Galactose-containing Glycosylphosphatidylinositols in *Trypanosoma brucei**

Satyajit Mayor‡, Anant K. Menon, and George A. M. Cross

From The Rockefeller University, New York, New York 10021

Many eukaryotic surface glycoproteins, including the variant surface glycoproteins (VSGs) of *Trypanosoma brucei*, are synthesized with a carboxyl-terminal hydrophobic peptide extension that is cleaved and replaced by a complex glycosylphosphatidylinositol (GPI) membrane anchor within 1-5 min of the completion of polypeptide synthesis. We have reported the purification and partial characterization of candidate precursor glycolipids (P2 and P3) from *T. brucei*. P2 and P3 contain ethanolamine-phosphate-Manα1–2Manα1–6Manα1-GlcN linked glycosidically to an inositol residue, as do all the GPI anchors that have been structurally characterized. The anchors on mature VSGs contain a heterogenously branched galactose structure attached α1–3 to the mannose residue adjacent to the glucosamine. We report the identification of free GPIs that appear to be similarly galactosylated. These glycolipids contain diacylglycerol and α-galactosidase-sensitive glycan structures which are indistinguishable from the glycans derived from galactosylated VSG GPI anchors. We discuss the relevance of these galactosylated GPIs to the biosynthesis of VSG GPI anchors.

The covalent linkage of a glycosylinositol phospholipid (GPI)[1] to the carboxyl-terminal amino acid of many eukaryotic cell surface glycoproteins provides the sole means by which these proteins are attached to cell membranes (Ferguson and Williams, 1988; Low, 1989; Cross, 1990). In many cases some fraction of these proteins can be released from the membranes by treatment with a phosphatidylinositol-specific phospholipase C (PI-PLC). Chemical structures of GPI membrane anchors of a trypanosome variant surface glycoprotein (VSG) (Ferguson et al., 1988a), rat brain Thy-1 (Homans et al., 1988), human erythrocyte acetylcholinesterase (E^{hu}-AChE) (Roberts et al., 1988a; Roberts et al., 1988b), and a *Leishmania major* surface protease, PSP (Schneider et al., 1990), are known. These GPI anchors have a common backbone structure of ethanolamine-phosphate-6Manα1–2Manα1–6Manα1–4GlcN linked α1–6 to an inositol phospholipid. The carboxyl terminus of the mature protein is attached to the glycolipid via an amide linkage to ethanolamine. The different anchors appear to have both protein and cell-type-specific modifications branching from the core backbone structure and variations in the composition and linkage of fatty acids (reviewed by Thomas et al., 1990). The GPI anchors of bloodstream stage *Trypanosoma brucei* VSGs have a branched α-galactose structure, containing variable numbers of residues, attached to the O-3 position of the mannose residue adjacent to the glucosamine (Ferguson et al., 1988a; Fig. 1).

Studies on the biosynthesis of VSGs of *T. brucei* suggest that the cDNA-predicted carboxyl-terminal hydrophobic sequence (absent from the mature protein) is replaced within 1 min of completion of protein synthesis by a GPI anchor (Bangs et al., 1985; Ferguson et al., 1986), which is then processed further by the addition of galactose residues (Bangs et al., 1988). The rapidity of the GPI addition suggested the existence of a prefabricated precursor glycolipid that could be transferred *en bloc* to the newly synthesized protein, probably in the endoplasmic reticulum (ER). Studies using a yeast secretory mutant (*sec*18) support an ER location for the addition of the GPI anchor (Conzelmann et al., 1988). The addition of some, if not all, of the galactose residues on the VSG GPI anchor takes place later in the secretory route of the VSG, on its way to the cell surface (Bangs et al., 1988).

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 bloodstream *T. brucei*. The structures of P2 and P3 are shown in Fig. 1. 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 an 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; Doering et al., 1989; Masterson et al., 1989; Masterson et al., 1990; Menon et al., 1990a, 1990b). We have recently demonstrated that these GPIs can be transferred to VSG polypeptides *in vitro*, confirming that they are indeed authentic precursors of the VSG GPI anchor (Mayor et al., 1991).

In earlier biosynthetic labeling studies (Mayor et al., 1990b) we noted that, in addition to P2 and P3, several minor lipids were also labeled when trypanosomes were incubated with [^{3}H]GlcN. In this paper we report the partial characterization of these lipids and show that they are galactose-containing GPIs. The implications of these results for the biosynthesis of VSG and other GPI anchors are discussed.

‡ To whom correspondence should be addressed at present address: Room 15-420, Dept. of Pathology, College of Physicians and Surgeons of Columbia University, 630 W. 168th St., New York, NY 10032. Tel.: 212-305-4091; Fax: 212-305-5498.

[1] The abbreviations used are: GPI, glycosylinositol phospholipid; PI-PLC, phosphatidylinositol-specific phospholipase C: GPI-PLD, GPI-specific phospholipase D; VSG, variant surface glycoprotein; AHM, 2,5-anhydromannitol; Inos, inositol; TLC, thin layer chromatography; HPLC, high-performance liquid chromatography; ER, endoplasmic reticulum.
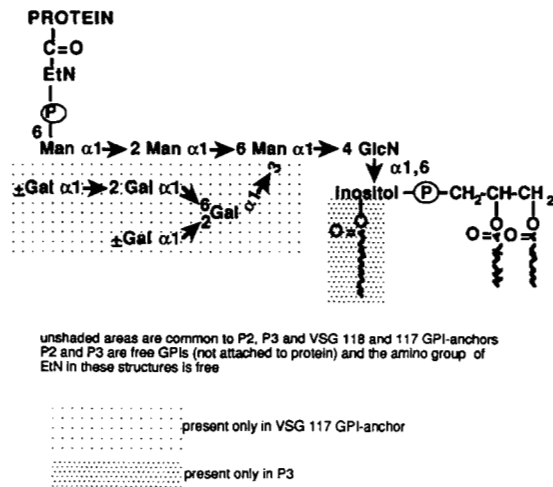
FIG. 1. **Structures of GPIs in bloodstream-form *T. brucei.*** The *unshaded area* is the conserved core sequence common to the GPI anchor of VSGs from trypanosome clones 117 and 118 and free GPIs, P2 and P3. The diacylglycerol moiety in all the three GPIs is dimyristylglycerol. The linkages EtN-P-6Man, Manα1–4GlcN, and GlcNα1–6Inos have not been explicitly described in P2 and P3 but are likely to be identical to the core sequence. ±, heterogeneity in the VSG 117 galactose branch (Ferguson *et al.*, 1988a). VSG 118 has only a trace amount of galactose (Holder, 1985) and may be regarded as a nongalactosylated GPI anchor (Mayor *et al.*, 1990b).

EXPERIMENTAL PROCEDURES AND RESULTS[2]

The trypanosome GPI-anchor precursors, P2 and P3, can be biosynthetically labeled by incubating trypanosomes with [3H]myristic acid, [3H]ethanolamine, [3H]Man, [32P]phosphate, or [3H]GlcN. Both these lipids can be recovered by extracting labeled trypanosomes with a polar solvent [chloroform/methanol/water (10/10/3, v/v)] and can be separated from labeled water-soluble material by drying the extract and partitioning the residue between water and *n*-butanol: the lipids quantitatively partition into the upper butanol-rich phase (Mayor *et al.*, 1990b). [3H]Man and [3H]GlcN labeling of these lipids is insensitive to the *N*-glycosylation inhibitor, tunicamycin (Mayor *et al.*, 1990b) and in all sugar-labeling experiments described below trypanosomes were preincubated with tunicamycin prior to the addition of radiolabeled sugar. Preliminary analyses of glycans derived from tunicamycin-insensitive [3H]GlcN-labeled glycolipids showed that, in addition to the P2 and P3-derived glycan, structures that co-chromatographed with galactosylated core glycan standards, derived from the GPI-anchor of VSG 117, were also present (Mayor *et al.*, 1990b). The results presented below confirm the existence of galactose-containing GPIs in trypanosome variants expressing both galactosylated and nongalactosylated VSG GPI anchors (117 and 118 VSG, respectively).

Presence of GPIs More Polar than P2—Polar lipids were extracted from biosynthetically labeled trypanosome variants 117 and 118 and chromatographed on thin layer plates (Fig. 2). In addition to P2 and P3, lipid species more polar than P2 (Fig. 2; regions X and Y) and a lipid species that chromatographs between P2 and P3 (Fig. 2; region Z) could be labeled with [3H]myristic acid, [3H]ethanolamine, [3H]Man, or [3H]GlcN. Attempts to biosynthetically label these lipids with either [3H]glucose or [3H]galactose were unsuccessful; the
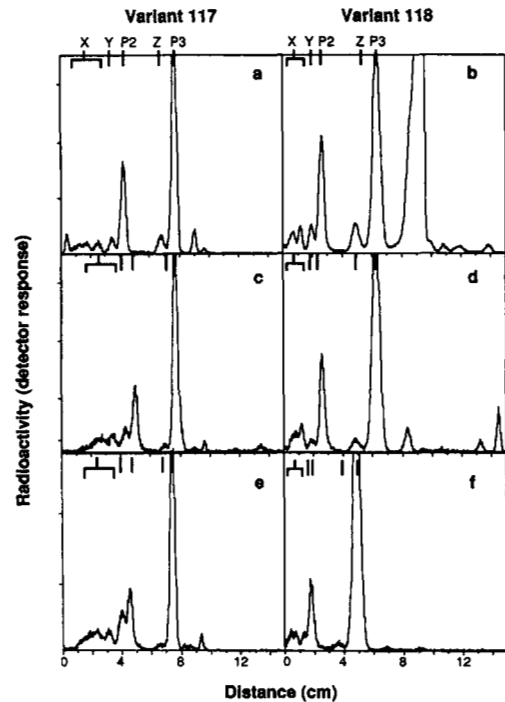
FIG. 2. **TLC analysis of glycolipids purified from trypanosomes radiolabeled with [3H]myristic acid (*a, b*), [3H]ethanolamine (*c, d*), [3H]GlcN (*e*), and [3H]Man (*f*).** Trypanosomes, variant 117 (*a, c,* and *e*) and variant 118 (*b, d,* and *f*), were biosynthetically labeled in the absence (*a, b, c, d*) or presence (*e, f*) of tunicamycin and labeled glycolipids were extracted into the butanol phase as described in the text. The butanol phases were then chromatographed on Kieselgel-60 thin-layer plates using solvent system *a*. Radioactivity was detected using a Berthold TLC scanner. P2 and P3 are previously characterized GPI anchor precursors (see Fig. 1). *X* and *Y* refer to labeled species migrating between P2 and the origin of the chromatogram. *Z* refers to the peak of labeled material migrating in the region of the chromatogram between P2 and P3. Origin, 0 cm; front, 16 cm.

little radioactivity (10^2–10^3 cpm/10^8 cells) that was incorporated into organic phase could not be distinguished from free radio label contamination. Data presented below and in the miniprint demonstrate that the glycolipids in regions X and Y are PI-PLC-sensitive GPIs that contain diacylglycerol, while the region Z glycolipid is PI-PLC-resistant and is probably monogalactosylated P3.

Analyses of Neutral GPI-glycans Generated from VSG 117 and Region X and Region Y Glycolipids—α-Galactosidase digestion of deaminated and NaB3H4-reduced GPI anchor glycans from VSG 117 (Fig. 3A, panel a) generated a major peak (~90%) that co-chromatographed with Man3AHM (Fig. 3A, panel b; 0), consistent with the presence of galactosylated GPI-glycans in the 117 VSG GPI-anchor (Ferguson *et al.*, 1988a). A minor peak (~10%) with a retention time of 21 min was also observed (Fig. 3A, panel b; *). This minor species chromatographs close to the Gal1Man3AHM standard, but is resistant to further digestion with α-galactosidase and jack bean α-mannosidase (data not shown). This suggests that it may be derived from an as yet uncharacterized glycan (15%) present in VSG 117 (Ferguson *et al.*, 1988a) that may be modified at the terminal mannose residue.

[3H]Man-labeled region X and Y GPIs from variant 118 trypanosomes were purified by TLC and neutral glycans were generated by HF dephosphorylation followed by deamination and reduction. Anion-exchange HPLC analyses of these neutral glycans are shown in Fig. 3B. The chromatogram of the region Y-derived material contained two peaks, one at 24.2
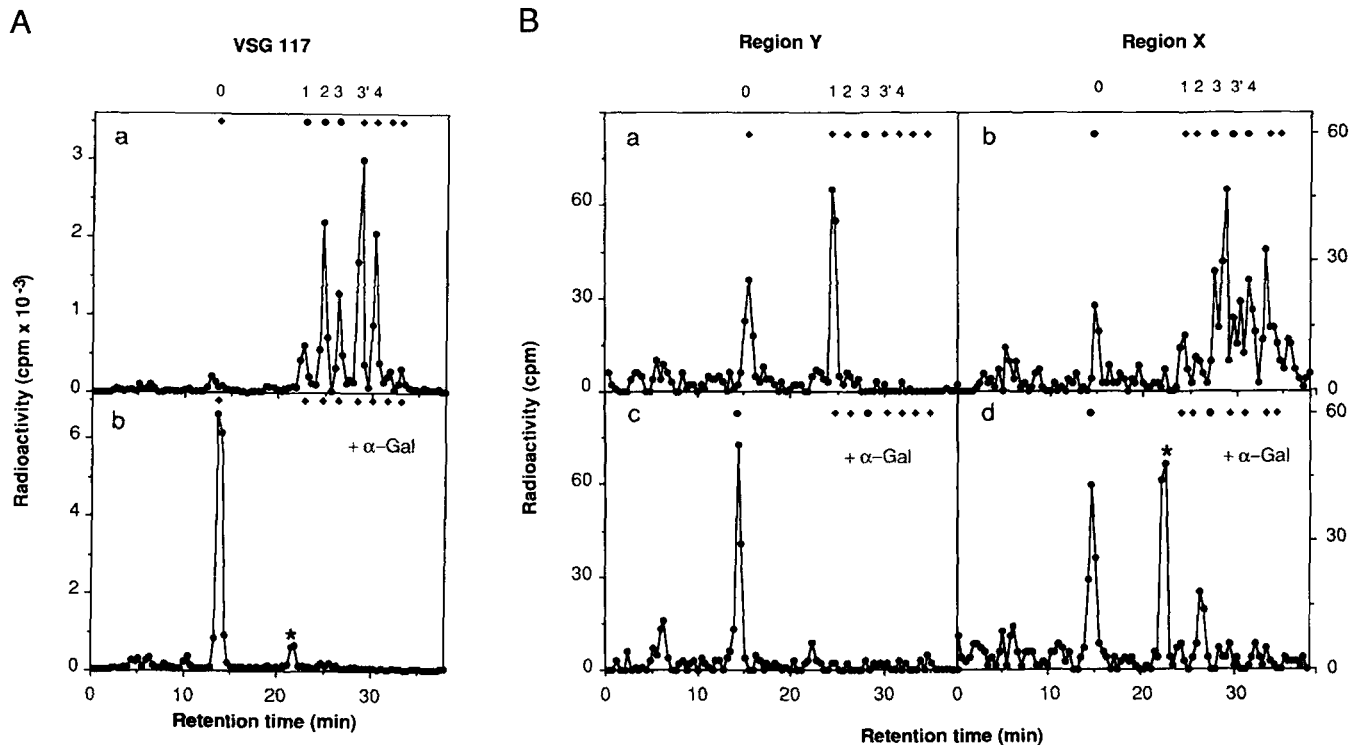
Galactosylated GPIs in Trypanosoma brucei

FIG. 3. α-Galactosidase digestion of neutral glycans derived from VSG 117 GPI anchor (A) and [³H] Man-labeled region X and Y lipids (B). Radiolabeled neutral glycans were prepared after HF dephosphorylation followed by deamination and reduction from VSG 117 (20,000 cpm; A, panels a and b) as described (Mayor et al., 1990b) and from [³H]Man-labeled region X (5000 cpm; B, panels b and d) and Y (3000 cpm; B, panels a and c) GPIs which were TLC-purified from variant 118 trypanosomes. Each sample was divided into two aliquots and each aliquot was incubated in 0.2 M NaOAc, pH 5.0, either with α-galactosidase (+α-Gal, A, panel b; B, panels c and d) or without any additions (A, panel a; B, panels a and b), prior to analyses by anion-exchange HPLC. The standards indicated at the top of each panel were included in each run and correspond to the heterogenously galactosylated neutral glycans from the membrane anchor of purified VSGs, i.e. GalₙMan₃(2,5-anhydromannitol) generated via deamination and reduction of the HF-dephosphorylated glycans from purified 117 and 118 VSGs, where n = 0, 1, 2, 3, and 4 (indicated as 0, 1, 2, 3, 3', and 4). 3 and 3' correspond to the two observed isomers of the Gal₃ structures (Ferguson et al., 1988a). The additional symbols (♦) indicate the retention times of uncharacterized galactosylated glycans derived from VSG 117 GPI-anchor.[3]

min cochromatographing with Gal₁Man₃AHM (Fig. 3B, panel a), and a second peak at 15-min cochromatographing with Man₃2,5-anhydromannitol (Man₃AHM; Fig. 3B, panel a) (probably derived from contaminating P2 that is present in the TLC-purified region Y material (data not shown). α-Galactosidase treatment of the region Y-derived glycans quantitatively (>95%) generated Man₃AHM (Fig. 3B, panel c), confirming that the region Y-derived glycan is Gal₁Man₃AHM.

Many peaks chromatographing in the Gal₂₋₄Man₃AHM region and were observed in the HPLC analysis of region X-derived glycans, all of which were susceptible to α-galactosidase treatment (Fig. 3B, compare panels b and d). However, unlike the region Y-derived glycans, Man₃AHM was not the sole product of the α-galactosidase digestion. About 56% of the α-galactosidase digestion product, marked by an asterisk (Fig. 3B, panel d), cochromatographed with the minor α-galactosidase digestion product (retention time, 21 min) generated from VSG 117-derived neutral glycans (Fig. 3A, panel b). Similar data were obtained from [³H]GlcN-labeled, TLC-purified region X and Y-derived neutral glycans (data not shown). The extra peak (retention time ~26 min) in Fig. 3B, panel d, probably represents a partial digestion product since it disappears upon incubations with a second aliquot of the enzyme and is not consistently observed (data not shown).

These data show that the region X and Y GPIs contain

galactosylated glycans similar to those derived from the GPI anchor of VSG 117.

Synthesis of Galactosylated GPIs in Vitro—GPI synthesis has been demonstrated in cell free systems from T. brucei (Menon et al., 1988b; Masterson et al., 1989; Menon et al., 1990b). In these systems, the synthesis of GPI biosynthetic intermediates as well as the mature GPIs, P2 and P3, is dependent on the presence of GDP-Man and UDP-GlcNAc (Masterson et al., 1989; Menon et al., 1990a). In order to determine whether region X, Y, or Z GPIs could be labeled in vitro directly via UDP-[³H]Gal, membranes were incubated with UDP-[³H]Gal under conditions that support GPI biosynthesis. TLC analysis of the glycolipids labeled via UDP-[³H] Gal in reactions containing membranes prepared from variant 118 trypanosomes (Fig. 4a), or membranes prepared from variant 117 (Fig. 5) revealed a spectrum of radiolabeled lipids that chromatographed in regions X, Y, and Z. All the labeled lipids were susceptible to deamination by nitrous acid; >70% of the glycolipid-associated radioactivity was released into the aqueous phase and the radioactivity remaining in the butanol phase did not comigrate with the starting material, confirming that the lipids were completely susceptible to deamination (Mayor et al., 1990a). In addition to UDP-[³H]Gal-labeled PI-PLC-sensitive GPIs chromatographing in regions A and B, a PI-PLC-resistant GPI, chromatographing in region Z, was also observed in reactions containing membranes prepared
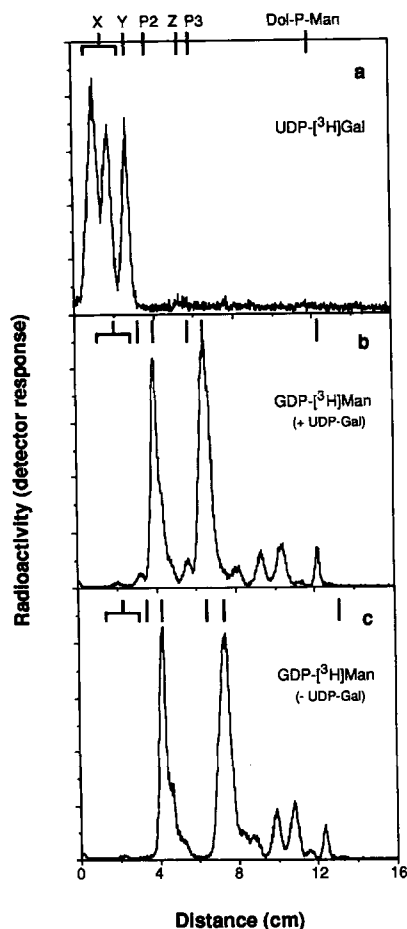
FIG. 4. TLC analysis of glycolipids synthesized in vitro. Membranes from variant 118 trypanosomes were incubated *in vitro* with UDP-[³H]Gal (*a*) or GDP-[³H]Man (*b*, *c*) in the presence of UDP-GlcNAc (1 mM, GDP-Man (1 mM; *a*) and UDP-Gal (1 mM; *b*). The reactions were incubated for 60 min at 37 °C and glycolipids were extracted and analyzed by TLC as described in Fig. 2. Origin, 0 cm; front, 16 cm.

FIG. 5. PI-PLC treatment of in vitro synthesized UDP-[³H] Gal-labeled lipids. Membranes from variant 117 trypanosomes were incubated *in vitro* with UDP-[³H]Gal as described in Fig. 4. The labeled glycolipids (1000 cpm), extracted into butanol, were incubated in PI-PLC buffer without (*top panel*) or with PI-PLC (*bottom panel*). The reaction mixture was then partitioned between water and butanol and the butanol phase analyzed as described in Fig. 2. Origin, 0 cm; front, *f*.

from variant 117 (Fig. 5), while in reactions containing membranes from variant 118 (Fig. 4*a*) the level of this species was considerably lowered. The reason for this difference is not known. A similar GPI species has been observed in biosynthetically labeled 117 and 118 trypanosome variants *in vivo* (Fig. 2; region Z).

UDP-[³H]Gal-labeled GPIs from five *in vitro* reactions were pooled and subjected to neutral glycan analyses as described for the region X and Y GPIs. About 70% of the radioactivity associated with UDP-[³H]Gal-labeled GPIs was recovered in the aqueous phase after HF dephosphorylation and butanol phase partitioning. Anion-exchange HPLC analysis of the deaminated and reduced material in the aqueous phase showed a profile (miniprint section; Fig. 8, *panels A* and *B*) similar to the neutral glycans derived from region X and Y GPIs. α-Galactosidase digestion released most of the radioactivity as galactose (retention time ~5.8 min); a minor proportion (<20%) was recovered at a retention time of 20.2 min, similar to the minor α-galactosidase digestion product (retention time, 21 min) generated from VSG 117-derived neutral glycans (Fig. 3*A*, *panel b*). The radioactivity recovered in the butanol phase of the HF dephosphorylation reaction was also analysed by anion-exchange HPLC after deamination and reduction. Most (95%) of the radioactivity chromatographed at a retention time of 21 min but was resistant to α-galactosidase digestion (Fig. 8, *panels C* and *D*).
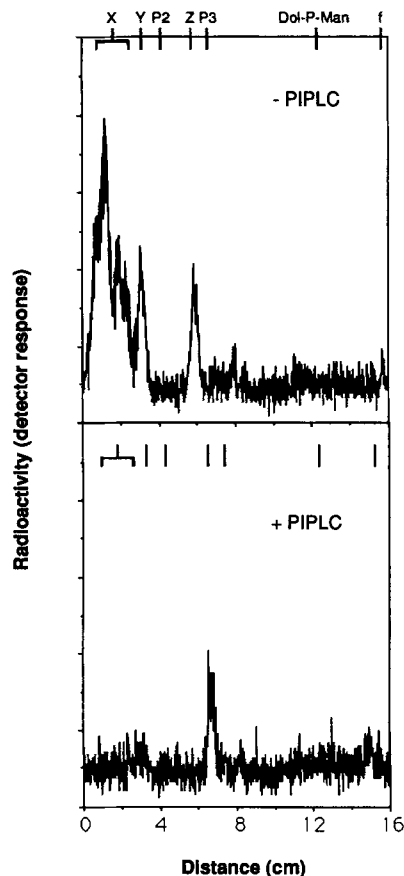
When GDP-[³H]Man-labeled GPIs from *in vitro* reactions were analyzed by TLC, minor GPI species (~5% of the radioactivity chromatographing as P2) corresponding to region Y and Z GPIs was observed, only when nonradioactive UDP-Gal was included in the reaction (compare Figs. 4*b* and 4*c*). Essentially no region X GPIs were observed in the presence or absence of UDP-Gal. Anion-exchange HPLC analyses of the HF-dephosphorylated GDP-[³H]Man-labeled GPI glycans that partition into the aqueous phase confirmed the presence of a neutral glycan that cochromatographed with a Gal₁Man₃AHM standard (data not shown), suggesting that a GPI similar to region Y GPI could be formed *in vitro* from *de novo* synthesized GPIs. Anion-exchange HPLC analyses of the GDP-[³H]Man-labeled GPI-derived glycans, that partition into the butanol phase after HF dephosphorylation, showed the presence of a neutral glycan that cochromatographed with a Gal₁Man₃AHM standard only in reactions that were supplemented with UDP-Gal (data not shown). These data are consistent with the synthesis of mono-galactosylated P2 (*region Y*) and mono-galactosylated P3 (*region Z*) *in vitro*.

In order to resolve the differences between the synthesis of region X, Y, and Z GPIs *in vitro*-labeled via GDP-[³H]Man in the presence of UDP-Gal (Fig. 4*b*) and directly by UDP-[³H]Gal *in vitro* (Figs. 4*a* and 5*a*), membranes were prepared from trypanosomes biosynthetically labeled with [³H]GlcN. The membranes were incubated in the *in vitro* reaction as

described in Fig. 6. Region X, Y, and Z GPIs were enriched only when UDP-Gal and Mn^{2+} were included in the reaction mixture. The identity of these enriched species were confirmed by anion-exchange HPLC analyses (before and after α-galactosidase treatment) of the HF-dephosphorylated, deaminated, and reduced-neutral glycans generated from GPIs shown in Fig. 6 (data not shown). These analyses suggest that the glycans generated from the in vivo-labeled, in vitro-enriched region X, Y, and Z GPIs are identical to the glycans generated from GPIs that are labeled directly via UDP-[³H]Gal in vitro. These results suggest that biosynthetically labeled GPIs are transported in vivo to galactosyltransferase-containing compartments and are able to act as substrates of this enzyme in vitro, while in vitro-synthesized de novo GPIs are not accessible to the whole spectrum of galactosyltransferases. This would account for the difference in the labeling patterns seen between the two incubation conditions. In support of this hypothesis is the observation that the labeling of region X, Y, and Z GPIs via UDP-[³H]Gal was not dependent on the de novo synthesis of P2 and P3; the presence or absence of millimolar GDP-Man and or UDP-GlcNAc had no effect on the incorporation of radioactivity into these GPIs ($\sim$300 cpm/10^7 cell equivalents of membrane). However, the labeling
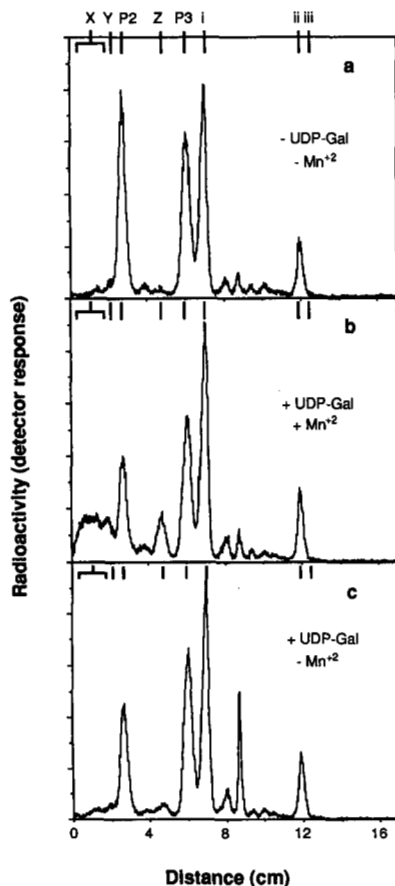

FIG. 6. *In vitro* synthesis of region X, Y, and Z GPIs in membranes prepared from [³H]GlcN-labeled trypanosomes. Membranes were prepared from 117 trypanosomes biosynthetically labeled with [³H]GlcN (35 μCi/ml; 50 min at 37 °C) and were incubated (4 × 10^7 cell equivalents/reaction) *in vitro* in the absence of Mn^{2+} (*a*) or in the presence of Mn^{2+} and UDP-Gal (*b*), or in the presence of UDP-Gal alone (*c*). The reactions were incubated for 90 min at 37 °C and glycolipids were extracted and analyzed by TLC as described in Fig. 2. Origin, 0 cm; front, 16 cm. *i*, Man₅GlcNAc₂-P-P-Dol (Low *et al.*, 1991; Mayor *et al.*, 1990b); *ii*, GlcN-PI; *iii*, trypanosome Dol-P-Man (Low *et al.*, 1991).

was dependent on the presence of Mn^{2+} in the reaction mixture; 10-fold less radioactivity was incorporated into region X, Y, and Z GPIs when membranes were incubated with UDP-[³H]Gal in the absence of Mn^{2+}.

Effect of Galactosylation on Transfer of GPIs to VSG in Vitro—Membrane preparations similar to those described above have been used to demonstrate the transfer of GPIs to protein acceptors *in vitro* (Mayor *et al.*, 1991). In order to determine whether the synthesis of galactosylated GPIs influenced the transfer of *in vitro*-synthesized GPIs to endogenous VSG acceptors, the protein fraction was analysed. SDS-PAGE followed by autoradiography was performed on the solubilized protein pellets obtained from *in vitro* reactions containing membranes prepared from VSG 117 expressing trypanosome variant, as described (Mayor *et al.*, 1991). No difference was observed in the transfer of radioactivity to protein ($\sim$4000 cpm/10^7 cell equivalents) when the GDP-[³H]Man-containing reactions were performed in the presence or absence of UDP-Gal. Omitting Mn^{2+} from the assay buffer abolished the synthesis of the galactosylated GPI that is generated in the presence of UDP-Gal, without any effect on the synthesis of GDP-[³H]Man-labeled GPIs or on the transfer of these lipids to protein. Analysis of the VSG-associated radioactivity by methods described earlier (Mayor *et al.*, 1991) showed that the glycan transferred to protein *in vitro* in the presence of UDP-Gal was not galactosylated; all ($>$97%) of the VSG-associated radioactivity (obtained from *in vitro*-labeled VSG that was purified by electroelution from SDS-PAGE and then HF-dephosphorylated, deaminated, and reduced to generate a neutral glycan) chromatographed with Man₃AHM, the neutral glycan derived from a nongalactosylated VSG membrane anchor.

DISCUSSION

In this paper we demonstrate the presence of a spectrum of galactose-containing GPIs in trypanosome variants 117 and 118. These glycolipids have been designated as region X, Y, and Z GPIs. Region X and Y GPIs are PI-PLC-sensitive, contain galactosylated glycans similar to those present on the GPI anchor of VSG 117, and have sn-1,2-diacylglycerol as their lipid moiety. The glycan moiety of these GPIs appears to be chromatographically indistinguishable from the heterogenous glycan present on the VSG 117 GPI-anchor. It is likely that the arrangement of the galactose residues on the GPIs is also identical to that of the VSG GPI anchor but limiting amounts of material ($<$50 pmol/10^{11} cells) preclude a complete structural analysis. Region Z GPI is PI-PLC-resistant, and may be monogalactosylated P3, however, the arrangement of the galactose residue on this GPI species was not determined due to limiting amounts of material. These galactose-containing GPIs are not degradation products of the VSG-attached GPI anchor, since their synthesis is unaltered when protein synthesis is inhibited by cycloheximide treatment[3] and they can be synthesized *in vitro* from radiolabeled sugar nucleotides and endogenous lipid.

A number of α-galactose-containing GPIs, such as the lipophosphoglycan and the glycoinositolphospholipids (GIPLs) in *Leishmania* (Rosen *et al.*, 1989; Turco *et al.*, 1989; McConville *et al.*, 1990a, 1990b), have been characterized in protozoan parasites. They are mainly cell-surface glycoconjugates and contain variously galactosylated versions of the core glycan Gal$f\alpha$1–3Manα1–4GlcNα1–6Inos, but do not contain ethanolamine. Another example of a major galactosylated GPI is the lipopeptidophosphoglycan from *Trypanosoma cruzi*

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[3] S. Mayor, unpublished observations.

which lacks ethanolamine but contains the sequence Manα1-2Manα1-6Manα1-GlcNα1-6Inos, common to the conserved core glycan present on all characterized GPI anchors (Previato *et al.*, 1990). In contrast the galactose-containing GPIs described here are ethanolamine-containing minor GPIs in *T. brucei*. The location of the ethanolamine residue on the GPIs has yet to be determined but it is likely that these lipids also contain the sequence, EtN-PO$_4$-Manα1-2Manα1-6Manα1-GlcNα1-Inos, common to P2 and P3 (as well as PP1, a GPI that resembles lyso-P3 present in the insect-stage trypanosome; Field *et al.*, 1991). This is supported by the observation that these GPIs have a common biosynthetic route that is inhibited to the same extent by 2-fluoro-2-deoxyglucose, an inhibitor of GPI biosynthesis *in vivo* (Schwarz *et al.*, 1988).[4]

Although the direct precursors of the galactose residues are not known, our data show that UDP-Gal or a UDP-Gal-derived donor is involved in the addition of the galactose residues *in vitro*. We have been unable to incorporate [^{3}H] glucose or [^{3}H]galactose into these glycolipids *in vivo* because these sugar molecules are not efficiently taken up into the cell and metabolized into sugar donors or because a large pool of galactose or UDP-Gal exists inside the cells.

In a previous paper we raised the possibility that galactosylated GPI species were the immediate precursors of the GPI membrane anchor of VSG (Mayor *et al.*, 1990b). Data presented here suggest that galactosylation of P2 is not necessary for efficient transfer of the GPI to protein *in vitro*. However, the data do not rule out the possibility that galactosylated forms of P2 are transferred to VSG and then rapidly degalactosylated following transfer *in vivo*, similar to transfer of glucosylated dolichol-linked oligosaccharides to protein in glycoprotein biosynthesis (Turco *et al.*, 1977).

The biosynthesis of a VSG (ILTat 1.3) containing a galactosylated GPI anchor has been studied (Bangs *et al.*, 1986; Bangs *et al.*, 1988), and the data presented suggest that some or all of the galactose residues are added after attachment of the glycolipid ($t_{1/2}$ for galactose addition ~15 min compared to ~1-2 min for glycolipid attachment). The detection of free galactosylated GPIs is not inconsistent with these observations. Considering the lack of stimulation of UDP-[^{3}H]Gal-labeled GPIs synthesized *in vitro* by the *de novo* synthesis of GPIs, it is possible that galactosylation takes place in a subcellular location (*e.g.* Golgi membranes) that is not the site of GPI synthesis. The formation of galactosylated GPIs may be due to the transport of P2 and P3 from the presumed site of synthesis, the ER, to the site(s) of galactosylation *in vivo*. This would account for the presence of limited amounts of nongalactosylated GPIs in the vicinity of the galactosyl transferase *in vitro*. Two observations support this hypothesis: i) GPIs synthesized *de novo in vitro* are inefficiently galactosylated; the only products appear to be monogalactosylated P2 and P3. ii) When membranes are prepared from [^{3}H]GlcN-labeled trypanosomes and incubated with Mn^{2+} and UDP-Gal at 37 °C, the *in vivo* labeled GPIs are efficiently galactosylated presumably because some free labeled GPIs are transported from the ER to the site of galactosylation during the *in vivo* labeling period. Transport of GPI anchor precursors from the ER in other systems may generate free GPIs that have cell-type-specific side-chain GPI-anchor modifications. In support of this hypothesis is the detection of minor ethanolamine-containing, nitrous acid, and GPI-PLD-sensitive lipids more polar than PP1 in procyclic trypanosomes (Field *et al.*, 1991), where the GPI anchor of the procyclic acidic

repetitive protein appears to be extensively modified.[5]

Mn^{2+}-dependent galactosyltransferase activities have been localized to isolated Golgi fractions in *T. brucei* (Grab *et al.*, 1984). The synthesis of galactosylated GPIs in the crude membrane preparation used here may reflect the extent of mixing of different subcellular compartments; however, since these GPIs are also synthesized *in vivo* they are authentic products of the appropriate galactosyltransferases. Further characterization involving fractionation of the membrane preparation will be necessary to confirm the intracellular location of the specific α-galactosyltransferases and the site of GPI synthesis.

VSGs, classified by the carboxyl-terminal amino acid residue (Asp, Ser, or Asn) of the mature polypeptide and carboxyl-terminal peptide homology, contain a subclass-dependent number of galactose residues attached to the GPI anchor (Holder, 1985). For example, VSG 117 (a class I variant) contains 0-5 galactose residues, VSG 221 (a class II variant) contains up to 8 galactoses, while VSG 118 (a class III variant) contains only trace amounts of galactose (Ferguson *et al.*, 1988a; Mayor *et al.*, 1990b). In VSG 117 the galactose residues are arranged in a branched structure attached to the mannose adjacent to the glucosamine, with >60% of the structures containing 3 or 4 residues (Ferguson *et al.*, 1988a). VSGs form a dense coat on the surface of the trypanosome, and this serves as a macromolecular diffusion barrier for the parasite (Ferguson and Homans, 1988). The predicted three-dimensional structure of the GPI suggests that it could make a significant contribution to the integrity of the surface coat as a diffusion barrier (Ferguson *et al.*, 1988b). Since the level of galactosylation correlates with subclass it may be that the galactose residues play a space-filling role to accommodate the differences in the three-dimensional structures of the different carboxyl-terminal domains. This may allow more efficient packaging of the VSG anchor at the cell surface. Our data show that both class I (117) and class III (118) VSG-expressing trypanosomes have comparable levels of galactosylated GPIs *in vivo* as well as comparable galactosyl transferase activities *in vitro*. This suggests that the extent and heterogeneity of galactosylation of an individual GPI anchor reflects the accessibility of the relevant α-galactosyltransferases. This feature of GPI anchors is unique to trypanosomes and the interruption of galactosylation could compromise the integrity of the surface coat, providing a useful target for drugs based on compounds that selectively inhibit galactosylation of trypanosome GPIs. Investigation of the specific galactosyltransferases may be facilitated by the observation that P2 is a substrate for the transferases and this could provide a convenient functional assay for these enzymes.

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SUPPLEMENTAL MATERIAL TO
Galactose-containing Glycosylphosphatidylinositols in *Trypanosoma brucei*
by
Satyajit Mayor@, Anant K. Menon and George A. M. Cross

EXPERIMENTAL PROCEDURES

Materials—D-[6-³H]GlcN (35 Ci/mmol), D-[2-³H]glucose (23 Ci/mmol), D-[6-³H]galactose (30 Ci/mmol), D-[6-³H]Man (35 Ci/mmol), [9,10-³H]palmitic acid (55.2 Ci/mmol) and [1-³H]ethanolamine hydrochloride (29.8 Ci/mmol) were purchased from Amersham Corp. Uridine diphosphate D-[4,5-³H]galactose (38.5 Ci/mmol), guanosine diphosphate D-[3,4-³H]Man (16 Ci/mmol), and [9,10-³H]myristic acid (22.4 Ci/mmol) was purchased from Du Pont-New England Nuclear. Lipid standards were from Sigma Chemical Co. PI-PLC from *Bacillus thuringiensis* was a gift from Dr. M. G. Low (Columbia University) and rabbit serum (used as a source of GPI-specific -phospholipase D, GPI-PLD, (Davitz *et al.*, 1987; Low and Prasad, 1988) was a gift from Mr. A. Zychlinksy (Rockefeller University). All solvents were either reagent- or HPLC-grade. Ion-exchange resins were purchased from Bio-Rad or Pharmacia. All solvents were analytical or high performance liquid chromatography (HPLC) grade.

Extraction and Purification of Biosynthetically Labelled Glycolipids—Rats were inoculated with trypanosome variants of the Molteno Institute Trypanozoon antigenic type (MITat) 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 labelled and glycolipids were extracted into butanol as described previously (Mayor and Menon, 1990).

Solvent Systems for Thin Layer Chromatography—All solvent mixtures are given as volume ratios. *a* : chloroform/methanol/H₂O, (10: 10: 2.7), *b* : petroleum ether/diethyl ether/acetic acid (70:30:1), c : chloroform/methanol/90 % formic acid (50:30:7).

Generation of Biosynthetically Labelled Glycans for HPLC analysis—Glycolipids (total butanol extracts from biosynthetically labelled trypanosomes or TLC-purified labelled glycolipids) were treated with cold aqueous HF and after 48 h the reaction was neutralized as described (Mayor and Menon, 1990). The neutralized aqueous solution was readjusted to pH 4.0, extracted twice with an equal volume of water-saturated n-butanol and the butanol-rich upper phase and the aqueous-rich lower phase were separated. The aqueous phase was desalted by passing over 0.4 ml AG3X4A(OH⁻) resin, and the eluate and washings were pooled and taken for deamination and reduction or N-acetylation (Mayor and Menon, 1990). Before deamination and reduction or N-acetylation, the butanol phase was treated with NH₃/methanol (1:1) for 3-12 h at ambient temperature. After N-acetylation or deamination followed by reduction the samples were desalted by passing them over a tandem ion-exchange column consisting of 0.2 ml Chelex100(Na⁺), over 0.4 ml AG50WX12(H⁺). In each case the eluate and washings were pooled and dried, and the residue flash evaporated with 3 x 50 µl toluene, resuspended and filtered through a 0.2 µm filter and stored at -20 °C until required for analysis.

Anion Exchange HPLC Analysis of Labelled Glycans—Desalted glycans were analysed by anion exchange chromatography on a Dionex Basic Chromatography System (Dionex Corporation, Sunnyvale, CA, USA). The separation was accomplished by gradient elution using a HPIC AS6 column preceeded by a guard column (HPIC AG6) filled with the same ion-exchange matrix. Gradient elution program ; 100 % Buffer A (0.1 M NaOH), 0 % Buffer B (0.1 M NaOH, 0.5 M NaOAc) up to 6 min after injection, then an increase of Buffer B to 15 % at 36 min. The eluant was neutralized by an anion micromembrane suppressor (Dionex Corp.) with 0.1 M H₂SO₄ as the counter flow regenerant. Fractions were collected directly into scintillation vials and the radioactivity was detected by liquid scintillation counting. Neutral glycan standards derived from VSG membrane anchors were included in each run and detected using pulsed amperometric detection (Dionex Corp.).

VSG GPI-anchor Glycan Standards—Neutral glycan standards corresponding to the glycan portion of the membrane anchor of VSGs were prepared from purified soluble form VSG (sVSG) from *T. brucei* variants 117 and 118 as described previously (Mayor *et al.*, 1990b). The glycan portion of the membrane anchor from sVSG 118 has been shown to be identical to the de-galactosylated, deaminated, reduced and dephosphorylated glycan from VSG 117 (Mayor *et al.*, 1990b). The neutral glycans were deaminated and reduced or N-acetylated as described above to generate the GalnMan₃AHM or GalnMan₃GlcNAcInos standards

respectively, where n=0 for the glycan generated from sVSG 118 (Mayor *et al.*, 1990b) and n=2-5 for the neutral glycans generated from sVSG 117 (Ferguson *et al.*, 1988a). Radiolabelled VSG GPI-anchor glycans were generated in a similar fashion except that the deaminated glycans were reduced with NaB³H₄ and purified as described previously (Ferguson *et al.*, 1988a).

Galactosidase Digestion of Neutral Glycans—Neutral glycans were generated as above and resuspended in 20 µl 0.2 M NaOAc, pH 5.0 containing coffee bean α-galactosidase (60 U/ml, Boehringer Mannheim) and incubated for 12-16 h at 37 °C The galactosidase digestion was terminated by heating the reaction at 100 °C for 1 min and the digest was passed over a 0.2 ml column of AG50WX12(H⁺). The column eluate and washings were pooled, filtered and analysed by anion exchange HPLC chromatography.

Phospholipase Treatments—Biosynthetically labelled, purified lipids were resuspended in 100 µl PI-PLC buffer (0.1 M Tris-HCl pH 7.4, 0.1 % Na deoxycholate) containing 2 µg *Bacillus thuringiensis* PI-PLC. The reaction was carried out for 2 h at 37 °C and terminated by adding 100 µl 0.1 M HOAc. The reaction mixtures were either extracted first with toluene and then with water-saturated n-butanol (for [³H]myristic acid-labelled lipids), or directly extracted with water-saturated n-butanol (for [³H]EtN and UDP-[³H]Gal-labelled lipids). The toluene extracts were chromatographed on Kieselgel-60 TLC plates along with lipid standards using solvent system *b*. The butanol phases were chromatographed on Kieselgel-60 plates using solvent system *a*.

GPI-PLD hydrolysis was carried out on TLC-purified lipids. The lipids were resuspended in 100 µl PI-PLC buffer containing 2.5 mM CaCl₂ and incubated at 37 °C for 2-4 h with 2 µl rabbit serum (source of GPI-PLD activity, (Davitz *et al.*, 1987; Low and Prasad, 1988)). The incubation was terminated by adding 100 µl 0.1M HOAc and the reaction products were extracted twice with 200 µl of water saturated butanol. The butanol phases were combined and chromatographed on Kieselgel-60 TLC using solvent system *a* or solvent system *c*.

In Vitro GPI Synthesis—Membranes were prepared from bloodstream trypanosomes as described (Mayor *et al.*, 1991). Reactions contained 5 x 10⁷ cell equivalents of dispersed membranes in a total volume of 100 µl of assay buffer (25 mM K-HEPES, pH 7.4, 75 mM KOAc, 5 mM MgOAc, 1 mM CoA, 200 ng/ml tunicamycin, 1 mM ATP, 10 mM creatine phosphate, 8 U/ml creatine kinase). 5 mM MnOAc was included in all reactions unless specifically mentioned otherwise. Radiolabelled precursors (1-2 µCi of GDP-[³H]Man or 5-10 µCi of UDP-[³H]Gal) were added to initiate the reaction and samples were incubated with millimolar amounts of the indicated unlabelled sugar nucleotides at 37 °C in a water bath. The *in vitro* synthesized GPI species were extracted as described earlier (Mayor *et al.*, 1991) and analysed by TLC on Kieselgel-60 plates (Merck) using solvent system *a*. The radioactivity was detected by a Berthold TLC scanner (LB-2842) and quantitated using the software provided by the manufacturer (Berthold Analytical Instruments, Inc.). Labelled protein was detected by SDS-PAGE followed by fluorography and the VSG-associated radioactivity was analysed as described (Mayor *et al.*, 1991).

RESULTS

Glycolipids in Region X and Y are GPI-lipids Containing sn-1,2 diacylglycerol—[³H]Ethanolamine-labelled region X and Y lipids were purified by TLC and the labelled glycolipids (1000 cpm) were incubated in PI-PLC buffer without or with *B. thuringiensis* PI-PLC. The reaction mixture was then partitioned between water and butanol and the butanol phase analysed as described in Fig. 2. All the radioactivity associated (>95%) with [³H]ethanolamine-labelled lipids in region X and Y could be released into the aqueous phase after treatment with PI-PLC (data not shown). Identical results were obtained with [³H]Man-labelled lipids (data not shown).

Table 1 shows that PI-PLC treatment of [³H]myristic acid-labelled region X and Y lipids generated lipid species that were soluble in the toluene phase. Thin layer chromatographic analysis (Kieselgel-60 TLC plates using solvent system *b*) of this phase showed that the toluene-soluble radioactivity co-migrated with sn-1,2-dimyristiylglycerol identical to the lipid species released from [³H]myristic acid-labelled P2 (data not shown). These results suggest that the lipid portion of the glycolipids in region X and Y is sn-1,2-diacylglycerol.

Glycolipid	PI-PLC Treatment	Total radioactivity (cpm)	Radioactivity (%) recovered # in	
			Toluene phase	Butanol phase
P2	—	4750	9.3%	90.7%
P2	+	3930	88.0%	12.0%&
Region X	—	4800	4.2%	95.8%
Region X	+	6600	78.7%	21.3%&
Region Y	—	2270	10.6%	89.4%
Region Y	+	3320	76.5%	23.5%&

After PI-PLC treatment the reaction mixture was sequentially extracted with toluene and water-saturated butanol as described in Experimental Procedures, an aliquot of each phase was taken for liquid scintillation counting and the remaining was analysed by TLC (see text).
& TLC analyses of the butanol phases of the PI-PLC-treated [3H]myristic acid-labelled-region X and Y lipids and P2 showed the presence of contaminating amounts of [3H]myristic acid-labelled-P3, present in the starting material (data not shown).

Rabbit serum contains a GPI-specific phospholipase D (GPI-PLD (Davitz et al., 1987; Low and Prasad, 1988)) that releases dimyristylphosphatidic acid from P2 and P3 (Mayor et al., 1990a). In order to show that region X and Y glycolipids were susceptible to this enzyme [3H]Myristic acid-labelled region X and Y lipids (5000 cpm each) were purified and incubated in PI-PLC buffer containing CaCl2 with rabbit serum or without any additions and after acidification the products were extracted into butanol and chromatographed along with lipid standards on Kieselgel-60 TLC plates using solvent system a, as described in Fig. 2. The phospholipase D treatment generated a product (>95 % of the starting material) in each case that quantitatively co-chromatographed with dimyristylphosphatidic acid (data not shown). Analyses of the GPI-PLD generated products by a separate TLC system (Kieselgel 60, solvent system c) confirmed that dimyristylphosphatidic acid was released from [3H]myristic acid-labelled-region X and Y lipids and P2 (data not shown). Taken together the above data suggest that the glycolipids in region X and Y are PI-PLC sensitive GPIs that contain lipid moieties identical to P2.

Galactosidase Sensitive Neutral Glycans Generated from Tunicamycin Resistant Polar Lipids—Glycolipids were isolated from [3H]Man-labelled trypanosomes (variant 117) and neutral glycans were prepared by previously described procedures (Mayor and Menon, 1990; Mayor et al., 1990b). The butanol phase extracts were dephosphorylated with aqueous HF and, following neutralization, the reaction products were partitioned between butanol and water. The radioactivity that partitioned into the aqueous phase was either N-acetylated or deaminated and reduced. The butanol-soluble radioactivity was first treated with mild base (NH3/MeOH; 1:1, v/v) before being either N-acetylated or deaminated and reduced. The neutral glycans were chromatographed on anion-exchange HPLC along with N-acetylated or deaminated and reduced neutral glycan standards prepared from VSG 117 and 118.

Anion-exchange HPLC (Fig. 7) showed that the aqueous-soluble radioactivity contained a major biosynthetically labelled glycan moiety (80-90%) and minor glycan moieties (10-20 %). The major species co-chromatographed with the non-galactosylated core VSG glycan (Man3GlcNAcInositol or Man3AHM; Figs 3 a and c) while the minor species co-chromatographed with identically prepared glycan standards from VSG 117 (Fig. 7 a and c), suggesting that these minor glycans species were derived from galactosylated GPIs. These minor species were susceptible to α-galactosidase digestion (Fig 7, b and d) but all the digested product (Fig. 7, b and d; peaks marked by *) did not co-chromatograph with the non-galactosylated core VSG glycan. This has been also observed for α-galactosidase-treated VSG 117 glycans (see Fig. 3 and Fig. 8).

Anion-exchange HPLC analysis of the deaminated and reduced material recovered in the butanol phase of neutralized HF dephosphorylation reaction, showed that, in addition to the P3-derived hydrophobic glycan (Mayor et al., 1990b), small amounts of galactosylated glycans (< 15% of that present in the aqueous phase) were also present. These species chromatographed with a retention time of ~21 min, close to the Gal1Man3AHM standard (data not shown). Further analysis of this species was not carried out due to the low abundance of material. This 'hydrophobic' glycan is probably derived from the glycolipid that chromatographs between P2 and P3 (Fig. 2, region Z). This glycolipid was susceptible to GPI-PLD but resistant to PI-PLC and could be labelled with [3H]palmitic acid (data not shown). These data suggest that a mono-galactosylated P3-like molecule is synthesized *in vivo*.

Galactosylated GPIs of identical structure were also obtained in similar yield from [3H]GlcN-labelled variant 118 trypanosomes, where the mature VSG-linked GPI anchor is not galactosylated (see Fig. 3).
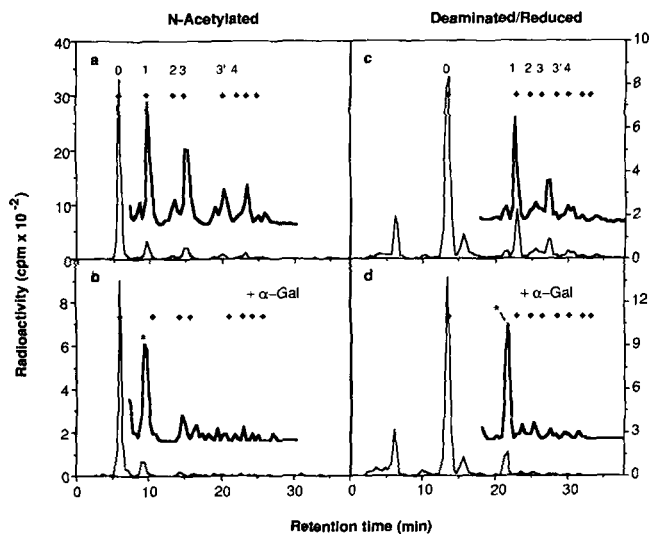

Aqueous Phase Glycans **Butanol Phase Glycans**

Retention time (min)

Figure 8. Anion-exchange HPLC analyses of neutral glycans generated from UDP-[3H]Gal-labelled glycolipids synthesized *in vitro*.:

Glycolipids were labelled via UDP-[3H]Gal *in vitro* (5 x 10^8 cell equivalents of trypanosomes, variant clone 117) as described in Fig.6 and extracted into butanol. An aliquot (1000 cpm) of the butanol-phase was analysed by TLC and the remainder (~6000 cpm) was HF-dephosphorylated, neutralized, phase separated. The radioactivity partitioning into the aqueous (A, B) and butanol (C, D) phases was deaminated and reduced and an aliquot (2/3) analysed by anion-exchange HPLC as described in Fig. 7. The remainder of each sample (1/3) was treated with α-galactosidase, prior to analyses by anion exchange HPLC (+α-Gal; B, D). The standards indicated at the top of each panel were included in each run and correspond to the deaminated and reduced neutral glycans from purified 117 and 118 VSGs as described in Fig. 7. i, galactitol; ii, galactose

N-Acetylated **Deaminated/Reduced**

Retention time (min)

Figure 7. Anion-exchange HPLC analyses of neutral glycans generated from [3H]Man-labelled glycolipids purified from variant 117 trypanosomes.

[3H]Man-labelled glycolipids were HF-dephosphorylated, neutralized, phase separated and the radioactivity partitioning into the aqueous phase was either N-acetylated (a, b) or deaminated and reduced (c, d). An aliquot (1/2-1/3) of each sample was treated with α-galactosidase (+α-Gal; b, d) prior to analyses by anion exchange HPLC. The standards indicated at the top of each panel were included in each run and correspond to the heterogenously galactosylated neutral glycans from the membrane anchor of purified VSGs, i. e. GalnMan3(2,5-anhydromannitol) generated via deamination and reduction (in panels a and b) or GalnMan3GlcNAcInos generated via N-acetylation (in panels c and d) of the HF-dephosphorylated glycans from purified 117 and 118 VSGs, where n=0, 1, 2, 3 and 4 (indicated as 0, 1, 2, 3, 3' and 4). 3 and 3' correspond to the two possible isomers of the Gal3 structures (Ferguson et al., 1988a). The additional symbols (*) indicate the retention times of uncharacterized galactosylated glycans derived from VSG 117 GPI-anchor3. Insets show the corre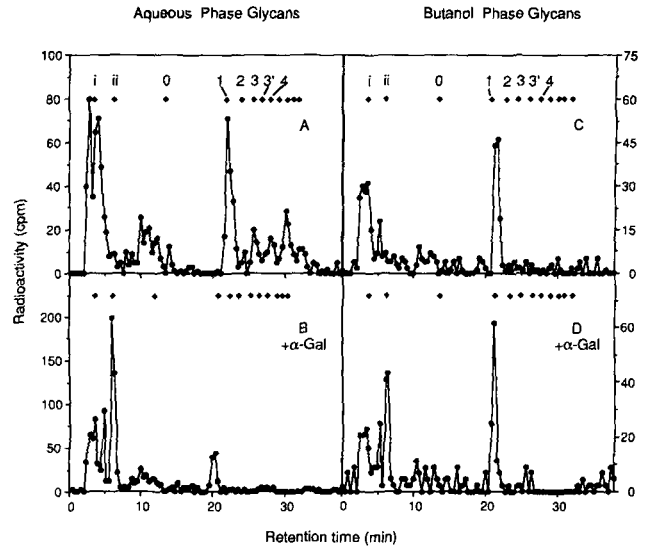sponding regions on a ~2.5 times expanded scale.