and that their synthesis in the short term does not require fresh translation of biosynthetic enzymes. Based on the specific activity of the [2-3H] mannose used and the extent of radiolabeling of H8 and H7, it can be estimated that thymoma cells contain >10^4 GPI molecules/cell, consistent with other estimates (4).

To determine the subcellular distribution of GPIs, thymoma cells were metabolically labeled with [2-3H]mannose for 2.5 h at 37 °C and disrupted by nitrogen cavitation. Following the removal of nuclei and unbroken cell debris by low speed centrifugation, the resulting homogenate was centrifuged at 10,000 × g to obtain pellet (P10) and supernatant (S10) fractions. The composition of the P10 and S10 fractions has been described previously (13, 14) and is summarized in Table I. Briefly, the S10 fraction is enriched in microsomal membranes, including the plasma membrane (PM), Golgi, and ER, whereas P10 consists mainly of lysosomes, lysosomes, and peroxisomes, as well as some ER and PM.

The distribution of phospholipids and H8 in the P10 and S10 fractions is shown in Fig. 1C. Both phospholipids and H8 were distributed roughly evenly between the S10 and P10 fractions. Phospholipid recovery (relative to the phospholipid content of the homogenate; Fig. 1C, bar H) was ~47 and ~40% in S10 and P10, respectively; H8 recovery (relative to the radiolabeled H8 content of the homogenate) was 49 and 31% in S10 and P10, respectively. The membrane concentration of H8, determined...
as radioactivity (counts (cts) as measured using a TLC radioactivity scanner/nmol phospholipid, was thus similar in the homogenate and S10 (139 and 144 cts/nmol phospholipid, respectively) with the level in P10 being somewhat lower (107 cts/nmol phospholipid). Because the organelle composition of the S10 and P10 fractions is quite different (Table I and below), these data suggest either (i) that H8 is similarly concentrated in all subcellular membranes or (ii) that the concentration of H8 in different membranes is quite different but coincidentally yields a roughly similar total amount in P10 and S10. To address these issues, S10 and P10 were subfractionated according to previously defined procedures (13, 14).

### Radiolabeled GPIs Generated in a Short (2.5 h) Labeling Pulse Are Roughly Equally Distributed in All Major Organelle Fractions Derived from S10—S10 was fractionated on a discontinuous sucrose gradient to yield three major fractions enriched in PM, Golgi (Fig. 2, lane G) and ER (Fig. 2). Measurements of organelle-specific markers as well as determinations of the phospholipid profile for each of the fractions support these assignments (Fig. 2, B and C, and Table I). For example, the PM fraction was characterized by high β-galactosyltransferase and α-mannosidase II activities (Table I). The GPI content of the S10-derived fractions was determined by lipid extraction and TLC. Fractions were obtained from cells labeled with [2-3H]mannose for 2.5 h. The data show that the membrane concentration of radiolabeled H8 (cts in H8/nmol phospholipid) is similar in all three fractions (Fig. 2C, left panel), but because the ER contributes most of the phospholipid content of the S10 the total amount of H8 is highest in the ER (Fig. 2C, right panel). An identical distribution was obtained for H7 in cells labeled for 2.5 h, as well as for radiolabeled H7 and H8 generated in much shorter labeling pulses (5 min or 1 h) (data not shown and Ref. 18).

A potential problem in interpreting the data presented above is the possibility that some redistribution of lipids occurs between the various S10 fractions during the lengthy fractionation procedure. However, the likelihood of general intermembrane lipid mixing occurring after cell homogenization is unlikely because the phospholipid profiles of the various fractions remain characteristically distinct (Fig. 2B). More importantly, in vitro experiments in which we tested the ability of radiolabeled H8 to be transferred from one vesicle population to another in the presence or absence of cytosol or purified glycolipid transfer protein (40) showed no detectable transfer; under the same in vitro assay conditions, both cytosol and glycolipid transfer protein were able to support intermembrane transfer of galactosylceramide. These data suggest that H8 is unlikely to “hop” between membranes during the subcellular fractionation procedure and that the distribution profile that we obtained represents the subcellular distribution of H8.

The purity of the subcellular fractions was also considered. Because the PM fraction contributes only about 10–15% to the total H8 recovered in S10 (Fig. 2C), cross-contamination from other organelar fractions, particularly the ER, could significantly affect estimates of PM H8 content. However, analysis of the data suggests that although cross-contamination accounts for some of the GPI content of the PM fraction, it cannot account for all PM-associated H8. To determine whether ER contamination alone accounts for the H8 content of the PM fraction, we deconvoluted the data for S10 shown in Table I and Fig. 2C using measurements of the total phospholipid content of each fraction and assuming that the various organelle-specific markers (Table I) are uniformly distributed in their respective membranes. Our calculations indicate that ER membrane accounts for ~52% of the membrane content of the PM fraction and ~92% of the membrane content of the ER fraction. Thus if H8 were solely confined to the ER, the relative H8 content of the ER and PM fractions would be in the proportion 7:1 (based on the amount of phospholipid recovered in each fraction). Our experimental measurements indicate that the proportion of H8 is ER:PM ~4:1 (Fig. 2C). Thus ER contami-
Data (representative of more than 10 fractionation experiments) are derived from Refs. 12 and 13 and the present paper. The recoveries (expressed as percentages of total in source) shown here are rounded numbers intended simply to guide the interpretation of the fractionation data presented in this paper. More detailed data are presented in Figs. 2 and 3. ND, not determined.

| Source | Fraction | Succ dehydrog (Mito) | β-hex (Lys) | catalase (Perox) | APDE I (PM) | α-man II (Golgi) | ribo I (ER) |
|--------|----------|----------------------|-------------|-----------------|-------------|-----------------|------------|
| H      | P10      | 85                   | 85          | 90              | 20          | 15              | 20         |
|        | S10      | 15                   | 15          | 5               | 70          | 80–90           | 60–80      |
| P10    | Mito     | 35                   | 35          | ND              | ND          | ND              | ND         |
|        | MAM      | 35                   | 35          | ND              | ND          | ND              | ND         |
| S10    | PM       | ND                   | ND          | ND              | ND          | ND              | ND         |
|        | Golgi    | ND                   | ND          | ND              | ND          | ND              | ND         |
|        | ER       | ND                   | ND          | ND              | ND          | ND              | 10         |

*Source for fractionation analysis. Recoveries are calculated in each case by setting activity in the source at 100.

GPIs in Enveloped Viruses Grown in MDCK Cells—In parallel with the subcellular fractionation analyses of GPI distribution in thymoma cells, we used enveloped viruses to probe the lipid composition of Golgi membranes and the PM in MDCK cells. MDCK cells were used because they support growth of the relevant viruses. The observation that particular classes of enveloped viruses assemble at specific cellular membranes has been exploited by a number of investigators to obtain pure samples of subcellular compartments (43–46). A variety of experimental observations indicate that virus envelopes are broadly representative of the lipid composition of the membranes from which they are derived (43, 46–48).

We metabolically labeled MDCK cell monolayers with [2-3H]mannose for 6–8 h and then infected the cells with VV or VSV. VSV buds from the PM (49), whereas VV undergoes a complex assembly process generating two intracellular forms, the intracellular mature virus (VV-IMV) and the intracellular enveloped virus (VV-IEV). VV-IMV corresponds to viral particles enwrapped in membranes derived from the cis-Golgi network (CGN), whereas VV-IEV particles correspond to IMVs that undergo an additional wrapping with membranes of the trans-Golgi network (TGN) (45, 50). After 15–24 h, viruses were purified from cell homogenates (in the case of VV) or the cell culture medium (in the case of VSV), extracted with organic solvent, and analyzed for the presence of radiolabeled GPIs. Preparations from identically incubated mock-infected cells were used for comparison.

All the virus preparations, VV-IMV, VV-IEV, and VSV, contained radiolabeled H8 and H7, indicating that these GPIs are constituents of the CGN, TGN, and PM, respectively. Fig. 4 shows the membrane concentration of GPIs (cts/nmol phospholipid) in the CGN, TGN, and PM relative to GPI concentration in bulk cellular membranes obtained via analysis of extracts of mock-infected cells (estimates of GPI concentration in the TGN were obtained by subtracting the contribution of CGN membranes (VV-IMV) to VV-IEV (35)). The data clearly show a concentration gradient of radiolabeled GPIs across the secretory pathway, starting at a level in the CGN comparable with that seen in bulk cellular membranes and approaching a 3-fold higher concentration at the PM. The quantitative differ-
Fig. 2. Fractionation of S10; characterization of fractions and distribution of metabolically radiolabeled H8. A, schematic presentation of S10 fractionation on a discontinuous sucrose gradient. S10 was layered over a series of sucrose density steps and centrifuged (100,000 × g for 2.5 h). Fractions were collected manually from the top of the gradient. Aliquots of each fraction were taken for lipid extraction and analysis, protein determination, and organelle-specific marker enzyme assays. The recovery of organelle-specific marker enzymes for each fraction is shown in Table I. Golgi, B, phospholipid composition of S10-derived fractions determined as described under “Experimental Procedures.” The amount of each phospholipid found in isolated membrane fractions is expressed as a percentage of total phospholipids present in that fraction. PE, phosphatidylethanolamine; PS, phosphatidylycerine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin. C, distribution of metabolically radiolabeled H8 in S10-derived fractions. S10 was obtained from thymoma cells metabolically labeled with [2-3H]mannose for 2.5 h. The left panel shows the membrane concentration of [3H]mannose-labeled H8 in the various fractions (concentration is expressed as H8 radioactivity (TLC scanner cts)/nmol phospholipid). The right panel shows total recovery of H8 in the different fractions expressed as a percentage of the H8 content of the S10.

ences in the data obtained here versus those generated from the subcellular fractionation approach are due to differences in the time frames used in the two experiments (see “Discussion”); the fractionation experiments were conducted after short labeling periods (~2.5 h), whereas virus production necessitated longer pulse labeling and chase times. The different time frames are reconciled in experiments described below showing that at early times (~5 h), GPI concentration at the PM of MDCK cells is similar to that seen in bulk cellular membranes, i.e. as seen in Fig. 2C but that with extended chase periods the PM complement of GIs becomes more concentrated, i.e. as seen in Fig. 4.

GPIs in Plasma Membrane Blebs—To estimate GPI concentration in the PM of MDCK and HeLa cells as a function of time, we used a plasma membrane vesiculation approach (36) that not only allowed us to isolate relatively pure plasma membrane blebs devoid of intracellular organelles but also permitted reasonable time resolution in the time frame that we wished to investigate. The protocol for generating blebs involves treating adherent cells with a buffer containing 2 mM DTT, 2 mM CaCl2, and 25 mM formaldehyde. The formaldehyde concentration used is at least 25–50-times lower than that used for fixation and causes no detectable aggregation of surface components as judged by lateral mobility measurements (51). Blebs have been previously extensively characterized in terms of their ultrastructure and their protein and lipid composition (36). Compositional data that we obtained with our preparations are consistent with these earlier characterizations. MDCK and HeLa blebs showed a 10-fold enrichment (over whole cells) of the PM marker 5'-nucleotidase activity and displayed only minor or undetectable contamination with the ER and mitochondrial markers, ribophorin I and Hsp70, respectively as determined by Western blot analysis (Fig. 5A). Also, as can be seen in data shown later (compare top and bottom panels at the right of Fig. 7A), certain radiolabeled lipids present in cells are not present in the blebs, confirming that bleb membranes are compositionally distinct from bulk cellular membranes.

Blebs prepared from [2-3H]mannose-labeled MDCK (Fig. 5) and HeLa cells (data not shown; see Figs. 7 and 8) contained radiolabeled GIs. The membrane concentration of GIs (H8 and H7) in blebs prepared from MDCK cells metabolically labeled with [2-3H]mannose for 2.5 h (2 h chase reflects blebbing time) was similar to that found in total cell extracts (Fig. 5C). When prelabeled MDCK cells were chased for 2 h before bleb formation, a dramatic increase (~5.4-fold) in the PM con-
concentration of labeled H8 was observed (Fig. 5C, left panel, black bars), whereas the PM concentration of H7 increased only slightly (~1.3-fold) (Fig. 5C, right panel, black bars). Similar results were obtained from experiments using [2-3H]mannose-labeled HeLa cells, although the concentration of H8 in blebs relative to cell membranes after labeling for 2.5 h was 0.25 ± 0.06, a lower ratio than observed in MDCK cells (0.93 ± 0.19). The reason for this discrepancy between MDCK and HeLa cells is unclear but could be due to differences in transport between polarized and nonpolarized cells. However, after longer chase times (24 h) the relative concentration of radiolabeled H8 in the PM compared with cell membranes was found to be similar in both HeLa and MDCK cells with ratios of 4.3 and 5.3 ± 0.46, respectively, reflecting an accumulation of radiolabeled H8 at the PM. The data indicate that pulse-labeled H8 and H7 initially populate the PM in MDCK and HeLa cells at a concentration similar to or somewhat lower than that seen in bulk cellular membranes. These results are similar to those obtained via subcellular fractionation analyses of metabolically radiolabeled thymoma cells (Fig. 2C). Over extended chase periods, H8 accumulates in bleb membranes consistent with the VSV data reported in Fig. 4. However, although the VSV data show a corresponding accumulation of H7 at the PM, the bleb results indicate that H7 concentration after a long chase period is only marginally in excess of that of bulk cellular membranes. The reason for this difference is unclear.

Fig. 5C also shows the membrane concentration of H8 and H7 in total cellular membranes from MDCK cells as a function of chase time following a 2.5-h labeling pulse. The concentration of H8 and H7 in total cellular membranes decreases 2- and 3.5-fold, respectively, over a chase period of 24 h, suggesting that bulk cellular GPIs are subject to turnover processes. At this point we have little information on the nature of the reactions involved in GPI turnover except to note that with the exception of GPI depletion through the protein-anchoring transamidation reaction (21), turnover reactions must involve dissociation of the GPI mannose core from the hydrophobic lipid moiety or other structural modifications such as removal of phosphoethanolamines to generate lipid species that are chromatographically distinct from H8 and H7. We conclude that the considerable relative (PM versus cells) enrichment of radiolabeled GPIs at the PM over long chase periods is due to the combined effects of two processes: accumulation of a PM pool and turnover of bulk cellular GPIs.

Slow Accumulation of GPIs at the Cell Surface—To gain insight into the transbilayer orientation of GPIs at the PM, we used membrane topological probes to assess the amount of GPIs present at the cell surface, i.e. the exoplasmic leaflet of

Fig. 5. Distribution of metabolically labeled GPIs in plasma membrane blebs. A, plasma membrane blebs were characterized by determining the content of organelle-specific marker enzymes in blebs compared with total cell membranes. PM enrichment was determined by 5'-nucleotidase activity (2.5 µg of protein/assay, activity given as arbitrary units/mg protein). ER and mitochondria content were determined by Western blot analysis for ribophorin I and Hsp70, respectively (4 µg of protein was loaded/ lane). Details are described under “Experimental Procedures.” B, thin layer chromatogram of radiolabeled lipids from [2-3H]mannose-labeled MDCK cells and blebs isolated from the labeled cells. MDCK cells were metabolically labeled with 10 µCi/ml of [2-3H]mannose for 2.5 h and chased for 22 h in complete medium (DMEM). Vesiculation, or blebbing, of the plasma membrane was performed on 2 × 10⁶ cells as described under “Experimental Procedures.” Lipids were extracted from blebs and mock-treated cells and analyzed by TLC using a linear analyzer. Arrothead denote the origin (o) and solvent front (f) as well as migration of GPI species H8 and H7. C, membrane concentration of [3H]mannose-labeled GPIs in plasma membrane blebs. Data were obtained from MDCK cells labeled for 2.5 h or labeled and then chased for a long (22 h) time period followed by blebbing of the PM. The short chase period of 2 h corresponds to the time taken for optimal bleb production. The amount of [3H]mannose-labeled GPIs was quantitated using integration software accompanying the TLC linear analyzer and normalized to the amount of total phospholipid. The data are expressed as membrane concentration (cts/nmol phospholipid) of labeled GPIs (H8 and H7) in bulk cellular membrane and blebs.

Cell Surface Display of GPI Lipids

Fig. 4. GPIs in the membranes of enveloped viruses. MDCK cell monolayers were metabolically labeled with 20 µCi/ml of [2-3H]mannose for 6 h. The cells were then infected with the IHD-J strain of VV at 5 plaque-forming units/cell for 24 h or with 10 plaque-forming units/cell of VSV (San Juan strain) for 8 h. The two intracellular forms of vaccinia virus (VV-IMV, which samples the CGN, and VV-IEV, which results from enwrapping VV-IMV with TGN membranes) and VSV were purified as described under “Experimental Procedures” and subjected to lipid extraction and analysis. Mock-infected cells were extracted in parallel and used for comparison. The data are presented as membrane concentration (cts/nmol) of [3H]mannose-labeled H8 (or H7) in the virus preparations relative to that in bulk cellular membranes (set at 1). Data for the TGN were obtained by subtracting the VV-IMV contribution to VV-IEV as described under “Results.”
Exoplasmic Leaflet—Experiments involving the oxidation of glycosides and glycoinositol phospholipids at the cell surface (22, 23, 52) and is useful for topological studies because the periodate ion is a large and negatively charged entity that is unlikely to permeate membranes passively, especially at lower temperatures.

MDCK and HeLa cells were metabolically labeled with [2-3H]mannose for 2.5 h, after which the labeling medium was replaced with normal growth medium (DMEM), and the cells were further incubated. The accessibility of labeled GPIs to NaIO₄ treatment was determined after different chase periods. Oxidation of H8 by NaIO₄ results in a molecular species that migrates differently from untreated GPIs by TLC analysis; chromatographic resolution of H7 and oxidized H7 was more difficult to achieve, and our results are therefore confined to analyses of H8. In MDCK cells the oxidized H8 co-migrated with other unknown radiolabeled species and could not be quantitated directly; thus for MDCK cell experiments the percentage of oxidized H8 was determined by quantitating residual H8 (data not shown). In HeLa cells, however, oxidized H8 could be visualized as a distinct region on the TLC (Fig. 6A, peak marked H8* most clearly seen in panel f) and quantitated as percentage of total H8 oxidized. For this reason detailed studies to determine GPI topology at the PM and GPI arrival at the cell surface were done using exclusively HeLa cells.

HeLa cells were labeled for 2.5 h and chased for 0, 2, or 18 h. At every chase time, samples were taken for cell surface periodate oxidation, and untreated samples were used as controls. The experiments showed that under conditions where 60–70% of free GPIs were modified in disrupted cells (data not shown), very little oxidation (<5%) of GPI species was detected in intact HeLa cells after the 2.5-h labeling period (0 h chase; Fig. 6A, panel b (+ periodate) versus panel a (− periodate)). These data indicate that radiolabeled H8 is predominantly intracellular over the 2.5-h labeling period and confirm that NaIO₄ does not permeate the PM. These results are also consistent with the data of Figs. 2C and 5C, which, together with the fact that the PM represents only ~10% of total cellular secretory membranes (42, 53), predict that only 10% of H8 at most should be oxidized after a 2.5-h labeling period. Indeed, for the specific case of HeLa cells (ratio of H8 concentration in PM versus that in whole cells after 2.5 h of labeling + 2 h blebbing is ~0.25), the prediction is maximally 2.5% oxidation of H8 after 2.5 h.

The fraction of GPIs accessible to periodate oxidation increased moderately after a 2-h chase (11.9%) and dramatically (48.2% in HeLa cells and up to 60% in MDCK cells; close to the maximum oxidation seen with disrupted cells) following a 18-h chase period during which cell viability remained unchanged as judged by trypan blue exclusion (Fig. 6B). In each case the amount of oxidized H8 plus native H8 (quantitated as cts/nmol phospholipid) added up to the amount of H8 in control cells. This confirmed that the accumulation of oxidized product was indeed the result of a loss in native H8. Because the maximum oxidation seen with disrupted cells is 60–70%, these results indicate that close to the entire complement of pulse-labeled GPIs can be chased to the cell surface over an extended time period. Identical results were obtained in experiments using a membrane-impermeant amine-reactive probe (sulfo-NHS-biotin) capable of modifying GPIs (data not shown). Thus, the slow accumulation of PM GPIs that was measured in the virus and bleb experiments (Figs. 4 and 5C) can be correlated with the appearance of GPIs at the cell surface.

GPIs in the Plasma Membrane Are Predominantly in the Exoplasmic Leaflet—Experiments involving the oxidation of cell surface GPIs on whole cells provided insight into the proportion of total GPIs that are accessible at the cell surface at early and late chase times. However, to define the topology of GPIs at the PM explicitly we looked at the orientation of GPIs in PM-derived blebs (Fig. 7). HeLa cells were metabolically labeled for 2.5 h with [2-3H]mannose or labeled and then analyzed by TLC. Panels a–f show thin layer chromatograms (visualized using a TLC linear analyzer) of [3H]mannose-labeled lipids from control (−) and periodate (+) treated cells for each chase period. The migration of H8 and oxidized H8 (H8*) and the origin (o) and solvent front (f) are indicated (arrowheads). B, quantitation of the percentage of total H8 oxidized was performed using integration software accompanying the TLC scanner and is shown in the bottom panel.

![Fig. 6. Detection of free GPIs at the cell surface by periodate oxidation](image-url)

**Fig. 6. Detection of free GPIs at the cell surface by periodate oxidation.** A. HeLa cells were metabolically labeled with 10 μCi/ml of [2-3H]mannose for 2.5 h at 37 °C. The labeling medium was removed, and cells were washed and chased for 2 or 18 h in complete medium (DMEM). At the end of the chase period cells were washed twice with PBS (pH 7.5) and incubated for 30 min on ice either PBS (control) or PBS containing 10 mM NaIO₄ for cell surface oxidation. The oxidation reaction was quenched by washing the cells several times with 0.15 M glycerol in PBS. Cells were then collected, and lipids were extracted and analyzed by TLC. Panels a–f show thin layer chromatograms (visualized using a TLC linear analyzer) of [3H]mannose-labeled lipids from control (−) and periodate (+) treated cells for each chase period. The migration of H8 and oxidized H8 (H8*) and the origin (o) and solvent front (f) are indicated (arrowheads). B, quantitation of the percentage of total H8 oxidized was performed using integration software accompanying the TLC scanner and is shown in the bottom panel.
der the same conditions, synthesis of [3H]mannose-labeled H8 of the given labeling period the cell surface was oxidized with 10 mM NaIO4 in PBS, the reaction was quenched, and the plasma membrane was induced to bleb for 2 h. Control cells were treated in parallel and mock-blebbled for comparison. Lipids were extracted from cell and bleb samples and analyzed by TLC. Thin layer chromatograms (visualized using a TLC linear analyzer) show [3H]mannose-labeled H8 and oxidized H8 (H8*) from cells chased for 2 or 18 h followed by cell surface oxidation and blebs obtained from the corresponding cells. The origin (o) and front (f) are indicated (arrowheads). B, percentage of H8 oxidized by periodate in the bleb samples after a 2- or 18-h chase was determined using integration software accompanying the TLC analyzer.

Proposal that, under our experimental conditions, periodate does not penetrate the PM to modify intracellular GPIs.

Examination of the radiolabeled lipid profiles in cells and blebs surface oxidized after a long (18 h) chase period shows that although the proportion of H8* in intact cells increases significantly (Fig. 7A, compare top panels), the relative amount of H8* in the PM blebs does not change (Fig. 7A, compare bottom panels). These data thus indicate that 75–85% of H8 (which can be interpreted as the entire PM pool if the efficiency of the oxidation reaction is factored into the calculation) at the PM is accessible to periodate oxidation i.e. in the exoplasmic leaflet and that this distribution is independent of the chase period (2 h versus 18 h, Fig. 7B). These data further suggest that H8 either arrives directly at the exoplasmic leaflet of the PM as would be the case for vesicular delivery of lumenally oriented GPIs or that it is immediately translocated to the outer leaflet upon arrival at the cytoplasmic leaflet of the PM.

Transport of GPIs to the Plasma Membrane Is Sensitive to Brefeldin A—To elucidate the mechanism by which GPIs are transported from their site of synthesis to the PM, experiments were performed to test the effect of BFA on cell surface exposure of H8. BFA is known to block transport of ER synthesized secretory proteins to distal points of the secretory pathway including the PM (54) and control experiments to test its efficacy in HeLa cells confirmed that the dose of inhibitor we were using did indeed block secretion into the extracellular medium of newly synthesized [3H]serine-labeled proteins (Fig. 8A). Under the same conditions, synthesis of [3H]mannose-labeled H8 was unaffected by BFA (Fig. 8B).

Strikingly, transport of [3H]mannose-labeled H8 to the cell surface (as detected by periodate oxidation) was completely abolished in cells treated with BFA (Fig. 8C; in some experiments the level of inhibition was ~90%; see below). Under the same conditions without BFA treatment approximately 50% of the labeled pool of H8 was accessible to oxidation by periodate in control cells (Fig. 8C). Thus, under conditions where protein secretion is blocked, transport of H8 to the cell surface is also inhibited. This result indicates that cell surface exposure of H8 depends on an intact vesicular transport pathway.

As an additional test to verify that BFA inhibits transport to the PM, HeLa cells were labeled for 2.5 h with [2-3H]mannose in the presence of BFA and induced to bleb (BFA was also present during the blebbing incubation). The relative concentration of radiolabeled H8 in the PM (ratio of H8 concentration in blebs to that in total cellular membranes) decreased dramatically in BFA-treated HeLa cells to levels of approximately 15% of that seen in control cells (Fig. 8D). The incomplete, although substantial, reduction in H8 delivery to the PM as judged by analysis of PM blebs may be due to (i) leakage of the BFA block under blebbing conditions or (ii) the presence of GPIs on the cytoplasmic face of the PM. These possibilities are not mutually exclusive. In experiments designed to test whether the BFA-induced transport block remains intact during bleb-
Age of total cellular H8 oxidized by periodate in cells chased at each temperature was performed as described under “Experimental Procedures.” The percentage of total cellular H8 oxidized represented occasional clumps of cells were seen to take up trypan blue but ability barrier was unaffected by exposure to blebbing buffer under these various conditions, confirming that the PM permeability barrier was unaffected by exposure to blebbing buffer (occasional clumps of cells were seen to take up trypan blue but these represented <5% of the total cells present). Under these conditions a low level of GPI transport to the cell surface was observed (~13% of that seen in cells not treated with BFA; data not shown) regardless of whether the cells were exposed to blebbing buffer or control buffer, indicating that in some instances the BFA transport block can be leaky. This leakiness may account for the level of GPIs found in PM blebs obtained from BFA-treated cells in the particular experiment shown in Fig. 8D, but more work needs to be done to be certain that this is the case. Possibility (ii) is considered under “Discussion.”

GPI Transport to the Cell Surface Is Sharply Dependent on Temperature—In addition to the use of pharmacologic agents, such as BFA, to disrupt the vesicular transport pathway, low temperature can also be utilized to block secretory transport. Specifically, transport vesicles budding from the ER are not able to fuse with the Golgi apparatus at temperatures below 15 °C, and transport beyond the TGN is inhibited at 20 °C (55–58). As an additional test of the proposal that GPIs are transported to the cell surface via a vesicular transport mechanism, we investigated the effect of lowered temperature on GPI transport to the cell surface. HeLa cells were metabolically pulse-labeled for 45 min at 37 °C and then chased for 4 h at 15, 22, 30, or 37 °C before cell surface oxidation with periodate. As shown in Fig. 9, cell surface arrival of GPIs was substantially decreased at 15 °C (2.7% H8 oxidized) and at 22 °C (3% H8 oxidized compared with 29% at 37 °C). These data are consistent with previous reports of the kinetics of soluble and membrane protein transport in other cell types (59–62). As an additional test of the proposal that GPIs are transported to the cell surface via a vesicular transport mechanism, we investigated the effect of lowered temperature on GPI transport to the cell surface. HeLa cells were metabolically pulse-labeled for 45 min at 37 °C and then chased for 4 h at 15, 22, 30, or 37 °C before cell surface oxidation with periodate. As shown in Fig. 9, cell surface arrival of GPIs was substantially decreased at 15 °C (2.7% H8 oxidized) and at 22 °C (3% H8 oxidized compared with 29% at 37 °C). These data are consistent with previous reports of the kinetics of soluble and membrane protein transport in other cell types (59–62). As an additional test of the proposal that GPIs are transported to the cell surface via a vesicular transport mechanism, we investigated the effect of lowered temperature on GPI transport to the cell surface. HeLa cells were metabolically pulse-labeled for 45 min at 37 °C and then chased for 4 h at 15, 22, 30, or 37 °C before cell surface oxidation with periodate. As shown in Fig. 9, cell surface arrival of GPIs was substantially decreased at 15 °C (2.7% H8 oxidized) and at 22 °C (3% H8 oxidized compared with 29% at 37 °C). These data are consistent with previous reports of the kinetics of soluble and membrane protein transport in other cell types (59–62).

Kinetics of GPI Transport to the Cell Surface—Because the transport of H8 to the cell surface is a BFA-sensitive and temperature-sensitive process most likely mediated by transport vesicles, additional experiments were performed to compare H8 transport with the vesicular transport of ER-translocated, soluble, secretory proteins destined for the extracellular medium. In HeLa cells metabolically labeled with [3H]serine, secretion of soluble [3H]serine-labeled proteins into the extracellular medium became detectable 1–2 h after addition of radiolabel (Fig. 10A). Quantitation of an SDS-PAGE fluorogram of [3H]serine-labeled proteins recovered in the medium at different time points showed that although the average lag time for secretion was ~30 min, several labeled proteins in the ~40-kDa molecular mass range were secreted after a somewhat longer lag time (>1 h after addition of label; data not shown). These data are consistent with previous reports of the kinetics of soluble and membrane protein transport in other cell types (59–62).

The arrival of free GPIs at the cell surface was monitored by periodate oxidation of [3H]mannose-labeled HeLa cells. Cells were labeled continuously for 9 h, during which time the synthesis of [3H]mannose-labeled H8 was linear (data not shown), and oxidation was performed at various time points. GPIs were first detected at the cell surface after a 2-h labeling period at which time approximately 4% of the labeled H8 was accessible at the cell surface (Fig. 10B). The maximum oxidation of H8 was 15–20% of the total labeled pool during the time frame where synthesis of H8 was linear. Extrapolation of these data indicates that the lag time for arrival of GPIs at the cell surface is approximately 1 h. Newly synthesized radiolabeled H8 can be detected in cells after a pulse labeling of as little as 5 min (data not shown). Therefore, the lag time for arrival at the PM can be interpreted as actual transport time because synthesis of GPIs occurs almost immediately upon introduction of [3H]mannose to the cells.

Data from the pulse-chase experiments shown above (Fig. 6) indicate that a large proportion of the pulse-labeled pool of free GPIs can be chased to the cell surface. This provides evidence for a vectorial transport process in which pulse-labeled GPIs become concentrated at the PM after a long chase period.
Under conditions where GPIs are being synthesized continuously, however, the amount of periodate accessible H8 plateaus between 5 and 7 h at about 15–20% of the labeled pool. This indicates that the PM pool of GPIs comprises approximately 15–20% of the entire cellular pool.

**DISCUSSION**

The results described in this paper indicate that the GPI lipids H8 and H7 are transported from their site of synthesis to populate the plasma membrane as well as a wide range of intracellular membranes. We analyzed the subcellular distribution of these lipids by metabolic radiolabeling in conjunction with cell surface derivatization methods and three independent membrane isolation techniques in three different cell lines. The results show that newly synthesized H8 and H7 are rapidly partitioned among the different subcellular membrane fractions analyzed (except for mitochondria where the membrane concentration of H8 and H7 is low) but that with extended chase periods following a pulse of metabolic radiolabeling, H8 (and to a lesser extent, H7) becomes concentrated in the exoplasmic leaflet of the plasma membrane. In concert with the cell surface accumulation of H8, levels of radiolabeled H8 in total cellular membranes decline, indicative of catabolic or other turnover processes from which the cell surface pool is excluded. We provide clear evidence that delivery of H8 to the cell surface occurs via a BFA-sensitive, temperature-dependent mechanism, presumably involving transport vesicles that transit through the Golgi apparatus.

**Vesicular Transport of GPI Lipids to the Cell Surface**—The initial steps in GPI biosynthesis occur on the cytoplasmic face of the ER (14–20); however, the addition of a GPI-anchor to newly synthesized proteins occurs via a luminaly oriented transamidation reaction (21). This topological inversion suggests that a GPI biosynthetic intermediate(s) must be transported across the ER membrane bilayer. Indeed a variety of data suggest that mannosylated GPIs, including mature GPIs such as H7 and H8, can undergo transbilayer movement in the ER. Importantly, H7 and H8, or their equivalents in other organisms, can be detected on the cytoplasmic face of intracellular membranes (15, 18, 63), even though they are consumed in the protein-anchoring reaction on the opposite face of the ER. Given that GPIs can occupy both leaflets of the ER membrane bilayer, redistribution from the ER to other membranes could occur via any of the traffic options available to lipids, i.e., protein-mediated transfer or contact-site mediated transfer between the cytoplasmic leaflets of cellular membranes or vesicular transport (64–66). Delivery to the cell surface could involve vesicular transport of luminaly oriented GPIs or membrane translocation of GPIs deposited by any of the proposed transport options in the cytoplasmic leaflet of the PM. Because the BFA sensitivity and temperature dependence of GPI transport to the cell surface argues against the latter possibility, we propose that GPI lipids arriving at the cell surface most likely originate from the luminal leaflet of the ER.

The 1-h lag before metabolically radiolabeled H8 can be detected at the cell surface is comparable with the lag time involved in the release of ER-translocated soluble secretory proteins into the extracellular medium. Because the protein secretion lag involves characteristic delays in protein folding and release from the ER quality control system in addition to actual vesicular transit time (59–62, 67), these data suggest that GPIs are inefficiently selected/packaged for secretion. This can be explained by suggesting (i) that vesicular transport of GPIs occurs via bulk flow rather than a forward-signal-mediated mechanism or (ii) that other transport steps, such as recruitment of cytoplasmically disposed GPIs to the ER lumen, are responsible for the lag. It is also possible that vesicular transport of GPIs to the cell surface occurs in concert with specific proteins, protein complexes, or membrane domains and that assembly of GPIs into these structures dictates the lag time. More work needs to be done to elucidate these points.

**Possibility of Alternate Transport Pathways**—Interestingly, fractionation studies with thymoma cells metabolically pulse-labeled for very short times (5 min) revealed that the subcellular distribution of GPIs after 5 min (data not shown) was identical to that found in cells labeled for 2.5 h. This uniformity in distribution was not due to inadequacies in our fractionation procedures because we were able to readily separate organelle-specific markers (Table I) and also establish, for example, that the total pool of labeled GPIs in the PM fraction could not be accounted for solely by cross-contamination of the PM with ER membranes (see “Results”). The observation that GPIs are present in all fractions at early times suggests that a rapid, nonvesicular transport option could be available to GPIs in thymoma cells and may act in concert with vesicular transport. We have not, however, been able to address whether an increase in the concentration of cell surface GPIs occurs over time in these particular cells for the following reasons: (i) catabolic turnover processes appear to be accelerated in thymoma cells, resulting in significant loss of radiolabel during relatively short chase times and (ii) the cells are maintained in suspension and thus cannot be blebbled to obtain pure plasma membrane vesicles. Thus, although the observed transport processes in HeLa and MDCK cells appear to be similar, thymoma cells may utilize alternate or accelerated transport and/or catabolic processes.

**Cell Surface Dynamics**—The accumulation of free GPIs at the cell surface over long chase periods parallels depletion of the bulk cellular pool of radiolabeled GPIs by about half. A possible interpretation of this is that GPIs at the cell surface are somehow protected from catabolic processes, whereas intracellular GPIs are turned over. Perhaps those GPIs that are luminaly oriented in the ER can be packaged into transport vesicles and displayed at the cell surface, whereas the cytosolically oriented GPIs are exposed to potential hydrolases and degraded. This would serve to explain the depletion of bulk cellular GPI concentration over time as well as the predominantly exoplasmic orientation of free GPIs. It is possible that turnover of the cell surface pool of GPIs could also occur by slow endocytosis and lysosomal degradation. However, given that GPIs accumulate at the cell surface, the rate of endocytic uptake must be lower than the rate of cell surface exposure of GPIs. GPI-anchored proteins are known to enter and exit the endocytic recycling pathway more slowly than proteins that utilize signal-mediated transport mechanisms (68), and it would be interesting to see whether free GPIs at the cell surface exhibit the same membrane dynamics as GPI-anchored proteins.

We estimate that at least 15–20% of the total free GPI pool exists at the PM from cell surface oxidation experiments under continuous labeling conditions. However, this estimate may be low because our data suggest that the intracellular GPI pool is susceptible to turnover during long chase periods, whereas the cell surface pool is protected. Therefore, although GPI lipids contribute only a portion fraction to bulk lipid amounts, a significant portion of the GPI pool exists at the cell surface. Overall, these results suggest that cells utilize both vectorial transport processes in conjunction with topology-specific turnover to concentrate GPI molecules at their surface. The functional significance of this cell surface pool of GPIs remains to be elucidated.

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