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

Jolanta Vidugiriene and Anant K. Menon

Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706

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)[1] (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_3-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 NH_2-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 NH_2-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 lumenal leaflet of the ER membrane bilayer giving rise to a product appropriately positioned for transfer to protein (Menon et al., 1990*b*; 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., 1990*b*; Menon et al., 1993]) required for GPI assembly are synthesized in the cytosol or in the cytoplasmic leaflet of the ER membrane

Address all correspondence to Dr. Anant K. Menon, Department of Biochemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706-1569. Telephone: (608) 262-2913; FAX: (608) 262-3453.

1. *Abbreviations used in this paper*: BiP, heavy chain binding protein or GRP78; dol, dolichol; EtN, ethanolamine; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; GPI, glycosylphosphatidylinositol; Man, mannose; PI, phosphatidylinositol; PI*, PI containing a fatty acid esterified to inositol; PI-PLC, phosphatidylinositol-specific phospholipase C; VSG, variant surface glycoprotein.

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-PI and GlcN-PI, 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 lumenal leaflet for transfer to protein.

Materials and Methods

Materials

GDP-[2-^{3}H]mannose and UDP-[6-^{3}H]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-D-mannopyranoside, 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 BSA-containing RPMI-1640 medium at $\leq 10^8$ 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 × 10^9 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× concentrated buffer A (buffer A: 25 mM Hepes/NaOH pH 7.5, 0.25 M sucrose, 1 μg/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 ≥1–2 × 10^9 cells/ml, and disrupted by two cycles of nitrogen cavitation (400 psi N_2 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 (1,000 *g* for 10 min at 4°C) and the resulting "post-nuclear" supernatant was fractionated by centrifugation at high speed (Beckman 70Ti rotor, 43,000 rpm, 2 h, 4°C) using either a discontinuous sucrose gradient (steps at 38%, 30%, and 20% sucrose, wt/vol) or a simple sucrose cushion (0.5 M) prepared in buffer A. In each case the pellet was resuspended in buffer A (4–5 × 10^9 cell equivalents [cell eq]/ml), frozen immediately, and stored at −70°C. The two preparations were used interchangeably in the experiments reported in this paper.

In Vitro Biosynthesis of GPIs

GlcNAc-PI and GlcN-PI were synthesized by incubating trypanosome microsomes (typically 2.5 × 10^7 cell eq) with UDP-[^{3}H]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 NH_4OH (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-[^{3}H]GlcNAc, the membranes (0.5 × 10^8 cell eq) were labeled with 1 μCi of UDP-[^{3}H]GlcNAc in 75 μl buffer B (buffer A + 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM ATP, 1 mM CoA, 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 × 10^7 cell eq) were incubated with 0.2–0.5 μCi of GDP-[^{3}H]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 desalted by 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 7.0 buffer containing 0.1% sodium deoxycholate [Low, 1992]) was obtained from Dr. M. G. Low (Columbia University, New York, NY). Microsomes radiolabeled via GDP-[^{3}H]mannose or UDP-[^{3}H]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 [^{3}H]Man- or [^{3}H]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 $MgCl_2$, 5 mM $MnCl_2$) 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 mg/ml prepared in buffer D: 25 mM Hepes/NaOH pH 7.5, 150 mM KCl, 5 mM $CaCl_2$). 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 1.7 ml chloroform-methanol (1:1, by volume) to generate a single organic phase (Masterson et al., 1989; Field and Menon, 1992). Insoluble material (serum proteins, Con A, and Con A-lipid complexes) was removed by centrifugation (1,500 *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-[^{3}H]mannose in buffer C as described above. Aliquots of the labeled membranes (typically 2 × 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 lumenal protein, as

the reporter. Membranes (2.5×10^6 cell eq in 100 μl buffer B) were mock-labeled for 30 min at 37°C and placed on ice. Aliquots of the sample (10 μl) were then diluted with 90 μl 25 mM Hepes/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 acetone containing 1% TCA, left at −70°C for 30 min, and then centrifuged again. The pellet was resuspended in SDS-PAGE sample buffer and analyzed 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 alkaline 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. Densitometry was performed by Kendrick Laboratories (Madison, WI) using a LGS-50 Laser Scanning Densitometer and QGEL software.

Transfer of GPIs to Endogeneous Protein Acceptors

Trypanosome microsomes (10^8 cell eq) were incubated at 37°C with 1.5 μCi GDP-[^{3}H]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 EN^{3}HANCE, DuPont-New England Nuclear). Exposure times of ~2 wk were usually required, and at least two different film exposures were obtained for densitometric analysis. Densitometry was performed as described above.

Results

In Vitro Synthesis of Radiolabeled GPIs

Labeling strategies were developed to probe the transbilayer distribution of GPIs in the ER membrane bilayer. Radiolabeled GPIs were synthesized by incubating trypanosome microsomes with radioactive sugar nucleotides (UDP-[^{3}H]-GlcNAc or GDP-[^{3}H]Mannose) in the presence of tunicamycin (to prevent synthesis of dolichol-PP-oligosaccharides derived from dolichol-PP-GlcNAc). Incubations were performed at 37°C. No incorporation of radioactivity was seen when microsomes were incubated with radiolabeled precursors on ice, nor did the spectrum or intensity of the radiolabeled GPIs change when a labeled sample was maintained on ice (not shown). In some experiments GPI biosynthesis was blocked at the GlcN-PI stage or the Man_3GlcN-PI stage by including EDTA or PMSF in the labeling medium (Vidugiriene and Menon, 1993; Masterson and Ferguson, 1991). At the end of the incubation lipids were extracted and analyzed by thin layer chromatography. The various labeled GPIs were identified by TLC mobility and by diagnostic treatments, including hydrolysis by PI-PLC. Representative chromatograms from GDP-[^{3}H]mannose- or UDP-[^{3}H]GlcNAc-labeling experiments are shown in Fig. 1. The structures and properties of the radiolabeled GPIs 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

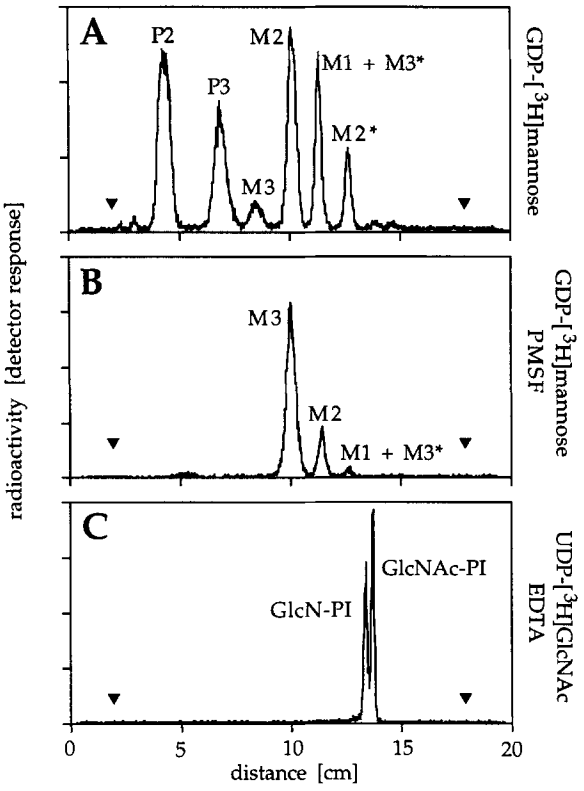

Figure 1. Thin layer chromatograms of in vitro labeled GPIs. Radiolabeled GPIs were synthesized by incubating trypanosome microsomes with GDP-[^{3}H]-mannose (*A* and *B*) or UDP-[^{3}H]GlcNAc (*C*). Assay mixtures contained either no inhibitors (*A*), or PMSF to block the GPI ethanolaminephosphotransferase (*B*), or EDTA to block mannosylation (*C*). Lipids were extracted and analyzed by TLC using chloroform-methanol-water, 10:10:2.5, by volume (*A* and *B*) or chloroform-methanol-1 M NH_4OH (10:10:3, by volume) (*C*). The distribution of radioactivity in the chromatograms was visualized using a TLC scanner. Labeling conditions were as follows. (*A* and *B*) 0.2 μCi GDP-[^{3}H]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-[^{3}H]-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.

microsomal GlcNAc-PI and GlcN-PI are hydrolyzed on exposing 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 preparation consists of a population of sealed vesicles (see below) and PI-PLC cannot traverse the membrane, these data indicate that GlcNAc-PI and GlcN-PI are primarily located in the external (cytoplasmic) leaflet of the microsomal vesicle membrane.

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

Table I. In Vitro Radiolabeled GPIs

Lipid	Structure	PI-PLC susceptibility	Con A binding
GlcNAc-PI	GlcNAc-PI	+	−
GlcN-PI	GlcN-PI	+	−
M1	Man_1GlcN-PI	+	−
M2	Man_2GlcN-PI	+	±
M3	Man_3GlcN-PI	+	+
M2*	Man_2GlcN-PI*	−	ND
M3*	Man_3GlcN-PI*	−	+
P2	EtN-P-Man_3GlcN-PI	+	+
P3	EtN-P-Man_3GlcN-PI*	−	+

The different lipids are biosynthetic intermediates representing partial structures and/or modified versions of the core GPI moiety: EtN-P-6Manα1-2Manα1-6Manα1-4GlcNα1-6Inositol-P-lipid (Masterson et al., 1989; Menon et al., 1990*a*). GPIs 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., 1990*a*), radioactivity in GlcNAc-PI and GlcN-PI was rapidly lost during the chase (Fig. 2 *B*, *closed squares*) as the precursors were converted into mannosylated GPI species (Fig. 2 *B*, *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. 2 *C*, *open diamonds*); changes in the amount of the PI-PLC-resistant fraction (Fig. 2 *C*, *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 mannose 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 lumenal leaflet during the chase, and is converted to M1 at about the same rate to give a constant steady-state amount of PI-PLC resistant GlcN-PI.

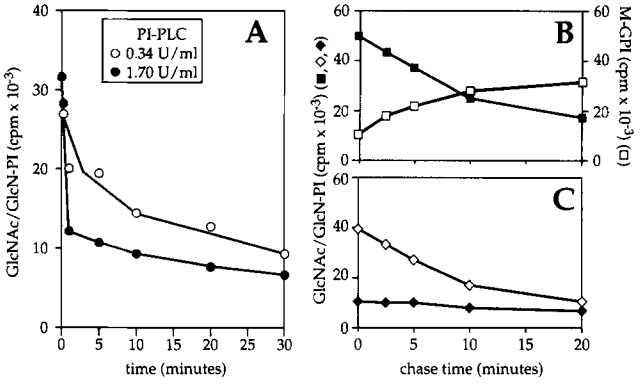

Figure 2. PI-PLC hydrolysis of GlcNAc-PI and GlcN-PI in intact microsomes. (*A*) Microsomes were labeled with UDP-[^{3}H]GlcNAc as described in Fig. 1 *C*, and then placed on ice and treated with 0.34 U/ml or 1.7 U/ml PI-PLC for different times. Lipids were extracted and taken for liquid scintillation counting and TLC analysis. (*B* and *C*) Microsomes were incubated with 1 μCi UDP-[^{3}H]GlcNAc for 10 min at 37°C in 75 μl buffer B, and then chased with non-radioactive UDP-GlcNAc. Lipids were extracted directly or after treatment with PI-PLC (0.34 U/ml, 20 min on ice). *B* indicates the total amount of radioactivity in GlcNAc-PI and GlcN-PI (*solid squares*) and in mannosylated GPIs (M-GPI, *open squares*). *C* indicates the result of PI-PLC analysis of microsomal GlcNAc-PI/GlcN-PI at each point in the chase: radioactivity in the PI-PLC accessible pool of GlcNAc/GlcN-PI is indicated by the open diamonds and residual radioactivity in GlcNAc/GlcN-PI after PI-PLC treatment is indicated by the closed diamonds.

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 lumenal 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; steric 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 steric 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 GPIs (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 GPI 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., 1990*a*). The M3 GPI species was probed in PMSF-treated microsomes (see Fig. 1 *B*); the other mannosylated GPI species were labeled at sufficiently high levels for analysis in normal microsomes (Fig. 1 *A*).

Microsomes were incubated with GDP-[^{3}H]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. 3 *A* 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. 3 *B*). The transbilayer distribution of microsomal P2 was also determined in the same experiments. Fig. 3 *B* 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-

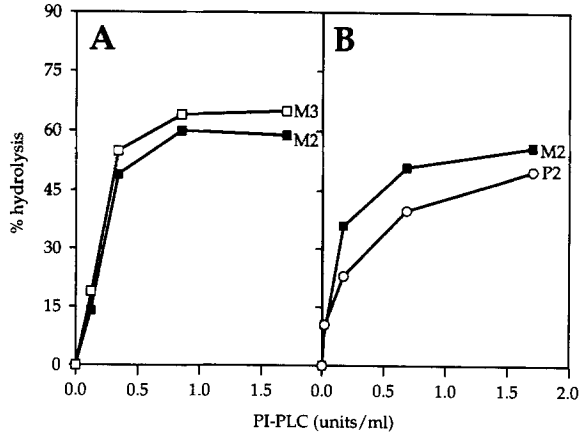

Figure 3. PI-PLC hydrolysis of in vitro labeled mannosylated GPIs in intact microsomes. Microsomes were incubated with GDP-[^{3}H]mannose in the presence (*A*) or absence (*B*) of PMSF as described in Fig. 1 *A* and *B*, and then treated with different concentrations of PI-PLC for 20 min on ice. Lipids 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 μl 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.

plete hydrolysis observed in our experiments suggests that there may be lumenal 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 lumenal ER protein, BiP, was protected from the action of exogenously added proteinase 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.

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

PI-PLC (U/ml)	Con A (mg/ml)	% lipid depleted P2	% lipid depleted M3
1.7	0	58.8	68.4
1.7	2.5	62.6	73.2
1.7	5.0	67.0	76.9
0	5.0	56.1	70.1

Microsomes (2.5×10^7 cell eq) were incubated with 0.25 μCi GDP-[^{3}H]mannose in 20 μl 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.

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,

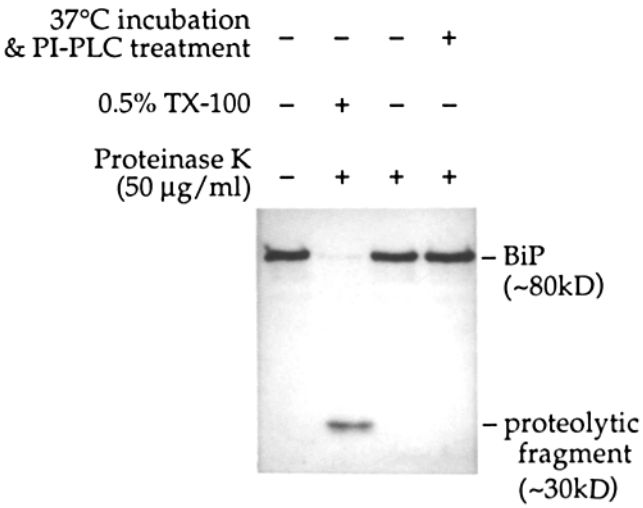

Figure 4. Proteolysis of BiP in intact and detergent-solubilized microsomes. Microsomes were treated with proteinase K (50 μg/ml) in the absence or presence of a membrane-disrupting concentration of Triton X-100. Where indicated, the microsomes were incubated at 37°C for 30 min followed by PI-PLC treatment (1.5 U/ml) for 20 min on ice (to mimic GPI radiolabeling and PI-PLC treatment procedures), before proteinase K was added. BiP was probed by Western blotting. Densitometric analysis gave the following results (in arbitrary units): (Lane *1*, control) 40420; (lane *4*, +mock incubation, +proteinase K) 48590 (120% of the control).

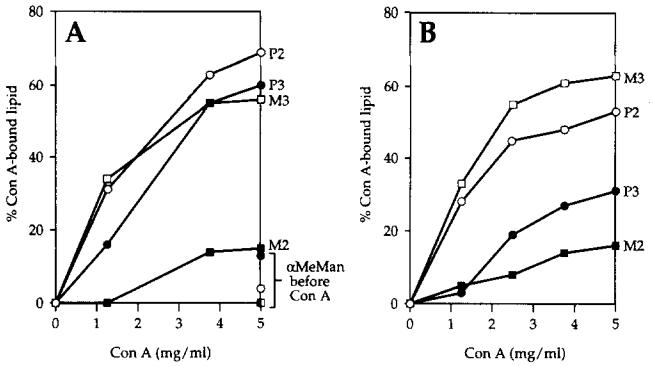

Figure 5. Con A binding to GPIs. (*A*) [^{3}H]Mannose-labeled GPIs (10,000 cpm/20 μl buffer C) were incubated with different amounts of Con A for 20 min on ice. The reaction was stopped with 5 μl 0.5 M methyl α-D-mannopyranoside (αMeMan) and GPIs were extracted as described in Materials and Methods and 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-[^{3}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.

1984). 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 (α-MeMan). 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-[^{3}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 last 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 >63% 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 carboxyl-terminal 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-[^{3}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 [^{3}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 lumenal 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 lumenal 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

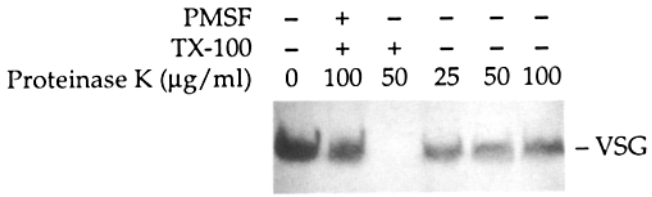

Figure 6. GPI-modification of VSG acceptors. GDP-[^{3}H]mannose-labeled membranes (10^8 cell eq) were incubated with the indicated concentrations of proteinase K for 30 min on ice. Proteolysis was stopped by adding PMSF and the samples were analyzed by SDS-PAGE on a 10% gel, followed by autoradiography of the dried gel. Densitometric analysis gave the following results (in arbitrary units): control sample (lane *2*, 100 μg/ml proteinase K in the presence of PMSF), 25595; protease-treated sample (lane *6*, 100 μg/ml proteinase K), 18530 (73% of control).

BiP protease protection assays, but retain the ability to synthesize and transfer GPI to VSG, giving rise to GPI-modified VSG that is accessible to protease digestion in the absence of detergent. We think this unlikely as BiP and other ER reticuloplasmins probably exist in two phases in the ER lumen: a mobile phase, free to diffuse in the lumenal space and a calcium-stabilized immobile matrix that is not easily removed by vesicle disruption (Booth and Koch, 1989). Indeed it is difficult to obtain quantitative release of reticuloplasmins such as protein disulfide isomerase by simply osmotically shocking the microsomes (Paver et al., 1989), and typically very little BiP is lost during membrane isolation procedures of the type we use (Bangs et al., 1993). The damaged vesicles would therefore be expected to retain some BiP and contribute a diagnostic proteolytic fragment in protease assays without detergent: no such fragment was detected (Fig. 4, lane *3*). An alternate hypothesis is that GPI transfer to protein occurs on the cytoplasmic face of the ER and that we detect a fraction of the VSG that is not translocated to the ER lumen after GPI modification. This possibility appears unlikely given current information on ER protein translocation mechanisms (Gilmore, 1993).

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 accessible to these reagents in intact microsomal vesicles (data summarized in Fig. 7). As it is difficult to assess the efficiency with which PI-PLC and Con A recognize membrane-bound substrates, the fraction of microsomal GPI detected using these probes necessarily represents a lower bound. Nevertheless the un-normalized data indicate that, with the exception of P3, all the in vitro synthesized GPIs probed are predominantly (>60%) located in the external leaflet of the microsomal vesicles (Fig. 7). The results suggest that the GPI transferases and the GlcNAc-PI de-N-acetylase have cytoplasmically oriented active sites. Indirect data in support of this proposal have been recently published (Mensa-Wilmot et al., 1994).

Bloodstream-form trypanosomes synthesize two forms of transfer-competent (phosphoethanolamine-containing) GPIs, P2, and P3, differing only in that P3 is inositol-acylated while P2 is not (Mayor et al., 1990*a*,*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., 1990*a*) 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-

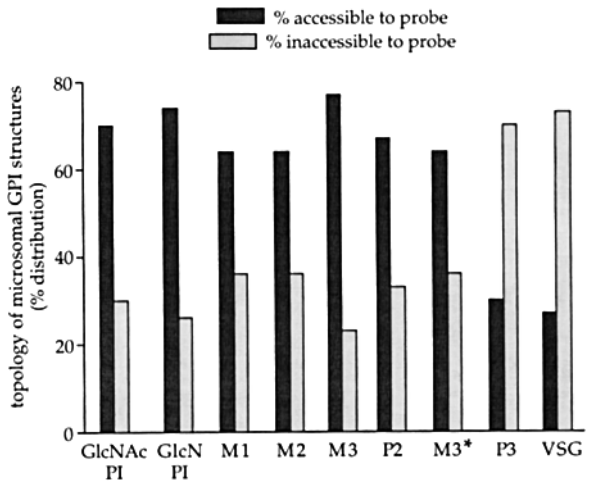

Figure 7. Orientation of GPI structures in microsomal membranes. The figure summarizes data on the accessibility of microsomal GPI structures to various topological probes. The microsomal orientation of newly GPI-modified VSG was determined by protease treatment (100 μg/ml proteinase K, 30 min on ice). The transbilayer distribution of lipid intermediates was probed with PI-PLC (1.7 U/ml, 20 min, ice) and/or Con A (5 mg/ml, 20 min, ice) as described in the text. The fraction of each lipid accessible to the probe (*dark bars*) is not normalized to probe efficiency and should therefore be taken as a lower limit of the amount of lipid present in the external (cytoplasmic) leaflet of the microsomal membrane. The % inaccessible (*light bars*) is obtained by subtraction. Variation in the data over several experiments was <10%. The data are taken from Fig. 1 *A* (GlcNAc-PI and GlcN-PI), Fig. 2 and the text (M2), Table II (P2 and M3, PI-PLC + Con A), Fig. 5 (P3), text (M1 and M3*, PI-PLC/Con A, microsomes without PMSF), and Fig. 6 (VSG).

ified P2 and P3 in organic solvents but not under milder, physiologically appropriate conditions (unpublished data). Data for another inositol acylated GPI (M3*) 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; Mensa-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 ($Man_5GlcNAc_2$-PP-dolichol) is synthesized on dolichol phosphate from sugar nucleotide donors on the cytoplasmic face of the ER, and then elongated with sugars (four mannose residues and three glucose residues) derived from dolichol-P-Man and dolichol-P-glucose donors on the lumenal 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 lumenal 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 GPIs. Our analyses of this sytem via protease protection experiments confirm that newly GPI-anchored VSG molecules are primarily lumenally 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 lumenal 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, $Man_5GlcNAc_2$-PP-dolichol is located predominantly in the cytoplasmic leaflet of the ER while $Man_6GlcNAc_2$-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 $Man_5GlcNAc_2$-PP-dolichol flips into the lumenal leaflet of the ER and is rapidly elaborated to $Man_6GlcNAc_2$-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 lumenal 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 flippase 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.

The experimental work described in this paper was carried out in The Rockefeller University's Laboratory of Molecular Parasitology, and we are grateful to the head of the laboratory, Professor George A.M. Cross for his support. We thank Adam Steinberg and Laura van der Ploeg for producing the figures, Jay Bangs for antibodies to trypanosome BiP, and Martin Low for PI-PLC. We are grateful to Claudia Abeijon, Jay Bangs, Jitu Mayor, Dave Nelson, Chris Nicchitta, and one of the Journal of Cell Biology reviewers for critical comments on the manuscript, and I. Menon, K. M. M., Ian Richardson, and Bob Dylan for stimulation.

This work was supported by National Institutes of Health grant AI28858, the Irma T. Hirschl Trust (New York), and the University of Wisconsin.

Received for publication 16 June 1994 and in revised form 27 July 1994.

References

Abeijon, C., and C. B. Hirschberg. 1992. Topography of glycosylation reactions in the endoplasmic reticulum. *Trends Biochem. Sci.* 17:32–36.

Amthauer, R., K. Kodukula, L. Brink, and S. Udenfriend. 1992. Phosphatidylinositol-glycan (PI-G)-anchored membrane proteins: requirement of ATP and GTP for translation-independent COOH-terminal processing. *Proc. Natl. Acad. Sci. USA.* 89:6124–6128.

Amthauer, R., K. Kodukula, L. Gerber, and S. Udenfriend. 1993. Evidence that the putative COOH-terminal signal transamidase involved in glycosylphosphatidyl-inositol protein synthesis is present in the endoplasmic reticu-

lum. *Proc. Natl. Acad. Sci. USA.* 90:3973–3977.
Bangs, J. D., T. L. Doering, P. T. Englund, and G. W. Hart. 1988. Biosynthesis of a variant surface glycoprotein of *Trypanosoma brucei*: processing of the glycolipid membrane anchor and N-linked oligosaccharides. *J. Biol. Chem.* 263:17697–17705.
Bangs, J. D., J. Uyetake, M. J. Brickman, A. E. Balber, and J. C. Boothroyd. 1993. Molecular cloning and cellular localization of a BiP homologue in *Trypanosoma brucei*: divergent retention signals in a lower eukaryote. *J. Cell Sci.* 105:1101–1113.
Bell, R. M., L. M. Ballas, and R. A. Coleman. 1981. Lipid topogenesis. *J. Lipid Res.* 22:391–403.
Booth, C., and G. L. E. Koch. 1989. Perturbation of cellular calcium induces secretion of luminal ER proteins. *Cell.* 59:729–737.
Brown, D. 1992. Interactions between GPI-anchored proteins and membrane lipids. *Trends Cell Biol.* 2:338–343.
Brown, D. 1993. The tyrosine kinase connection: how GPI-anchored proteins activate T cells. *Curr. Opin. Immunol.* 5:349–354.
Bülow, R., G. Griffiths, P. Webster, Y.-D. Stierhof, F. R. Opperdoes, and P. Overath. 1989. Intracellular localization of the glycosyl-phosphatidylinositol-specific phospholipase C of *Trypanosoma brucei*. *J. Cell Sci.* 93:233–240.
Cross, G. A. M. 1990. Glycolipid anchoring of plasma membrane proteins. *Annu. Rev. Cell Biol.* 6:1–39.
Doering, T. L., W. J. Masterson, P. T. Englund, and G. W. Hart. 1989. Biosynthesis of the glycosyl phosphatidylinositol membrane anchor of the trypanosome variant surface glycoprotein: origin of the nonacetylated glucosamine. *J. Biol. Chem.* 264:11168–11173.
Englund, P. T. 1993. The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annu. Rev. Biochem.* 62:121–138.
Field, M. C., and A. K. Menon. 1992. Biosynthesis of glycosyl-phosphatidylinositol membrane protein anchors. *In* Lipid Modifications of Proteins: A Practical Approach. N. M. Hooper and A. J. Turner, editors. IRL Press, Oxford. pp. 155–189.
Gilmore, R. 1993. Protein translocation across the endoplasmic reticulum: a tunnel with toll booths at entry and exit. *Cell.* 75:589–592.
Güther, M. L. S., W. J. Masterson, and M. A. J. Ferguson. 1994. The effects of phenylmethylsulphonyl fluoride on inositol acylation and fatty acid remodeling in African trypanosomes. *J. Biol. Chem.* 269:18694–18701.
Haselbeck, A., and W. Tanner. 1982. Dolichyl phosphate-mediated mannosyl transfer through liposomal membranes. *Proc. Natl. Acad. Sci. USA.* 79:1520–1524.
Higgins, J. A., B. W. Hitchin, and M. G. Low. 1989. Phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis* as a probe for the distribution of phosphatidylinositol in hepatocyte membranes. *Biochem. J.* 259: 913–916.
Hubbard, S. C., and R. J. Ivatt. 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 50:555–583.
Hutson, J. L., and J. A. Higgins. 1982. Asymmetric synthesis followed by transmembrane movement of phosphatidylethanolamine in rat liver endoplasmic reticulum. *Biochim. Biophys. Acta.* 687:247–256.
Jackson, R. C., and G. Blobel. 1977. Post-translational cleavage of presecretory proteins with an extract of rough microsomes from dog pancreas containing signal peptidase activity. *Proc. Natl. Acad. Sci. USA.* 74:5598–5602.
Kinoshita, T., and J. Takeda. 1994. GPI-anchor synthesis. *Parasitol. Today.* 10:139–143.
Krakow, J. L., T. L. Doering, W. J. Masterson, G. W. Hart, and P. T. Englund. 1989. A glycolipid from *Trypanosoma brucei* related to the variant surface glycoprotein membrane anchor. *Mol. Biochem. Parasitol.* 36: 263–270.
Lambert, N., and R. B. Freedman. 1983. The latency of rat liver microsomal protein disulphide isomerase. *Biochem. J.* 213:225–234.
Low, M. G. 1992. Phospholipases that degrade the glycosyl-phosphatidylinositol anchor of membrane proteins. *In* Lipid Modifications of Proteins: A Practical Approach, N. M. Hooper, and A. J. Turner, editors. IRL Press, Oxford. pp 117–154.
Low, M. G., and P. W. Kincade. 1985. Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. *Nature (Lond.).* 318:62–64 (also errata on page 687).
Masterson, W. J., T. L. Doering, G. W. Hart, and P. T. Englund. 1989. A novel pathway for glycan assembly: biosynthesis of the glycosyl-phosphatidylinositol anchor of the trypanosome variant surface glycoprotein. *Cell.* 56:793–800.
Masterson, W. J., and M. A. J. Ferguson. 1991. Phenylmethanesulphonyl fluoride inhibits GPI anchor biosynthesis in the African trypanosome. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:2041–2045.
Mayor, S., A. K. Menon, and G. A. M. Cross. 1990. Glycolipid precursors for the membrane anchor of *Trypanosoma brucei* variant surface glycoproteins. II. Lipid structures of phosphatidylinositol-specific phospholipase C sensitive and resistant lipids. *J. Biol. Chem.* 265:6174–6181.
Mayor, S., A. K. Menon, and G. A. M. Cross. 1991. Transfer of glycosyl-phosphatidylinositol membrane anchors to polypeptide acceptors in a cell-free system. *J. Cell Biol.* 114:61–71.
McConville, M. J., and M. A. J. Ferguson. 1993. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* 29:305–324.
Menon, A. K., R. T. Schwarz, S. Mayor, and G. A. M. Cross. 1990*a*. Cell-free synthesis of glycosyl-phosphatidylinositol precursors for the glycolipid membrane anchor of *Trypanosoma brucei* variant surface glycoproteins: structural characterization of putative biosynthetic intermediates. *J. Biol. Chem.* 265:9033–9042.
Menon, A. K., S. Mayor, and R. T. Schwarz. 1990*b*. Biosynthesis of glycosyl-phosphatidylinositol lipids in *Trypanosoma brucei*: involvement of mannosyl-phosphoryldolichol as the mannose donor. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4249–4258.
Menon, A. K., M. Eppinger, S. Mayor, and R. T. Schwarz. 1993. Biosynthesis of glycosylphosphatidylinositol anchors in *Trypanosoma brucei*: the terminal phosphoethanolamine group is derived from phosphatidylethanolamine. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1907–1914.
Mensa-Wilmot, K., J. H. LeBowitz, K.-P. Chang, A. Al-Qahtani, B. S. McGwire, S. Tucker, and J. C. Morris. 1994. A glycosylphosphatidylinositol (GPI)-negative phenotype produced in *Leishmania major* by GPI phospholipase C from *Trypanosoma brucei*: topography of two GPI pathways. *J. Cell Biol.* 124:935–947.
Nagel, S. D., and J. C. Boothroyd. 1989. The major surface antigen, P30, of *Toxoplasm gondii* is anchored by a glycolipid. *J. Biol. Chem.* 264:5569–5574.
Ogata, S., T. Muramatsu, and A. Kobata. 1975. Fractionation of glycopeptides by affinity column chromatography on Concanavalin A-sepharose. *J. Biochem.* 78:687–696.
Paver, J. L., H. C. Hawkins, and R. B. Freedman. 1989. Preparation and characterization of dog pancreas microsomal membranes specifically depleted of protein disulphide isomerase. *Biochem. J.* 257:657–663.
Perez, M., and C. B. Hirschberg. 1985. Translocation of UDP-N-acetylglucosamine into vesicles derived from rat liver rough endoplasmic reticulum and golgi apparatus. *J. Biol. Chem.* 253:7173–7178.
Roberts, W. L., J. J. Myher, A. Kuksis, M. G. Low, and T. L. Rosenberry. 1988. Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase: palmitoylation of inositol results in resistance to phosphatidylinositol-specific phospholipase C. *J. Biol. Chem.* 263:18766–18775.
Rodriguez-Boulan, E., and S. K. Powell. 1992. Polarity of epithelial and neuronal cells. *Annu. Rev. Cell Biol.* 8:395–427.
Snider, M. D., and P. W. Robbins. 1982. Transmembrane organization of protein glycosylation: mature oligosaccharide-lipid is located on the luminal side of microsomes from Chinese hamster ovary cells. *J. Biol. Chem.* 257: 6796–6801.
Snider, M. D., and O. C. Rogers. 1984. Transmembrane movement of oligosaccharide-lipids during glycoprotein synthesis. *Cell.* 36:753–761.
Stevens, V. L., and C. R. H. Raetz. 1991. Defective glycosyl phosphatidylinositol biosynthesis in extracts of three Thy-1 negative lymphoma cell mutants. *J. Biol. Chem.* 266:10039–10042.
Tanner, W., and L. Lehle. 1987. Protein glycosylation in yeast. *Biochim. Biophys. Acta.* 906:81–99.
van Meer, G. 1993. Transport and sorting of membrane lipids. *Curr. Opin. Cell Biol.* 5:661–673.
Vidugiriene, J., and A. K. Menon. 1993. Early lipid intermediates in glycosyl-phosphatidylinositol anchor assembly are synthesized in the ER and located in the cytoplasmic leaflet of the ER membrane bilayer. *J. Cell Biol.* 121: 987–996.
Zachowski, A. 1993. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem. J.* 294:1–14.