

Transfer of Glycosyl-Phosphatidylinositol Membrane Anchors to Polypeptide Acceptors in a Cell-free System

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Abstract. Glycosylinositol phospholipid (GPI) membrane anchors are the sole means of membrane attachment of a large number of cell surface proteins, including the variant surface glycoproteins (VSGs) of the parasitic protozoan, *Trypanosoma brucei*. Biosynthetic data suggest that GPI-anchored proteins are synthesized with carboxy-terminal extensions that are immediately replaced by GPI, suggesting the existence of preformed GPI species available for transfer to the na-

scient protein in the ER. Candidate precursor glycolipids having a linear sequence indistinguishable from the conserved core structure found on all GPI anchors, have been characterized in *T. brucei*. In this paper we describe the transfer of three GPI variants to endogenous VSG in vitro. GPI addition is not reduced by inhibitors of protein synthesis and does not require ATP or GTP, consistent with a transpeptidation mechanism.

GLYCOSYLINOSITOL phospholipid (GPI)¹ membrane anchors attach many proteins of varied function to eukaryotic cell membranes, almost always to the cell surface (for review see Ferguson and Williams, 1988; Low, 1989; Cross, 1990; Thomas et al., 1990). It appears that GPI membrane anchors share an evolutionarily conserved linear core structure, consisting of ethanolamine-P-6 man α 1-2 man α 1-6 man α 1-4 GlcN α 1-6 inositol. This linear sequence links the phospholipid moiety to the protein via an amide bond between ethanolamine and the carboxy-terminal amino acid. Different anchors have both protein and cell-specific side chain modifications and differ in phospholipid structure. Side chain modifications include carbohydrate (galactose, mannose, and GalNAc), extra ethanolamine phosphate, and an extra fatty acid attached to an inositol hydroxyl. Acylation of inositol confers resistance to PI-PLC, an enzyme often used to diagnose GPI anchoring (Roberts et al., 1988). The various GPI lipid moieties described include *sn*-1,2-dimyristylglycerol (*T. brucei* VSG; Ferguson et al., 1985), 1-alkyl-2-acyl glycerol (human erythrocyte acetylcholinesterase; Roberts et al., 1988 and *Leishmania major* promastigote surface protease; Schneider et al., 1990), monoacylglycerol (*T. brucei* procyclic acidic repetitive protein; Field et al., 1991), and ceramide (*Dictyostelium discoideum* contact site A glycoprotein; Sadeghi et al., 1988; Stadler et al., 1989). The functional significance of these structural variations is unclear.

Mature GPI-anchored proteins lack a carboxy-terminal peptide that is encoded in the cDNA sequence. Putative precursor proteins that contain this extension have been ob-

served for neural cell adhesion molecule, N-CAM 120 (He et al., 1987), and alkaline phosphatase (Takami et al., 1988). Biosynthetic studies suggest that the carboxy-terminal sequence is replaced within 1–5 min of completion of protein synthesis (Bangs et al., 1985; Ferguson et al., 1986; Conzelmann et al., 1987; He et al., 1987). The rapidity of this modification suggested the existence of a preformed precursor glycolipid that could be transferred en bloc to the newly synthesized protein concomitant with or after cleavage of the carboxy-terminal peptide extension. Recent studies using a yeast secretory mutant (*sec 18*) support an endoplasmic reticulum location for the GPI addition reaction (Conzelmann et al., 1988).

The abundance of VSG in *T. brucei* makes this protozoan parasite a good model for the study of GPI-anchor structure and biosynthesis. Two glycolipids, P2 and P3 (Menon et al., 1988a [presumably identical to lipids A and C; Krakow et al., 1986]), with characteristics expected of GPI anchor precursors have been identified in this parasite. P2 is PI-PLC sensitive and consists of a linear sequence identical to the conserved core sequence found on all GPI anchors (Menon et al., 1988a; Mayor et al., 1990b). P3 is PI-PLC resistant due to the acylation of the inositol hydroxyl by a fatty acid but is otherwise identical to P2 (Krakow et al., 1989; Mayor et al., 1990a).

The biosynthesis of these glycolipids has been studied in vivo (Schwarz et al., 1988) and in vitro (Menon et al., 1988b; Masterson et al., 1990; Doering et al., 1989; Masterson et al., 1989; Menon et al., 1990b). The simplest model (summarized in Fig. 1) suggested by the available data is that the lipids are assembled by the sequential glycosylation of PI followed by the addition of phosphoethanolamine. A peculiar feature of trypanosome GPI biosynthesis is the observation of heterogeneously acylated forms of P2 and P3,

1. **Abbreviations used in this paper:** GPI, glycosyl-phosphatidylinositol; Inos, inositol; pCMPSA, sodium *p*-chloromercuriphenylsulfonate; PLC, phospholipase C; VSG, variant surface glycoprotein.

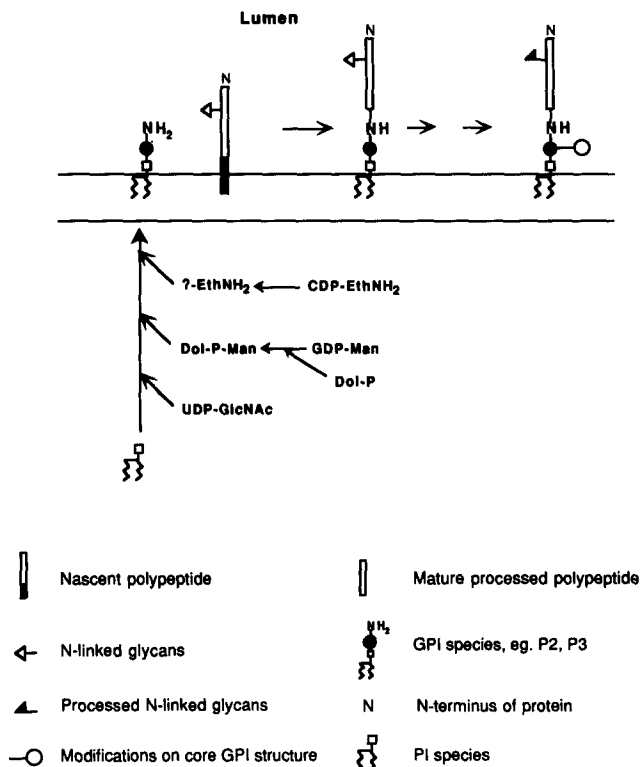


Figure 1. Model for GPI synthesis and transfer to protein. Glycosylphosphatidylinositols (see Table I for complete structures) that have properties characteristic of preformed precursors of GPI membrane anchors are proposed to be synthesized from PI species via appropriate, activated donor molecules, UDP-GlcNAc, (Doering et al., 1989), Dol-P-Man, (Menon et al., 1990a) and possibly phosphatidylethanolamine (γ -EtnNH₂; Menon, A. K., S. Mayor, and R. T. Schwarz, manuscript in preparation). The topography of these biosynthetic reactions is not known but completed GPIs are expected to be located, with the terminal ethanolamine facing the lumen of the ER (Lumen), available for transfer to newly synthesized protein acceptors containing the GPI addition signal. After GPI addition the protein and GPI anchor may be processed in the ER and/or during transport along the secretory pathway.

which are then converted to dimyristyl species via a series of fatty acid remodeling reactions (Masterson et al., 1990). The origin and function of the extra fatty acid on the inositol of P3 and other biosynthetic intermediates is unknown. Data consistent with the assembly of the free GPI species in the lumen of the ER have been presented (Menon et al., 1990a).

Besides their remarkable structural similarities with GPI anchors, there is no direct evidence that these free GPI species are transferred to protein. Pulse-chase experiments have proved inadequate in establishing their role in GPI anchoring because these lipids turn over rapidly in the presence or absence of protein synthesis inhibitors (Menon et al., 1988a; unpublished observations). Cell free systems to study GPI addition to *in vitro* translated polypeptides containing the carboxy-terminal extension have been described. In one set of experiments, using CHO cell membranes, placental alkaline phosphatase and site-directed mutants were selectively processed to cleaved forms lacking the carboxy-terminal hydrophobic sequence (Bailey et al., 1989; Micanovic et al., 1990). In a separate report, Thy-1 and decay accelerating fac-

tor were processed to PI-PLC sensitive forms (Fasel et al., 1989) in the presence of dog pancreas membranes. However, direct evidence for the transfer of GPI components to protein was not demonstrated in either of these systems.

In this paper, we report experiments demonstrating the transfer of radiolabeled GPI precursor molecules to VSG in a cell free system from *T. brucei*, providing direct evidence that the free GPI molecules are bona fide precursors of the membrane anchors of GPI-linked proteins. We also describe some properties of the GPI addition reaction.

Materials and Methods

Materials

Guanosine diphospho-[3,4-³H]mannose (20 Ci/mmol) and uridine diphospho-*N*-acetyl-D-[6-³H]glucosamine (26.8 Ci/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA). GDP-Man, UDP-GlcNAc, ATP, GTP, β - γ -imidoadenosine-5'-triphosphate, β - γ -methyleneadenosine-5'-triphosphate, β - γ -imidoguanosine-5'-triphosphate and guanosine 5'-*O*-(3-thiotriphosphate) were purchased from Sigma Chemical Co. (St. Louis, MO). Protein synthesis inhibitors (cycloheximide, puromycin, and anisomycin), protease inhibitors (TLCK, PMSF, leupeptin, aprotinin) and Coenzyme A were also purchased from Sigma. Endo- β -*N*-acetylglucosaminidase H, creatine phosphate, rabbit muscle creatine kinase, and proteinase K were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tunicamycin was purchased from Calbiochem-Behring Corp. (La Jolla, CA). PI-specific phospholipase C from *Bacillus thuringiensis* was a gift from Martin Low (Columbia University). Neutral glycan standards derived from glycolipid anchors of VSGs 117 and 118 and glucose oligomer standards were prepared as described elsewhere (Mayor et al., 1990b). Antibodies to VSGs 117 and 118 were raised against purified sVSG as previously described (Cross, 1979). 118 antiserum was used directly as a source of antibodies for 118 VSG and anti-117 antibodies were purified by affinity chromatography over sVSG 117 coupled to Sepharose as previously described (Ferguson et al., 1986). All other chemicals were of reagent grade or the highest purity obtainable unless otherwise mentioned.

Preparation of the Cell-free System

Rats were inoculated with trypanosome variants of the Molteno Institute Trypanozoon antigenic type 1.4 or 1.5 (clones 117 or 118, respectively) of *T. brucei* strain 427. "Buffy-coat" trypanosomes were purified by centrifugation and used directly, or after passage through a DEAE column as previously described (Cross, 1975). Trypanosomes were incubated with or without tunicamycin (400 ng/ml) at 1×10^8 cells/ml in RPMI medium for 30–60 min as previously described (Mayor et al., 1990a). The cells were pelleted by centrifugation, lysed, and frozen as described (Masterson et al., 1989). 1 ml of the frozen lysate (5×10^8 cell equivalents) was thawed at room temperature, transferred to 9 ml of ice-cold buffer 1 (25 mM K-Hepes pH 7.4, 75 mM KAc, 5 mM MgAc₂, 1 mM TLCK, 1 μ g/ml leupeptin, 1 or 2 mM DTT), and centrifuged at 10,000 g at 4°C. The pelleted membranes were washed twice in the same buffer and finally resuspended in ice-cold buffer 2 (buffer 1 containing 10 mM MnAc₂). The membranes were resuspended in a Dounce homogenizer equipped with a tight fitting pestle (10 strokes) and kept on ice until required.

Labeling of GPI Species and Endogenous Acceptors *In Vitro* via Sugar Nucleotides

A standard reaction contained 5×10^7 cell equivalents of dispersed membranes in a total volume of 100 μ l of buffer 3 (buffer 2 containing 1 mM CoA, 200 ng/ml tunicamycin, 1 mM ATP, 10 mM creatine phosphate, 8 U/ml creatine kinase). Radiolabeled precursors (1–2 μ Ci of GDP-[³H]Man or UDP-[³H]GlcNAc) were added to initiate the reaction and samples were incubated at 37°C in a water bath. In incubations with UDP-[³H]GlcNAc, 1 mM GDP-Man was included in the reaction, whereas 1 mM UDP-GlcNAc was added to incubations where the radiolabel used was GDP-[³H]Man. In some GDP-[³H]Man labeling experiments the reaction mixture was preincubated (10–15 min at 37°C) in the absence of the radiolabel but with 0.2 μ M GDP-Man. The labeling reaction was initiated by the addition of the

1–2 μCi of GDP-[^3H]Man followed by UDP-GlcNAc (1 mM final concentration). After the incubation an aliquot ($\sim 50\%$) of the reaction was transferred to an equal volume of solubilization buffer (0.375 M Tris-HCl, pH 6.8, 4% SDS) at 100°C and boiled for 5 min before being subjected to SDS-PAGE and fluorography. The remainder of the reaction mixture was analyzed for GPI synthesis as described (Masterson et al., 1989) except that TLC of the glycolipids was performed with Kieselgel-60 thin-layer plates (Merck, Darmstadt, FRG) using a modified solvent system, chloroform/methanol/water (4:4:1; vol/vol/vol) and the radioactivity in each lane detected as described previously (Mayor et al., 1990a).

SDS-PAGE and Quantitation of Radioactivity Incorporated into Protein

An aliquot of the solubilized reaction mixture was boiled (3 min, 100°C) with 0.2 vol of glycerol/DTT (50% glycerol, 0.5 M DTT, 0.25% bromophenol blue) and loaded on 16-cm 7.5% SDS polyacrylamide gels (Laemmli, 1970). The stacking gel was 4% acrylamide. The gels were run for 10–12 h, stained with Coomassie brilliant blue, impregnated with EN 3 HANCE (Du Pont-NEN) and exposed to preflashed Kodak XAR-5 film at -70°C . Molecular weight markers ("pre-stained" markers and SDS-VII) were from Sigma Chemical Co. To quantitate the radioactivity transferred to protein, the labeled bands were located on the gel and excised. The gel slices were dissolved in 80% Protosol (DuPont-NEN) at room temperature for 14 h, neutralized with glacial acetic acid, mixed with scintillation fluid, and taken for liquid scintillation counting (Packard Instrument Co., Downers Grove, IL).

Purification of Radiolabeled GPI Molecules for Use as Substrates for the Transfer Reaction

GPI species were labeled via GDP-[^3H]Man as described above and purified by TLC (Mayor et al., 1990a) or as described below. The purified lipids were rechromatographed and determined to be $>90\%$ radiochemically pure. (P2 and P3 samples were $>99\%$ pure [Mayor et al., 1990a,b], whereas impurities in P2' and P2'' were mainly P2'' and P2', respectively.) For some experiments glycolipids were purified by column chromatography (1.5 \times 25 cm; Iatrobeads, Iatron Laboratories, Japan) following the procedure described by Masterson et al. (1989) or by phase separation as described below. The radiolabeled glycolipids extracted into the butanol phase from an *in vitro* reaction were vacuum-dried in a Speed Vac (Savant Instruments, Hicksville, NY) and redissolved in 500 μl chloroform/methanol (CM; 2:1). 100 μl of 4 mM MgCl_2 solution was added to create two phases. The methanol-rich upper phase was separated and washed with an equal volume of artificial lower phase and the chloroform-rich lower phase was washed with an equal volume of artificial upper phase. The upper methanol-rich phase contains P2, P2' and P2'' ($>95\%$) while the lower chloroform-rich phase contains P3 and the less polar GPI species as well as dol-P-man. The upper methanol-rich phase was occasionally used without further purification as a substrate for the transfer reaction. P3 was purified (purity $>99\%$) from the lower phase either by thin layer or column chromatography before use as a substrate in the transfer reaction.

Transfer of Purified GPI Species to Endogenous Acceptors in the Cell-free System

Purified GPI species were dried *in vacuo* in conical centrifuge tubes. The lipids were resuspended in buffer 2 containing 10 times the final concentration (wt/vol) of Triton X-100. This suspension was sonicated for 10 min (bath sonicator) or gently vortexed to disperse the glycolipid and the membrane preparation (9 vol; 5×10^7 cell equivalents in buffer 3) was added to this dispersion. The tubes containing the reaction mixture in a final volume of 50 or 100 μl were transferred to 37°C and the reaction processed as above except that a larger aliquot (90%) of the reaction mixture was taken for SDS-PAGE analyses. In all instances the intact GPI species that were recovered at the end of the reaction were unchanged as assessed by TLC.

Enzymatic Treatments of the Labeled Polypeptides

Solubilized reaction mixtures from labeling reactions were pooled. The resulting pooled lysate was divided into two aliquots and precipitated with 10 vol of ice cold acetone at -20°C for 3 h and centrifuged at 4°C at 13,000 *g* for 15 min. The protein precipitate was recovered after being washed once with ice-cold acetone. The precipitates were solubilized in 100 μl PI-PLC

buffer (0.1 M Tris-HCl, pH 7.4, 0.1% sodium deoxycholate) and incubated at 37°C without or with 1 μg of *B. thuringiensis* PI-PLC for 3 h. Control incubations were performed with similarly treated [^{35}S]methionine-labeled mFVSG (Ferguson et al., 1986). At the end of the incubation the reactions were reprecipitated with acetone and the precipitates were analyzed by SDS-PAGE and fluorography. Analyses of PI-PLC treated VSG species by Triton X-114 detergent phase separation were carried out as described (Conzelmann et al., 1988). Endo H digestions of polypeptides labeled via GDP-[^3H]Man *in vitro* were carried out on solubilized reaction mixtures. 10 μl of 0.2 M DTT in 0.1 M Na-PO $_4$, pH 5.5 buffer was added to each of two aliquots of 10 μl of the solubilized mixture and boiled for 3 min. 170 μl of 0.1 M Na-PO $_4$, pH 5.5 containing protease inhibitors (2 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ aprotinin, 0.1 M PMSF) and 10 μg of BSA was added to each aliquot and incubated with (10 mU) or without enzyme at 37°C for 14–16 h, precipitated with ice-cold acetone, and analyzed by SDS-PAGE and fluorography.

Immunoprecipitation of the Labeled Polypeptides

Labeled polypeptides were directly immunoprecipitated from the solubilized labeling reactions. 30- μl aliquots of the solubilized reaction were added to 300 μl of TEN buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% NP-40 [wt/vol]) containing 10 μl of the specific purified antibody (1.0 mg/ml) or 20 μl of the relevant antisera and protease inhibitors. This solution was incubated for 1 h at room temperature, 100 μl of Protein A Sepharose CL-4 beads (Pharmacia Fine Chemicals, Piscataway, NJ) was added to each tube, and the samples were incubated for an additional 12 h at 4°C . The beads were pelleted and washed once with 1 ml of TEN buffer containing 0.6 M NaCl and 3 mg/ml BSA followed by 1 ml of TEN buffer containing 0.6 M NaCl and twice with 1 ml of TEN. The washed pellet was boiled with solubilization buffer containing 0.2 vol glycerol/DTT (50% glycerol, 0.5 M dithiothreitol, 0.25% bromophenol blue), centrifuged, and the supernatants were analyzed by SDS-PAGE followed by fluorography.

Characterization of the Labeled Moiety Transferred to Endogenous Acceptor

The solubilized lysates of two GDP-[^3H]Man labeling reactions were pooled and chromatographed on SDS-PAGE. The gels were stained with Coomassie brilliant blue and polypeptides migrating in the 48–58-kD molecular weight range were excised from the gel and electroeluted as described (Hunkapillar et al., 1983). The electroeluted protein was recovered in 300 μl of 0.02% SDS in 0.01 M NH_4HCO_3 . An aliquot (50 μl) was taken for analysis by SDS-PAGE and fluorography and the remaining was dried in a Speed-Vac. The dried residue was treated with ice-cold aqueous HF (50%) and the aqueous soluble radioactivity was deaminated and reduced to obtain a neutral glycan. The radiolabeled glycan was mixed with neutral glycan standards and analyzed by anion exchange HPLC (Mayor et al., 1990b).

Protease Protection

1.9×10^8 cell equivalents of the membrane preparation were incubated with GDP-[^3H]Man under standard conditions in a final volume of 275 μl for 90 min and then transferred to an ice-water mixture. CaCl_2 was added to a final concentration of 2.5 mM and the mixture divided into 20- μl aliquots. Proteinase K (20 U/mg) was added to each aliquot in the absence or presence of 1% Triton X-100. After incubation (final volume 30 μl) at 0°C for 45 min PMSF was added to a final concentration of 2 mM. An equal volume of boiling solubilization buffer was added to each tube and the solubilized mixture analyzed by SDS-PAGE and fluorography.

Protein Synthesis

Membranes ($2.5\text{--}5 \times 10^7$ cell equivalents) were incubated at 37°C for 60 min in buffer 3 containing 0.2–1 mM GTP along with [^{35}S]methionine (0.5 mCi/ml), a mixture of 19 unlabeled amino acids excluding methionine (at 30 μM each) and 1 mM GDP-Man. *De novo* protein synthesis in the *in vitro* system was measured by [^{35}S]methionine incorporation into protein species. Labeled samples were analyzed by SDS-PAGE and fluorography and quantitated by TCA precipitation and liquid scintillation counting as described elsewhere (Ferguson et al., 1986). The effect of protein synthesis inhibitors on GPI addition *in vitro* was determined by preincubating the membranes with protein synthesis inhibitors for 10 min at 0°C . In experiments involving the incorporation of GDP-[^3H]Man into GPI species GTP (1 mM final concentration) was added 15 min after the addition of the radiolabel as GTP appears to inhibit GDP-[^3H]Man incorporation into GPI species.

Results

Synthesis of GPIs *In Vitro* and Transfer to Endogenous Protein Acceptors

Several recent papers have described the cell free synthesis of GPI species using crude preparations of trypanosome membranes (Masterson et al., 1989; Menon et al., 1990*b*). Trypanosome membranes are also capable of synthesizing dolichol-linked oligosaccharides (Menon et al., 1990*b*) and transferring them to peptide substrates and endogenous protein acceptors (Ip, H. S., and A. K. Menon, unpublished observations), similar to the activities present in mammalian and yeast cell free systems (Parodi et al., 1972; Turco et al., 1977; Hart et al., 1979). To investigate specifically GPI biosynthesis and transfer and avoid complications in the protein labeling patterns due to N-glycosylation *in vitro*, membranes were prepared from cells treated with tunicamycin, a specific inhibitor of the first step in the synthesis of dolichol-linked oligosaccharides (Schwarz and Datema, 1982). Tunicamycin was also included in the *in vitro* assay to prevent *de novo* synthesis of dolichol-linked oligosaccharides. When membranes prepared in this way were incubated with UDP-[³H]GlcNAc, no incorporation of radioactivity into dolichol-linked oligosaccharide was observed (data not shown). However, synthesis of GlcN-PI and its mannosylated derivatives was not inhibited (data not shown and Doering et al., 1989). In all the experiments described below, unless otherwise mentioned, membranes were prepared from tunicamycin-treated trypanosomes (variant clone 117) and incubated in the presence of tunicamycin.

When membranes were incubated with GDP-[³H]Man in the absence of added UDP-GlcNAc, only dol-P-[³H]Man (Menon et al., 1990*a,b*) was synthesized in significant amounts (Fig. 2 *A, a*). The insignificant incorporation of radioactivity into GPI species and into high-mannose dolichol-linked oligosaccharides (Man₆₋₉GlcNAc₂-P-P-dol), under conditions where dol-P-[³H]Man was synthesized in an unimpaired fashion, indicated that the membranes were effectively depleted of precursors of the N-glycosylation pathway and of GlcN-PI (in many experiments, to ensure that the cell-free system was completely depleted of precursors of the mannose-containing dolichol-linked oligosaccharides that are synthesized via dol-P-Man [Chapman et al., 1980], the membranes were preincubated with low concentrations of GDP-Man [0.2 μM] for 5 min before labeling with GDP-[³H]Man). Incubation of membranes in the presence of UDP-GlcNAc resulted in the synthesis of a spectrum of GPIs, including P2, P3 and the fatty acid remodeling intermediates P2' and P2'' (Fig. 2 *A, b*; see Table I for structures).

To detect transfer of *in vitro* synthesized [³H]Man-labeled GPIs to endogenous acceptor polypeptides, the cell free system was incubated with GDP-[³H]Man as described above and the labeled proteins were analyzed by SDS-PAGE. No radiolabel was incorporated into any protein species if UDP-GlcNAc was omitted from the reaction (Fig. 2 *B, lane a*). In the presence of 1 mM UDP-GlcNAc, radioactivity was incorporated into protein species migrating in the molecular weight range (48–58 kD) expected for the differently glycosylated forms of membrane-form VSG (mfVSG) (Fig. 2 *B, lane b*).

Fig. 2 *C* shows the time course of incorporation of [³H]-

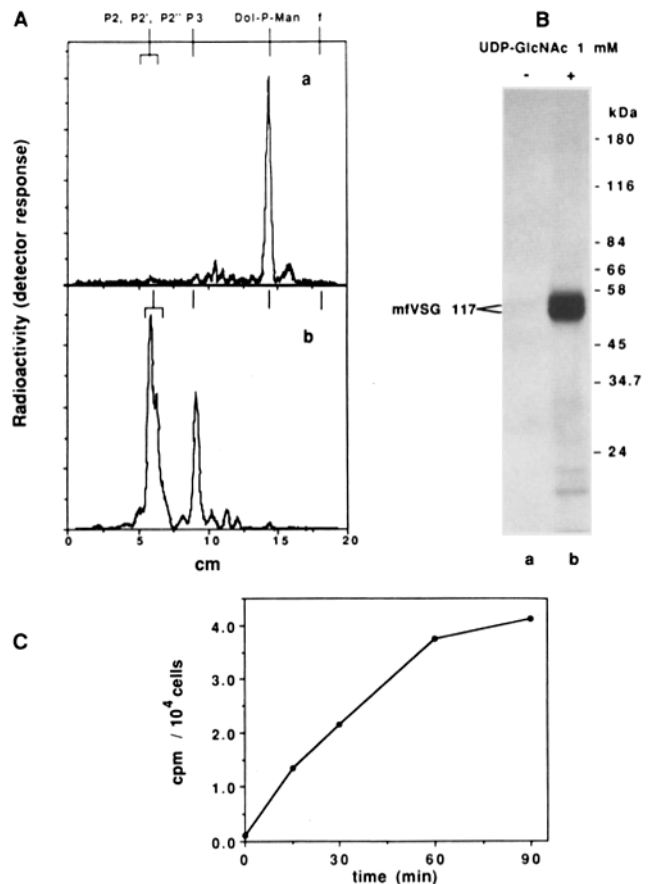


Figure 2. Labeling of GPI species and endogenous proteins *in vitro*. Trypanosome membranes (5×10^7 cell equivalents/reaction) from variant clone 117 were preincubated at 37°C in buffer 3 (Materials and Methods) for 5 min in the presence of $0.2 \mu\text{M}$ GDP-Man. The labeling reaction was initiated by adding $2 \mu\text{Ci}$ of GDP-[³H]Man ($1.4 \mu\text{M}$) and the incubation continued for an additional 15 min. UDP-GlcNAc (final concentration 1 mM) was then added to some of the tubes to initiate GPI synthesis and all the tubes were incubated for 90 min and processed as described (Materials and Methods). (*A*) TLC scans of aliquots of the organic extract containing glycolipids labeled via GDP-[³H]Man in the absence (*a*; 1.25×10^7 cell equivalents) or presence (*b*; 2.5×10^6 cell equivalents) of UDP-GlcNAc. P2, P2', P2'', and P3 are GPI molecules (see Table I), Dol-P-Man is dolichol-phosphomannose, the peaks chromatographing between P3 and Dol-P-Man are putative biosynthetic intermediates in the synthesis of P2 and P3 (Masterson et al., 1989, 1990; Menon et al., 1990*a*). Dol-PP-oligosaccharides (dol-PP-GlcNAc₂Man₆₋₉) chromatograph between P3 and the origin. *f*, front. (*B*) SDS-PAGE and fluorographic analyses of 8×10^6 cell equivalents of SDS solubilized reactions containing membranes prepared from tunicamycin-treated variant clone 117 and incubated in the presence or absence of 1 mM UDP-GlcNAc as indicated. (*C*) Time course of radioactivity incorporated into protein migrating in the region of 48–58-kD in SDS-PAGE analyses of analogous *in vitro* incubations as described above, except that the membranes were not preincubated with $0.2 \mu\text{M}$ GDP-Man prior to the addition of the radiolabel and UDP-GlcNAc. Radioactivity was quantitated as described in the text.

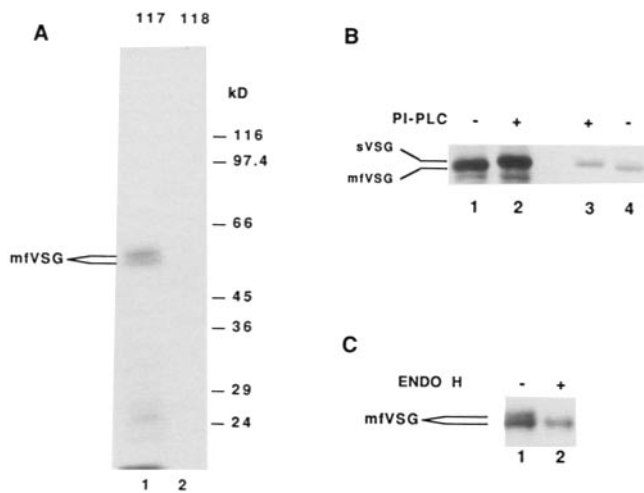


Figure 3. Characteristics of the labeled polypeptides. (A) Immunoprecipitation of 0.8×10^7 cell equivalents of a solubilized in vitro labeling reaction as described in Fig. 2 B with antibodies to VSG 117 (lane 1) and antiserum to VSG 118 (lane 2). (B) SDS-PAGE analyses of lysates from tunicamycin-treated 117 trypanosomes labeled with [35 S]methionine (lanes 1 and 2; 2×10^6 cell equivalents; as described in Ferguson et al. (1986), and from in vitro reactions (lane 3 and 4; 2×10^7 cell equivalents) as described in Fig. 2 B, which were incubated in the presence (lanes 2 and 3) or absence (lanes 1 and 4) of *B. thuringiensis*, PI-PLC. (C) SDS-PAGE analyses of protein species labeled in vitro as described in Fig. 2 B (lanes 1 and 2; 8×10^6 cell equivalents) and incubated in the presence (lane 2) or absence (lane 1) of Endo H.

Man into endogenous protein acceptors. The incorporation of radiolabel into protein ceased after ~ 60 min while GPI synthesis continued (data not shown).

Properties of the Labeled Polypeptide

The molecular weights of the in vitro labeled polypeptides suggested that they were glycosylated and unglycosylated mfVSG (variant 117, Fig. 2 B). To confirm their identity, it was shown that both the radiolabeled bands from a trypanosome 117 membrane incubation could be quantitatively precipitated with rabbit anti-VSG 117 antibodies but not with antiserum to a different VSG (Fig. 3 A).

Table I. Structures of Ethanolamine-containing GPIs in *T. brucei*

GPI species	Structure of GPI*
P2	E-P-Man ₃ GlcNInos-P-1, 2-dimyristylglycerol
P2'	E-P-Man ₃ GlcNInos-P-1-stearyl-2-acyl [‡] glycerol
P2''	E-P-Man ₃ GlcNInos-P-1, 2-diacyl [‡] glycerol
P3	E-P-Man ₃ GlcN acyl [§] Inos-P-1, 2-dimyristylglycerol
PP1	E-P-Man ₃ GlcN acyl [§] Inos-P-1-monoacyl glycerol

* The structures of GPIs are taken from the following references: P2 and P3, Menon et al., 1988 and Mayor et al., 1990a,b (presumably identical to lipids A and C, Krakow et al., 1986; Krakow et al., 1990). P2' and P2'' (presumably identical to lipids A'' and A', respectively), Masterson et al., 1990. PP1, Field et al., 1991.

[‡] Fatty acids other than myristic acid.

[§] The inositol residue is esterified with palmitic acid as determined by biosynthetic labeling (Mayor et al., 1990a; Field et al., 1991).

^{||} The glycerol fatty acid is stearic acid as determined by biosynthetic labeling (Field et al., 1991).

E, ethanolamine; Inos, inositol; P, phosphate.

When solubilized cell free reactions were incubated with *Bacillus thuringiensis* PI-PLC, the in vitro [3 H]Man-labeled VSGs displayed a mobility shift (Fig. 3 B), consistent with the labeled VSG species having PI-PLC-susceptible GPI anchors (Ferguson et al., 1986). Both the labeled VSG species partitioned into the detergent phase when analyzed by TX-114 detergent phase separation (Bordier, 1981). After PI-PLC treatment, the VSG species were recovered in the aqueous phase (data not shown).

Mature mfVSG 117 is completely susceptible to Endo H digestion resulting in a product that is indistinguishable by SDS PAGE analyses from non *N*-glycosylated mfVSG obtained from tunicamycin-treated trypanosomes (data not shown). Endo H digestion of the two in vitro labeled VSG species generated one band corresponding to the faster migrating species that cochromatographed with mfVSG 117 from tunicamycin treated trypanosomes (Fig. 3 C). These results confirm that the slower migrating species corresponds to fully glycosylated mfVSG and the faster migrating species corresponds to unglycosylated mfVSG, suggesting that both glycosylated and unglycosylated forms of the acceptor VSG polypeptide are present in this membrane preparation.

To characterize the radiolabeled moiety transferred to VSG in vitro, [3 H]Man-labeled VSG was purified by electroelution from SDS gels. Re-analysis of an aliquot of the purified material showed that all the radiolabel chromatographed as two bands comigrating with glycosylated and unglycosylated mfVSG (data not shown). The remainder of the purified VSG was subjected to glycan analysis (Mayor et al.,

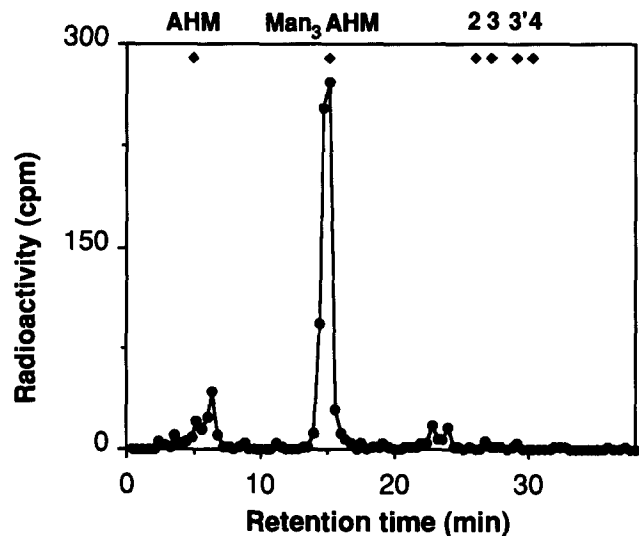


Figure 4. HPLC analysis of the radiolabeled moiety transferred to endogenous VSG acceptors. Polypeptides labeled in vitro from 2.5×10^7 cell equivalents were gel-purified and the recovered radioactivity (3,000 cpm) was dephosphorylated, deaminated, and reduced. The resultant material was deionized, and chromatographed on a high pressure anion-exchange HPLC system (Dionex) along with neutral glycan standards as described previously (Mayor et al., 1990b). The symbols (\diamond) at the top of the chromatogram indicate the retention time of neutral glycan standards derived from the membrane anchor of purified VSGs, i.e., Man₃(2,5-anhydromannitol) (Man₃AHM) from nongalactosylated 118 VSG and Gal_nMan₃AHM, where $n = 2, 3, 4$ (indicated as 2, 3, 3', and 4) from heterogeneously galactosylated VSG 117 (Mayor et al., 1990b). AHM, 2,5-anhydromannitol.

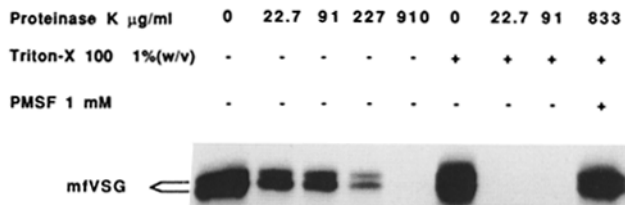


Figure 5. Protease protection of in vitro labeled VSG. Equal aliquots of GDP- ^3H Man-labeled membranes (1.3×10^7 cell equivalents) were incubated (see Materials and Methods) with indicated concentrations of PMSF, proteinase K, and Triton-X (1% wt/vol final concentration) in a final volume of $30 \mu\text{l}$, at 0°C for 45 min. Then PMSF (2 mM, final concentration) was added to each reaction and all the samples were analyzed by SDS-PAGE and fluorography as described. The regions of the gel corresponding to the migration position of mfVSG 117 species (shown above) were excised and the radioactivity in each gel slice was quantitated. Radioactivity in lane 1 is 4,760 cpm and in lane 9 (control; protease treatment in the presence of PMSF) is 3,475 cpm.

1990b). The sample was treated with aqueous HF (a procedure expected to release a glycan in phosphodiester linkage) and the released aqueous-soluble glycan was deaminated and reduced. Greater than 95% of the radioactivity associated with the labeled VSG species was recovered from this procedure as a neutral species. Analysis of this material by anion-exchange HPLC (Mayor et al., 1990b) revealed a single peak with a retention time of 13.3 min (Fig. 4). This peak co-chromatographs with a glycan moiety, $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4(2,5\text{-anhydromannitol})$, that is derived by an identical procedure from the non-galactosylated GPI anchor of VSG 118 as well as the glycolipids P2 and P3 (Mayor et al., 1990b). This result confirms that a glycan, corresponding to the core glycan of a non-galactosylated GPI anchor, is transferred to endogenous protein acceptors in this in vitro system and provides further evidence that the ^3H Man-labeling of the VSG species is confined exclusively to its GPI membrane anchor.

To address the possibility that the GPI addition reaction observed in vitro could be the result of an exchange reaction, a pulse-chase experiment was performed. The inability of excess unlabeled GDP-Man (1 mM) to chase out the radioactivity already incorporated into VSG during a 30-min GDP- ^3H Man (1 μM) labeling pulse suggests that an exchange reaction where newly synthesized GPIs are exchanged into GPI anchors of preexisting GPI-anchored VSG is not involved in GPI addition to VSG in vitro. In a control experiment, the membrane preparation was shown to be capable of synthesizing and transferring GPIs to the endogenous VSG acceptors during the "chase period": after a 30-min preincubation in the absence of GDP-Man, addition of GDP- ^3H Man for 30 min resulted in equally efficient radiolabeling of acceptor polypeptides.

Protease protection experiments were performed to determine the location of in vitro-labeled VSG (Fig. 5). Coomassie staining of SDS-PAGE gels of protease treated membranes showed no stained bands in the molecular weight range expected for VSG, whereas the most prominent stained protein in the untreated membranes corresponded to VSG (data not shown). At 22.7, 91, and 227 $\mu\text{g/ml}$ protease concentration, 22, 24, and 11% of the labeled polypeptides, respectively, were protected from proteolytic degradation. A small but significant portion (3%) of the labeled protein bands was

resistant to digestion by the protease even at the highest protease concentration (910 $\mu\text{g/ml}$) used. In the presence of detergent (1% wt/vol Triton X-100), even at the lowest concentration of protease (22.7 $\mu\text{g/ml}$), there was no detectable protease protection of the labeled polypeptide species. The protease could be inactivated by including 1 mM PMSF in the incubation (Fig. 5, lane 9). The data indicate that some of the in vitro labeled (GPI-anchored) VSG molecules reside in sealed compartments in this cell-free system, and the amount of protease-protected VSG constitutes a very small portion of the bulk (Coomassie staining) VSG population.

Transfer of Purified P2 to Endogenous VSG Acceptors

To detect transfer of exogenously added GPI species to endogenous VSG acceptors, P2 labeled via GDP- ^3H Man in an in vitro reaction was purified and added back to membranes in the presence of different detergent concentrations. When the reactions were analyzed by SDS-PAGE and fluorography, radiolabeled bands chromatographing with authentic mfVSG species were observed only in a narrow range of detergent concentrations (0.01 and 0.02% wt/vol, Triton X-100; Fig. 6). No radioactivity was incorporated into protein if the reaction contained <0.005% or >0.02% Triton X-100. As with the GDP- ^3H Man-labeled VSG described above, these radiolabeled proteins were PI-PLC sensitive, and could be im-

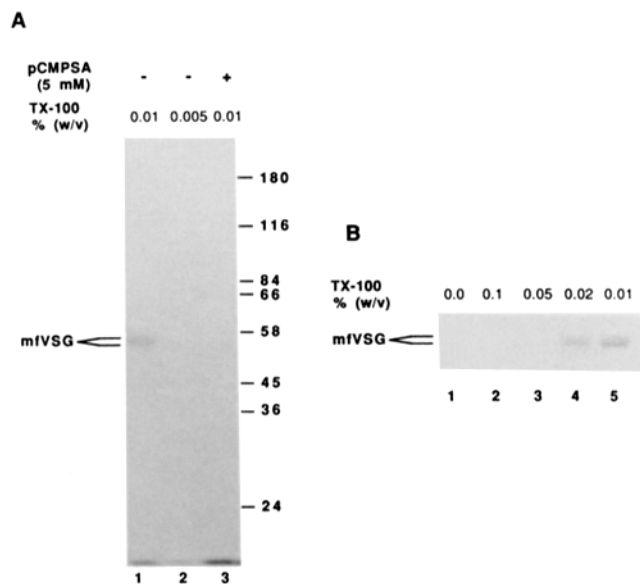


Figure 6. Transfer of purified ^3H Man-labeled P2 to endogenous VSG acceptors. (A) P2, labeled via GDP- ^3H Man in an in vitro system was purified as described and 25,000 cpm of the purified lipid (^3H -P2) was dispersed in $25 \mu\text{l}$ of 0.02% (lanes 1 and 3) or 0.01% (lane 2) of Triton X-100 (wt/vol) in buffer 3. Membranes from tunicamycin-treated variant clone 117 ($25 \mu\text{l}$; 5×10^7 cell equivalents) were preincubated at 0°C for 10 min without (lane 1 and 2) or with pCMPSA (GPI-PLC-inhibitor; lane 3) in buffer 3 without DTT, added to the above dispersion, and incubated for 90 min at 37°C . The reactions were solubilized and an aliquot (2×10^7 cell equivalents) analyzed by SDS-PAGE and fluorography. (B) ^3H -P2 (20,000 cpm) was dispersed by sonication in $5 \mu\text{l}$ buffer 3 containing 10 times the indicated detergent concentration (0–1.0% w/v Triton X-100) and $45 \mu\text{l}$ of buffer 3 containing membranes from tunicamycin-treated variant clone 117 (5×10^7 cell equivalents) was added to each dispersion and processed as described above.

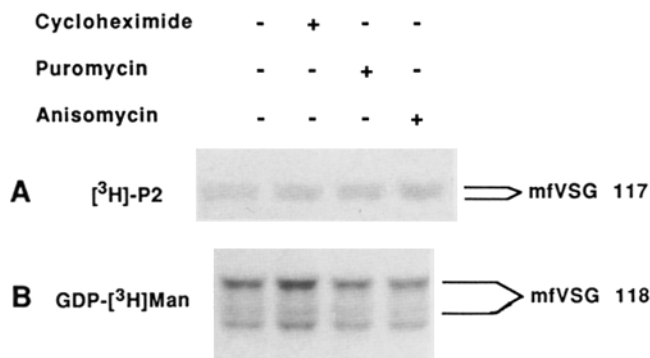


Figure 7. Protein synthesis is not required for GPI transfer. (A) Membranes (50 μl ; 5×10^7 cell equivalents) from trypanosomes (variant clone 117) were incubated in the absence or presence of protein synthesis inhibitors (250 μM cycloheximide, 1 mM puromycin, and 50 μM anisomycin; final concentration) then added to an equal volume of detergent solubilized $[^3\text{H}]\text{P2}$ (25,000 cpm, 0.04% Triton X-100 in buffer 2 containing 2 mM CoA, 400 ng/ml tunicamycin, 0.4 mM GTP, 2 mM ATP, 16 U/ml creatine kinase, 20 mM creatine phosphate) and processed for SDS-PAGE and fluorography as described in Fig. 6 A. (B) Membranes from trypanosomes (variant clone 118; 50 μl ; 2.5×10^7 cell equivalents) were pre-incubated with protein synthesis inhibitors as above and 2 μCi of GDP- $[^3\text{H}]\text{Man}$ was added to each tube. After incubation of the reaction for 10 min at 37°C UDP-GlcNAc and GTP (1 mM each, final concentration) were added. The reaction (100 μl in buffer 3) was processed as described in Fig. 1 B.

munoprecipitated with antibodies to VSG 117 but not with antisera to VSG 118 (data not shown).

T. brucei membrane lysates contain a GPI-specific phospholipase C (GPI-PLC) that is capable of hydrolyzing P2 and releasing GPI-anchored proteins (Bulow and Overath, 1986; Fox et al., 1986; Hereld et al., 1986). This enzyme has been localized to the cytoplasmic surface of membrane-bound organelles but is absent from the plasma membrane, including the region lining the flagellar pocket (Bulow et al., 1989). Addition of detergent to solubilize the dried, labeled lipid and deliver it to VSG may result in a hydrolytic destruction of $[^3\text{H}]\text{-P2}$ and prevent its utilization for the transfer reaction. To determine the extent of hydrolysis of exogenous $[^3\text{H}]\text{-P2}$ by GPI-PLC during the transfer reaction, an aliquot of the reaction mixture was analyzed for release of radioactivity from the butanol-soluble $[^3\text{H}]\text{-P2}$ into the aqueous phase as described previously (Mayor et al., 1990a). Hydrolysis was dependent on detergent concentration even though the lowest detergent concentration adequately solubilized (verified by determining radioactivity obtained in an aliquot of the reaction buffer in the absence of membranes) the lipid in the reaction buffer. Detergent concentrations of 0.1, 0.05, 0.02, 0.01, and 0.005% wt/vol Triton X-100 resulted in 97, 92, 27, 27, and 6%, respectively, of the PI-PLC destruction of $[^3\text{H}]\text{-P2}$. In the absence of detergent, $[^3\text{H}]\text{-P2}$ was not solubilized in the reaction mixture and insignificant hydrolysis (<5% aqueous soluble radioactivity) was observed. Other detergents (NP-40, *n*-octyl glucoside, or deoxycholate) at 0.02% (wt/vol) were able to promote the hydrolytic destruction of $[^3\text{H}]\text{-P2}$, but failed to facilitate its delivery to VSG (data not shown). $[^3\text{H}]\text{-P2}$ was efficiently solubilized in the reaction buffer in the presence of ethanol (3% vol/vol), but neither the transfer reaction nor GPI-PLC hydrolysis were observed. Efforts to inactivate the GPI-PLC by sodium *p*-chloromer-

curiphenylsulphonate (pCMPSA) treatment (5 mM; Hereld et al., 1986) or heat treatment (47°C, 10 min; Bulow and Overath, 1986) resulted in the inactivation of the transfer reaction (Fig. 6 A, lane 3 and data not shown). The low level of radioactivity transferred to VSG was detected by fluorography and the optical density of the labeled bands was taken as an estimate of the transfer reaction. Quantitation of the small amount of radioactivity transferred to VSG via exogenously added lipid was not possible due to background scintillation of the protosol-solubilized gel slices.

These data show that exogenously added $[^3\text{H}]\text{-P2}$ can be transferred to VSG *in vitro* and that the magnitude of the transfer reaction is probably a compromise between "delivery" of the GPI, GPI-PLC-mediated hydrolysis, and disruption of membrane integrity.

Protein Synthesis Is Not Required for GPI Transfer

The incorporation of $[^{35}\text{S}]\text{methionine}$ into TCA-precipitable material was very poor under conditions (0.02% Triton X-100) that permitted the transfer of exogenously added $[^3\text{H}]\text{-P2}$ to endogenous acceptors, and no radiolabeled proteins were detected by SDS-PAGE and fluorographic analysis. Under these conditions, the addition of cycloheximide (250 μM), puromycin (1 mM) or anisomycin (50 μM) had no effect on the incorporation of $[^{35}\text{S}]\text{methionine}$, nor was there any effect on the radiolabeling of the endogenous VSG acceptors via exogenously added $[^3\text{H}]\text{-P2}$ (Fig. 7 A). These compounds effectively inhibit protein synthesis in translationally active trypanosome extracts (Moreno et al., 1991). Incubation of the membrane preparation with protein synthesis inhibitors had no effect on the transfer of GPIs, labeled via GDP- $[^3\text{H}]\text{Man}$, into endogenous acceptor VSGs from 118 (Fig. 7 B) and 117 trypanosome membranes (data not shown). These data suggest that the transfer reaction does not require ongoing protein synthesis and that GPIs are transferred to small amounts of presynthesized acceptor VSG molecules trapped in the membrane preparation.

Energy Requirement of the Transfer of $[^3\text{H}]\text{-P2}$ to Endogenous VSG Acceptors *In Vitro*

The presence of ATP in the lumen of the ER has yet to be confirmed. A protein that is involved in "proofreading" newly synthesized proteins in the secretory pathway (BiP or glucose regulated protein 78) is an ATPase (Munro and Pelham, 1986), suggesting that ATP may cross the membrane of the ER and participate in luminal reactions (Dorner et al., 1990). To determine whether the transfer of GPI molecules to endogenous VSG acceptors requires an exogenous chemical energy source, membranes were incubated along with detergent solubilized $[^3\text{H}]\text{-P2}$ in the absence or presence of an ATPregenerating system, or nonhydrolyzable ATP analogues. No effect was observed on the transfer of $[^3\text{H}]\text{-P2}$ to endogenous VSG under these conditions (Fig. 8). The presence of 0.02% Triton X-100 was sufficient to abolish protease protection of VSG labeled *in vitro* via GDP- $[^3\text{H}]\text{Man}$ (data not shown), indicating that the lumen of the ER is accessible to the various additions. The effect of ATP and GTP hydrolysis on $[^3\text{H}]\text{-P2}$ addition to endogenous acceptors was also determined in the absence of added GTP by including ATP analogues (1 mM $\beta\text{-}\gamma\text{-methyladenosine-5'-triphosphate}$ and 1 mM $\beta\text{-}\gamma\text{-imidoadenosine-5'-triphosphate}$), GTP analogues

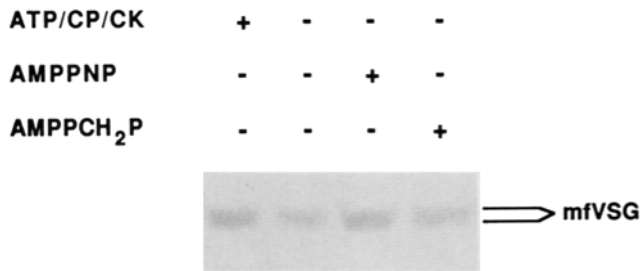


Figure 8. ATP hydrolysis is not required for GPI transfer. Membranes (50 μ l; 5×10^7 cell equivalents) from trypanosomes (variant clone 117) were added to an equal volume of detergent solubilized [³H]-P2 (25,000 cpm, 0.04% Triton X-100 in buffer 2 containing 2 mM CoA, 400 ng/ml tunicamycin, and 0.4 mM GTP) in the presence of various additions as indicated and incubated for 90 min at 37°C and processed for SDS-PAGE and fluorography as in Fig. 6 A. ATP/CP/CK, 1 mM ATP, 10 mM creatine phosphate, and 8 U/ml creatine kinase; AMPPCH₂P, 2 mM β - γ -methyleneadenosine-5'-triphosphate; AMPPNP, 2 mM β - γ -imidoadenosine-5'-triphosphate.

(1 mM β - γ -imidoguanosine-5'-triphosphate, 1 mM guanosine 5'-O-(3-thiotriphosphate)) or in the presence of an ATP-depleting system (7.5 U/ml hexokinase and 2 mM glucose). In the absence of GTP, but in the presence of an ATP-regenerating system, nonhydrolyzable ATP analogues, an ATP-depleting system, or nonhydrolyzable analogues of GTP, similar amounts of radioactivity, as detected by fluorography, were incorporated into endogenous VSG acceptors compared to that observed in the presence of GTP (data not shown).

These observations suggest that the transfer reaction does not require an exogenous source of energy.

Transfer of Other GPI Species to Endogenous Acceptors

[³H]-P3, exogenously added to the membrane system, was capable of being transferred to endogenous acceptors (Fig. 9 A). Similar to the transfer of [³H]-P2, the transfer of [³H]-P3 was dependent on the presence of a narrow range of detergent concentrations (0.01 and 0.02% Triton X-100), independent of ATP or ATP hydrolyses (data not shown), and inhibited by pCMPSA. The VSG species labeled with [³H]-P3 were PI-PLC resistant: PI-PLC treatment did not generate a gel mobility shift and the labeled VSG failed to become aqueous soluble upon Triton X-114 detergent phase partitioning after PI-PLC treatment (data not shown).

Other ethanolamine-containing GPI species that contain the linear conserved sequence common to all of the GPI anchors but do not have *sn*-1,2-dimyristyl glycerol as the lipid moiety (Table I) were also transferred to VSG in vitro. The fatty acid remodeling intermediates P2' and P3'', and an insect stage-specific GPI molecule (PPI), similar to lyso-P3 (Field et al., 1991), were purified and added to the membrane preparation in the absence of ATP and CoA. Comparable radioactivity (as detected by fluorography; see above) was incorporated into endogenous VSG species on addition of 6,500–20,000 cpm of the different GPI species, in the presence of 0.02% Triton X-100 (Fig. 9 B). No incorporation of radioactivity into VSG was detected if <2,000 cpm of the

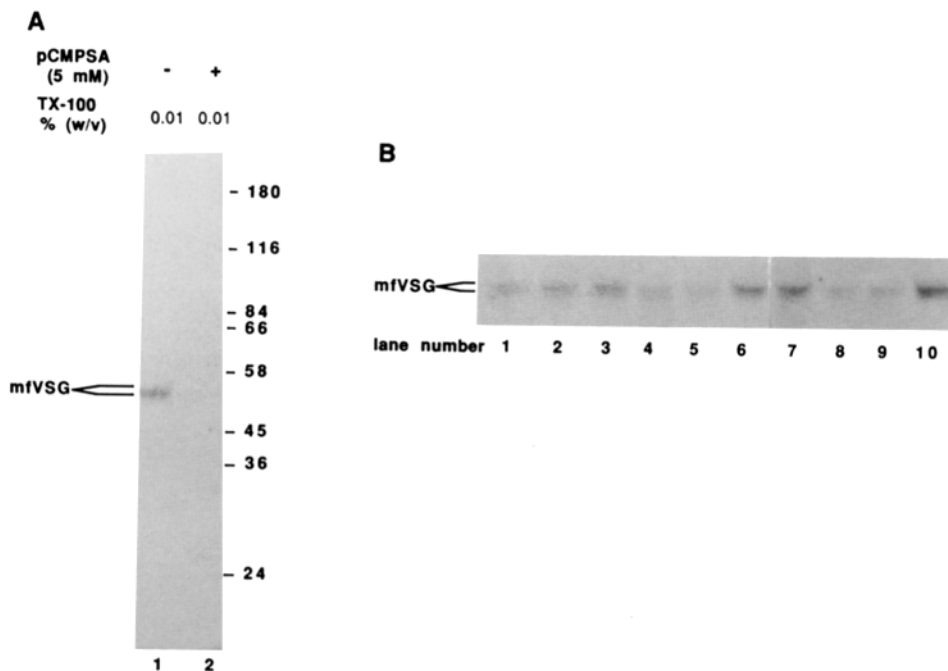


Figure 9. P3 and other GPI molecules are substrates for the GPI transfer reaction. (A) P3, labeled via GDP-[³H]Man in an in vitro system was purified as described and 25,000 cpm of the purified lipid ([³H]P3) was dispersed in 25 μ l of 0.02% (wt/vol) Triton X-100 in buffer 3. Membranes were added to the above dispersion as described in Fig. 6 A and incubated for 90 min at 37°C. The reactions were solubilized and an aliquot (2×10^7 cell equivalents) analyzed by SDS-PAGE and fluorography. (B) [³H]-labeled GPI-species were dispersed by sonication in 5 μ l buffer 3 containing 10 times the final detergent concentration (0.01% wt/vol Triton X-100 in buffer 2) and 45 μ l of buffer 3 containing membranes from tunicamycin treated variant clone 117 (5×10^7 cell equivalents) was added to each dispersion and processed as described as above.

Lane 1, [³H]ethanolamine-labeled P2 (13,000 cpm); **lane 2,** [³H]Man-P2 (13,000 cpm); **lane 3,** [³H]Man-P2 (16,000 cpm); **lane 4,** [³H]Man-P2' (16,000 cpm); **lane 5,** [³H]Man-P2'' (6,500 cpm); **lane 6,** [³H]Man-P2'' (19,600 cpm); **lane 7,** [³H]Man-P3 (16,000 cpm); **lane 8,** [³H]Man-P3 (16,000 cpm); **lane 9,** [³H]ethanolamine-labeled P3 (16,000 cpm); **lane 10,** [³H]ethanolamine-labeled insect stage GPI (PPI; 8,000 cpm). GPIs in lanes 1, 2, 8, and 9 were TLC-purified from [³H]Man or [³H]ethanolamine-labeled bloodstream trypanosomes, GPIs in lanes 3, 4, 5, and 7 were TLC purified from lipids labeled in vitro via GDP-[³H]Man, [³H]Man-P2'' (lane 6) was purified by phase separation of lipids labeled in vitro via GDP-[³H]Man in the absence of ATP, and PPI in lane 10 was TLC-purified from insect stage trypanosomes radiolabeled with [³H]ethanolamine (Field et al., 1991).

lipids, [^3H]P2 or [^3H]P3, were used in the reaction (data not shown), ruling out the possibility that the labeling of VSG observed in Fig. 9 B (lanes 4–10) was due to contaminating amounts of other GPIs. P3 and the PPI are intrinsically resistant to GPI-PLC hydrolysis: 30–45% of the added P2, P2', and P2'' but <5% of P3 and PPI were hydrolyzed by the endogenous GPI-PLC in the reactions described above. TLC analyses of the intact lipids at the end of the reaction showed that the lipid moiety of each of the GPI species was unchanged (data not shown).

The data indicate that the transferase is capable of transferring P2, heterogeneously acylated forms of P2 (P2' and P2''), PPI, and P3 to VSG *in vitro*, and suggest that the transferase does not require a specific lipid moiety in the GPI molecule to facilitate the transfer reaction. The transfer of [^3H]-P2 and P3, labeled in the ethanolamine (Fig. 9 B; lanes 1 and 9), mannose (Fig. 9 B; lanes 2, 3, 7, and 8) and glucosamine (data not shown) residues to endogenous VSG, provides evidence that the GPI is added *en bloc* to VSG.

Discussion

In this paper we describe the transfer of free GPIs to endogenous VSG in a cell free system containing unfractionated membranes prepared from tunicamycin-treated trypanosomes. The incorporation of radiolabel from GDP-[^3H]Man into GPI species and into VSG was totally dependent on the addition of exogenous UDP-GlcNAc; lack of significant glycolipid and polypeptide labeling in the absence of added UDP-GlcNAc indicated that VSG molecules were not labeled directly via GDP-[^3H]Man or via dol-P-[^3H]Man. The labeling of two protein species was attributed to the presence of glycosylated and unglycosylated forms of VSG acceptors in the membrane due to the preparation of membranes from tunicamycin-treated trypanosomes. *In vitro* labeled VSGs were protected from proteolytic degradation to a significant extent (20–25%), consistent with a luminal location for the newly translocated VSG acceptor polypeptides. However, the data do not rule out the unlikely possibility that the reaction takes place before the translocation of newly synthesized VSG. Failure to observe a greater amount of proteolytic protection of the labeled polypeptides may be due to the method of preparation of the membranes, which involved hypotonic cell lysis.

Analyses of the [^3H]Man-labeled moiety derived from gel-purified *in vitro*-labeled mfVSG species showed that all the radioactivity (>95%) associated with VSG was present in a glycan fragment that cochromatographed with the neutral glycan species from a nongalactosylated VSG membrane anchor. The radioactivity incorporated into mfVSG 117 at the end of a 90-min incubation ($\sim 4,000$ cpm/ 10^7 cell equivalents) provided an estimate of the amount of VSG acceptor in this system. Taking the specific activity of the GPI species as three times that of GDP-[^3H]Man (due to the presence of three mannoses in the GPI anchor and assuming no dilution of the specific radioactivity) there appear to be 3,600 molecules/cell (~ 60 fmol/ 10^7 cells) of acceptor VSG species present in the membrane preparation. Surprisingly, the level of acceptor VSG appears to be consistently ~ 10 -fold lower in membranes made from a different variant clone (118 trypanosomes, three separate observations; Mayor, S., unpublished observations). At the present time it is difficult to find a reason for this quantitative difference in acceptor levels. No at-

tempt was made to enhance the level of acceptor polypeptides (and possibly the efficiency of the labeling reaction) by coupling either of the membrane preparations to an mRNA translation system, since, despite considerable effort in this laboratory, it has been impossible to observe processing of proteins synthesized in the presence of trypanosome membranes (Ip, H., unpublished observations), or processing of proteins translated *de novo* from mRNA added to a trypanosome lysate (Moreno et al., 1991).

Transfer of purified, exogenously added, labeled P2 ([^3H]-P2) to endogenous VSG acceptors was also observed in this cell free system. The labeled polypeptides were indistinguishable from the VSG species that were labeled by GPI species synthesized *in situ*. These protein species were PI-PLC susceptible and could be immunoprecipitated by antibodies to VSG. This observation directly demonstrates the precursor-product relationship between P2 and the GPI anchor of mfVSG. Pulse-chase experiments show that the reaction observed *in vitro* is not a simple exchange reaction between newly synthesized GPIs and preexisting GPI-anchored VSG, and transfer of [^3H]-P2 to endogenous VSG indicates that the GPI is transferred *en bloc* to VSG. However, the data do not rule out the unlikely possibility of more complex exchanges between GPI fragments (GlcN-PI or any fragment of the same). In this context, the observation of partial anchors on a fusion protein containing the carboxy terminus of Thy-1 may result from anchor degradation or complex exchanges rather than partial anchor component addition as suggested previously (Kaetzel et al., 1990).

The transfer of exogenous GPI species to VSG was dependent on the presence of detergent in a narrow concentration range. The requirement for proper membrane integrity has been observed as a prerequisite for a number of membrane-associated enzymes (Fleischer and Fleischer, 1967) and is likely to be the reason for the lack of transfer at the higher detergent concentrations. Oligosaccharyl transferases from a variety of sources are resident ER enzymes that also display such a requirement (Kaplan et al., 1987; Chalifour and Spiro, 1988).

The absence of the effect of protein synthesis inhibitors indicates that the cell free transfer reaction takes place on polypeptide acceptors that have already been translated *in vivo* and are present in the membrane preparation. The lack of an energy requirement for the transfer reaction supports a transpeptidation reaction mechanism involving cleavage of the carboxy-terminal extension of the protein and attachment of the free amine group of the GPI substrates to the carboxy-terminal amino acid of the mature protein. However, at this stage it is not clear whether the acceptor VSG molecules contain the carboxy-terminal extension. Transpeptidases have been described in the penicillin-sensitive peptidoglycan polymerization reaction involved in cell wall formation of prokaryotes, mainly Gram-negative *Escherichia coli* (Izaki et al., 1968) and gram positive *Staphylococcus aureus* (Tipper and Strominger, 1968). Similar transpeptidation reactions are carried out by a variety of peptidases (Lutek et al., 1988; Berne et al., 1990) and γ -glutamyl transpeptidases (Walsh, 1979). Maturation of Concanavalin A into a carbohydrate binding protein in jackbean cotyledons (Bowles et al., 1986) and the "splicing" of the 119-kD yeast TFP1 gene product to a 69-kD subunit of the vacuolar proton-translocating adenosine triphosphatase (Kane et al., 1990), are also possible ex-

amples of such a reaction, but no definite mechanistic proof for transpeptidation exists.

PI-PLC-sensitive and -resistant GPIs (P2 and P3) are synthesized *in vivo* (Krakow et al., 1986; Menon et al., 1988a) and in cell-free GDP-[³H]Man-labeling experiments (Masterson et al., 1989; Menon et al., 1990b) but only PI-PLC-sensitive VSGs are detected *in vivo* and *in vitro*. VSG is expressed only in the bloodstream stage of the parasite; insect stage parasites express a major PI-PLC-resistant GPI-anchored cell surface glycoprotein (Clayton and Mowatt, 1989) and recent analyses show that this stage of the parasite synthesizes only PI-PLC-resistant free GPI species of which the most prominent, PPI, is similar to lyso P3 (Field et al., 1991). The results presented here show that, besides P2, P2', and P2'', the PI-PLC-resistant GPIs, P3, and PPI, were all transferred to endogenous VSG acceptors *in vitro*. The P3-labeled VSG was PI-PLC resistant. These data suggest that the protein sequence does not determine the nature of the GPI moiety transferred to the polypeptide. The low level of incorporation of radioactivity into endogenous VSG prohibited a kinetic analysis of the transfer of the different GPI species.

Some possible models to account for these observations are proposed. Firstly, the cell free system may not reflect the true selectivity of the transfer process or the actual K_m s of the transferase for the different lipid substrates are vastly different *in vivo*. Alternatively, both PI-PLC-sensitive and -resistant GPIs are equally good substrates but an inositol deacylase, active only in the VSG-expressing bloodstream stage, is inactivated *in vitro*, possibly due to the addition of detergent. A third possibility is that PI-PLC sensitive and resistant GPIs are synthesized via parallel pathways but in the bloodstream stage only the PI-PLC-sensitive forms are delivered to the transferase and in the insect stage PI-PLC-resistant lipids become accessible to the transferase (either by the removal of physical constraints or K_m differences). The first and third possibilities are not mutually exclusive. The transfer of P3 and PPI to VSG *in vitro* supports the second possibility and is consistent with the proposal of a developmentally regulated deacylase, active in the bloodstream stage and inactive in the insect stage. However, at this stage there is no evidence for such an enzyme, nor has the PI-PLC susceptibility of the PPI-labeled VSG been determined.

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