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Developmental Variation of Glycosylphosphatidylinositol Membrane Anchors in *Trypanosoma brucei*

IN VITRO BIOSYNTHESIS OF INTERMEDIATES IN THE CONSTRUCTION OF THE GPI ANCHOR OF THE MAJOR PROCYCLIC SURFACE GLYCOPROTEIN*

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The African trypanosome, *Trypanosoma brucei*, expresses two abundant stage-specific glycosylphosphatidylinositol (GPI)-anchored glycoproteins, the procyclic acidic repetitive protein (PARP or procyclin) in the procyclic form, and the variant surface glycoprotein (VSG) in the mammalian bloodstream form. The GPI anchor of VSG can be readily cleaved by phosphatidylinositol (PI)-specific phospholipase C (PI-PLC), whereas that of PARP cannot, due to the presence of a fatty acid esterified to the inositol. In the bloodstream form trypanosome, a number of GPIs which are structurally related to the VSG GPI anchor have been identified. In addition, several structurally homologous GPIs have been described, both *in vivo* and *in vitro*, that contain acyl-inositol. *In vivo* the procyclic stage trypanosome synthesizes a GPI that is structurally homologous to the PARP GPI anchor, *i.e.* contains acyl-inositol. No PI-PLC-sensitive GPIs have been detected in the procyclic form.

Using a membrane preparation from procyclic trypanosomes which is capable of synthesizing GPI lipids upon the addition of nucleotide sugars we find that intermediate glycolipids are predominantly of the acyl-inositol type, and the mature ethanolamine-phosphate-containing precursors are exclusively acylated. We suggest that the differences between the bloodstream and procyclic form GPI biosynthetic intermediates can be accounted for by the developmental regulation of an inositol acylhydrolase, which is active only in the bloodstream form, and a glyceride fatty acid remodeling system, which is only partially functional in the procyclic form.

A variety of eukaryotic organisms express membrane glycoproteins that are bound to lipid bilayers by covalent attachment of a glycosylated phosphatidylinositol (GPI)[1] to the carboxyl terminus of the polypeptide (Ferguson and Williams, 1988; Cross, 1990). A conserved headgroup, EthN-P-6Manα1-2Manα1-6Manα1-4GlcNα1-, links the carboxyl-terminal amino acid to phosphatidylinositol (PI) in all protein GPI anchors studied to date (Thomas *et al.*, 1990). Because these structures contain PI, a common method for detecting GPI-anchored proteins is susceptibility to bacterial PI-specific phospholipase C (PI-PLC) (Ferguson and Williams, 1988). However, a number of proteins and lipids contain the GPI moiety and are resistant to PI-PLC, probably because of a fatty acid esterified to the inositol (Roberts *et al.*, 1988; Walter *et al.*, 1990; Clayton and Mowatt, 1989; Sadeghi *et al.*, 1988; Stadler *et al.*, 1989; Mayor *et al.*, 1990b; Krakow *et al.*, 1989; Field *et al.*, 1991a, 1991b; deGasperi *et al.*, 1990; Sugiyama *et al.*, 1991). These structures retain sensitivity to mammalian serum GPI-PLD (Roberts *et al.*, 1988; Huang *et al.*, 1991; Mayor *et al.*, 1990b; Masterson *et al.*, 1989; Field *et al.*, 1991a).

GPIs are constructed by sequential glycosylation of PI with 1 glucosamine from UDP-GlcNAc (Doering *et al.*, 1989), 3 mannose residues from dolichol phosphorylmannose (Dol-P-Man) (Menon *et al.*, 1990b; Orlean, 1990; deGasperi *et al.*, 1990), and finally ethanolamine phosphate (reviewed by Doering *et al.*, 1990; Field and Menon, 1991). In bloodstream form (BSF) trypanosomes, both glycerol-linked fatty acids are remodelled to myristate to form the mature GPI structures P2 and P3 (glycolipids A and C, Masterson *et al.*, 1989, 1990). P2 and P3 are identical except that P3 contains a palmitate esterified to the inositol (Mayor *et al.*, 1990b), possibly derived from a phospholipid donor (Field *et al.*, 1991b).

Studies with the procyclic stage of *T. brucei* have revealed some interesting stage-specific features of GPI biosynthesis in this organism. The major GPI in procyclics, PP1, contains an identical headgroup to P2 and P3, but differs in the arrangement and composition of fatty acids. In contrast to P2 and P3, PP1 contains an *sn*-1, monostearoylglycerol, rather than dimyristylglycerol (Field *et al.*, 1991a). PP1 also has a palmitate esterified to the inositol (Field *et al.*, 1991a), similar to P3 (Mayor *et al.*, 1990b). Three lines of evidence indicate that PP1 is the PARP GPI anchor precursor; the structure of PP1 and the PARP GPI anchor are identical within the phosphatidylinositol moiety (Field *et al.*, 1991b), treatment of procyclics with mannosamine results in a coordinate shutdown of PP1 biosynthesis and addition of a GPI anchor to PARP, suggesting that biosynthesis of PP1 and the PARP anchor are parts of the same process (Lisanti *et al.*, 1991),

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[1] The abbreviations used are: GPI, glycosylated phosphatidylinositol; AHM, 2-anhydromannitol; BSF, bloodstream form; CoA, coenzyme A; CHAPS, 3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Dol, dolichol; EthN, ethanolamine; FCS, fetal calf serum; GlcNAc, *N*-acetylglucosamine; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; HPLC, high-performance liquid chromatography; PARP, procyclic acidic repetitive protein (procyclin); PI, phosphatidylinositol; PI-PLC, PI-specific phospholipase C; PLA_2, phospholipase A_2; SDS, sodium dodecyl sulfate; TLC, thin layer chromatography; VSG, variant surface glycoprotein.

and PP1 has been added to VSG in an *in vitro* system prepared from bloodstream trypanosomes (Mayor *et al.*, 1991) demonstrating that it is a substrate for the transfer machinery.

We have continued these investigations by exploiting a procyclic trypanosome cell-free system, similar to those developed for BSF trypanosomes (Masterson *et al.*, 1989; Menon *et al.*, 1990a). In this report we show that the intermediates in the synthesis of PP1 are considerably more inositol-acylated than their BSF counterparts, and that the immediate PP1 precursor appears to be a diacyl-GPI (PP3) also containing acyl-inositol. Pulse-chase studies support the hypothesis that PP3 is the precursor of PP1, and that less elaborate mannosylated GPIs are the precursors of PP3.

MATERIALS AND METHODS

Cells and Membranes—Procyclic stage *T. brucei* strain 421 were grown in SDM-79 medium (Brun and Schonenberger, 1979), containing 10% heat-inactivated fetal calf serum and 7.5 μg/ml haemin, at 27 °C. Cells were routinely grown to 1–2 × 10^7 parasites/ml, harvested, and washed three times with cold phosphate-buffered saline. Membranes were prepared from the cells by hypotonic lysis following the method of Masterson *et al.* (1989), except that preincubation with tunicamycin was omitted. Membranes were stored at 1 × 10^9 cell equivalents/ml at −80 °C. Immediately before use the membranes were thawed, washed (Masterson *et al.*, 1989), and resuspended at 5 × 10^8 cell equivalents/ml. Aliquots of the washed membranes (100–200 μl) were then used for the *in vitro* reactions. Bloodstream form and procyclic form 100,000 × *g* *T. brucei* microsome membranes were made exactly as described previously (Menon *et al.*, 1990a). Procyclic cells were labeled *in vivo* with [2,6-^{3}H]mannose (Amersham, 30–60 Ci/mmol) in modified RPMI 1640 (Speciality Media, Inc.) as described (Field *et al.*, 1991a).

In Vitro Glycosylphosphatidylinositol Biosynthesis—Membranes were labeled essentially as described by Menon *et al.* (1990b) with a typical reaction volume of 100–200 μl. Biosynthesis of Dol-PP-linked oligosaccharides was inhibited by the inclusion of 100 ng of tunicamycin (Calbiochem) added from a 1 mg/ml stock in 95% ethanol in each reaction. For [^{3}H]mannose-labeling, 2 μl each of stock solutions of 0.1 M ATP, CoA, dithiothreitol, and UDP-GlcNAc (all from Sigma) were added to the reaction tube containing 2 μCi of dried [^{3}H]GDP-mannose (Du Pont-New England Nuclear, 15 Ci/mmol). Tunicamycin was added followed by the membranes. For labeling with [^{3}H]GlcNAc, 2 μl of 0.1 M GDP-mannose (Sigma) was substituted for the UDP-GlcNAc, and 2 μCi [^{3}H]UDP-GlcNAc (Du Pont-New England Nuclear, 19 Ci/mmol) was added instead of radiolabeled GDP-mannose. In some experiments, the cold GDP-mannose was omitted. Preliminary experiments using 100,000 × *g* pelleted membranes indicated that incorporation of [^{3}H]GDP-Man into organic solvent extractable material was maximal at 27 °C (data not shown). Therefore, all incubations were routinely performed at this temperature.

In experiments where biosynthesis of [^{3}H]GPI was made dependent on [^{3}H]Dol-P-Man, radiolabeled Dol-P-Man was synthesised *in situ* as described by Menon *et al.* (1990b). Amphomycin (a gift from Dr. R. T. Schwarz, Philipps-Universitaet Marburg, Germany), was used at varying concentrations, and was added to the reaction mix from a 10 mg/ml stock suspension in water.

Extraction of Lipids and Proteins—Following incubation, the membranes were extracted with either 1.0 ml of chloroform/methanol, followed by chloroform/methanol/water as described by Menon *et al.* (1990a) or a single extract of chloroform/methanol/water (final composition, 10:10:3, v/v) (Masterson *et al.*, 1989). Residual material was solubilized with 5% sodium dodecyl sulfate as described (Field *et al.*, 1991b).

Analysis of Lipid Extracts—Lipid extracts were analyzed by thin layer chromatography using glass-backed Silica 60 plates (Merck) developed in presaturated tanks with chloroform/methanol/water (10:10:3, v/v, system 1), chloroform/methanol/water (10:10:2.5, v/v, system 2) or chloroform/methanol/1 M NH_4OH (aq) (10:10:3, v/v, system 3). Chromatograms were analyzed with a Berthold LB 2842 linear radioactivity scanner. In some experiments lipids were recovered from the TLC plate by scraping off the silica support and extracting with chloroform/methanol/water (10:10:3, v/v). The extracts were then dried and partitioned between water and *n*-butanol.

Analysis and Purification of Protein—Protein was analyzed by SDS-polyacrylamide gel electrophoresis on 15% gels (Laemmli, 1970). Molecular weight standards were from Sigma. Fixed gels were impregnated with En^{3}Hance (Du Pont) and dried. Fluorography was performed at −80 °C with two intensifying screens and Kodak X-Omat AR film.

Enzyme and Chemical Treatments—PI-PLC from *Bacillus thuringiensis* (gift of Dr. M. G. Low, Columbia University), rabbit serum as a source of GPI-PLD, and PLA_2 from *Crotalus adamanteus* (Sigma) were all used as described in Mayor *et al.* (1990a, 1990b). 50% aqueous HF, sodium nitrite deamination, sodium borohydride reduction, and base treatments were all performed using the methods described previously (Mayor *et al.*, 1990a).

Liquid Chromatography—Dionex HPLC was performed with a HPIC-AS6 anion-exchange column using a Dionex chromatography

TABLE I

Incorporation of [^{3}H]mannose and glucosamine into organic extracts and GPI species

Experiment	Radiolabel[a]	Chloroform/methanol	CMW	CMW[b]	PP1	PP3
			cpm[c]			
1	GDP-Man	0.95	16.0	—[d]	6.3	3.0
2	UDP-GlcNAc			0.5	0.13	0.1
3	UDP-GlcNAc (no GDP-Man)	4.5	0.65		0.0	0.0
4	GDP-Man			50.0	20.0	6.2
5	GDP-Man (+ 0.2 μg/ml amphomycin)			5.6	2.4	0.79
6	GDP-Man (+ 0.8 μg/ml amphomycin)			0.68	0.05	0.03

[a] ^{3}H-labeled sugar nucleotide is indicated. Additions or deletions from the standard reaction supplements are given in parentheses.

[b] Single step extraction protocol (chloroform/methanol extraction omitted) (CMW, chloroform/methanol/water) (see "Materials and Methods").

[c] Radioactivity incorporated is expressed as cpm (counts per minute) per 10^4 cell equivalents.

[d] Not determined.

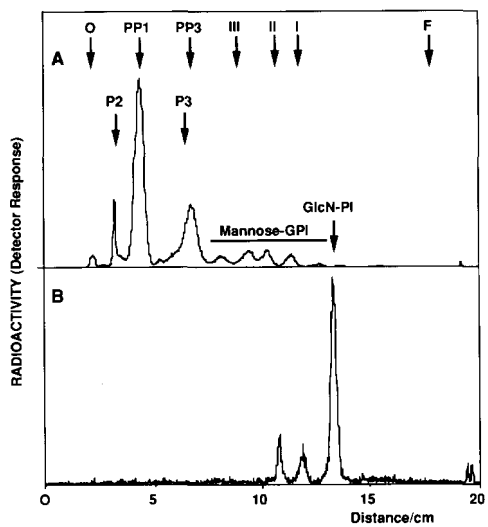

FIG. 1. **Thin layer chromatograms of *in vitro*-labeled glycolipids.** *A*, species labeled with [^{3}H]GDP-mannose in a reaction supplemented with UDP-GlcNAc. *B*, species labeled with [^{3}H]UDP-GlcNAc in the absence of GDP-mannose. TLC plates were developed using system 2. *Arrows* indicated the migration positions of PP1 and PP3 (procyclic form GPIs) (this study and Field *et al.*, 1991a), P2, P3, I (ManGlcNPI), II (Man_2GlcNPI), III (Man_3GlcNPI), and GlcNPI (bloodstream form trypanosome GPIs (Mayor *et al.*, 1990a, 1990b; Menon *et al.*, 1990a)). *O* and *F* indicate the positions of the origin and front, respectively. "Mannose-GPI" indicates the migration positions of the less polar GPIs corresponding to Man_{1-3}GlcNPI species.

TABLE II

Structural analysis of the two major [^{3}H]mannose-labeled GPI lipids synthesised by procyclic membranes

TLC-purified (system 2) polar mannose-labeled lipids were treated with the various reagents detailed below to determine structure.

Treatment	Cleavage/result	
	PP1 (R_F = 0.14)	PP3 (R_F = 0.29)
B. thuringiensis PI-PLC[a]	−	−
Rabbit serum GPI-PLD[b]	+	+
Nitrous acid[c]	58.2	49.6
C. adamanteus PLA_2[d]	−	+
Headgroup[e]	EPM_3AHM	EPM_3AHM

[a] No release of radioactivity as an aqueous soluble fragment, and no alteration to the organic soluble reaction products by TLC analysis.

[b] Complete cleavage and conversion to a hydrophobic glycan seen by TLC analysis.

[c] Data shown for the percent converted to aqueous soluble material. TLC analysis of the organic phase following deamination indicated that all the original material had been converted, but that the release was incomplete. Similar observations have been documented for P2 and P3 (Mayor *et al.*, 1991a).

[d] PP3, after PLA_2 treatment, comigrates with PP1 on TLC system 1. PP1 is unaffected.

[e] Deaminated and reduced headgroup analyzed by Dionex HPLC. Cochromatography with the headgroup from P2 and P3 (EPM_3AHM, ethanolamine-P-$[Man]_3$-anhydromannitol) obtained.

system. The column was eluted with 100 mM NaOH for 3 min, followed by a linear gradient to 225 mM sodium acetate in 100 mM NaOH at 33 min, with a flow rate of 1 ml/min. The eluant was neutralized with a membrane suppressor (Dionex Corp.), and 0.4-ml fractions were collected for scintillation counting.

RESULTS

Incorporation of ^{3}H-Labeled Sugars into Lipids—The simple hypotonic lysate employed by Masterson *et al.* (1989) was more efficient at incorporating ^{3}H-labeled sugars into glycolipid than the 100,000 × *g* membranes,[2] and therefore the former type of membranes were used in this study.

The incorporation of ^{3}H-labeled sugars into lipid extracts is shown in Table I. Tunicamycin had little effect on the total incorporation of [^{3}H]GlcNAc or mannose (data not shown). The level of [^{3}H]glycolipid biosynthesis by the procyclic membranes was similar to that found for BSF hypotonic lysate membranes (Menon *et al.*, 1990b). Addition of amphomycin to the reaction caused almost complete inhibition of the synthesis of GPIs at 0.8 μg/ml (Table I) consistent with Dol-P-Man as the direct mannosyl-donor, as shown previously for the BSF trypanosome (Menon *et al.*, 1990b), mammalian cells (DeGasperi *et al.*, 1990), and yeast (Orlean, 1991).

The *in vitro* reaction with [^{3}H]GDP-Man resulted in the labeling of a major protein that migrated as a tight doublet on SDS-polyacrylamide gel electrophoresis about 5–7 kDa smaller than *in vivo* [^{3}H]mannose-labeled PARP (data not shown). This protein was extractable with 2% CHAPS, did not bind to SP-Sephadex (data not shown), and is presumably *in vitro* glycosylated PARP (Clayton and Mowatt, 1989). Amphomycin also inhibited the incorporation of radioactivity into the protein (see "Discussion").

The in Vitro System Synthesizes Two Major GPI Lipids—When membranes were labeled with [^{3}H]GDP-Man, in the presence of UDP-GlcNAc, CoA, and ATP, two major polar lipid species were recovered in the chloroform/methanol/water organic extract (Fig. 1*A*). In a separate experiment, these lipids were included in the second extract of a two-step extraction, which is similar to the behavior of PP1, P2, and P3 (Mayor *et al.*, 1990a, Field *et al.*, 1991a). Furthermore, the least mobile species cochromatographed with PP1 on TLC (systems 1 and 2, R_F = 0.14 on system 2). The more mobile species chromatographed similarly to P3 (R_F on system 2 of 0.28) on TLC (systems 1 and 2), but always ran just ahead of it. This lipid was designated PP3 (procyclic P3, R_F = 0.29 on system 2). Structural analysis of the two lipids (results summarized in Table II) confirmed that the slower migrating species was PP1, and the faster-migrating lipid, PP3, contained a diacylglycerol but was otherwise identical to PP1. We were unable to detect a PI-PLC-sensitive lipid species in this region of the chromatogram (the region where P2, the VSG GPI anchor homologue migrates), consistent with the absence of PI-PLC-sensitive mature ethanolamine-phosphate containing GPI lipids in the procyclic-labeled *in vivo* (Field *et al.*, 1991a). In the BSF, P3 contains two myristates, due to remodelling of the original fatty acid substituents (originally stearate at *sn*-1, Masterson *et al.*, 1990). The observation that PP3 is slightly more hydrophobic than P3 is consistent with our data on PP1, where the fatty acid at *sn*-1 is stearate (Field *et al.*, 1991a), suggesting that PP3 is not remodelled in the procyclic, but is converted to PP1 by the action of a specific PLA_2.

Inositol Acylation of Mannose-containing GPI-lipids—Analysis of glycans obtained after deamination and reduction of total lipids labeled via [^{3}H]GDP-Man identified four species that cochromatographed with the $Man_{1-3}AHM$ and EthN-P-Man_3AHM standards on Dionex HPLC, indicating that the headgroup structures of the procyclic were identical to the BSF (data not shown). Labeling of membranes with [^{3}H]UDP-GlcNAc in the presence of GDP-Man produced the same spectrum of nitrous acid-cleavable GPI glycan headgroups as seen with the mannose label, except for an additional species (AHM) derived from GlcN-PI was seen (data not shown).

We next investigated the level of inositol acylation in the earlier intermediates in the PP1 biosynthetic pathway, *i.e.*

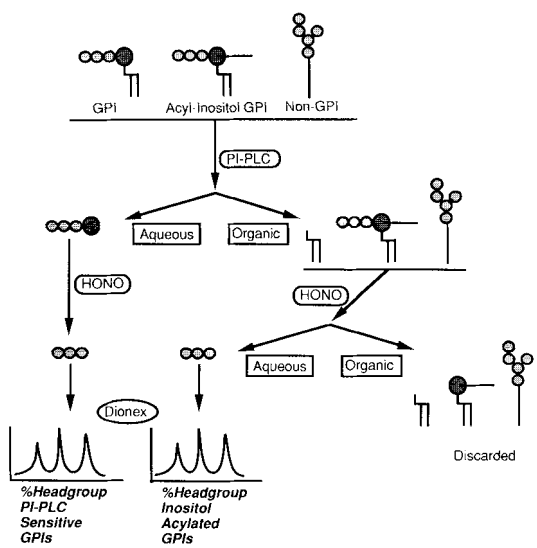

FIG. 2. **Schematic of protocol used to estimate incidence of inositol acylation in GPI lipids.** A mixture of metabollically radiolabeled glycolipids contains GPIs, acyl-inositol GPIs, and non-GPI glycolipids (*e.g.* Dol-PP-GlcNAc, sphingolipids). Headgroup glycans can be cleaved from the GPIs by PI-PLC, and recovered by a phase-separation between water and butanol. The acyl-inositol GPIs remain unreacted, and their headgroups can be released by a deamination reaction, and similarly recovered by phase partitioning. Lipids that are not GPIs are unaffected by these two procedures. The respective headgroups from the GPIs and acyl-inositol GPIs are separately worked up and analyzed by Dionex anion-exchange HPLC.

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

those lacking ethanolamine. Membranes were labeled with [^{3}H]GDP-Man and extracted with chloroform/methanol/water. The total extract was chromatographed on a TLC plate (system 2), and the lipid species migrating ahead of PP3 were isolated and extracted (R_F = 0.36 and 0.70 on system 2). After partitioning between water and butanol the organic phase was dried and treated with *B. thuringiensis* PI-PLC (two additions of enzyme, total digest time 4 h, 50-μl reaction volume) to cleave noninositol-acylated headgroups. The digestion products were then partitioned between water and butanol, the phases separated and aliquots scintillation counted. The aqueous phase was adjusted to pH 3.7 (with sodium acetate), treated with nitrous acid followed by sodium borohydride (after adjustment to pH 10), and the glycans analyzed by Dionex HPLC (schematically illustrated in Fig. 2). The PI-PLC digest organic phase was dried, treated with nitrous acid to release PI-PLC-resistant GPI headgroups (inositol-acylated), and again partitioned between water and butanol. The aqueous phase was reduced with sodium borohydride and then analyzed by Dionex HPLC. A standard mix of BSF GPIs (labeled via [^{3}H]GDP-Man *in vitro*) were processed in parallel with the procyclic mannosylated GPIs. The analysis (Fig. 3) shows quite clearly that the degree of inositol acylation increases markedly with a greater headgroup size, attaining ~75% by the Man_3 stage, and completion once the ethanolamine phosphate has been added (PP1 and PP3, see above). This is in marked contrast to the situation in BSF, where the mannose-GPIs are only 10% inositol-acylated, and where only 30–50% of the metabolically labeled mature GPI precursors (P3, not P2) contain a fatty acid on the inositol.

Analysis of Nonmannosylated GPI Species—Two early species lacking mannose substitution have been identified in BSF trypanosomes; GlcNAc-PI and GlcN-PI (Doering *et al.*, 1989; Menon *et al.*, 1990a). GlcNAc-PI is deacetylated to give GlcN-PI (Doering *et al.*, 1989). If GDP-Man was omitted from the reaction, essentially all radioactivity was recovered in the CM extract (Table I and Fig. 1*B*). A single predominant species was seen (R_F = 0.71 on system 2), which cochromatographed with GlcN-PI from BSF on TLC (systems 2 and 3), and two minor, more polar, species (R_Fs = 0.54 and 0.62 on system 2). GlcNAc-PI and GlcN-PI were discriminated from GlcNAc-PP-Dol (which chromatographs similarly) and each other as follows. The total extract was digested with *B. thuringiensis* PI-PLC, releasing >80% of the radioactivity. Analysis of the organic soluble products of the digestion showed that >95% of the putative GlcN-PI peak had been lost, with selective retention of the more polar species. These compounds were also not cleaved by GPI-PLD (GlcN/GlcNAc-PI is partially sensitive to the PLD, and is cleaved at about 50% under the conditions used here), and are therefore unlikely to be GPI lipids (data not shown). An aliquot of the PI-PLC-released material was *N*-acetylated. Analysis by Dionex HPLC detected a single species, cochromatographing with the glucose-12 standard, and presumed to be GlcNAc-inositol-P. A second aliquot was treated with nitrous acid and reduced, to cleave GlcN-inositol-P (not GlcNAc-inositol-P) and convert it to AHM. Analysis of this material by Dionex HPLC showed that >80% of the radioactivity eluted in the region of AHM, while the remainder eluted at the same position as GlcNAc-inositol-P. Therefore at least 80% of [^{3}H] glucosamine-labeled GPI was present as GlcN-PI, with a smaller amount of GlcNAc-PI. As no significant radiolabeled material migrated ahead of the GlcN-PI peak (Fig. 1*B*) little or no GlcN(Ac)-acylPI was synthesized under these conditions, suggesting that acylation of the inositol occurs after addition of GlcNAc.

Pulse-Chase Analysis—A pulse-chase experiment was performed to provide direct evidence of a product/precursor relationship between PP1 and PP3, and PP3 and the smaller mannose-containing GPIs. Membranes were first preincubated with a small amount of GDP-Man to complete synthesis of any endogenous GPI species, and then pulsed with 2 μCi of [^{3}H]GDP-Man at 27 °C for 10 min. This results in the near exclusive labeling of Dol-P-Man (Menon *et al.*, 1991b and Fig. 4). The membranes were chased by adding an excess of GDP-Man and UDP-GlcNAc to allow *de novo* synthesis of GPIs (Menon *et al.*, 1991b). Reactions were quenched by addition of CM to the tube at the relevant time and extracts analyzed by TLC (system 2, Fig. 4). At zero chase time essentially only Dol-P-Man was labeled (Fig. 4, *top*, R_F = 0.79). After a 60-min chase many GPI species, including PP1 and PP3 had been labeled. The data (Table III) clearly show that radioactivity in the less polar GPIs rises rapidly (the Man_{1-3} species), peaks at 1 min, and then falls again, commensurate with increased radiolabeled PP3. PP3 levels are maximal at 5 min, and as the amount of this species begins to diminish, PP1 is seen to slowly increase (Table III). PP1 attains a plateau at 30 min. Analysis of the protein residue after lipid extraction was not informative, due to insufficient incorporation. These data are consistent with the following scheme;

$$[Man_{1-3}]GlcN\text{-}PI \rightarrow \underset{[PP3]}{E\text{-}P\text{-}[Man_{1-3}]GlcN\text{-}PI^*} \rightarrow \underset{[PP1]}{E\text{-}P\text{-}[Man_{1-3}]GlcN\text{-}lysoPI^*}$$

where PI* is inositol-acyl-PI, and where inositol acylation increases in the order $Man_1 < Man_2 < Man_3$. The data from this experiment rule out the possibility that PP1 is converted to PP3.

DISCUSSION

In this report we provide further information on the developmental variation of GPI biosynthesis in the African trypan-

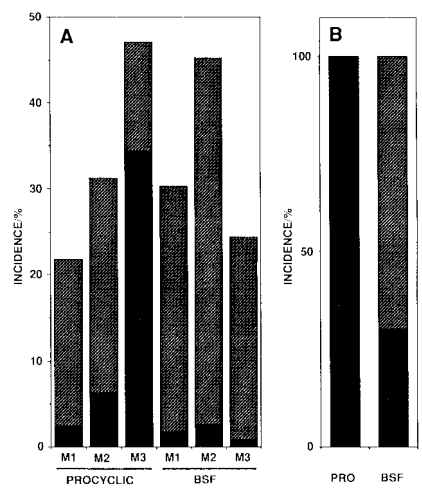

FIG. 3. **Incidence of acyl-inositol in procyclic GPI biosynthetic intermediates.** The *bar graph* summarizes data on the incidence of inositol acylation in BSF compared to procyclic form GPIs as determined by resistance to PI-PLC cleavage. *A*, mannosylated species (see text for experimental details). Incidence is normalized to 100% for the Man_{1-3}GlcN-containing species. The *height* of the *bar* represents the total incidence of each headgroup, and the *solid region* the fraction of each headgroup that contains acyl-inositol. *B*, mature GPI species (EthN-P-M_3GlcN headgroup). BSF data from the same experiment as described for *A*. Pro (procyclic) data from the analysis of PP1 and PP3.

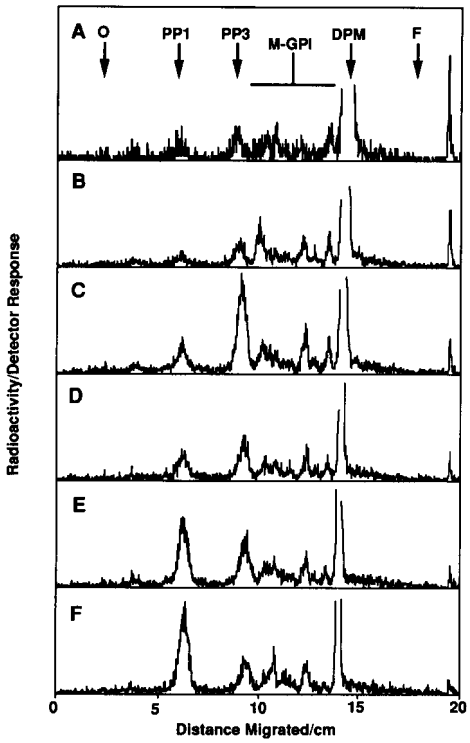

FIG. 4. **Pulse-chase analysis of GPI intermediates synthesized by procyclic cell free system.** *A–F*, thin layer chromatograms (system 2) of species labeled *in vitro* via dolicholphosphomannose (see text for experimental details). *A*, labeled species following the 10-min pulse; *B-F*, labeled species following 1, 5, 15, 30, and 60 min chase, respectively, in the presence of UDP-GlcNAc. The migration positions of PP1, PP3, mannosylated GPIs (MGPI), and dolichoylphosphomannose (*DPM*) are indicated. *O* and *F* indicated the origin and front of the chromatogram, respectively. The total cpm for each chromatogram has been scaled to 200 for comparison. Note that recovery of radioactivity from the 15-min time point (*D*) was 20% less than predicted.

TABLE III

Pulse-chase analysis of biosynthesis of procyclic GPI intermediates

Data from the chromatograms shown in Fig. 4 were quantitated by integration and are presented below.

Chase time	Total	Dol-P-Man	Man-GPI	PP3	PP1	Ratio PP1/PP3
min			*cpm*			
0.0	23,650	22,090	910	375	280	0.75
1.0	10,700	7,160	2,340	770	440	0.57
5.0	6,750	3,640	1,120	1,560	430	0.28
15.0[a]	4,250	1,950	910	855	540	0.63
30.0	5,350	2,210	1,070	870	1,200	1.37
60.0	4,150	1,410	960	560	1,210	2.17

[a] Recovery of counts from this sample is about 20% lower than expected and artifactually implies a lag between the decrease in counts in PP3 and synthesis of PP1. As label was added individually to each tube, this probably reflects a small pipetting error, and it is clear from the ratio of counts recovered in PP1 to PP3 that there is a progression of radioactivity from PP3 to PP1 without a lag. The majority of the rapid fall in Dol-P-Man radioactivity is independent of GPI biosynthesis and has been discussed elsewhere (Menon *et al.*, 1990b).

osome. Our data can be summarized as follows. The very earliest glycosylated PI species are GlcNAc-PI and GlcN-PI as in the BSF trypanosomes. Unlike reports in mutant T cells (Sugiyama *et al.*, 1991) and Dol-P-Man synthase-deficient yeast (Orlean, 1991), we were unable to detect forms of these lipids carrying an acyl group on the inositol, and therefore in the trypanosome the GlcN-PI is either not a substrate for the inositol-specific acyltransferase, or is very rapidly converted to the Man_1GlcN-PI following addition of the acyl group to the inositol, and is therefore not seen by our analysis. At present it is not possible to discriminate between these two possibilities in the normal physiological system. The latter possibility is, however, made less likely as we do not see acylGlcN(Ac)PI even when mannosylation is prevented by omitting GDP-Man from the labeling reaction. We failed to detect significant levels of PI-PLC-sensitive GPIs containing the complete headgroup, EthN-P-Man_3GlcN. This observation is consistent with our previous data demonstrating that all GPI-anchored proteins in procyclics are insensitive to PI-PLC (Field *et al.*, 1991b).

As expected, the mannose moieties are derived from Dol-P-Man, as reported for the BSF trypanosome (Menon *et al.*, 1990b). PP1, PP3, and the earlier intermediates are labeled in a reaction where [^{3}H]mannose is provided from endogenously synthesized Dol-P-[^{3}H]Man, and synthesis is inhibited by inclusion of amphomycin in the reaction. We cannot draw strong conclusions about the effect of amphomycin on protein labeling, *i.e.* if the inhibition of incorporation of radiolabel is due to effects on the GPI, because our attempts to release the GPI glycan from the *in vitro*-labeled PARP were unsuccessful. However, we did note that the *in vitro*-labeled PARP was significantly smaller and less heterogenous than the tunicamycin-treated PARP labeled with [^{3}H]mannose *in vivo*. In addition, we saw no evidence of a large heterogenous glycan as seen for the PARP anchor,[3] and therefore it is possible that the *in vitro* glycosylated protein does not contain an elaborated GPI glycan. Further work will be required to clarify this point.

In contrast to BSF, a large proportion of the mannosylated GPIs in procyclics contain acyl inositol. Our data suggest that the larger headgroups are better substrates for an inositol acyltransferase, which is supported by our observation that the ethanolamine phosphate-containing lipids, PP1, and PP3, are exclusively found as acyl-inositol species. An alternate explanation would be that the activity of an inositol acylhydrolase decreases with increased headgroup size, but from the data reported here and previously there is no evidence for such an activity in the procyclic trypanosome as no mature nonacylated intermediates or GPI-anchored proteins have been detected in the procyclic (Field *et al.*, 1991a, 1991b; Engstler *et al.*, 1991). It cannot be ruled out that the mannosylated species *without* acylated inositol are either abortive intermediates (*i.e.* they cannot be processed further until the inositol becomes acylated) or artefacts generated by the cell-free preparation (*i.e.* some microsome vesicles may have been produced that contain the glycosyltransferases but not the acyl or ethanolaminylphosphate transferase activities). More elaborate pulse-chase studies will be needed in order to determine the true relationships between these species.

Our characterization of PP3 suggests that a form of fatty acid remodelling does take place in the procyclic trypanosome. The pulse-chase experiment provides strong evidence for a precursor-product relationship between PP3 and PP1 and suggests that removal of the fatty acid at *sn*-2 is the final maturation step in the procyclic GPI pathway. We have previously reported that the lyso-PI structure is preserved in the PARP anchor (Field *et al.*, 1991b). By analogy with the BSF pathway, and the evidence that PP3 contains a diacylPI, it is most likely that the earlier intermediates in the procyclic are also diacyl. The similarity to the BSF remodelling is striking, in that the mature GPI species P2 and P3 also begin with stearate at the *sn*-1 position, with the first remodelling

[3] M. C. Field, unpublished observations.

reaction being the removal of the *sn*-2 fatty acid (Masterson *et al.*, 1990). Therefore, the difference between the BSF and procyclic could be accounted for by the loss of expression of the myristoyltransferase involved in reacylation at the *sn*-2 position. Presumably if this reaction cannot take place then further processing of the *sn*-1 fatty acid does not occur. The pulse-chase data also suggest that the PLA_2 that acts on PP3 is unable to act on the earlier GPI intermediates, as it is not until after these species have been chased into PP3 that significant amounts of PP1 appear, and that the ethanolamine phosphate moiety may be a structural requirement for recognition by the PLA_2 involved in the remodelling process.

The presence of P3 in the BSF trypanosome remains enigmatic. A simple scheme whereby an inositol acyltransferase is restricted to expression in the procyclic stage is clearly ruled out by the presence of this GPI. A recent observation, that P3 synthesis can be inhibited without affecting VSG GPI anchor addition, suggests that P3 may not be intimately involved in the BSF pathway (Masterson and Ferguson, 1991). However, in the procyclic pathway PP1 is important in GPI anchoring of PARP. The most likely interpretation of these data is that an acylhydrolase is under developmental control, *i.e.* present in BSF, but not in the procyclic.

In T cell hybridomas, the majority of the early intermediates and the mature GPI anchor precursor are resistant to PI-PLC, but sensitive to GPI-PLD (DeGasperi *et al.*, 1990; Sugiyama *et al.*, 1991), and therefore structurally similar to the procyclic intermediates. However, the GPI-anchored proteins from these cells are PI-PLC-sensitive, raising the possibility that removal of the acyl moiety from the inositol may take place following transfer to protein, unlike the case of PARP where it remains in place. It is formally possible that a similar mechanism is operating in VSG biosynthesis, but from current data it is not possible to draw definitive conclusions. It will be of interest to analyze the structures of GPI biosynthetic intermediates in mammalian cells that synthesise PI-PLC-resistant GPI-anchored proteins, and to compare these with the procyclic and the T cell lipids.

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