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Developmental Variation of Glycosylphosphatidylinositol Membrane Anchors in *Trypanosoma brucei*

IDENTIFICATION OF A CANDIDATE BIOSYNTHETIC PRECURSOR OF THE GLYCOSYLPHOSPHATIDYLINOSITOL ANCHOR OF THE MAJOR PROCYCLIC STAGE SURFACE GLYCOPROTEIN*

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The major surface antigen of the mammalian bloodstream form of *Trypanosoma brucei*, the variant surface glycoprotein (VSG), is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor. The VSG anchor is susceptible to phosphatidylinositol-specific phospholipase C (PI-PLC). Candidate precursor glycolipids, P2 and P3, which are PI-PLC-sensitive and -resistant respectively, have been characterized in the bloodstream stage. In the insect midgut stage, the major surface glycoprotein, procyclic acidic repetitive glycoprotein, is also GPI-anchored but is resistant to PI-PLC.

To determine how the structure of the GPI anchor is altered at different life stages, we characterized candidate GPI molecules in procyclic *T. brucei*. The structure of a major procyclic GPI, PP1, is ethanolamine-PO_4 - Manα1 - 2Manα1 - 6Manα1 - GlcN - acylinositol, linked to lysophosphatidic acid. The inositol can be labeled with [^{3}H]palmitic acid, and the glyceride with [^{3}H]stearic acid. We have also found that all detectable ethanolamine-containing GPIs from procyclic cells contain acylinositol and are resistant to cleavage by PI-PLC. This suggests that the procyclic acidic repetitive glycoprotein GPI anchor structure differs from that of the VSG by virtue of the structures of the GPIs available for transfer.

Many membrane proteins are now known to be tethered to lipid bilayers by the covalent attachment of a glycosylated phosphatidylinositol (GPI)[1] of novel structure to the carboxyl terminus of a protein via an ethanolamine-phosphate bridge (Ferguson and Williams, 1988; Cross, 1990; Thomas *et al.*, 1990). This modification has been demonstrated in a wide variety of eucaryotic species (Cross, 1990). Despite a large amount of speculation, it has been difficult to substantiate specific reasons for using GPI anchors. Some evidence for a vectorial role in membrane protein trafficking has been reported (Lisanti *et al.*, 1988).

The complete structures of GPI anchors from a small number of proteins and several related glycoconjugates have been reported. Partial information is available for several more proteins. Protein-linked GPIs contain a conserved glycan, Man_3GlcN, linking ethanolamine to the inositol phospholipid (see Thomas *et al.* (1990) for a recent summary of GPI structures). In the mammalian bloodstream stage of the protozoan parasite *Trypanosoma brucei*, the structure of the GPI anchor on the abundant variant surface glycoprotein (VSG, Ferguson *et al.*, 1988) and the structure of two glycolipids, P2 and P3, with the expected structural properties of GPI anchor precursors, have been determined (Krakow *et al.*, 1986; Mayor *et al.*, 1990a, 1990b; Menon *et al.*, 1988). P2 and P3 contain the Man_3GlcNinositol core glycan identified in the VSG GPI anchor coupled to ethanolamine phosphate at the nonreducing terminus (Mayor *et al.*, 1990a) and dimyristoylglycerol (Mayor *et al.*, 1990b). The sole difference between P2 and P3 is the additional attachment of palmitic acid to inositol in P3 (Mayor *et al.*, 1990b), which is similar to a modification seen in the GPI anchor of human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988). The presence of this fatty acid is responsible for the PI-PLC resistance of both P3 and the human erythrocyte acetylcholinesterase (Mayor *et al.*, 1990b; Roberts *et al.*, 1988). The variable α-galactose branch, which is observed as part of the GPI anchor of some VSGs (Ferguson *et al.*, 1988), is absent from P2 and P3 and is probably constructed once the GPI moiety is attached to the protein (Bangs *et al.*, 1988).

It has been proposed that the mature VSG anchor is constructed by sequential glycosylation of PI, followed by addition of ethanolamine phosphate and remodeling of the glyceride fatty acids. In support of this model, a number of glycosylPI lipids with 0–3 mannose residues have been identified (Masterson *et al.*, 1989; Menon *et al.*, 1990a). The glucosamine on glycosylPI originates from UDP-GlcNAc (Doering *et al.*, 1989), whereas the three mannose residues are derived from mannose-phosphoryldolichol (Menon *et al.*, 1990b). Both fatty acids in P2 and P3 are remodeled to myristate; the substituent originally at the C1 position has been identified as stearic acid (a common fatty acid in this position in mammalian PI), but that on the C2 position is unknown (Masterson *et al.*, 1990; reviewed in Doering *et al.*, 1990). The lipid is then probably transferred *en bloc* to the nascent VSG polypeptide within the endoplasmic reticulum

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[1] The abbreviations used are: GPI, glycosylated phosphatidylinositol; AHM, 2-anhydromannitol; GPI-PLD, glycosylPI-specific phospholipase D; HPLC, high performance liquid chromatography; NAhg, nitrous acid-released headgroup; PARP, procyclic acidic repetitive protein (procyclin); PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; VSG, variant surface glycoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPTLC, high performance TLC; PLA_2, phospholipase A_2. All monosaccharide residues reported in this paper are in the pyranose D configuration.

shortly after the polypeptide is synthesized (Bangs *et al.*, 1985; Ferguson *et al.*, 1986).

In the bloodstream form of *T. brucei*, the VSG GPI anchor is exclusively of the PI-PLC-sensitive P2 type (Ferguson *et al.*, 1988). In the procyclic (insect midgut form) trypanosome, VSG is not expressed, but the major cell surface protein, the procyclic acidic repetitive protein (PARP, Roditi *et al.*, 1989), has the properties of a PI-PLC-resistant GPI-anchored protein (Clayton and Mowatt, 1989).

The occurrence of different GPI anchor structures on two developmentally restricted proteins expressed in the same organism clearly raises the question of how the structure of the anchor is controlled. The biosynthetic relationship between P2 and P3 is not known; some evidence suggests that acylation of the inositol may occur early in the pathway, as glycosylacylinositol species without ethanolamine and with only 2 or 3 mannose residues have been identified (Masterson *et al.*, 1989; Menon *et al.*, 1990). The question of whether both lipids are effective substrates for a putative glycolipid-polypeptide transferase *in vivo* has not been addressed. If P3 can be transferred to VSG *in vivo*, it must subsequently be deacylated. A second possibility is that the structure of the GPI anchor that is added is in some way specified by the structure of the polypeptide itself, so that VSG will accept only P2-type anchors, and PARP only P3-type.

To determine which mechanism operates in the procyclic trypanosome, it is necessary to determine the structures of potential precursors and the mature GPI anchor of PARP. In this paper, we report the structure of a novel GPI species in procyclic trypanosomes.

EXPERIMENTAL PROCEDURES

Trypanosomes—Culture-adapted procyclic *T. brucei* strain 427 were grown at 27 °C in SDM-79 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and 7.5 μg/ml hemin (Brun and Schonenberger, 1979). Cells were maintained at a density of 5×10^5–1×10^7 parasites/ml.

Metabolic Labeling—Cells were harvested, washed three times with phosphate-buffered saline, pH 7.5, and resuspended in labeling medium at 1–2 × 10^7 parasites/ml. For [^{3}H]ethanolamine labeling, cells were resuspended in fresh SDM-79, 10% fetal calf serum, 7.5 μg/ml hemin, with 25 μCi/ml [1-^{3}H]ethanolamine (3–30 Ci/mmol, Amersham Corp.). For sugar labeling, cells were resuspended in glucose-free RPMI 1640 (Speciality Media Inc.) supplemented with 7.14 mg/ml HEPES, 0.6 mg/ml sodium citrate, 0.6 mg/ml L-proline, pH 7.3, and 1 mg/ml defatted bovine serum albumin (Boehringer Mannheim), 7.5 μg/ml hemin; with 40 μCi/ml D-[6-^{3}H]glucosamine (20–40 Ci/mmol, Amersham Corp.); or with 40 μCi/ml D-[2,6-^{3}H]mannose (30–60 Ci/mmol, Amersham Corp.). Labeling with myristic acid was performed in SDM-79 with 7.5 μg/ml hemin and 0.5 mg/ml bovine siderophilin (Sigma). [^{3}H]Myristic acid (10–60 Ci/mmol, Du Pont-New England Nuclear) was coupled 1:1 with defatted bovine serum albumin and added to the culture at 200 μCi/ml. Labeling was performed in each case for 4–16 h, cells were harvested and washed three times with cold phosphate-buffered saline, and lipids were then extracted. For the sugar labeling experiments, it was necessary to remove adherent cells from the culture vessel with a rubber pestle. In experiments where tunicamycin was used, cells were preincubated with the inhibitor at 1–2 μg/ml for at least 1 h before the addition of radiolabel. Cells were inspected under a light microscope following labeling to assess viability.

Lipid Extractions—Lipids were extracted into two separate fractions as described previously (Menon *et al.*, 1990a). The first extract, obtained with chloroform/methanol (2:1) (v/v) (extract 1), contains the phospholipid species together with less polar GPI lipids, whereas the second extract, obtained with chloroform/methanol/water (10:10:3) (v/v/v) (extract 2), contains the more polar ethanolamine-containing GPI lipids. Folch washing of extract 1 and partitioning of extract 2 between butanol and water was performed as described previously (Menon *et al.*, 1990a).

Enzyme and Chemical Treatments—Nitrous acid deamination, sodium hydroxide/methanol base hydrolysis, ammonia/methanol (mild) base hydrolysis, sodium borohydride reduction, aqueous 50% HF phosphate ester hydrolysis, and enzyme digestions with GPI-phospholipase D (rabbit serum), *Bacillus thuringiensis* PI-PLC (gift of Dr. M. Low, Columbia University, New York), *Crotalus adamanteus* phospholipase A_2 (Sigma), and *Canavalia ensiformis* α-mannosidase (Sigma) were all performed as described previously (Mayor *et al.*, 1990a, 1990b). Where necessary, reaction products were partitioned between water and water-saturated butanol. The reaction mixture was acidified with 10 μl of glacial acetic acid and made up to a total volume of 0.5 ml with distilled water. The sample was then extracted twice with 0.5 ml of water-saturated butanol, and the organic phases were pooled.

Monosaccharide linkage analysis was performed as described previously (Ciucanu and Kerek, 1984; Mayor *et al.*, 1990a). After TLC of the partially methylated mannose residues, the radioactivity was visualized by spraying the plate with EN^{3}HANCE (Du Pont), followed by fluorography.

Mild acid hydrolysis to release the glycans from oligosaccharyl-PP-dolichol lipids was achieved by incubating the sample in 500 μl of 2 M HCl with 500 μl of propanol-1 at 55 °C for 15 min. The sample was then dried and the products were partitioned between water and butanol. Released glycans were recovered in the aqueous phase. Generation of fatty acid methyl esters was achieved by acid methanolysis. The sample was dried in a Reacti-Vial (0.3 ml, Pierce Chemical Co.) and flash-evaporated with methanol (three times). The sample was methanolyzed in 0.5 M methanolic HCl (Supelco) at 70 °C for 16 h and was then dried in a Speedvac and resuspended in a small volume of hexane before chromatography.

Chromatography—All thin layer solvent compositions are given as v/v. Separation on thin layer plates was performed with glass-backed Si60 Silica plates (Merck) in chloroform/methanol/water (10:10:3) (system 1), chloroform/methanol/water (4:4:1) (system 2), chloroform/methanol/acetic acid/water (25:15:4:2) (system 3), chloroform/methanol/water (65:25:4) (system 4), or with glass-backed Si50000 Silica plates (Merck) in chloroform/methanol/30% ammonia/1 M ammonium acetate/water (90:70:4.5:4:11.5) (system 5). Reverse-phase TLC was performed with HPTLC RP18 plates (Merck) in methanol/acetonitrile (1:1) (system 6). Ethanolamine was demonstrated not to have been interconverted by total hydrolysis of extracted lipids in 6 M HCl at 100 °C and chromatography in butanol-1/pyridine/0.1 M HCl (100:60:40) on plastic-backed MN300 cellulose plates (Merck) (system 7). After chromatography, the plates were air-dried and scanned with a Berthold LB 2842 linear scanner. For visualization of phosphatidic acid standards, plates were sprayed with molybdenum blue reagent (Sigma) following radioactivity scanning. Fatty acid methyl esters were visualized by spraying the TLC plate with 10% phosphomolybdic acid (in ethanol) and heating the plate at 110 °C for 10 min. Where appropriate, lipids were recovered from the TLC plate by scraping off the relevant region and extracting three times with chloroform/methanol/water (10:10:3), followed by water-saturated butanol. In the case of the acylated lipid headgroup from PP1 (see below), the silica was extracted with chloroform/methanol/30% ammonia/1 M ammonium acetate/water (90:70:4.5:4:11.5). Combined extracts were dried and resuspended in water-saturated butanol. Anion exchange HPLC (Dionex) was performed using the conditions described by Mayor *et al.* (1990a). Bio-Gel P4 (−400 mesh, Bio-Rad) gel filtration was performed with a 1.5 × 100-cm column eluted with 50 or 100 mM sodium acetate buffer, pH 5.5, at 6 ml/h, except for chromatography of the [^{3}H]ethanolamine-labeled NAhg from PP1 (Fig. 3*A*), for which a 1.0 × 100-cm column, eluted at 3.6 ml/h was used. Anion exchange with Mono-Q (Pharmacia LKB Biotechnology Inc.) was performed as described by Field *et al.* (1989).

Miscellaneous Methods—Protein was assayed with the BCA reagent (Pierce Chemical Co.). Liquid scintillation spectroscopy was performed with ReadySafe scintillation fluid (Beckman). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was as described by Laemmli (1970). After electrophoresis, gels were stained with Coomassie Blue and destained in 40% methanol, 10% acetic acid (v/v) and then impregnated with EN^{3}HANCE (Du Pont) and dried for fluorography. TLC plates for fluorography were sprayed with EN^{3}HANCE reagent. Fluorography was performed at −80 °C with two intensifying screens and Kodak X-Omat AR film. Glycolipid and neutral glycan standards for TLC, Dionex, and Bio-Gel P4 chromatography were prepared from bloodstream trypanosomes as described previously (Mayor *et al.*, 1990a). Some of the glycolipid standards were the kind gift of S. Mayor of this laboratory. Lysophosphatidic acid standards were obtained by digestion of phosphatidic acid standards (Sigma) with *C. adamanteus* PLA_2. Fatty acid methyl ester

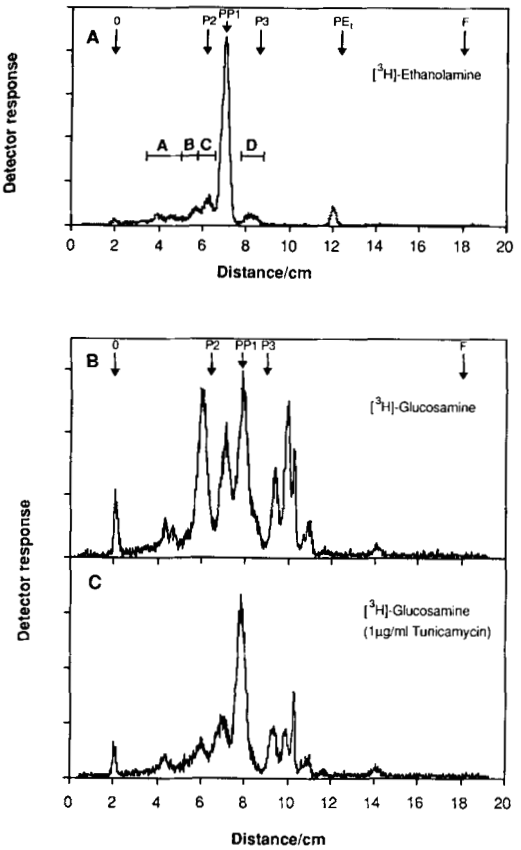

FIG. 1. **Thin layer radiochromatograms of chloroform/methanol/water extracts of metabolically labeled procyclic trypanosomes.** TLC plates developed with system 1 are shown. *A*, [^{3}H]Ethanolamine. [^{3}H]Glucosamine in the absence (*B*) or presence (*C*) of 1 μg/ml tunicamycin is also shown. *P2* and *P3*, mobility of GPIs isolated from bloodstream trypanosomes; PE_T, trypanosome phosphatidylethanolamine; *PP1*, major procyclic GPI; *O*, origin; *F*, front. *Bars A–D* indicate the regions of the chromatogram that were isolated for analysis in Table IV.

standards were from Sigma. Glucose oligomers were obtained by partial acid hydrolysis of dextran (Sigma). ^{3}H-Reduced glucose oligomers were obtained by reduction of the partial dextran hydrolysate with sodium borotritide (12 Ci/mmol, Du Pont-New England Nuclear).

RESULTS

Identification of Glycosylinositol Lipids

When procyclic *T. brucei* cells were metabolically labeled with [^{3}H]ethanolamine, extract 2 (see "Experimental Procedures" for nomenclature) contained a number of species that could be resolved by TLC (Fig. 1*A*). The predominant species (R_f 0.43 on system 1) was designated *PP1* and accounted for about 65% of the radioactivity in this extract (Table I). No significant labeling of glycolipids with the mobilities of P2 or P3 was seen in this extract. [^{3}H]mannose- (data not shown) or glucosamine- (Fig. 1*B*) labeled cells produced a large number of peaks, one of which cochromatographed with [^{3}H] ethanolamine-labeled PP1. When cells were labeled with ^{3}H-sugars in the presence of tunicamycin at 1 μg/ml (observed to be the most effective concentration for selective suppression of synthesis of dol-PP-GlcNAc, without significantly altering the labeling of PP1 or cell viability),[2] the majority of these peaks was lost (Fig. 1*C*), indicating that these species were dol-PP-GlcNAc-containing glycolipids. Tunicamycin at this concentration did not affect the labeling of lipids with [^{3}H] ethanolamine, as demonstrated for P2 and P3 in bloodstream form trypanosomes (Mayor *et al.*, 1990a). Mild acid hydrolysis of the tunicamycin-sensitive [^{3}H]mannose- or glucosamine-labeled lipids released 66–80% of the radiolabel as aqueous-soluble species. Subsequent analysis by Bio-Gel P4 gel filtration indicated that the released glycans had hydrodynamic volumes between 12.5 and 9.2 glucose units (in order of increasing R_f), with identical chromatograms for both sugar labels, which is consistent with their assignment as dol-PP-$GlcNAc_2Man_{6-9}$ species (Table II). These species were not investigated further.

Treatment of [^{3}H]ethanolamine-labeled extract 2 with nitrous acid clearly converted the radiolabel from a lipophilic to an aqueous-soluble form (Table III), suggesting that the ethanolamine was a component of glycosylPI-type compounds (Mayor *et al.*, 1990a). Similar data were obtained with the sugar-labeled extracts (data not shown). Base treatment of the labeled species in extract 2 released the radiolabel as a aqueous-soluble fragment, suggesting that the hydrophobic moieties are fatty acids in ester linkage.

Analysis of extract 1 from the [^{3}H]ethanolamine-labeled cells revealed that the vast majority (>95%) of radioactive incorporation was into phosphatidylethanolamine (alkyl-acyl phosphatidylethanolamine, data not shown). Sugar-labeling identified a spectrum of lipids in extract 1 which were not susceptible to PI-PLC, GPI-PLD, or nitrous acid treatments,

[2] M. C. Field and A. K. Menon, unpublished observations.

TABLE I

Incorporation of radiolabeled compounds into procyclic trypanosome lipids in vivo

Cells were labeled with the indicated compounds under the conditions described in the text. Similar levels of incorporation were observed on different occasions with a culture maintained in the laboratory. Incorporation into PP1 was determined by integration of the radiochromatogram obtained after running an aliquot of extract 2 in system 1 or 2.

^{3}H-Compound	Extract 1 (CM)[a]	Extract 2 (CMW)	PP1
		cpm/10^7 cells	
Ethanolamine	6.2×10^6	1.4×10^4	9.4×10^3
Mannose	1.0×10^5	2.9×10^4	1.2×10^4
Glucosamine	7.3×10^4	1.5×10^4	6.6×10^3
Myristic acid	5.8×10^7	4.4×10^5	1.6×10^4

[a] See "Experimental Procedures" to solvent compositions.

TABLE II

Characterization of glycan fragments from tunicamycin-sensitive glycolipids by Bio-Gel P4 gel filtration

[^{3}H]Mannose- or [^{3}H]glucosamine-labeled glycolipids, identified as tunicamycin-sensitive, were isolated by TLC (system 1) and quantitated by integration of the radiochromatogram obtained with the glucosamine-labeled lipids. The liberated glycans were analyzed on a Bio-Gel P4 column. V_e (relative retention volume) was calculated using coinjected bovine serum albumin and [^{3}H]mannose as V_o and V_t markers. Hydrodynamic volume (HDV) was calculated by reference to the V_e of ^{3}H-reduced glucose oligomers run in a separate experiment. Note that the HDV derived for both sugar labels was the same for each peak analyzed.

Peak	Incidence	R_f	% Released by acid[a]	V_e	HDV/glucose units
1	26.1	0.33	76/74	0.36	12.5
2	16.8	0.37	66/76	0.38	11.8
3 (PP1)	29.4	0.43	ND[b]	ND	ND
4	9.5	0.48	80/88	0.42	10.7
5	18.2	0.52	78/72	0.48	9.2

[a] The first number refers to glucosamine and the second to mannose-labeled lipids.

[b] ND, not determined.

TABLE III

Cleavage of ^{3}H-labeled PP1 with lipases, and nitrous and hydrofluoric acids

All experiments were performed with ethanolamine-labeled PP1, except the HF cleavage, which was done with mannose-labeled material. The purified lipid was treated with the indicated reagents as detailed under "Experimental Procedures," and products were partitioned between water and butanol. Data are presented as percent release above control (controls typically less than 5% of total counts). From the proposed structure of PP1 (Fig. 4), it is expected that only the nitrous acid cleavage would give efficient release of radiolabel as an aqueous-soluble fragment. Analysis of the aqueous-soluble material released by HF by Dionex HPLC indicated that very little material with the predicted retention time of the PP1 glycan was present. Note that even though GPI-PLD does not alter the partitioning of the radioactivity, PP1 has been cleaved (see Fig. 2).

Treatment	% Release over control
50% HF	38.0
Rabbit serum (GPI-PLD)	1.5
B. thuringiensis PI-PLC	1.0
Nitrous acid	71.6

indicating that glycosylPI and ethanolamine-containing glycolipids were not present, and therefore these extracts were not analyzed further.

Analysis of PP1

Because of the high abundance of this lipid and its unique R_f compared with P2 and P3, PP1 was subjected to detailed structural analysis.

Components—The [^{3}H]ethanolamine-labeled lipid designated PP1 chromatographed between P2 and P3 on TLC (systems 1 and 5) and cochromatographed with the major [^{3}H]mannose- and -glucosamine-labeled species from extract 2 on the same TLC systems. Labeling of cells with [^{3}H]myristic acid also produced a species that cochromatographed with [^{3}H]ethanolamine-labeled PP1 (systems 1 and 2). That the radiolabels were not converted to other components was verified as follows. Total acid hydrolysis of the [^{3}H]ethanolamine-labeled second extract and analysis of the products by TLC (system 7) identified only ethanolamine. Jack bean α-mannosidase digestion of the neutral glycan produced from [^{3}H]mannose-labeled PP1 by HF digestion released all the radiolabel as free mannose. Digestion of the [^{3}H]glucosamine-labeled PP1 neutral glycan with the α-mannosidase produced a fragment identified as anhydromannitol (see below). Analysis of the radiolabeled fatty acids in PP1 indicated that elongation of the myristate had occurred but that the radioactivity remained as fatty acid (see below).

It was concluded that PP1 contains ethanolamine, mannose, glucosamine, and fatty acid. Analysis of the incorporation of [^{3}H]ethanolamine over time showed that the radiolabel reached a steady state following a 2-h labeling period. Therefore, a *lower* limit estimate of the amount of PP1/cell can be made from the radioactivity recovered as PP1 when labeling is performed for greater than 2 h, assuming that no dilution of the [^{3}H]ethanolamine has occurred. 9.4×10^3 cpm of PP1 were recovered from 10^7 cells after 16 h of labeling, and from this it is estimated that there are at least 2×10^4 molecules of PP1/cell. This is a level similar to that reported for P3 in bloodstream trypanosomes (Mayor *et al.*, 1990a).

Fatty Acid Components—The lipophilic components of PP1 were determined to be in ester linkage by the complete solubilization of the [^{3}H]ethanolamine label on base hydrolysis. [^{3}H]Myristic acid-labeled PP1 was purified following TLC in system 2. Treatment of PP1 with PI-PLC was without effect, but digestion of PP1 with GPI-PLD generated two labeled species (Fig. 2). The more polar species remained close to the origin of the TLC plate ($R_f = 0.03$, system 2), whereas the less polar species cochromatographed with lysophosphatidic acid standards ($R_f = 0.56$), which were clearly resolved from the diacylphosphatidic acid standards ($R_f = 0.72$, Fig. 2). The behavior of the more polar species was similar to that observed for the GPI-PLD-generated headgroup of P3, suggesting that an acylglycoinositol fragment had been generated from PP1 by the action of the GPI-PLD (Mayor *et al.*, 1990b). From these data, it was concluded that PP1 contains a monoacylglycerol moiety. The absence of a peak corresponding to free fatty acid in the chromatogram shown in Fig. 2, *upper panel*, demonstrates that the lyso-species is not an artifact of the GPI-PLD digestion generated by a contaminating phospholipase A. Fatty acid chromatographs at the solvent front in this TLC system.

To confirm the arrangement of fatty acid on the glycerol, [^{3}H]ethanolamine-labeled PP1 was treated with *C. adamanteus* PLA_2.[3] No alteration in the migration position of PP1 was observed by this treatment. Under identical digestion conditions, P3 was converted to a lyso-lipid, which then comigrated with PP1 on TLC system 1 ($R_f = 0.43$, not shown). To ensure that an inhibitor was not present in the PP1 preparation, preventing the action of PLA_2, P3 was digested in a mixture containing an equal amount of ^{3}H activity of PP1. P3 was converted to lysoP3, which then ran with PP1 on the TLC plate. These data confirm the assignment of the glyceride of PP1 as a mono-acyl lipid, with chromatographic properties similar to those of lysoP3, having a fatty acid ester at the *sn*-1 position. It cannot be formally ruled out that there is not a small substituent at the *sn*-2 position, *e.g.* an acetyl group, which would not affect the migration position of PP1 as analyzed by the TLC systems used here.

The individual fatty acids on the headgroup and the glyceride of PP1 were identified by isolating fatty acids on GPI-PLD-derived PP1 fragments and fractionating their methyl ester derivatives on TLC (system 6, Fig. 2, *lower panels*). The analysis clearly showed that the labeled fatty acid linked to glycerol was exclusively stearic acid ($R_f = 0.27$), whereas the major ^{3}H-fatty acid on the inositol was palmitic acid ($R_f = 0.34$). Very small amounts of stearic and myristic acid ($\leq$10% of the total) were also recovered. Similarly to P3, the specificity of the fatty acid addition to the headgroup appears to be less rigid than that to the glycerol as an almost identical result was obtained for the headgroup of P3 when it was labeled with myristate (Mayor *et al.*, 1990b). Only stearate and palmitate were recovered from intact PP1, indicating that further fatty acid modifications that had been removed during the GPI-PLD treatment were not present. As a control to ensure that efficient elongation of the added myristic acid had taken place during the labeling period, the fatty acids present in the phospholipids from the first extract were analyzed. Efficient conversion of the input label to palmitate and stearate was seen, with less than 10% of the radiolabel recovered from these compounds as [^{3}H]myristate (data not shown).

Headgroup Glycan Structure—[^{3}H]Ethanolamine- and sugar-labeled PP1 were purified by TLC (system 1). As shown in Table III, nitrous acid treatment of [^{3}H]ethanolamine-labeled PP1 released the radioactivity as an aqueous-soluble fragment, but neither PI-PLC nor GPI-PLD altered the par-

[3] Interestingly, during the course of this analysis it was observed that bee venom PLA_2 (Sigma) was unable to act on P3 or P2, under conditions where it was capable of completely converting *T. brucei* [^{3}H]ethanolamine-labeled phosphatidylethanolamine to lysophosphatidylethanolamine, so that this latter enzyme may not be suitable for probing the structure of glycosylPIs.

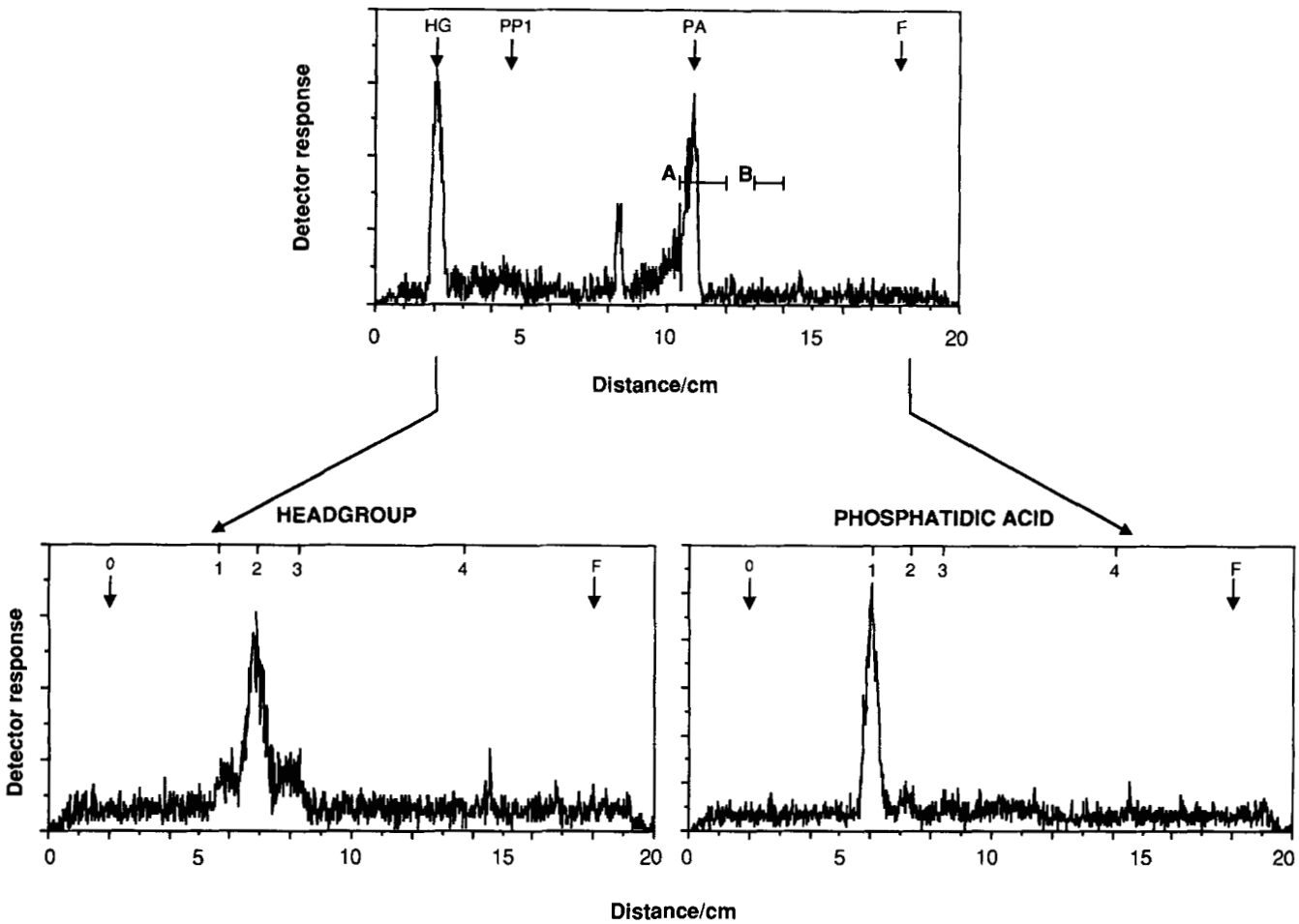

FIG. 2. **Analysis of [^{3}H]myristic acid-labeled PP1.** *Upper panel*, radiochromatogram (system 2) of [^{3}H] myristic acid-labeled PP1 following cleavage with GPI-PLD. Migration positions of GPIs are indicated as in Fig. 1. The chromatogram origin was at 2 cm. *HG*, lipophilic acylinositolglycan from PP1; *PA*, phosphatidic acid fragment from PP1. The positions of lysophosphatidic acid and phosphatidic acid standards run on the same plate are indicated by the *bars A* and *B*, respectively. The *small peak* at 8.2 cm was present in the original material. *Lower panels*, reverse-phase thin layer radiochromatograms (system 6) of the fatty acid methyl esters derived from the PP1 headgroup (*left*) and the lysophosphatidic acid (*right*) fragments. *1*, methylstearate; *2*, methylpalmitate; *3*, methylmyristate; *4*, methylcaprylate; *O*, origin; *F*, front.

titioning of the radiolabel between water and butanol. However, analysis of the butanol-soluble reaction products showed that GPI-PLD had cleaved PP1, releasing a lipophilic headgroup (as already shown for the fatty acid-labeled PP1), which remained in the organic phase. This polar fragment comigrated on TLC (systems 1 (R_f = 0.03) and 5 (R_f = 0.42)) with the GPI-PLD-released headgroup (ethanolamine-P-Man_3-GlcN-inositol-palmitate (Mayor *et al.*, 1990a)) obtained from P3, suggesting that the structures of these two fragments are similar.

The [^{3}H]ethanolamine-labeled nitrous acid-released headgroup (NAhg) was generated by mild base treatment followed by nitrous acid deamination and reduction with sodium borohydride (Mayor *et al.*, 1990a). When this species was analyzed by Bio-Gel P4 gel filtration chromatography in 50 mM sodium acetate buffer, the fragment coeluted with the NAhg from P3, with an approximate hydrodynamic volume of 7 glucose units (V_e = 0.57, Fig. 3*A*). Dionex HPLC of this fragment also demonstrated cochromatography between the NAhg from PP1 and P3 (Fig. 3*B*). Identical results were obtained on the Dionex analysis if PP1 NAhg labeled with [^{3}H]mannose or -glucosamine was used (data not shown).

Treatment of the [^{3}H]mannose-labeled NAhg with α-mannosidase did not alter the retention time of this material on the Dionex column, but treatment with HF converted the fragment to a species that comigrated with Man_3AHM and which was susceptible to α-mannosidase digestion, releasing all the radioactivity as free mannose (data not shown). When the [^{3}H]glucosamine NAhg was sequentially treated with HF and α-mannosidase, the radioactivity was recovered as a species that coeluted with AHM on the Dionex column (data not shown). These data demonstrated that the core glycan is Man_3-GlcN-inositol and that the nonreducing terminus is blocked by an ethanolamine residue, linked by a phosphodiester to the core glycan. Coelution of the NAhg fragment, both before and after HF treatment, with the corresponding P3 fragment, strongly suggests that the linkages between the monosaccharides in the core glycan are the same for both molecules, as the Dionex system is very sensitive to linkage position (Hardy and Townsend, 1988).

As further proof of the identity of the PP1 and P3 core glycans, [^{3}H]mannose-labeled PP1 was treated with HF to release the glyceride fragment and the ethanolamine phosphate group. The reaction products were partitioned between butanol and water, and the material recovered in the organic phase was processed further. The lipophilic headgroup was

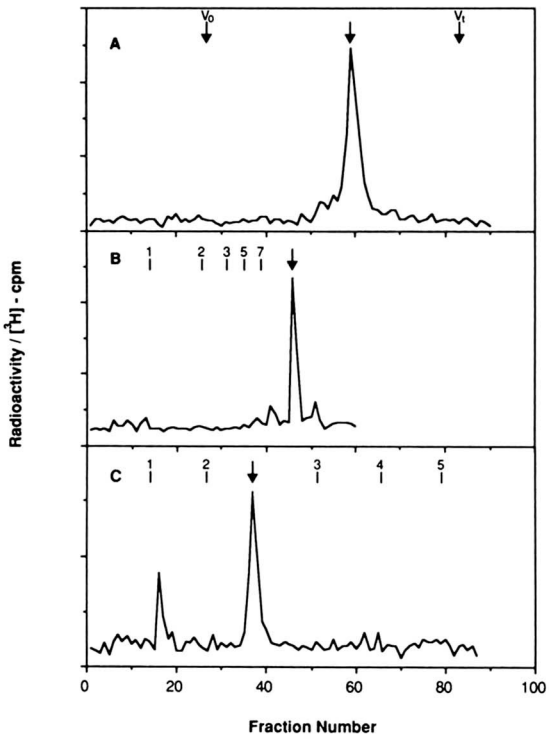

FIG. 3. **Analysis of the headgroup from PP1.** Bio-Gel P4 gel filtration (*A*) and Dionex HPLC (*B*) chromatograms of the NAhg derived from [^{3}H]ethanolamine-labeled PP1. *Arrow*, NAhg from P3. *1–7*, glucose oligomers; V_t and V_0, included volume and void volume, respectively. Dionex chromatography conditions were as follows: buffer A (100 mM NaOH) and buffer B (100 mM NaOH with 500 mM sodium acetate): gradient: 0–3 min 100% buffer A, 3–33 min linear gradient to 100% buffer B; flow rate 1 ml/min. For the gel filtration, 0.5-ml fractions (8.33 min) were collected, and for the HPLC, 0.4-min fractions were collected. *C*, Dionex HPLC chromatogram of the neutral glycan from [^{3}H]mannose-labeled PP1, generated by HF cleavage of the NAhg. 0.4-min fractions were collected. This chromatogram is of the same material that was used for the methylation analysis in Fig. 4. *Arrow*, Man_3AHM; *1–5*, glucose oligomers. Gradient: 0–6 min 100% A buffer, 6–36 min linear gradient to 15% buffer B; flow rate 1 ml/min.

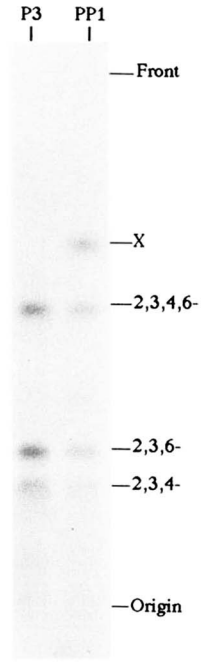

FIG. 4. **Fluorogram of TLC separation of partially *O*-methylated [^{3}H]mannoses derived from the NAhg of PP1 and P3.** The migration positions of the expected methyl mannose derivatives are indicated by *2,3,4,6-* for the terminal mannose derivative, *2,3,4-* for the 2-linked derivative, and *2,3,6-* for the 6-linked derivative. *X*, additional peak in PP1. *Origin* and *front* positions are indicated. *Note Added in Proof*—The numbers *2,3,6-* on the figure should be *2,3,4-*; the numbers *2,3,4-* on the figure should be *3,4,6-*.

treated with base and then converted to Man_3AHM by deamination and reduction. The fragment was shown by Dionex to be the expected species, *i.e.* Man_3AHM, together with approximately 30% of the radioactivity, which eluted from the column as a second component at 6 min (*fraction 16*, Fig. 3*C*). The material, containing both species, was taken for linkage analysis. The three expected methylmannoses were identified by fluorography of the TLC (2,3,4,6-tetramethylmannose, 3,4,6-trimethylmannose, and 2,3,4-trimethylmannose) in a chromatogram almost identical to that obtained from mannose-labeled P3 (Fig. 4). A fourth component was also seen in the PP1 sample, which ran between tetramethylmannose and hexamethylmannose, and was probably a pentamethylmannose, derived from the second species seen in the Dionex chromatogram. The presence of this second component in the Dionex chromatogram has been observed previously in analysis of the headgroups from P2 and P3 and is probably a degradation product generated at some point during the release and subsequent workup of the neutral glycan fragment from the GPI.

From the combined data, it is concluded that PP1 has the structure shown in Fig. 5. The lipid is clearly very similar to P3, with the exception that there is only a single fatty acid present on the glycerol, and this is stearic acid on PP1 rather than myristic acid as found on P3.

Analysis of the Less Abundant Lipids in Extract 2

The observation that only a single highly abundant ethanolamine-containing GPI lipid is seen in the procyclic trypanosome, in contrast to two in the bloodstream form, suggested that a further analysis of the minor ethanolamine-labeled lipids in extract 2 was warranted. Nitrous acid treatment of [^{3}H]ethanolamine-labeled extract 2 and analysis of the remaining radioactivity in the organic phase indicated that other lipids were also affected by this treatment (data not shown), suggesting that PP1 was not the only ethanolamine-containing GPI present. The remaining [^{3}H]ethanolamine-labeled lipids were resolved by TLC into five fractions (Table IV, Fig. 1) and treated with PI-PLC and GPI-PLD. Very little radioactivity could be converted to an aqueous form by either enzyme (Table IV and data not shown), and analysis of the reaction products following PI-PLC indicated that the lipids were unaffected by the enzyme. The high level of radioactivity recovered in the aqueous phase for both the PI-PLC and control for the region A lipids is probably due to the high polarity of these lipids (which contain hydrophilic headgroups considerably larger than PP1 (see below)). We have observed similar partial solubility in the aqueous phase of water-butanol partitioning experiments involving lysoP2. In an analogous manner to that found for PP1, GPI-PLD was observed to have cleaved the lipids, generating labeled lipophilic headgroups from each fraction, which had chromatographic properties similar to GPI-PLD-released headgroups from PP1 and P3 on TLC (systems 1 and 5). Therefore, we were unable to detect P2-type lipids in procyclic cells at a significant level.

As the resolution of the TLC plates was poor for the released headgroups, it was not possible to obtain information about possible structures for these fragments. Therefore, aliquots of the fractionated lipids were treated with nitrous acid and reduction and analyzed on Bio-Gel P4 in 100 mM sodium

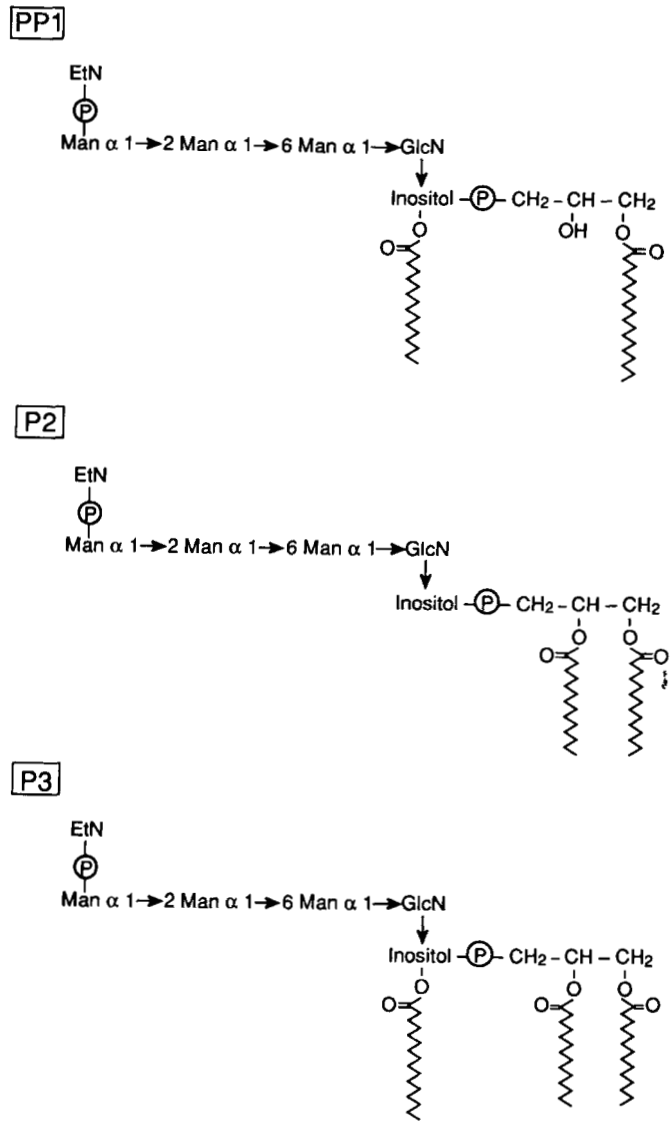

FIG. 5. **Proposed structures of P2, P3 (Mayor *et al.*, 1990a, 1990b), and PP1 (this paper).** Note that in P2 and P3 the fatty acids linked to glycerol have been identified as myristic acid, whereas the fatty acid in PP1 is stearic acid by radiolabeling. For both P3 and PP1, the fatty acid on the inositol has been labeled with palmitic acid.

acetate buffer (Table V). The NAhg derived from regions C and D were of a similar size to the PP1 headgroup (note that the PP1 NAhg apparent size is decreased compared with Fig. 3 as the Bio-Gel P4 is here eluted with 100 mM buffer). The lipid from region D was shown to cochromatograph with P3 on TLC systems 1 and 5 and therefore is probably P3 (the fatty acid composition of this minor species was not investigated). Region B lipids showed heterogeneity in the NAhg, but all the fragments were larger than the PP1 fragment, whereas the region A NAhg were larger still.

Anion exchange chromatography of the NAhg on Mono-Q demonstrated that all the fragments carried the same charge as PP1 (data not shown), and therefore the additional hydrodynamic volumes of the region A and B lipid headgroups are not due to additional ethanolamine-phosphate substituents. Minor GPIs in bloodstream trypanosomes have been detected that possess α-galactose residues in addition to mannose and glucosamine[4] and which migrate on TLC with mobilities

[4] S. Mayor, A. K. Menon, and G. A. M. Cross, unpublished observations.

TABLE IV

Cleavage of [^{3}H]ethanolamine-labeled procyclic glycolipids with B. thuringiensis PI-PLC

Isolated lipids (ca. 2000 cpm) were dried in Eppendorf tubes and digested with *B. thuringiensis* PI-PLC for 5 h at 37 °C. After digestion, the reaction was stopped by the addition of 10 μl of acetic acid, and the products were partitioned between water and butanol (see "Experimental Procedures"). Results are expressed as percent release into the aqueous phase, +/− enzyme. Data shown are the mean of two determinations, where the variability was less than 1.1%, except A, which was more variable (see text) and B and C, which are single determinations.

Chromatogram region	Incidence[a]	PI-PLC	% Released to aqueous phase
	%		
A	10.6	−	30.0
		+	32.8
B	8.9	−	3.7
		+	6.7
C	10.0	−	5.2
		+	4.5
B + C	18.9	−	2.9
		+	3.6
PP1	65.4	−	2.9
		+	3.9
D	5.1	−	5.3
		+	5.6
P2		−	2.4
		+	92.6
P3		−	0.9
		+	2.0

[a] Incidence calculated from integration of the radiochromatogram produced with system 1.

TABLE V

Characterization of ethanolamine-containing glycolipids

[^{3}H]Ethanolamine-labeled lipids were isolated as in Table IV. The NAhg was generated by nitrous acid treatment and reduction. Incidence, V_e, and HDV were estimated as described in Table II.

Region	Incidence	R_f	V_e	HDV/glucose units
A	10.6	0.10–0.26	0.16	>15.0
			0.38	11.2
B	8.9	0.26–0.33	0.52	8.3
			0.59	7.0
C	10.0	0.33–0.38	0.63	6.2
PP1	65.4	0.42	0.62	6.2
D	5.1	0.45–0.51	0.63	6.2

similar to the region A and B lipids seen here. Therefore, it is possible that these lipids are also substituted with additional monosaccharides.

The GPI Anchor of PARP Is Similar to PP1

When procyclic cells were labeled with [^{3}H]myristic acid and extensively extracted with chloroform/methanol and chloroform/methanol/water, several proteins were visualized after fractionation of the delipidated residue by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The most heavily labeled band migrated with the same apparent molecular weight as PARP (approximately 50 kDa, Clayton and Mowatt, 1989, data not shown). Greater than 80% of the radiolabel was released with mild base treatment. In order to obtain some structural information about the arrangement of the fatty acids in PARP, the butanol-soluble [^{3}H]myristic acid-labeled nitrous acid cleavage products of PARP and PP1 were compared. PP1 was quantitatively deaminated, but more than one product was obtained (Fig. 6, *top*). The yield of the least polar product was improved by two separate additions of sodium nitrite during a 20-h reaction

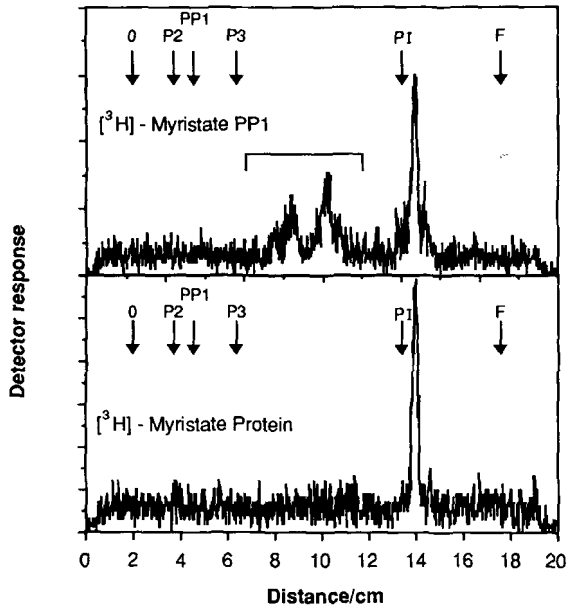

FIG. 6. **Examination of the GPI anchor of PARP.** Thin layer radiochromatograms (system 2) of the butanol-soluble deamination products from [^{3}H]myristic acid-labeled PP1 (*top*) or total delipidated cellular protein (*bottom*). Migration positions of *P2, P3, PP1,* and 1-stearyl,2-arachidonylphosphatidylinositol (*PI*) are indicated. *O,* origin; *F,* front. The additional more polar deamination products obtained with the free lipid are bracketted.

period, compared with a single addition, suggesting that the more polar species were reaction intermediates, possibly involving deamination adducts of the ethanolamine and the glucosamine, and that the least polar species is the expected final product, PI (see Mayor *et al.*, 1990b). Nitrous acid treatment of the delipidated labeled proteins released 80% of the radioactivity as a single lipophilic species, which on TLC systems 1 (Fig. 6) and 4 (data not shown) is a single species migrating just ahead of 1-stearyl,2-arachidonylPI. Control experiments where the sodium nitrite addition was omitted released less than 10% of the radiolabel into the organic phase. The least polar species obtained by deamination of PP1 cochromatographed with the lipid released from the procyclic proteins. These data strongly suggest that the phosphatidylinositol fragment from the procyclic proteins is similar to that from PP1, containing only two fatty acids, and is consistent with the proposal that PP1 is transferred to protein. Further characterization of the protein-linked lipid will be required to confirm these observations.

DISCUSSION

The results presented in this report can be summarized as follows. First, PP1, the major GPI species (at least 2×10^4 molecules/cell) identified in procyclic trypanosomes, is a lyso-GPI lipid of the P3-type, in contrast to the situation in bloodstream form trypanosomes, where two GPIs with a diacyl structure are seen. The headgroup of PP1 is the same as that of P2 and P3, in agreement with current data on the conservation of GPI core structures (Thomas *et al.*, 1990). The fatty acid of PP1 on the inositol can be labeled with palmitate, as seen in P3 (Mayor *et al.*, 1990b) and human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988), and the glycerol can be labeled with stearate. Other fatty acids may also be present at both of these sites but would not have been detected in this analysis if they could not be generated by the trypanosomes from myristate. This structure is clearly different from P2 and P3, but it is probable that, in the case of the bloodstream GPIs, the lipid at carbon 1 of the glycerol starts out as stearate before remodeling (Masterson *et al.*, 1990). In fact, the glyceride portion of PP1 has the structure expected of an aborted remodeling intermediate following removal of the first fatty acid in the pathway (on carbon 2 of the glycerol (Masterson *et al.*, 1990)). This structure is novel, as lyso-species containing glycosylPI have only been reported in *Leishmania* spp. (*e.g.* LPG, lysoGIPL3 (McConville *et al.*, 1990)), but in this case the hydrophobic moiety is in ether linkage to the glycerol. Lyso-P3 is barely seen in bloodstream trypanosomes *in vivo*, and therefore the alteration of the predominant GPI structure from P2/P3 to PP1 represents a distinct structural change that correlates with the parasite life cycle. The structural similarity to a remodeling intermediate may suggest that the enzymology for the remodeling in the bloodstream trypanosome is partially functional in the procyclic stage. The absence of myristic acid from the glyceride moiety of PP1 demonstrates that the myristyltransferases that act on P2 and P3 are not active in the insect stage. The reason for fatty acid remodeling in the VSG anchor is not known and, it is also not possible to propose a reason for the lack of such remodeling in the procyclic stage based on current data.

Second, in cultured procyclic trypanosomes, the ethanolamine-containing GPIs are all PI-PLC-resistant, confirming the exclusive presence of acylinositolGPIs in this life stage. Partial characterization of the less abundant lipids showed that their headgroup sizes were variable; most were similar to PP1, but a significant percentage had larger headgroups. Elaboration of the GPI core glycan in the absence of transfer to protein is difficult to assess but may be due to a small amount of transport of the GPI into the median and trans Golgi, where the lipid becomes exposed to various glycosyltransferases (Kornfeld and Kornfeld, 1985). The physiological relevance of headgroup elaboration is difficult to evaluate. In *Leishmania*, a family of GPI lipids are also predominantly intracellular (McConville *et al.*, 1990), and larger GPI lipids may represent a small amount of leakage of GPI from the endoplasmic reticulum into the Golgi stack. It should be noted that the minor GPIs analyzed did not appear to contain additional ethanolamine phosphate substituents as judged by anion exchange of the NAhg. More than one ethanolamine has been found on the GPI anchor of Thy-1 (Homans *et al.*, 1988) and human erythrocyte acetylcholinesterase (Roberts *et al.*, 1988). The GPI anchor of PARP may also contain more than one ethanolamine (Clayton and Mowatt, 1989). The observation that the GPI NAhg fragment in procyclic cells can be further processed by addition of neutral but not charged components suggests that, if subsequent ethanolamine addition does occur, there may either be a requirement for linkage to protein or that this modification takes place in a compartment to which the protein-linked GPI, but not the free GPI, has access. In bloodstream form, *T. brucei* α-galactose residues may be added to a small but significant fraction of the free GPI population.[4] The addition of α-galactose to the VSG GPI anchor, however, probably occurs after transfer of the GPI to protein (Bangs *et al.*, 1988).

Third, by analysis of the phosphatidylinositol released by nitrous acid from total procyclic proteins labeled with [^{3}H] myristate, we provide preliminary evidence that the lipid structure of the GPI anchors in procyclic cells is the same as that for PP1. We have recently obtained data that demonstrate the arrangement of fatty acids in the PARP GPI anchor to be identical to that shown here for PP1. PP1 labeled with [^{3}H]ethanolamine is apparently transferred to VSG *in vitro*. Although the structure of the transferred GPI anchor was not

investigated in this case, similar experiments using P2 and P3 indicated that the lipids were transferred to protein unchanged. These observations (Mayor *et al.*, 1991) also demonstrate that the protein plays no role in selecting the type of GPI anchor that is added to the carboxyl terminus *in vitro*, but a P3-type anchor has not been seen on mfVSG *in vivo*. In the case of PARP, selectivity may be restricted by the lipid substrates available for addition because, as we have shown, only P3-type GPIs are detectable in the procyclic cell. If P2-type anchors represent a default situation, that is they will be added if present in favor of other potential substrates, then the control of the GPI anchor added to VSG or PARP could be simply obtained by preventing the synthesis of P2 in the procyclic cell, possibly by the repression of an inositol deacylase activity. Masterson *et al.* (1990) have speculated that the remodeling of P2 fatty acids involves a P3 species, perhaps to ensure stability of a monomyristate glycolipid within the membrane. Biosynthesis of PP1 may therefore represent the product of an incomplete remodeling process. However, our data suggest that PP1 is not simply a biosynthetic intermediate and that it probably represents a *bona fide* GPI anchor precursor in the procyclic-form trypanosome.

The procyclic trypanosome provides a good model system in which to extend studies into the biosynthesis of GPI anchors. The cells can be maintained in culture, allowing greater flexibility for experimental and genetic manipulation (Bellafatto, 1990).

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