The GPI Anchor of Cell-Surface Proteins Is Synthesized on the Cytoplasmic Face of the Endoplasmic Reticulum

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Abstract. Glycosylphosphatidylinositol (GPI) membrane protein anchors are synthesized from sugar nucleotides and phospholipids in the ER and transferred to newly synthesized proteins destined for the cell surface. The topology of GPI synthesis in the ER was investigated using sealed trypanosome microsomes and the membrane-impermeant probes phosphatidylinositol-specific phospholipase C, Con A, and proteinase K. All the GPI biosynthetic intermediates examined were found to be located on the external face of the microsomal vesicles suggesting that the principal steps of GPI assembly occur in the cytoplasmic leaflet of the ER. Protease protection experiments showed that newly GPI-modified trypanosome variant surface glycoprotein was primarily oriented towards the ER lumen, consistent with eventual expression at the cell surface. The unusual topographical arrangement of the GPI assembly pathway suggests that a biosynthetic intermediate, possibly the phosphoethanolamine-containing anchor precursor, must be translocated across the ER membrane bilayer in the process of constructing a GPI anchor.

Many eukaryotic cell-surface proteins are known to be anchored in the plasma membrane by covalent linkage to glycosylphosphatidylinositol (GPI) (for reviews see McConville and Ferguson, 1993; Englund, 1993). Although the biological significance of protein modification by GPI remains enigmatic, emerging clues suggest that GPI-anchored proteins may be markers of membrane structural domains that are functionally important in intracellular membrane traffic (Brown, 1992) and transmembrane signaling (Brown, 1993). In some polarized cells and neurons, the GPI anchor also acts as a dominant targeting signal serving to deliver GPI-anchored proteins to specific plasma membrane domains (Rodriguez-Boulan and Powell, 1992). GPIs have been found in all eukaryotes examined to date, and a wide spectrum of functionally diverse proteins rely on a GPI anchor for membrane association.

GPIs are constructed in the ER by the action of at least seven enzymes. The simplest scheme for GPI assembly involves sequential addition of components (monosaccharides and phosphoethanolamine) to phosphatidylinositol (PI), leading to the formation of a glycolipid with the minimal structure EtN-P-Man-GlcN-PI (see Table I). The completed GPI moiety is transferred to newly synthesized proteins containing a carboxyl-terminal GPI-attachment signal sequence (Cross, 1990).

In parallel with other modifications of translocated proteins such as cleavage of the NH2-terminal signal peptide (Jackson and Blobel, 1977), N-glycosylation (Hubbard and Ivatt, 1981), and formation of disulfide bonds (Lambert and Freedman, 1983), it is widely assumed that GPI attachment to the polypeptide chain occurs in the ER lumen (Amthauer et al., 1993). The recipient proteins are targeted during synthesis to the ER via a conventional NH2-terminal signal sequence and translocated across the ER membrane bilayer. GPI is then attached (via ethanolamine) to the carboxyl-terminal amino acid exposed upon removal of the carboxyl-terminal signal peptide. By analogy with other ER glycosylation reactions, most notably those involved in the final stages of assembly of the dolichol (dol)-linked oligosaccharide precursor of N-linked sugars, it has also been suggested that the assembly of the GPI structure occurs in the luminal leaflet of the ER membrane bilayer giving rise to a product appropriately positioned for transfer to protein (Menon et al., 1990b; Abeijon and Hirschberg, 1992). Despite considerable progress in elucidating the biochemical steps involved in the construction of the GPI moiety, evidence concerning the topology of this pathway is lacking.

The phospholipid substrate, PI, and the direct reaction donors (UDP-GlcNAc, dol-P-Man, and phosphatidylethanolamine [Doering et al., 1989; Menon et al., 1990b; Menon et al., 1993]) required for GPI assembly are synthesized in the cytosol or in the cytoplasmic leaflet of the ER membrane.
bilayer, but can be transported to the ER lumen (Bell et al., 1981; Hutson and Higgins, 1982; Haselbeck and Tanner, 1982; Higgins et al., 1989; Perez and Hirschberg, 1985; Abeijon and Hirschberg, 1992). Thus, based on substrate availability, GPI assembly is not restricted to either side of the ER membrane. Recent data show that early GPI biosynthetic lipid intermediates (GlcNAC-P and GlcN-P, see Table I) are located in the external membrane leaflet of ER vesicles suggesting that GPI assembly is initiated in the cytoplasmic leaflet of the ER (Vidugiriene and Menon, 1993). Sequences of genes encoding proteins involved in GlcNAC transfer to PI are less informative but are nevertheless consistent with this hypothesis (Kinoshita and Takeda, 1994). No information is available on the topology of the reactions involved in elaborating GlcN-PI to generate the GPI anchor structure. In this paper, we show that the principal steps of GPI assembly are confined to the cytoplasmic face of the ER membrane. It seems likely that the product of these reactions, a phosphoethanolamine-containing GPI, is then translocated across the bilayer into the luminal leaflet for transfer to protein.

Materials and Methods

Materials

GDP-2-[3H]mannose and UDP-6-[3H]GlcNAC (20 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). Proteinase K and Staphylococcus aureus nuclease were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). UDP-GlcNAc, GDP-mannose, ATP, methyl-α-Dmannopyranoside, Con A (type VI) were from Sigma Chem. Co. (St. Louis, MO). Tunicamycin was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Bacillus thuringiensis phosphatidylinositol-specific phospholipase C (PI-PLC) was a gift from Dr. Martin G. Low (Columbia University, New York), and antibodies raised against Trypanosoma brucei heavy chain binding protein or GRP78 (BiP) were generously provided by Dr. James D. Bangs (University of Wisconsin-Madison, Madison, WI). The Rad-Free™ kit for chemiluminescent detection of Western blots was obtained from Schleicher & Schuell (Keene, NH), and silica 60 thin layer plates were from Merck (Gibbstown, NJ).

Preparation of Trypanosoma brucei Microsomes

Bloodstream forms of T. brucei strain 427 (clone 117) were isolated from the blood of infected rats as described previously (Field and Menon, 1992). Purified trypanosomes were resuspended in HSA-containing RPMI-1640 medium at 4 x 10^6 cells/ml, and incubated with tunicamycin (400 ng/ml) for 15-30 min at 37°C. The cells were washed twice with PBS, and membrane fractions were prepared after disrupting the cells by hypotonic lysis (Bangs et al., 1993) or nitrogen cavitation (Vidugiriene and Menon, 1993) after previously described procedures. Briefly, washed cells were resuspended at 0.5-1 x 10^7 cells/ml in hypotonic buffer (1 mM Hepes/NaOH pH 7.5, 1 mM EDTA,) and, after a 10-min incubation on ice, the lysate was passed through a 25 gauge needle three times. The lysate was adjusted to isotonicity by adding 10 x concentrated buffer A (buffer A: 25 mM Hepes/NaOH pH 7.5, 0.25 M sucrose, 1 mg/ml leupeptin, 0.1 mM TLCK, 1 mM DTT) and cell disruption was completed with five strokes in a Dounce homogenizer equipped with a tight fitting pestle. Alternatively, washed cells were resuspended in buffer A at 2 x 10^7 cells/ml, and disrupted by two cycles of nitrogen cavitation (400 psi N2 pressure), in a mini-bomb disruption chamber (Kontes, Vineland, NJ) followed by three strokes of a tight pestle Dounce homogenizer.

After lysis, cell ghosts, nuclei, and microbodies were removed by low speed centrifugation (1,500 x g, 20 min, 4°C) and the supernatant was carefully removed, hence be depleted from the lipid extract. A are expected to precipitate with the lectin during lipid extraction and hence be depleted from the lipid extract. For experiments in which Con A was tested for its ability to bind GPIs in intact microsomes, radiolabeled GPIs were synthesized by incubating trypanosome membranes with GDP-[3H]mannose in buffer C as described above. Aliquots of the labeled membranes (typically 2 x 10^7 cell eq in 20 μl) were mixed with dilutions of Con A (80 μl, prepared in buffer D) and buffer D, and after a 20-min incubation on ice, lipids were extracted into chloroform-methanol-water (10:10:3, by volume) and analyzed as described above.

In Vitro Biosynthesis of GPIs

GlcNAC-P and GlcN-P were synthesized by incubating trypanosome microsomes (typically 2.5 x 10^7 cell eq) with UDP-[3H]GlcNAC (0.25-0.5 μCi) in 50 μl buffer A containing 5 mM EDTA at 37°C. At the end of the incubation, samples were placed on ice and lipids were extracted as described (Stevens and Raetz, 1991). Lipid extracts were analyzed by TLC on silica 60 using chloroform-methanol-1 M NH4OH (10:10:3, by volume). 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 individual lipid species was determined using integration software supplied with the scanner in conjunction with liquid scintillation counting.

For pulse-chase experiments with UDP-[3H]GlcNAC, the membranes (typically 10^7 cell eq) were labeled with 1 μCi of UDP-[3H]GlcNAC in 75 μl buffer B (buffer A + 5 mM MgCl2, 5 mM MnCl2, 1 mM ATP, 1 mM CaO4, 200 ng/ml tunicamycin) for 10 min at 37°C. Then, non-radioactive sugar nucleotides (GDP-mannose and UDP-GlcNAC, 1 mM each final concentration) were added and the samples were incubated further for 0-45 min as indicated. At the end of the incubation lipids were extracted and analyzed as above.

Mannosylated GPI precursors were synthesized and analyzed as described previously (Field and Menon, 1992). Briefly, trypanosome microsomes (2.5 x 10^7 cell eq) were incubated with 0.2-0.5 μCi of GDP-[3H]mannose and 1 mM non-radioactive UDP-GlcNAC in 20-100 μl buffer 2 for 5-60 min at 37°C. Where indicated, 0.5 mM PMSF was included in the labeling medium. The reaction was stopped by placing the sample on ice and lipids were extracted in chloroform-methanol-water (final composition 10:10:3, by volume). The extract was dried under butanol-water partitioning and analyzed by TLC (silica 60, chloroform-methanol-water, 10:10:2.5, by volume).

PI-PLC Treatment of Microsomes:

Analysis of GPI Hydrolysis

PI-PLC (1,700 U/ml, 1 U = the amount of enzyme capable of hydrolyzing 1 μmol of phosphatidylinositol per minute at 37°C in pH 7 buffer containing 0.1% sodium deoxycholate [Low, 1992]) was obtained from Dr. M. G. Low (Columbia University, New York, NY). Microsomes radiolabeled via GDP-[3H]mannose or UDP-[3H]GlcNAC were placed on ice; PI-PLC (0-5 U/ml) was added and after incubation on ice (typically 20 min), lipids were extracted and analyzed as described above. No hydrolysis was observed during lipid extraction, i.e., after addition of organic solvents to the sample.

Con A Binding to Mannosylated GPIs

Extracts containing [3H]Man- or [3H]GlcN-labeled GPIs were dried and the residue was dissolved in buffer C (25 mM Hepes/NaOH pH 7.5, 0.1 M sucrose, 150 mM KCl, 5 mM MgCl2, 5 mM MnCl2) containing 0.5% Triton X-100. Aliquots of the lipid solution (20 μl, 5,000-10,000 cpm) were mixed with different dilutions of Con A (80 μl, 0.6-4 mg/ml) prepared in buffer D: 25 mM Hepes/NaOH pH 7.5, 150 mM KCl, 5 mM CaCl2). After 20 min on ice, the samples were processed by adding 5 μl of 0.5 M methyl α-D-mannopyranoside (to block further binding), 50 μl of calf serum (to ensure 100% precipitation of organic insoluble material during lipid extraction [see below]) and 95 μl buffer D. Lipids were extracted by adding 17 ml chloroform-methanol (1:1, by volume) to generate a single organic phase (Masterson et al., 1988; Field and Menon, 1992). Insoluble material (seum proteins, Con A, and Con A-lipid complexes) was removed by centrifugation (1,500 x g, 20 min, 4°C) and the supernatant was carefully removed, dried, and analyzed by TLC as described above. GPIs recognized by Con A are expected to precipitate with the lectin during lipid extraction and hence be depleted from the lipid extract.

For experiments in which Con A was tested for its ability to bind GPIs in intact microsomes, radiolabeled GPIs were synthesized by incubating trypanosome membranes with GDP-[3H]mannose in buffer C as described above. Aliquots of the labeled membranes (typically 2 x 10^7 cell eq in 20 μl) were mixed with dilutions of Con A (80 μl, prepared in buffer D) and buffer D, and after a 20-min incubation on ice, lipids were extracted into chloroform-methanol-water (10:10:3, by volume) and analyzed as described above.

Integrity of ER Vesicles

The integrity of isolated trypanosome microsomal vesicles was determined by protease protection experiments using BiP, an ER luminal protein, as
the reporter. Membranes (2.5 × 10^6 cell eq in 100 μl buffer B) were mock-
treated for 30 min at 37°C and placed on ice. Aliquots of the sample (10 μl) 
were then diluted with 90 μl 25 mM Heps/NaOH pH 7.5, 0.25 mM 
sucrose with or without proteinase K and Triton X-100 (50 μg/ml and 0.5 % 
final concentrations, respectively). Where indicated, the membranes were 
treated with PI-PLC (on ice or at 37°C) before proteinase K addition. After 
30 min on ice, proteolysis was stopped by adding 300 μl of 4 mM PMSF in 
water (diluted from a 0.2 M stock solution in 95% ethanol just before use). Proteins were precipitated by adding 400 μl of ice-cold 14% (wt/vol) 
TCA. After a 30-min incubation on ice, the sample was centrifuged at 
12,000 g for 15 min at 4°C. The pellet was washed with 500 μl ice-cold 
aqueous containing 1% TCA, left at −70°C for 30 min, and then centrifuged 
again. The pellet was resuspended in SDS-PAGE sample buffer and ana-
yzed by 10% SDS-PAGE. After electrophoretic separation, proteins were 
transferred to Rad-Free™ membrane using standard procedures. The blots 
were probed with antibodies raised against Trypanosoma brucei BIP (Bangs 
et al., 1993) and alkaline phosphatase-conjugated secondary antibodies. 
Immunoreactive bands were visualized by adding a chemiluminescent alka-
line phosphatase substrate supplied with the Rad-Free™ kit and exposing the 
blots to X-ray film. At least two different film exposures were obtained to 
verify linearity of the film response for densitometric analysis. Densi-
tometry was performed by Kendrick Laboratories (Madison, WI) using a 
LGS-50 Laser Scanning Densitometer and QGEL software.

Transfer of GPls to Endogeneous Protein Acceptors

Trypanosome microsomes (10^6 cell eq) were incubated at 37°C with 1.5 
μCi GDP-[^3H]mannose and 1 mM non-radioactive UDP-GlcNAc in 100 μl 
buffer B. For protease protection experiments, labeled microsomes were 
subjected to proteinase K treatment as described above, before analysis. 10% 
of the sample was taken for lipid extraction and TLC. The remainder 
of the sample was analyzed by SDS-PAGE and fluorography (using 
EN3HANCE, DuPont-New England Nuclear). Exposure times of ~2 wk 
were usually required, and at least two different film exposures were ob-
tained for densitometric analysis. Densitometry was performed as de-
scribed above.

Results

In Vitro Synthesis of Radiolabeled GPls

Labeling strategies were developed to probe the transbilayer 
distribution of GPls in the ER membrane bilayer. Radi-
labeled GPls were synthesized by incubating trypanosome 
microsomes with radioactive sugar nucleotides (UDP-[^3H]- 
GlcNAc or GDP-[^3H]mannose) in the presence of tunica-
mycin (to prevent synthesis of dolichol-PP-oligosaccharides 
derived from dolichol-PP-GlcNAc). Incubations were per-
formed at 37°C. No incorporation of radioactivity was seen 
when microsomes were incubated with radiolabeled pre-
cursors on ice, nor did the spectrum or intensity of the radiolabeled GPls change when a labeled sample was maintained 
on ice (not shown). In some experiments GPl biosynthesis 
was blocked at the GlcN-PI stage or the Man:GlcN-PI stage 
by including EDTA or PMSF in the labeling medium (Vidu-
giriene and Menon, 1993; Masterson and Ferguson, 1991). 
At the end of the incubation lipids were extracted and ana-
yzed by thin layer chromatography. The various labeled 
GPls were identified by TLC mobility and by diagnostic treat-
ments, including hydrolysis by PI-PLC. Representative chroma-
igrams from GDP-[^3H]mannose- or UDP-[^3H]GlcNAc-
labeled experiments are shown in Fig. 1. The structures and properties of the radiolabeled GPls are summarized in 
Table I.

Non-Mannosylated GPI Lipids Are 
Located in the External Leaflet of the Microsomal Membrane Bilayer

Fig. 2 A shows that significant amounts of radiolabeled 
microsomal GlcNAc-PI and Glc-N-PI are hydrolyzed on ex-
posing trypanosome microsomal vesicles to relatively low 
concentrations of PI-PLC on ice (~75% hydrolysis after 30 
min with 1.7 U/ml PI-PLC). Since the microsome prepara-
tion consists of a population of sealed vesicles (see below) 
and PI-PLC cannot traverse the membrane, these data indi-
cate that GlcNAc-PI and Glc-N-PI are primarily located in 
the external (cytoplasmic) leaflet of the microsomal vesicle 
membrane.

We next investigated whether these cytoplasmically ori-
ented pools of GlcNAc-PI and Glc-N-PI represent biosynthet-
ically active precursors that are used in subsequent steps of 
GPl assembly. EDTA was omitted from the reaction mixture, 
and conversion of GlcNAc/Glc-N-PI into mannosylated GPl 
species was measured using a pulse-chase protocol. The membranes were labeled with UDP-[^3H]GlcNAc for 10 
min, and then chased by adding a 1,000-fold excess of non-
radioactive UDP-GlcNAc and GDP-mannose. As expected

Figure 1. Thin layer chromatograms of in vitro labeled GPls. 
Radiolabeled GPls were synthesized by incubating trypanosome 
microsomes with GDP-[^3H]-mannose (A and B) or UDP-
[^3H]GlcNAc (C). Assay mixtures contained either no inhibitors 
(A), or PMSF to block the GPI ethanolaminephosphotransferase 
(B), or EDTA to block mannosylation (C). Lipids were extracted 
and analyzed by TLC using chloroform-methanol-water, 10:10:3, by volume (A and B) or chloroform-methanol-1 M 
NH4OH (10:10:3, by volume) (C). The distribution of radioactivity in the 
chromatograms was visualized using a TLC scanner. Labeling con-
ditions were as follows. (A and B) 0.2 μCi GDP-[^3H]mannose, 
2 × 10^7 cell eq of microsomes in 20 μl buffer B (+0.5 mM 
PMSF), 20-min incubation, 37°C; (C) 0.35 μCi UDP-[^3H]- 
GlcNAc, 3 × 10^7 cell eq of microsomes in 50 μl buffer A (+0.5 
mM EDTA), 30-min incubation, 37°C. Arrowheads denote the 
origin and solvent front. Lipid nomenclature is given in Table I.
Table I. In Vitro Radiolabeled GPls

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Structure</th>
<th>PI-PLC susceptibility</th>
<th>Coa. A binding</th>
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<tbody>
<tr>
<td>GlcNAc-PI</td>
<td>GlcNAc-PI</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>GlcN-PI</td>
<td>GlcN-PI</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M1</td>
<td>Man_GlcNAc-PI</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M2</td>
<td>Man_GlcN-PI</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>M3</td>
<td>Man_GlcN-PI</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M2*</td>
<td>Man_GlcN-PI*</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>M3*</td>
<td>Man_GlcN-PI*</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>P2</td>
<td>EtN-P-Man_GlcNAc-PI</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>P3</td>
<td>EtN-P-Man_GlcNAc-PI</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The different lipids are biosynthetic intermediates representing partial structures and/or modified versions of the core GPI moiety: EtN-P-Man3GlcNAc as described in Fig. 1C, closed squares); as this lipid chromatographs identically with PI-PLC-resistant M3* in several TLC systems that we tested (Menon et al., 1990a), GPls containing PI* are resistant to hydrolysis by PI-PLC (Roberts et al., 1988; Krakow et al., 1989; Mayor et al., 1990). ND, not determined.

(Masterson et al., 1989; Menon et al., 1990a), radioactivity in GlcNAc-PI and GlcN-PI was rapidly lost during the chase (Fig. 2B, closed squares) as the precursors were converted into mannosylated GPl species (Fig. 2B, open squares). Interestingly, topological analysis of GlcNAc/GlcN-PI during the chase indicated that loss of radioactivity occurred mainly in the PI-PLC-sensitive pool (Fig. 2C, open diamonds); changes in the amount of the PI-PLC-resistant fraction (Fig. 2C, closed diamonds) were minor. These data are consistent with the hypothesis that cytoplasmically oriented GlcN-PI is a substrate for the first GPI mannosyltransferase and that mannos transfer probably occurs on the external (cytoplasmic) face of microsomal vesicles. More elaborate explanations of this result include the possibility that cytoplasmically oriented GlcN-PI flips into the luminal leaflet during the chase, and is converted to M1 at the same rate to give a constant steady-state amount of PI-PLC resistant GlcN-PI.

The PI-PLC-resistant fraction of GlcNAc/GlcN-PI represents a pool of lipid that is protected from enzyme action. The ER membrane is an obvious permeability barrier and the PI-PLC-resistant fraction could correspond to a luminal pool of GlcNAc/GlcN-PI. On the other hand the PI-PLC-resistant fraction may represent cytoplasmically oriented GlcNAc/GlcN-PI molecules that are located in sterically constrained environments which prevent PI-PLC access; sterical constraints could be generated through non-specific interactions with other lipids or proteins, or specific interactions with proteins such as the various transferases involved in constructing the GPI glycan. This interpretation is supported by speculation elsewhere that sterical constraints are probably responsible for the inefficient cleavage of membrane-bound GPI-anchored proteins by PI-PLC (Low and Kincade, 1985; Nagel and Boothroyd, 1989), although in these cases the GPI-linked polypeptide chain itself may be a factor in hindering enzyme access. These hypotheses concerning the PI-PLC-resistant fraction of microsomal GlcNAc/GlcN-PI are not mutually exclusive and neither can be directly ruled out in our experiments.

**Significant Amounts of PI-PLC-Sensitive Mannosylated GPls (M2, M3, and P2) Are Located in the External Leaflet of Microsomal Vesicle Membranes**

To characterize the topological distribution of the GPI mannosylation reactions further, we used PI-PLC to probe the distribution of mannosylated GPl species in microsomal vesicles. M2, M3, and P2 were examined as these lipids are substrates for PI-PLC in detergent solutions. We did not study the distribution of a fourth PI-PLC-sensitive lipid, M1, as this lipid chromatographs identically with PI-PLC-resistant M3* in several TLC systems that we tested (Menon et al., 1990a). The M3 GPI species was probed in PMSF-treated microsomes (see Fig. 1A); the other mannosylated GPl species were labeled at sufficiently high levels for analysis in normal microsomes (Fig. 1A).

Microsomes were incubated with GDP-[3H]mannose in the presence or absence of PMSF for 20 min at 37°C, and then treated with different concentrations of PI-PLC for 20 min on ice before lipid extraction and analysis. Fig. 3A shows that up to 65% of M3 and 60% of M2 in PMSF-treated microsomes were hydrolyzed by PI-PLC (1–1.5 U/ml). Similar experiments performed in the absence of PMSF showed approximately the same level of hydrolysis of microsomal M2 (64 ± 7.5% (n = 11)) by 1.7 U/ml PI-PLC (Fig. 3B). The transbilayer distribution of microsomal P2 was also determined in the same experiments. Fig. 3B shows that ~50% of in vitro synthesized P2 was hydrolyzed by PI-PLC (1.7 U/ml) under standard conditions (see also Table II). The average percent hydrolysis of microsomal P2 determined from several experiments was 52 ± 6.5% (n = 11).

Since the three lipids are hydrolyzed efficiently (80–90%) once the microsomes are solubilized in detergent, the incom-

![Figure 2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1427466/bin/JOCL671089/Figure2.jpg)
Figure 3. PI-PLC hydrolysis of in vitro labeled mannosylated GPIs in intact microsomes. Microsomes were incubated with GDP-[\(^{3}H\)mannose in the presence (A) or absence (B) of PMSF as described in Fig. 1 A and B, and then treated with different concentrations of PI-PLC for 20 min on ice. Lipids were extracted and analyzed as described in Materials and Methods. 10% of the lipid extract was taken for liquid scintillation counting. The remainder of the sample was dried, dissolved in 20 \(\mu\)l water-saturated butanol, and analyzed by TLC as in Fig. 1 A. Lipid hydrolysis was calculated from the amount of radioactivity incorporated into individual lipid species before and after PI-PLC treatment.

Complete hydrolysis observed in our experiments suggests that there may be luminal pools of the lipids protected from enzyme action by a membrane barrier, and/or that PI-PLC hydrolysis of lipids located in the external leaflet of the microsomal membrane is inefficient under the experimental conditions employed. As discussed above, neither explanation can be ruled out. It was possible to increase the level of hydrolysis (up to 90% and 75% for M2 and M3, respectively) while retaining microsomal vesicle intactness (see below) by performing PI-PLC incubations at 37°C, but uncertainties in assessing the contribution of ongoing lipid metabolism and the effect of PI-PLC on the flux through the biosynthetic pathway under these conditions make the interpretation of this result unclear. Nevertheless, the overall data indicate that significant amounts of all three mannosylated GPIs (M2, M3, and P2) are located in the external (cytoplasmic) leaflet of the microsomal membrane bilayer.

Intactness of Microsomal Vesicles

Verification of the intactness of the microsomal membrane barrier is critical for the interpretation of the experiments described above. Since GPI biosynthesis occurs in the ER (Vidugiriene and Menon, 1993), it was important to determine the quality of the ER vesicles in the microsomal fraction. The intactness of the ER-derived microsomal vesicles before and after PI-PLC treatment was monitored by determining the extent to which a luminal ER protein, BiP, was protected from the action of exogenously added protease K. Published data show that very little BiP is released during hypotonic lysis and microsome preparation (Bangs et al., 1993) indicating that disruption of the microsomes during these procedures is minimal. Fig. 4 shows that BiP (determined by SDS-PAGE, Western blotting and densitometry) was quantitatively protected from proteolysis in the absence of detergent. Importantly, mock-incubation of the microsomes at 37°C followed by PI-PLC treatment on ice had no measurable effect on BiP proteolysis. Similar protease protection results were obtained if the microsomes were treated with PI-PLC for 30 min at 37°C (not shown). These data indicate that the microsomal vesicles are intact and that their quality is unaffected by PI-PLC treatment, validating the use of PI-PLC as a probe of transbilayer lipid distribution.

Both PI-PLC-Sensitive and Resistant Mannosylated GPIs Bind Con A in Intact Microsomes

The transmembrane distribution of labeled GPIs was investigated with Con A as a membrane-impermeant probe, a technique that has been successfully used to determine the orientation of dolichol-linked oligosaccharides in microsomal vesicles (Snider and Robbins, 1982; Snider and Rogers, 1984) and GPIs in microsomal membranes (Snider and Rogers, 1985). Table II shows that PI-PLC and Con A interact with the same pool of microsomal GPIs. Microsomes (2.5 x 10^7 cell eq) were incubated with 0.25 \(\mu\)Ci GDP-[\(^{3}H\)]mannose in 20 \(\mu\)l buffer C for 20 min at 37°C. PMSF (0.5 mM) was included to obtain data on M3. After labeling, the membranes were treated with PI-PLC on ice in the presence of different concentrations of Con A (20-min incubation) and lipids were extracted as described in the Con A-binding protocol (Materials and Methods). The results are presented as the % lipid (P2 or M3) lost from the lipid extract due to PI-PLC hydrolysis and/or Con A binding.

Table II. PI-PLC and Con A Interact with the Same Pool of Microsomal GPIs

<table>
<thead>
<tr>
<th>PI-PLC (U/ml)</th>
<th>Con A (mg/ml)</th>
<th>% lipid depleted</th>
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<tr>
<td>1.7</td>
<td>0</td>
<td>58.8</td>
</tr>
<tr>
<td>1.7</td>
<td>2.5</td>
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<td>1.7</td>
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</tr>
<tr>
<td>0</td>
<td>5.0</td>
<td>56.1</td>
</tr>
</tbody>
</table>

Microsomes (2.5 x 10^7 cell eq) were incubated with 0.25 \(\mu\)Ci GDP-[\(^{3}H\)]mannose in 20 \(\mu\)l buffer C for 20 min at 37°C. PMSF (0.5 mM) was included to obtain data on M3. After labeling, the membranes were treated with PI-PLC on ice in the presence of different concentrations of Con A (20-min incubation) and lipids were extracted as described in the Con A-binding protocol (Materials and Methods). The results are presented as the % lipid (P2 or M3) lost from the lipid extract due to PI-PLC hydrolysis and/or Con A binding.

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Figure 5. Con A binding to GPIs. (A) [H]Mannose-labeled GPIs (10,000 cpm/20 µl buffer C) were incubated with different amounts of Con A for 20 min on ice. The reaction was stopped with 5 µl 0.5 M methyl α-D-mannopyranoside (αMeMan) and GPIs were extracted as described in Materials and Methods and analyzed by liquid scintillation counting and TLC. In control assays, αMeMan was added to the sample before Con A addition. (B) Microsomes (2 × 10^7 cell eq in 20 µl buffer C) were incubated with 0.2 µCi GDP-[H]mannose for 20 min at 37°C, and then treated with different concentrations of Con A for 20 min on ice. Lipids were extracted and analyzed as described in Materials and Methods. The extent of the Con A-binding reaction was determined from the amount of individual lipids recovered in the organic extract.

Since previous work (Bangs et al., 1988) showed that protein-linked GPIs bind to Con A, we first needed to determine which of the GPI biosynthetic intermediates would also bind. Detergent solutions of radiolabeled GPIs were incubated with different amounts of Con A on ice, and then lipids were extracted and analyzed. During lipid extraction, GPIs recognized by Con A are expected to precipitate with the lectin, whereas GPI species not bound to Con A are expected to be recovered quantitatively in the organic extract. As shown in Fig. 5 A, all GPI species with three mannose residues (M3, P2, and P3) were significantly depleted (60-70% maximum efficiency) from the lipid extract indicating that they were bound to the lectin. The binding efficiency was unaffected by inositol acylation as P2 and P3 were equally well recognized. Binding of the M2 species was much weaker, and binding of all species was specifically inhibited by α-D-methylmannopyranoside (αMeMan). These results are consistent with the known structural requirements for Con A-ligand binding (Ogata et al., 1975) and indicate that GPI biosynthetic intermediates are specifically recognized by Con A.

To determine the extent to which microsomal GPIs bind Con A, GDP-[H]mannose-labeled membranes were incubated on ice with different amounts of Con A. Lipids were then extracted and analyzed by TLC. Fig. 5 B shows that significant amounts (55, 65, and 30%) of P2, M3, and P3 were depleted from lipid extracts of Con A-treated microsomes indicating that all three lipids are accessible on the cytoplasmic face of the microsomal vesicles. The results for P2 and M3 are quantitatively similar to those obtained with PI-PLC. This observation is substantiated by the results summarized in Table II. When Con A (up to 5 mg/ml) was included in standard PI-PLC treatments of labeled microsomes (1.7 U/ml PI-PLC, 20-min incubation on ice), very little (<10%) additional P2 or M3 was depleted from the lipid extract indicating that both probes interact with the same microsomal pool of P2 and M3. An additional point of interest concerns the significant difference in Con A reactivity of microsomal P2 and P3 (Fig. 5 B). Since both P3 and P2 bind Con A equally well in detergent solution (Fig. 5 A), the difference in binding seen with intact vesicles suggests that significantly larger quantities of P3—compared to P2—may be localized in the lumenal leaflet of the ER.

The Con A/PI-PLC experiments also generated information on M1 and M3* (Table I), two GPI structures that we were unable to study conveniently using PI-PLC alone because they comigrated in several TLC systems. M1 is PI-PLC-sensitive but is not recognized by Con A; M3* is PI-PLC-resistant but Con A reactive. In PMSF-treated microsomes, 56% of M1/M3* was hydrolyzed by PI-PLC (1.7 U/ml), 30% was bound to Con A (5 mg/ml), and 76% was depleted by a combination of the probes. Although the precise proportion of M1 and M3* in M1/M3* is unknown, these data indicate that at least 80% of M1 and 68% of M3* is located on the external face of the microsomes. Data obtained from microsomes without PMSF were similar, indicating distributions of >65% for M1 and >65% for M3* in the external leaflet of the vesicle membrane.

Newly GPI-modified Trypanosome Variant Surface Glycoprotein Is Located in the Lumenal Leaflet of ER Microsomes

Mature phosphoethanolamine-containing GPIs are transferred to newly synthesized proteins possessing a carboxyterminal GPI-attachment signal sequence. It has been shown previously that trypanosome variant surface glycoprotein (VSG) polypeptides left unprocessed at the time of cell lysis and microsome preparation are capable of being modified by in vitro synthesized GPIs (Mayor et al., 1991). We have taken advantage of this endogenous pool of acceptors to investigate the topology of GPI transfer to protein. Microsomal membranes were radiolabeled with GDP-[H]mannose and TCA-insoluble material was analyzed by SDS-PAGE. The transfer of a GPI anchor to VSG was followed by assessing the incorporation of [H]mannose into the polypeptide chain. Since these experiments were carried out with microsomes prepared from tunicamycin-treated cells and by including tunicamycin in the assay mixture, no protein-associated radioactivity can be ascribed to N-glycosylation (Mayor et al., 1991). As shown in Fig. 6, only one major radiolabeled band of ~55 kD was detected, corresponding to trypanosome VSG (variant 117). To examine the localization of in vitro labeled VSG, we tested the extent to which the labeled VSG polypeptide was protected from exogenously added proteinase K. Fig. 6 shows that the majority (73%) of newly GPI-modified VSG was protected from proteinase K digestion, indicating a luminal orientation (compare lanes 2 and 6, densitometric data are provided in the legend to Fig. 6).

We are unable to provide a simple explanation for the observation that a fraction of the GPI-modified VSG (27%) is susceptible to protease (Fig. 6) even though the luminal marker BiP is completely protected in the same experiments (Fig. 4). One possibility is that some vesicles become unsealed during microsome preparation. It might be anticipated that these vesicles lose BiP and escape detection in the
Discussion

**GPIs Are Synthesized on the Cytoplasmic Face of the ER**

The construction of the GPI anchor precursor is a multistep process involving at least four glycosyltransferases, one inositol acyltransferase, one deacetylase, and one ethanolaminephosphotransferase. The enzymes are located in the endoplasmic reticulum but the arrangement of enzyme active sites and hence the topology of the assembly process has not been described. In this paper we use two membrane-impermeant probes, Con A and phosphatidylinositol-specific phospholipase C, to analyze the transbilayer distribution of GPI biosynthetic lipid intermediates. All the GPIs probed (GlcNAc-PI, GlcN-PI, M1, M2, M3, P2, M3*, and P3) were transfer-competent (phosphoethanolamine-containing) GPIs, P2, and P3, differing only in that P3 is inositol-acylated while P2 is not (Mayor et al., 1990a,b). The two lipids appear to exist in dynamic equilibrium, undergoing interconversion through inositol acylation and acyl chain hydrolysis (Güther et al., 1994). Although the precise role of inositol acylation in GPI assembly remains to be defined, it has been suggested that it may provide a critical stereochemical constraint essential for glycan assembly (Menon et al., 1990a) or function as a tag for GPI reservoirs or excess GPI pools destined for catabolism (Güther et al., 1994). Our data show that while both P3 and P2 are equally recognized by Con A in detergent solution and both lipids are accessible to Con A in intact microsomal vesicles, significantly less microsomal P3 reacts with the lectin. We were unable to pursue this observation further with membrane-impermeant probes directed against the amino group of ethanolamine in both lipids. We tested trinitrobenzenesulfonic acid and the N-hydroxysuccinimide ester of biotin: both reagents mod-
ified P2 and P3 in organic solvents but not under milder, physiologically appropriate conditions (unpublished data). Data for another inositol acylated GPI (M*) indicate that at least 65% of this species is present on the external face of the microsomal vesicles, suggesting that inositol acylation alone is not responsible for the relative inaccessibility of microsomal P3. The significance of the oppositely skewed distributions of P2 and P3 in microsomal vesicles (Fig. 7) is not clear, but the observation may be relevant to an understanding of the possible effect on GPI metabolism of the GPI-specific phospholipase C expressed in bloodstream trypanosomes and located on the cytoplasmic face of otherwise undefined membrane vesicles (Bülow et al., 1989; Menz-Wilmot et al., 1994).

The GPI pathway may be compared to other ER lipid glycosylation pathways involving dolichol phosphate. The synthesis of dolichol-linked oligosaccharide structures in the ER proceeds in two phases. The initial seven-sugar structure (Man6GlcNAc~2-PP-dolichol) is synthesized on dolichol phosphate from sugar nucleotide donors on the cytoplasmic face of the ER, and then elongated with sugars (four mannoside residues and three glucose residues) derived from dolichol-P-Man and dolichol-P-glucose donors on the luminal face of the ER (Abeijon and Hirschberg, 1992). In this reaction sequence, as in yeast O-mannosylation (Tanner and Lehle, 1987), dolichol-P-mannose-dependent glycosylation reactions occur on the luminal face of the ER. Thus, despite the many points of similarity between GPI assembly and other ER glycosylation reactions, the results described in this paper suggest that GPI biosynthesis uniquely involves the use of a lipid-linked sugar (dolichol-P-mannose) by a cytoplasmically oriented eukaryotic glycosyltransferase.

**Newly GPI-modified VSG Is Mainly Located in the Lumenal Leaflet of the ER**

Despite the now considerable body of information on GPI assembly, the enzymology of protein modification by GPI is poorly understood. It has been generally assumed that a pseudotranspeptidation (transamidation) reaction is involved, and that the cleavage of the protein carboxyl-terminal GPI-directing signal sequence and attachment of GPI occurs in concerted fashion without external energy inputs. Since GPI modification occurs in the absence of ongoing protein translation (Mayor et al., 1991; Amthauer et al., 1992) and the GPI-directing signal sequence is at the carboxyl terminus of the protein (Cross, 1990), it is likely that GPI attachment occurs after protein translocation across the ER membrane, possibly after release of the polypeptide from the translocation apparatus. We investigated the GPI addition reaction by exploiting the fact that trypanosome microsomes contain at least 4,000 copies of unprocessed VSG per cell equivalent (Mayor et al., 1991) that are available for modification by in vitro synthesized GPs. Our analyses of this system via protease protection experiments confirm that newly GPI-anchored VSG molecules are primarily lumenerally oriented, consistent with their eventual expression at the cell surface. These results do not directly address the issue of the topology of GPI transfer to protein, and the statement that transfer occurs in the luminal leaflet must be regarded as simply a possibility, albeit a likely one, until further data are obtained.

**Transbilayer Movement of Glycolipids**

In the dolichol pathway of N-glycosylation, Man6GlcNAc~2-PP-dolichol is located predominantly in the cytoplasmic leaflet of the ER while Man6GlcNAc~2-PP-dolichol and more elaborate species are located primarily in the lumenal leaflet (Snider and Rogers, 1984). These data are best accounted for by the proposal that a certain fraction of Man6GlcNAc~2-PP-dolichol flips into the lumenal leaflet of the ER and is rapidly elaborated to Man6GlcNAc~2-PP-dolichol. The synthesis of gangliosides in the Golgi may also involve flipping of a partially glycosylated lipid precursor, since the biosynthetic reactions involve synthesis of glucosylceramide (and possibly lactosylceramide) on the cytoplasmic face and subsequent processing on the luminal face (van Meer, 1993). The data presented in this paper suggest that the GPI end-products, P2 and P3, may undergo similar transbilayer movement. Thus experimental characterizations of lipid glycosylation in the ER and Golgi are best explained by postulating transbilayer movement (flip-flop) of glycosylated lipids. In the dolichol-P and PI glycosylation pathways, lipid species with headgroups of considerable polarity must be translocated across the ER membrane bilayer. It has been clearly established that transport of phospholipids across membrane bilayers does not occur spontaneously, and that in some biological membranes, particularly those possessing lipid biosynthetic capability, transport is facilitated by protein catalysts termed flippases or lipid translocases. Studies with glycerophospholipid substrates have demonstrated several characteristics of such an activity (Zachowski, 1993), but no flip-fapse has been isolated. It remains to be seen whether specific flippases exist for these various substrates, or whether species that are flipped are those that are available for transport because they have relatively long half lives or because they are not involved in stable lipid–lipid or lipid–protein interactions. It is conceivable that partially assembled GPI structures may not be available to flippases (but nevertheless accessible to topological probes) because they may be bound to one of the GPI biosynthetic transferases.

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**References**


