Early Lipid Intermediates in Glycosyl-phosphatidylinositol Anchor Assembly Are Synthesized in the ER and Located in the Cytoplasmic Leaflet of the ER Membrane Bilayer

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Abstract. Glycosylated phosphoinositides serve as membrane anchors for numerous eukaryotic cell surface glycoproteins. Recent biochemical and genetic studies indicate that the glycolipids are assembled by sequential addition of components (monosaccharides and phosphoethanolamine) to phosphatidylinositol. The biosynthetic steps are presumed to occur in the ER, but formal proof of this is lacking. We describe experiments designed to establish the subcellular location of the initial steps in glycosyl-phosphatidylinositol (GPI) anchor biosynthesis and to define the transmembrane distribution of early biosynthetic lipid intermediates. The experiments were performed with the thymoma cell line BW5147.3. A subcellular fractionation protocol was used to show that early biosynthetic steps in GPI assembly, i.e., synthesis and deacetylation of N-acetylglucosaminyl phosphatidylinositol, occur in the ER. GPI biosynthetic intermediates were synthesized by incubating the microsomes with UDP-[^{3}H]GlcNAc, and the transmembrane distribution of the labeled lipids was probed with phosphatidylinositol-specific phospholipase C (PI-PLC). Treatment of the radiolabeled microsomes with PI-PLC showed that >70% of the N-acetylglucosaminyl phosphatidylinositol and glucosaminyl phosphatidylinositol could be hydrolyzed, indicating that the two lipids were primarily distributed in the cytoplasmic (outer) leaflet of the microsomes. Similar cleavage results were obtained using Streptolysin O-permeabilized thymoma cells. When permeabilized cells were incubated with UDP-[^{3}H]GlcNAc and treated with PI-PLC, ∼85% of the radiolabeled N-acetylglucosaminyl phosphatidylinositol and glucosaminyl phosphatidylinositol could be cleaved, indicating that they were accessible to the enzyme. The cumulative data indicate that early GPI intermediates are primarily located in the cytoplasmic leaflet of the ER, and are probably synthesized from PI located in the cytoplasmic leaflet and UDP-GlcNAc synthesized in the cytosol.

GLYCOLIPIDS containing the structural motif Manα1-4GlcNα1-6*myo* Inositol-1-P-lipid are ubiquitous in the eukaryotes (Ferguson et al., 1992). These lipids, termed glycosyl-phosphatidylinositols (GPIs)[1], were originally discovered covalently linked to eukaryotic cell-surface glycoproteins and recognized to be an important alternate mechanism for anchoring proteins to cell membranes (Low et al., 1986). All protein-linked GPIs contain the extended structure Ethanolamine-P-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo* Inositol-1-P-lipid. The EtN residue is amide linked to the carboxyl-terminal amino acid of the protein, and the trimannose core glycan can be modified by a variety of substituents. Several lines of evidence indicate that GPI addition to protein occurs in the ER in a reaction involving cleavage of a carboxyl-terminal GPI signal sequence from newly synthesized proteins and attachment of a presynthesized GPI moiety to the newly exposed carboxyl-terminal amino acid.

Recent biochemical and genetic studies indicate that GPIs are assembled by sequential addition of components (monosaccharides, phosphoethanolamine) to phosphatidylinositol (PI). Synthesis is initiated by transferring GlcNAc from UDP-GlcNAc to PI to form GlcNAc-PI, which is then de-N-acetylated to give GlcN-PI. GlcN-PI is elaborated by inositol acylation, and addition of mannose residues and one or more phosphoethanolamine moieties (reviewed in Cross, 1990; Field and Menon, 1992; Englund, 1993). These reactions have been demonstrated in microsomal membranes from trypanosomes and mammalian cells (Menon et al., 1990; Hirose et al., 1992), but the microsomes have not been further fractionated to determine the subcellular distribution of the GPI biosynthetic enzymes, nor has the topological arrangement of the different reaction steps been described.

1. *Abbreviations used in this paper*: 5BrdUMP, 5-bromo-2′-deoxyuridine 5′-monophosphate; GPI, glycosyl-phosphatidylinositol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; PNS, post nuclear supernatant; SLO, streptolysin O; TLC, thin layer chromotography; TLCK, *N-p* -tosyl-L-lysine chloromethyl ketone; UDP-GlcNAc, uridine 5′-diphospho N-acetylglucosamine.

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Information on the compartmental availability of the various biosynthetic ingredients involved provides few clues as to the transmembrane arrangement of the different steps in GPI assembly. For example, UDP-GlcNAc required for the initial step in GPI assembly, is synthesized in the cytosol (Coates et al., 1980) but can be transported into the ER lumen via a protein carrier (Cecchelli et al., 1985; Perez and Hirschberg, 1985). Also, PI, dolichol-P-mannose (donor of the core GPI mannose residues), and phosphatidylethanolamine (PE; donor of the capping phosphoethanolamine moiety) are all synthesized on the cytoplasmic face of the ER, but are available in the luminal leaflet through transbilayer movement or "flip-flop" (Bell et al., 1981; Higgins et al., 1989; Haselbeck and Tanner, 1982; Lennarz, 1987; Hirschberg and Snider, 1987; Tanner and Lehle, 1987; Hutson and Higgins, 1982). Since all the biosynthetic ingredients (PI, UDP-GlcNAc, dolichol-P-mannose, and PE) required for GPI assembly are available on both sides of the ER membrane, the different steps of GPI assembly could, in principle, occur in either one or both leaflets of the ER membrane bilayer.

In this paper we focus on the initial steps in GPI assembly and describe experiments aimed at defining the subcellular location of GlcNAc-PI synthesis and de-N-acetylation, and the transmembrane distribution of GlcNAc-PI and GlcN-PI. We show that GlcNAc-PI and GlcN-PI are synthesized in the ER and located primarily in the cytoplasmic leaflet of the ER membrane bilayer.

Materials and Methods

Materials

GDP-[^{3}H]mannose (20 Ci/mmol) was purchased from DuPont New England Nuclear (Wilmington, DE), UDP-[^{3}H]GlcNAc (25 Ci/mmol) from American Radiolabeled Chem. (St. Louis, MO) and streptolysin O was from VWR Scientific. Proteinase K and *Staphylococcus aureus* nuclease were obtained from Boehringer Mannheim. Dolichol-phosphate, 5Brd-UMP, dimercaptopropanol, *Bandeiraea simplicifolia* lectin BS-II, and 4-methylumbelliferyl-α-D-mannopyranoside were from Sigma Immunochemicals (St. Louis, MO). *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C was a gift from Dr. Martin Low (Columbia University, New York), and anti-BiP antibodies were generously provided by Dr. David Bole (University of Michigan, Ann Arbor, MI). HRP-conjugated goat anti-rat IgG was obtained from Pierce (Rockford, IL) and silica 60 thin layer plates were from Merck (Darmstadt, Germany).

Cell Culture

The Thy-1$^+$ mouse thymoma cell line BW5147.3 was maintained in suspension culture in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37°C.

Preparation of Subcellular Fractions

BW5147.3 thymoma cells (2–5 × 10^8) were collected by centrifugation (1,000 *g* for 5 min), washed twice with PBS, and once with 5 ml of buffer A (0.25 M sucrose, 10 mM Hepes/NaOH, pH 7.5, 1 mM DTT, 1 mM PMSF). The cells were resuspended in 10 ml buffer A (supplemented with 1 μg/ml leupeptin, and 0.1 mM TLCK), transferred to a 15 ml nitrogen cavitation bomb (Kontes Glass Company, Vineland, NJ) and held at 400 psi N_2 pressure for 30 min. The cavitated cells were disrupted further with three strokes of a tight pestle dounce homogenizer, and treated with *S. aureus* nuclease (1.5 units/ml) for 20 min on ice. The resulting lysate was clarified by centrifugation at 10,000 *g* for 15 min at 4°C. The pellet contained ~35% of the lysate protein, most (70–90%) of the lysosomal and peroxisomal marker enzyme activities (β-hexosaminidase and catalase, respectively, assayed as described by Storrie and Madden, 1990, and some plasma membrane (~35%), Golgi (~5%), and ER (~30%) (assayed as described below). The supernatant (PNS) was layered on top of a series of sucrose steps: 3.6 ml 38% sucrose, 1.8 ml 30% sucrose, and 1.8 ml 20% sucrose (all prepared in 10 mM Hepes/NaOH, pH 7.5, 1 mM DTT), and fractionated by centrifugation in a rotor (70Ti; Beckman Instruments Inc., Fullerton, CA) at 43,000 rpm for 2 h. Fractions were collected from the top of the tube and the pellet was resuspended in 3 ml of buffer A. The protein content of each fraction was measured with the Micro BCA Protein Assay Reagent (Pierce Chemical Co.). The fractions were frozen and stored at −70°C.

Marker Enzyme Assays

Subcellular fractions were analyzed for the following marker enzyme activities: alkaline phosphodiesterase I (plasma membrane), α-mannosidase II (Golgi), and dolichol-P-mannose synthase (ER). The assays are described below.

Alkaline phosphodiesterase I activity was assayed according to Storrie and Madden (1990), using 25 μl of the cell lysate or PNS, or 100 μl of each fraction from the sucrose density step centrifugation.

Golgi α-mannosidase II activity was measured by a modification of the procedure described by Storrie and Madden (1990). The assays were performed in 96-well microtiter plates. Briefly, a 10-μl aliquot of each fraction was diluted with 50 μl of PBS containing 2.5 μg of 4-methylumbelliferyl-α-D-mannopyranoside (from a 10 mg/ml stock solution in DMSO), 0.1% Triton X-100, and 0.25 mg BSA, and the sample was left at ambient temperature for 2 to 5 h. The reaction was stopped by adding 150 μl of 0.5 M glycine, 0.5 M Na_2CO_3. Product formation was monitored by measuring the fluorescence (excitation 355 nm, emission 480 nm) of the samples using a Titertek Fluoroscan II (ICN Radiochemicals, Irvine, CA) plate reader.

Dolichol-phosphomannose synthase (ER) was assayed by a modification of the procedure described by Braell (1988). Briefly, aliquots of each fraction (25–50 μl, corresponding to ~100–200 μg protein) were diluted with 50 mM Hepes/NaOH, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$ to a final volume of 200 μl. The reaction was initiated by adding 2 μl dolichol phosphate (2 mg/ml in chloroform/methanol, 1:2, vol/vol) and 2 μl of GDP-[^{3}H]mannose (1 μCi/10 μl). After incubation for 30 min at 37°C, the reaction was stopped by adding 1 ml chloroform/methanol (1:2, vol/vol). The resulting two-phase mixture was centrifuged to separate the phases and the lipid-containing lower phase was washed with 500 μl of mock upper phase. The lipid-containing lower phase was taken for liquid scintillation counting. Thin layer chromatographic analysis of the lipid phase showed only radiolabeled dolichol-P-mannose.

In vitro *Biosynthesis of GlcNAc-PI and GlcN-PI*

GlcNAc-PI and GlcN-PI were synthesized in vitro by incubating ER fractions (typically 0.2 mg protein) with UDP-[^{3}H]GlcNAc (1 μCi) in buffer (50 mM Hepes/NaOH (pH 7.5), 5 mM EDTA, 1 μg/ml leupeptin, and 0.1 mM TLCK), in a total volume of 100 μl. After incubation at 37°C (for up to 2 h), the reaction was stopped by placing on ice, and a single phase lipid extract was obtained by adding 300 μl water and 1.5 ml ice-cold chloroform/methanol (1:2, vol/vol). A two phase mixture was induced by adding 0.5 ml chloroform and 0.5 ml water. The lipid containing chloroform-rich lower phase was washed several times with 0.5 ml mock upper phase to remove any contaminating UDP-[^{3}H]GlcNAc or breakdown products. 10% of the lipid-containing phase was taken for liquid scintillation counting. The remainder of the sample was dried, dissolved in 20 μl water-saturated butanol, and analyzed by thin layer chromatography (silica Gel 60 thin layer plates, using chloroform/methanol: 1 M ammonium hydroxide 10:10:3, vol/vol/vol as the solvent system). After chromatography, the plates were air dried and scanned for radioactivity with a Berthold LB 2842 automatic scanner (Berthold Analytical Instruments, Inc., Nashua, NH). Incorporation of radioactivity into the individual lipid species was determined using the integration software supplied with the scanner in conjunction with liquid scintillation counting.

PI-PLC Treatment

Purified phosphatidylinositol-specific phospholipase C (PI-PLC) was obtained from Dr. Martin Low (Columbia University College of Physicians and Surgeons, New York). The enzyme was isolated from culture supernatants of *Bacillus subtilis* (BG2320) transfected with the PI-PLC gene from *B. thuringiensis*, and was assayed using [^{3}H]phosphatidylinositol as substrate at pH 7.0 in 0.1% sodium deoxycholate (Henner et al., 1988; Low

et al., 1988; Low, 1992). The preparation obtained was ~1,700 U/ml and had a specific activity of ~1,000 U/mg (1 U = 1 μmol/min). ER membranes (radiolabeled via UDP-[^{3}H]GlcNAc) were treated with different concentrations of PI-PLC in the presence or absence of 0.1% sodium deoxycholate (final concentration). After an incubation on ice (up to 60 min, typically 20 min), lipids were extracted as described above and analyzed by thin layer chromatography.

Integrity of ER Vesicles

The integrity of isolated ER membrane vesicles was determined by performing protease protection experiments. The membranes were tested for their ability to protect BiP, an ER luminal protein, from proteinase K treatment; control proteolysis was performed in the presence of a membrane-disrupting concentration of detergent. Membranes (10 μl, ~20 μg protein) were treated with different amounts of proteinase K for 30 min on ice. To determine the effect of 37°C incubation and PI-PLC treatment on membrane integrity, membranes were incubated at 37°C for 1 h, and then treated with PI-PLC (1.5 U/ml) for 20 min on ice before proteinase K treatment. Proteolysis was stopped by adding PMSF (from a 0.2 M stock solution in ethanol) to a final concentration 3 mM. Proteins were precipitated with 7% TCA, and the precipitate was washed with ice-cold acetone and analyzed by SDS-PAGE using 10% gels. After electrophoretic separation, proteins were transferred from the gel to a nitrocellulose membrane (Biotrace™ NT; Gilman Science, Ann Arbor, MI) using standard procedures. The nitrocellulose sheets were blocked with a 3% solution of BSA (in PBS) for 30 min, washed several times with PBS, and then incubated with a 1:20 dilution of a monoclonal rat anti-BiP antibody in PBS for 30 min. The blots were washed three times with PBS, then incubated with HRP-conjugated goat anti-rat IgG and developed using 4-chloronaphthol as described by the manufacturer (Pierce).

Plasma Membrane Permeabilization with Streptolysin O

Thymoma cells (0.5-1 × 10^7) were harvested and washed once with ice-cold PBS. Permeabilization was achieved using a two-step procedure. In the first step, the cells were incubated for 20 min on ice with a pre-chilled solution of activated streptolysin O (SLO) (SLO was activated by incubation with 2 mM DTT for 10 min at ambient temperature at a concentration of 50–150 U per 10^7 cells) (SLO units are based on lysis of human erythrocytes, and it is assumed that each vial of SLO) (catalog no. DF0482-60; VWR Scientific) contains 100–120 haemolytic units (Gravotta et al., 1990). During this procedure some proportion of the SLO is expected to bind/insert into the plasma membrane. Excess SLO was removed by centrifuging the cells at 4°C and washing the cell pellet once with ice-cold PBS. The washed cells were resuspended at a concentration of 10^7 cells/ml in ice-cold PBS containing 8 mM EDTA, 4 mM DTT, 1 mM PMSF, 0.1 mM TLCK, and 1 μg/ml leupeptin. GlcNAc-PI and GlcN-PI synthesis was initiated by adding 1 μCi of UDP-[^{3}H]GlcNAc to 0.1 ml of SLO-treated cells and shifting the temperature to 37°C to induce SLO pore formation (cell viability was occasionally compromised during this treatment, so that up to 25% of maximal lipid labeling could be observed in the absence of SLO in some experiments; viability was consistently higher if the cells were suspended at <10^7 cells/ml). After incubation for 90 min, the cells were placed on ice, and lipids were extracted as described above. In some experiments, the permeabilized, labeled cells were treated with *B. thuringiensis* PI-PLC on ice before lipid extraction.

Results

In vitro *Synthesis of GlcNAc-PI and GlcN-PI*

Thymoma cell lysates were prepared as described by Stevens and Raetz (1991), or by nitrogen cavitation. The lysates were incubated with UDP-[^{3}H]GlcNAc in the presence of EDTA (to suppress synthesis of dolichol-PP-GlcNAc$_{1-2}$) at 37°C and lipids were extracted and analyzed by thin layer chromatography (TLC) (Fig. 1 *B*). Two major radiolabeled lipid species could be resolved by TLC, both chromatographing slightly behind phosphatidylinositol. The lipids were isolated from the thin layer plate and analyzed by enzymatic and chemical treatments. Both lipids could be hydrolyzed (>95%; hydrolysis of untreated samples was <0.5%) by PI-PLC, and the more polar lipid could be efficiently (>70%) converted to the faster moving species by N-acetylation. These diagnostic parameters, taken together with TLC mobilities (Fig. 1 *B*) and the extensive characterization of these lipid species reported in the recent literature (Doering et al., 1989; Menon et al., 1990; Stevens and Raetz, 1991; Hirose et al., 1991; Sugiyama et al., 1991), indicate that the faster-moving lipid is GlcNAc-PI and the slower-moving species is GlcN-PI (Fig. 1 *A*).

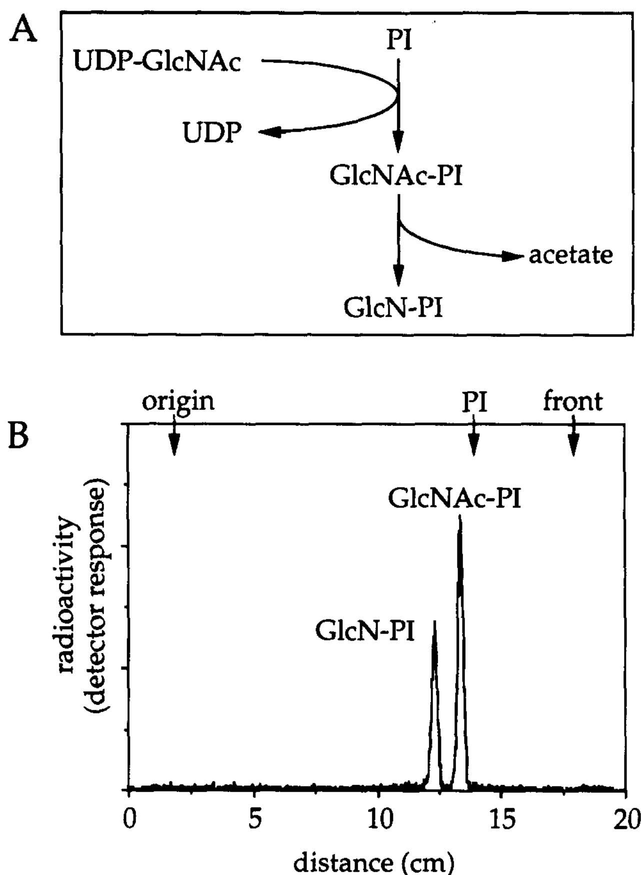
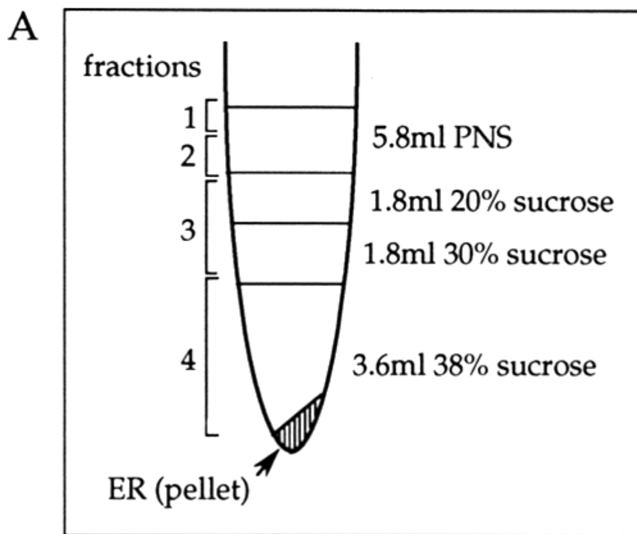


Figure 1. Biosynthesis of GlcNAc-PI and GlcN-PI. (*A*) The figure shows the first steps GPI biosynthesis. GPI assembly is initiated by transferring GlcNAc from UDP-GlcNAc to PI. GlcNAc-PI is de-N-acetylated to form GlcN-PI. (*B*) A thymoma cell lysate (0.5 × 10^7 cell equivalents) was incubated with UDP-[^{3}H]GlcNAc (1 μCi) in buffer (50 mM Hepes/NaOH, pH 7.5, 5 mM EDTA, 1 μg/ml leupeptin, and 0.1 mM TLCK) in a total volume 100 μl for 2 h at 37°C. Lipids were extracted and analyzed by thin layer chromatography as described in Material and Methods. The chromatogram was scanned for radioactivity using a Berthold TLC scanner. The migration of rat liver phosphatidylinositol (*PI*) is indicated.

The reaction conditions were assessed by varying microsomal protein concentration and incubation time, and subsequent experiments were performed in a linear range (0–0.8 mg protein, 0–2 h incubation period) with respect to these two parameters. The extent of reaction was not limited by the radiolabeled substrate (UDP-[^{3}H]GlcNAc), and attempts to prevent possible degradation of the substrate by including the nucleotide pyrophosphatase inhibitor dimercaptopropanol



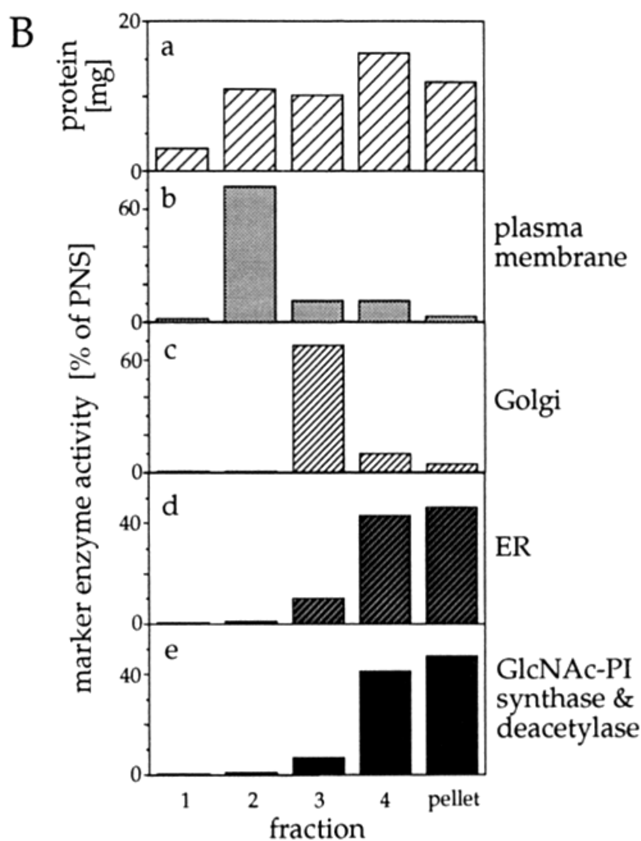


Figure 2. Distribution of organelle-specific marker enzymes and GPI biosynthesis in subcellular fractions. Thymoma cells (2 × 10^8) were broken by nitrogen cavitation and the resulting lysate was clarified by centrifugation (10,000 *g* for 15 min). The supernatant (PNS) was layered over a series of sucrose density steps and centrifuged (100,000 *g*; 2 h). Fractions were collected from the top of the tube and the pellet was resuspended in 3 ml of 0.25 M sucrose in 10 mM Tris/HCl, pH 7.5, containing 1 mM DTT, 1 mM PMSF, 0.1 mM TLCK, and 1 μg/ml leupeptin. The four fractions and pellet were characterized by assaying for organelle specific marker enzymes as described in Material and Methods. The enzyme activity in each fraction was calculated as a percentage of total activity in the PNS. To assay GlcNAc-PI and GlcN-PI biosynthetic activity 50–100 μl (0.2–0.4 mg protein) of each fraction was used. (*A*) Sucrose density steps and definition of the fractions collected. (*B*) Protein and enzyme activity distribution amongst the various fractions: (*a*) protein, (*b*) alkaline phosphodiesterase (plasma membrane marker), (*c*) α-mannosidase II (Golgi marker), (*d*) dolichol-P-mannose synthase (ER marker), and (*e*) distribution of GlcNAc-PI synthase and deacetylase.

(Faltynek et al., 1981) in the reaction mixture did not increase the product yield.

GlcNAc-PI and GlcN-PI Are Synthesized in the ER

To define the subcellular localization of GlcNAc-PI and GlcN-PI biosynthesis, thymoma cells were disrupted by nitrogen cavitation, nuclei and microbodies were removed by centrifugation, and the post-nuclear supernatant (PNS) was fractionated by centrifugation through a series of sucrose density steps (Fig. 2 *A*). Isolated fractions were tested for the presence of various marker enzyme activities. The distribution of the three marker enzymes showed essentially no overlap (Fig. 2 *B*, *b–d*): plasma membrane alkaline phosphodiesterase activity was recovered mainly in fraction 2 (70% of activity in the PNS, Fig. 2 *B*, *b*), Golgi α-mannosidase II activity was found primarily in fraction 3 (67% of the total activity in the PNS, Fig. 2 *B*, *c*) and the ER marker enzyme, dolichol-P-mannose synthase, was distributed roughly equally between fraction 4 and the pellet (totally 89% of the activity in the PNS, Fig. 2 *B*, *d*). The pellet presumably corresponds to rough ER, while fraction 4 presumably contains ER-derived microsomes without bound ribosomes. The distribution of other organelle-specific enzymes followed the pattern shown in Fig. 2 *B*. For example, the Golgi marker enzyme galactosyltransferase was recovered primarily in fraction 3, and ethanolaminephosphotransferase, an ER enzyme responsible for PE biosynthesis via the CDP-ethanolamine pathway, was found in fraction 4 and the pellet (data not shown). Thus the sucrose density centrifugation protocol permits clear resolution of major organelles in the PNS.

Individual fractions were incubated with UDP-[^{3}H]-GlcNAc to assay GlcNAc-PI and GlcN-PI biosynthesis. Significant lipid synthesis was observed only in the two ER fractions (Fig. 2 *B*, *d* and *e*), which together contained ~90% of the GlcNAc-PI/GlcN-PI biosynthetic activity of the PNS. Approximately 10% of the total GlcNAc/GlcN-PI biosynthesis activity was recovered in the Golgi fraction (fraction 3). Since this fraction also contained ~10% of the ER marker enzyme activity (Fig. 2 *B*, *c* and *d*), the detection of GlcNAc-PI/GlcN-PI biosynthesis in fraction 3 can be directly attributed to ER contamination of the Golgi fraction. The cumulative data are consistent with the proposal that GPI synthesis is initiated in the ER.

GlcNAc/GlcN-PI Biosynthesis Is Unaltered in Microsomes Depleted of Luminal Contents and Peripheral Proteins

A widely applied method to study the topology of membrane proteins is to assay their sensitivity to treatment with various membrane-impermeant protein-modification reagents. For membrane-bound enzymes loss of activity on protease treatment of sealed membrane vesicles is usually taken to mean that the catalytic domain of the enzyme is exposed on the

vesicle exterior. After treatment of ER microsomes with proteinase K (50 μg/ml, 20 min on ice), GlcNAc/GlcN-PI synthesis was reduced to ~30% of the amount synthesized by untreated microsomes. However, since it has been previously demonstrated that UDP-GlcNAc can be transported across the ER membrane via a protein carrier known to be sensitive to protein modification reagents applied to intact microsomes (Perez and Hirschberg, 1985; Cecchelli et al., 1985; Abeijon and Hirschberg, 1992), these results do not provide any topological information since the reduction in GlcNAc/GlcN-PI biosynthetic activity could be indirectly due to an effect on the UDP-GlcNAc transporter.

In other experiments, isolated ER microsomes were stripped of peripheral membrane proteins and depleted of luminal proteins by salt washing (with 0.5 M KOAc) and permeabilization (with 0.1% deoxycholate in the presence of 100 mM KOAc). The depleted vesicles were recovered by centrifugation and found to possess 80–90% of the GlcNAc/GlcN-PI biosynthetic activity of the untreated microsome preparation. These data indicate that extrinsic and luminal ER proteins are probably not essential for GlcNAc/GlcN-PI biosynthesis.

GlcNAc-PI and GlcN-PI Are Distributed Primarily in the Cytoplasmic Leaflet of the ER Membrane Bilayer

In the absence of information concerning the arrangement of the GlcNAc/GlcN-PI biosynthetic enzymes in the ER membrane, we pursued an alternate strategy to obtain topological information and examined the transbilayer distribution of the two lipids themselves. Since both GlcNAc-PI and GlcN-PI are susceptible to cleavage by PI-PLC, we used PI-PLC as a membrane-impermeant probe to assay the distribution of the two lipids. After incubating the membranes with UDP-[^{3}H]-GlcNAc to generate radiolabeled GlcNAc-PI and GlcN-PI, the sample was placed on ice (to stop further lipid synthesis) and treated with PI-PLC. Both lipids could be hydrolyzed suggesting that the majority of the in vitro synthesized GlcNAc-PI and GlcN-PI was situated in the cytoplasmic (outer) leaflet of the ER and therefore accessible to PI-PLC.

PI-PLC treatment was investigated by varying the concentration of enzyme and the length of incubation. The results are shown in Fig. 3. Maximal cleavage was achieved within 10 min, and although the extent of cleavage increased with increasing PI-PLC concentration, the bulk of the effect could be reproduced with relatively small concentrations (0.5 U/ml) of the enzyme. The clear time dependence of the cleavage reaction, as well as the observation that addition of PI-PLC after addition of solvent did not result in lipid hydrolysis (data not shown), ruled out the possibility that cleavage occurred during the lipid extraction process. Cleavage in the presence of membrane-disrupting concentrations of detergent was invariably ~20% higher, suggesting that a small fraction of the lipid substrates was inaccessible to PI-PLC in intact microsomes.

The incomplete cleavage of GlcNAc-PI and GlcN-PI by PI-PLC treatment of intact microsomes may be explained in two ways. Since inefficient cleavage of membrane-anchored substrates by PI-PLC has been recognized in the past (see for example, Low and Kincade, 1985), it is possible that the uncleaved fractions represent cytoplasmically oriented pools

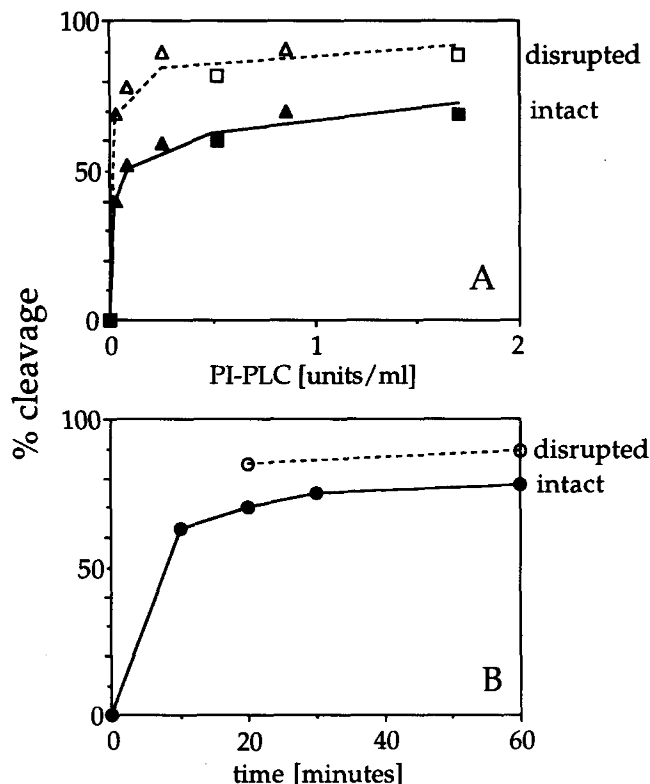


Figure 3. PI-PLC treatment of intact ER microsomes: susceptibility of GlcNAc-PI and GlcN-PI. GlcNAc-PI and GlcN-PI were synthesized by incubating 50 μl (0.2 mg protein) of ER microsomes (pellet fraction, Fig. 2) with 1 μCi UDP-[^{3}H]GlcNAc for 2 h at 37°C. The reaction was stopped by cooling on ice. The samples were treated on ice in the presence (○, □, △) or absence (●, ■, ▲) of detergent (0.1% deoxycholate). (*A*) Samples incubated with different concentrations of PI-PLC for 20 min (triangles and squares represent two different experiments) (*B*) Samples treated with 1.5 U/ml PI-PLC for different periods of time. Lipids were extracted as described in Material and Methods and 10% of the lipid extract was taken for liquid scintillation counting. The remainder of the sample was dried, dissolved in 20 μl water-saturated butanol and analyzed by TLC (as in Fig. 1).

of GlcNAc-PI and GlcN-PI that are somehow protected from PI-PLC cleavage through non-covalent interactions with other lipids or proteins. Another possibility is that some GlcNAc-PI and GlcN-PI are located in the luminal leaflet of the ER. This luminal pool of lipids may be derived via limited transbilayer transport (flipping) of lipids synthesized in the cytoplasmic leaflet. Alternatively, the luminal fraction may arise from lipid synthesis in the luminal leaflet, followed by incomplete transfer into the cytoplasmic leaflet.

We tested the possibility that GlcNAc-PI and GlcN-PI are synthesized in the cytoplasmic leaflet of the ER, and a fraction of each species is flipped into the luminal leaflet. Pulse–chase experiments (Table I) showed no change in the levels of GlcNAc-PI and GlcN-PI, and only a slight increase in the extent of PI-PLC cleavage after chase periods of 60–90 min. The results indicate that there is essentially no turnover of the lipids during the course of the experiments, and that transbilayer redistribution corresponds to transfer of $<5\%$ of the labeled pool from the inner to the outer leaflet in a 60–90-min period. However, as discussed above it is likely

Table I. Metabolic Turnover and Transbilayer Movement of GlcNAc-PI and GlcN-PI

Labeling time	Chase time	PI-PLC	DOC	GlcNAc-PI + GlcN-PI#	Percent cleavage*
(min)	*(min)*			*(cpm)*	
30	0	–	–	12,890	
		+	–	4,050	68.6
30	90	–	–	12,800	
		+	–	3,350	73.8
60	0	–	–	25,090	
		+	–	8,140	67.8
60	60	–	–	22,080	
		+	–	5,720	74.1
		+	+	3,220	85.4

ER microsomes (0.2 mg protein) were labeled with 1 μCi of UDP-[³H]GlcNAc and chased by adding 2.5 mM non-radioactive UDP-GlcNAc at the time indicated in the table. At the end of the chase period, samples were treated with 1.5 U/ml of PI-PLC for 20 min on ice. For the data shown in the last line, membranes were disrupted with detergent (0.1% deoxycholate), after the chase period, before PI-PLC treatment.

* Both lipids were hydrolyzed to the same extent at each time point, hence, the data are presented simply in terms of total recovery of labeled lipid and total percent cleavage.

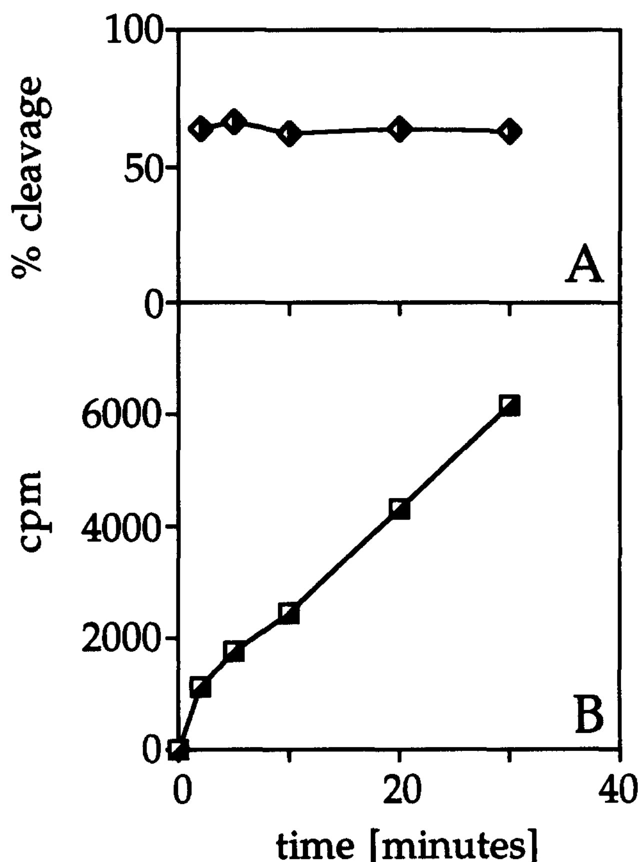


Figure 4. Transmembrane distribution of newly synthesized GlcNAc-PI and GlcN-PI. ER microsomes (0.4 mg protein) were incubated with UDP-[³H]GlcNAc (1 μCi) as described in Materials and Methods. Samples were taken at the indicated times and analyzed (each sample was divided into two aliquots, one aliquot was treated with PI-PLC, then lipids were extracted and quantitated by scintillation counting). *B* indicates the extent of incorporation of radioactivity into GlcNAc-PI and GlcN-PI. *A* indicates the percentage of the two lipids cleaved by PI-PLC in intact microsomes.

that the slight increase in cleavage efficiency after the chase period reflects the release of a small additional fraction of the lipids from environments in which the phospholipase cleavage site is obscured. Additional kinetic experiments in which we analyzed the transbilayer distribution of GlcNAc-PI and GlcN-PI in short labeling periods showed that the same proportion of the two lipids could be cleaved by PI-PLC after <5 min of labeling, as after 30 min of labeling (Fig. 4). These results make it unlikely that the incomplete cleavage of GlcNAc-PI and GlcN-PI in intact microsomes is due to luminal pools of the two lipids.

The analysis of the transbilayer distribution of GlcNAc-PI and GlcN-PI described above is critically dependent on the intactness of the ER microsomal vesicles used in the experiments. We established that the microsomal vesicles were intact by examining their ability to protect an abundant luminal protein, BiP, from digestion by an exogenous protease. In the presence of a membrane-disrupting concentration of detergent, BiP was sensitive to proteinase K treatment (Fig. 5, compare lane *1* with lanes *4–6*). In the absence of detergent, no degradation of BiP was detected even at the highest concentration of protease used (Fig. 5, compare lanes *1* and 2). Also, incubation of the microsomes for 1 h at 37°C (as in radiolabeling incubations with UDP-[³H]GlcNAc), followed by PI-PLC treatment on ice, had no effect on membrane integrity (Fig. 5, compare lanes *1* and *3*). These data indicate that the ER-derived microsomal preparation consists of vesicles that are impermeable to large molecules such as Proteinase K, and that the radiolabeling incubation and PI-PLC treatment have no detectable effect on their intactness.

GlcNAc-PI and GlcN-PI Can Be Synthesized by Incubating Streptolysin O–Permeabilized Cells with UDP-[³H]GlcNAc

SLO, a streptococcal pore-forming exotoxin, has found numerous applications in experiments requiring permeabilization of the plasma membrane without damaging intracellular membranes (Ahnert-Hilger et al., 1989). The toxin is generally added to cells at 4°C to allow binding to the cell surface, and excess toxin is removed by washing the cells at 4°C. Pore formation is induced by shifting the cells to 37°C. The SLO-derived pores can be as large as 30 nm in diameter (Bhakdi et al., 1985), and are large enough to permit cytosolic proteins of >140 kD to escape into the extracellular medium (Gravotta et al., 1990; Tan et al., 1992).

When SLO-permeabilized thymoma cells were incubated with UDP-[³H]GlcNAc, both GlcNAc-PI and GlcN-PI were labeled. The amount of radiolabeled lipid synthesized depended on SLO concentration, and maximum synthesis was achieved at a concentration of 60 U SLO per 10^7 cells. Beside the two major radiolabeled lipids, a third relatively minor radiolabeled species (lipid X) was also detected (Fig. 6 *A*). X chromatographed just ahead of GlcNAc-PI, and available data (see below) suggest that X is GlcN-acyl PI, i.e., GlcN-PI with a fatty acid ester linked to the inositol residue (Menon et al., 1990; Urakaze et al., 1992; Costello and Orlean, 1992). Although X was consistently detected in permeabilized cells after UDP-[³H]GlcNAc labeling, X was only occasionally identified when microsomes or post nu-

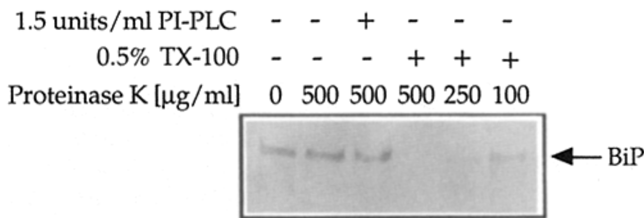


Figure 5. Proteolysis of BiP in intact, PI-PLC-treated, or detergent-disrupted microsomes. ER-derived microsomes were tested for their ability to protect BiP, a luminal protein, from digestion by exogenous protease. Microsomes were also tested after incubation at 37°C for 1 h followed by PI-PLC treatment (1.5 U/ml PI-PLC, 15 min on ice), or after disruption with 1% Triton X-100. Proteinase K treatment was performed as described in Materials and Methods. BiP (78 kD) was detected by Western blotting.

clear supernatants were incubated with UDP-[^{3}H]GlcNAc (see Fig. 1 *B*). This may be because the microsomal preparations, unlike the permeabilized cells, are significantly depleted of palmitoyl-CoA which is required to convert GlcN-PI to X (Costello and Orleans, 1992; Stevens, V. L., unpublished data).

GlcNAc-PI and GlcN-PI Are Accessible to PI-PLC in Streptolysin O-permeabilized Cells

The PI-PLC polypeptide is small enough (~40 kD) to enter cells via SLO pores. We therefore investigated the transbilayer distribution of GlcNAc-PI and GlcN-PI by treating SLO permeabilized, UDP-[^{3}H]GlcNAc–labeled cells with PI-PLC. Table II shows that up to 80% of GlcNAc-PI and 85% of GlcN-PI are cleaved in such an experiment. (In Table II, the amount of lipid synthesized after permeabilization with 70 U SLO/10^7 cells is approximately five times more than that synthesized after permeabilization with 20 U SLO/10^7 cells [114,120 cpm vs. 23,290 cpm]. Furthermore, when the amount of lipid synthesis is high the relative amount of GlcNAc-PI is also high [75% vs. 55%], and the amount of enzyme relative to the lipids is correspondingly low. Under these circumstances, hydrolysis of GlcNAc-PI is less than that of GlcN-PI, possibly because GlcNAc-PI is a poorer substrate or, more attractively, because GlcNAc-PI is protected by the de-N-acetylase enzyme [even in the presence of 0.1% deoxycholate] and therefore cannot be accessed by PI-PLC). Addition of detergent (0.1% DOC) to disrupt the membrane permeability barrier raised the cleavage efficiency only slightly. These results indicate that the two lipids are located predominantly in the cytoplasmic leaflet of an intracellular organelle, and support the conclusions obtained via PI-PLC analysis of radiolabeled ER-derived microsomes (Fig. 3 and 4). TLC data for one experiment are shown in Fig. 6. Lipid X is clearly visible in the TLC profiles, and is resistant to PI-PLC, regardless of the presence of detergent. Since inositol acylated GPIs are resistant to PI-PLC (Roberts et al., 1988), this observation is consistent with the identification of lipid X as GlcN-acyl PI.

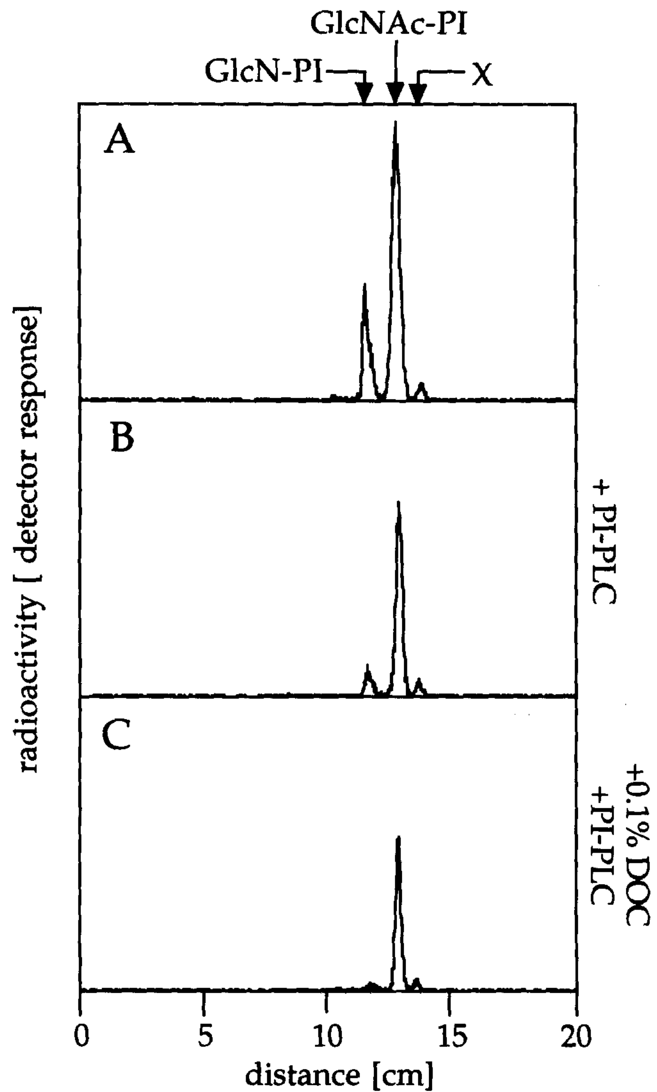


Figure 6. TLC analysis of the transmembrane distribution of GlcNAc-PI and GlcN-PI in SLO-permeabilized cells. Thymoma cells were permeabilized with SLO, and labeled with UDP-[^{3}H]GlcNAc. Samples were taken directly for lipid extraction (*A*), or lipids were extracted after the cells had been treated with PI-PLC (1.5 U/ml) in the presence (*C*) or absence (*B*) of detergent. The extracted lipids were analyzed by TLC as described in Materials and Methods, and quantitated by integration using the software provided with the scanner in conjunction with liquid scintillation counting. The profiles correspond to the data presented in Table II for permeabilization at 70 U SLO/10^7 cells.

Discussion

GPI anchoring is directed by a carboxy-terminal GPI signal sequence that is cleaved from newly synthesized proteins and replaced with a pre-synthesized GPI moiety soon (1–5 min) after completion of protein synthesis (reviewed in Ferguson and Williams, 1988). These kinetic data, together with analyses of GPI anchoring in a *S. cerevisiae* secretory mutant (*sec18*) blocked in ER to Golgi transport (Conzelmann et al., 1988) suggested that GPI addition to protein occurred in a pre-Golgi compartment, probably the ER. The identification of the ER as the site of GPI addition to protein was further supported by experiments demonstrating carboxy-terminal signal cleavage in in vitro translation–translocation assays using total microsomes from a variety of mammalian cells (Kodukula et al., 1992), and rough microsomes from canine pancreas (Vidugiriene, J., A. K. Menon, and C. V. Nicchitta, unpublished data). These data also suggested that the

Table II. Accessibility of GlcNAc-PI and GlcN-PI to PI-PLC in SLO-permeabilized Cells

	70 U SLO/10^7 cells			20 U SLO/10^7 cells		
	GlcNAc-PI	GlcN-PI	X	GlcNAc-PI	GlcN-PI	X
	(*cpm* × 10^{-2}/10^7cells)					
Control	840	273	28.2	129.5	91.7	11.7
PI-PLC	300	42.9	24.1	25.7	13.7	12.5
(Percent cleavage)	(64.3)	(84.3)	(14.6)	(80)	(85.1)	(0)
PI-PLC	207	15.3	23.5	14	3.8	11.7
(0.1% DOC)	(75.4)	(94.4)	(16.7)	(89)	(95.9)	(0)

3 × 10^6 SLO-loaded thymoma cells (treated with SLO on ice, washed, resuspended at a concentration of 10^7 cells/ml in PBS) were diluted with 300 μl two times concentrated buffer (see Materials and Methods) and incubated with 1.5 μCi UDP-[^{3}H] GlcNAc for 90 min at 37°C. The reaction was stopped by cooling on ice, and 200-μl aliquots were left untreated (control sample) or treated with 1.5 U/ml PI-PLC for 20 min on ice in the presence or absence of 0.1% deoxycholate. Lipids were extracted and analyzed as described in Materials and Methods.

GPI moiety itself was synthesized in the ER, but it remained a formal possibility that GPIs were synthesized elsewhere and transported to the ER.

The subcellular fractionation data described in this paper clearly indicate that the initial steps in GPI biosynthesis, i.e., the synthesis and deacetylation of GlcNAc-PI, occur in the ER. The GPI biosynthesis assay used in these experiments consisted of incubating subcellular fractions with UDP-[^{3}H]GlcNAc, and extracting and analyzing the labeled lipids. No PI was added to the incubation mixtures since in our experience it is impossible to glycosylate exogenously added PI or even PI synthesized in vitro by incubating the microsomes with [^{3}H]inositol. These observations have been noted previously (Menon et al., 1990) and remain to be explained, although in other experiments using total cell lysates it was reportedly possible to stimulate GlcNAc-PI/GlcN-PI synthesis approximately fivefold by adding PI to the incubation mixture (Stevens and Raetz, 1991). For the data presented here it is important to note that the PI content of the major subcellular fractions in the PNS varies by no more than a factor of 2, and in every case the PI is roughly equally distributed between the luminal and cytoplasmic leaflets of the microsomal vesicles (Higgins et al., 1989; White, 1973). Thus absence of an appropriately located endogenous PI acceptor from non-ER fractions is unlikely to be responsible for the observed concentration of the GlcNAc-PI/GlcN-PI biosynthetic activity in the ER fractions.

Analyses of the transbilayer distribution of GlcNAc-PI and GlcN-PI showed that the two lipids were distributed primarily in the cytoplasmic leaflet of the ER membrane bilayer since up to 85% of GlcNAc-PI and GlcN-PI could be hydrolyzed by PI-PLC treatment of microsomes or SLO-permeabilized cells. The extent of hydrolysis remained constant even as lipid synthesis continued, and kinetic analyses gave no indication of any transbilayer movement. Thus the bulk of the GlcNAc-PI synthase and de-N-acetylase activity appears to be located on the cytoplasmic leaflet of the ER, giving rise to lipid products oriented towards the cytoplasm and hence susceptible to PI-PLC. The small fraction of lipid (~15%) that is apparently inaccessible to PI-PLC may be the result of additional (albeit minor) synthesis in the luminal leaflet by a second set of GPI biosynthetic enzymes. It is more likely that the uncleaved fraction is the result of incomplete or inefficient cleavage, possibly due to protection of the lipid cleavage site by other molecules. In this context it is worth noting that PI-PLC treatment of intact cells frequently results in incomplete release of cell-surface GPI-anchored proteins, even when the solubilized protein is known to be completely susceptible to the enzyme. Again, this phenomenon is best explained by steric constraints that prevent enzyme access.

Our conclusions concerning the transverse distribution of early GPI intermediates are based on accessibility of the lipids to PI-PLC in an intact organelle. The use of phospholipases as topological probes has been questioned in the past, particularly in situations where the substrates for the enzyme constitute major components of the target membrane and the consequent possibility of membrane disruption is high (van Meer, 1986; Higgins and Hutson, 1986). However, for a variety of reasons we think it unlikely that PI-PLC perturbs the lipid bilayer to gain access to substrates in the luminal leaflet, thus providing a false estimate of the transverse distribution of GlcNAc-PI and GlcN-PI. Firstly, our assays are conducted for only a short time (<20 min) with the membranes on ice, minimizing changes of microsomal deterioration. Secondly, PI is a relatively minor component (~10%; White, 1973; Higgins et al., 1989) of the spectrum of ER phospholipids, and PI-PLC is specific for the inositol headgroup (Low, 1992), so no bulk lipid degradation is expected. Thirdly, measurements of microsomal intactness after PI-PLC treatment showed that the microsomes were still able to protect a luminal protein (BiP) from proteolytic cleavage. Fourthly, exhaustive treatment of canine pancreas rough microsomes with PI-PLC did not affect targeting and translocation of preprolactin, nor did it affect the degree to which the translocated protein was protected from exogenous protease (C.V. Nicchitta, personal communication). Finally, in related studies where PI-PLC was used to probe PI asymmetry in rat liver ER, treatment of microsomes with PI-PLC for 60 min at 37°C did not cause any reduction in the latency of mannose-6-phosphatase (Higgins et al., 1989). These data support the use of PI-PLC as a membrane-impermeant probe of the transverse distribution of PI-containing lipids, and indicate that the considerable cleavage of GlcNAc-PI and GlcN-PI in microsomes and permeabilized cells is due to the presence of these lipids in the accessible (cytoplasmic) leaflet of the ER membrane bilayer.

As part of a search for topological probes other than PI-PLC, we examined the possibility that the amino group of glucosamine (albeit a secondary amine) could be derivatized under physiological conditions so as to be able to use membrane-impermeant amine-reactive probes to determine the distribution of GlcN-PI. However, no reaction was seen when detergent-solubilized GlcN-PI was reacted at pH 8 with sulfosuccinimidobiotin, even though ethanolamine-containing GPIs and PE were clearly biotinylated (data not shown). After the experiments of Snider and Robbins (1982) and Snider and Rogers (1984) in which the jackbean lectin Con A was used as a probe of the transverse distribution of dolichol-PP-GlcNAc$_2$Man$_{1-9}$ in microsomes, we tested whether incubation of intact or detergent-disrupted microsomes with an α-GlcNAc-binding lectin (Iyer et al., 1976) would deplete GlcNAc-PI from organic lipid extracts. The

results were essentially negative: either the lectin did not recognize GlcNAc-PI, or the lectin–lipid interaction did not survive organic solvent extraction (data not shown). Furthermore, following related experiments by Abeijon and Hirschberg (1990), we examined GlcNAc-PI synthesis in the presence of 5-bromo-2′-deoxyuridine 5′-monophosphate (5BrdUMP), a compound known to block the carrier-mediated transport of UDP-GlcNAc into the ER lumen. Although we were able to demonstrate the UDP-GlcNAc transport block in the presence of 5BrdUMP, preliminary experiments suggested that 5BrdUMP also inhibited the GlcNAc transferase itself and could not therefore be used to probe the sidedness of GlcNAc-PI synthesis (data not shown).

Further support for our finding that GlcN-PI is situated in the cytoplasmic leaflet of the ER comes from a consideration of the next step in GPI assembly. In mammalian cells and yeast (Urakaze et al., 1992; Costello and Orlean, 1992), and possibly in trypanosomes (Menon et al., 1990), GlcN-PI is acylated on the inositol ring before elaboration with mannose and phosphoethanolamine. Experiments with yeast and thymoma cell lysates indicate that the donor of the inositol-linked fatty acid is palmitoyl CoA (Costello and Orlean, 1992; Stevens, V. L., unpublished data; see also Field et al., 1991). Since palmitoyl–CoA does not normally permeate microsomal membranes (Polokoff and Bell, 1978), inositol acylation of GlcN-PI must occur on the cytoplasmic side of the ER, implying that GlcN-PI must be available in the cytoplasmic leaflet.

The presence of GlcNAc-PI and GlcN-PI in the cytoplasmic leaflet of the ER suggests that the two lipids are synthesized from PI located in the cytoplasmic leaflet and UDP-GlcNAc present in the cytosol. The data further suggest that the protein-mediated UDP–GlcNAc transport into the ER lumen (Perez and Hirschberg, 1985; Abeijon and Hirschberg, 1992) is unnecessary for GlcNAc-PI biosynthesis. In this context, it is interesting to note that three gene products are required in the synthesis of GlcNAc-PI since thymoma cell mutants belonging to three complementation groups (classes A, C, and H; Hyman, 1988) are unable to synthesize GlcNAc-PI (Stevens and Raetz, 1991; Sugiyama et al., 1991). Beyond the obvious requirement for at least one gene product, i.e., the GlcNAc transferase, and the possibility that one or more of the genes may encode the UDP-GlcNAc transporter, this observation has yet to be clarified. Since the arguments presented above suggest that UDP-GlcNAc transport is not required for GlcNAc-PI biosynthesis, defects in genes encoding the UDP-GlcNAc transporter are unlikely to explain any of the three mutant phenotypes.

There are many points of similarity between the assembly of GPI anchors and the construction of the dolichol-linked oligosaccharide precursor of asparagine-linked sugars. Both pathways are localized to the ER, and both involve the sequential addition of components to a lipid substrate. An important postulated feature of the dolichol-PP–oligosaccharide biosynthetic scheme is that glycosylation is initiated in the cytoplasmic leaflet of the ER and completed in the luminal leaflet after transbilayer movement of a partially glycosylated intermediate (dolichol-PP-$GlcNAc_2Man_5$) (reviewed by Lennarz, 1987; Hirschberg and Snider, 1987; Abeijon and Hirschberg, 1992). A similar transport step may have to be invoked in the GPI pathway, possibly after the synthesis of GlcN-acyl PI. Since dolichol-P-mannose-dependent glycosylation processes occur in the ER lumen (Hirschberg and Snider, 1987; Tanner and Lehle, 1987), it seems likely that GlcN-acyl PI is mannosylated (via dolichol-P-mannose) after transport across the ER membrane into the luminal leaflet. The data presented in this paper offer no insight into this possibility since the transverse distribution of GlcN-acyl PI cannot be probed by PI-PLC (nor by any of the other methods discussed). We are currently investigating the distribution of ethanolamine-containing mannosylated intermediates (Hirose et al., 1992; Kamitani et al., 1992; Puoti and Conzelmann, 1992) in an effort to address this question.

The implied transbilayer movement of biosynthetic intermediates in the assembly of GPIs and dolichol-linked oligosaccharides may occur through the action of specific protein carriers in the ER membrane, similar to the postulated protein-mediated flipping of glycerophospholipids from their site of synthesis in the cytoplasmic leaflet of the ER to the luminal leaflet (Bretscher, 1974; Bishop and Bell, 1985; Backer and Dawidowicz, 1987; Devaux, 1992). Since transbilayer movement of lipids is central to the process of creating and maintaining the lipid asymmetry of the ER membrane bilayer, and since diverse lipid classes seem to undergo this type of transport, it would appear that the ER membrane contains a number of specific lipid "flippases," or that transport is nonspecific but physical constraints such as charge and membrane curvature determine the precise compositional asymmetry of the bilayer. Much more experimental data needs to be considered before these options can be resolved.

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