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**

(Received for publication, September 3, 1991)

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The African trypanosome, *Trypanosoma brucei*, expresses two abundant stage-specific glycosylphosphatidylinositols (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-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.

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A variety of eukaryotic organisms express membrane glycoproteins that are bound to lipid bilayers by covalent attachment of a glycosylated phosphatidylinositol (GPI) to the carboxyl terminus of the polypeptide (Ferguson and Williams, 1988; Cross, 1990). A conserved headgroup, EthN-P-6Manal-2Manol-6Manol-4GlcNaol-, 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, 1988; 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 PI-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 dolichyl 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 procyclins, PP1, contains an identical headgroup, EthN-P-6Manal-2Manol-6Manol-4GlcNaol-, 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, 1988; 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 PI-PLD (Roberts et al., 1988; Huang et al., 1991; Mayor et al., 1990b; Masterson et al., 1989; Field et al., 1991a).

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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 GPs 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 x 10⁸ 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 x 10⁸ cell equivalents/ml at -80 °C. Immediately before the use membranes were thawed, washed (Masterson et al., 1989), and resuspended at 5 x 10⁶ 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 x g truc cells were labeled in vivo with [2,6-3H]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. Bioynthesis of Dol-PP-linked oligosaccharides was inhibited by the inclusion of 100 µg of tunicamycin (Calbiochem) added from a 1 mg/ml stock in 95% ethanol in each reaction. For [3H]mannose labeling, 2 µCi 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 µg of dried [3H]DGG-mannose (Du Pont-New England Nuclear, 15 Ci/mmol). Tunicamycin was added followed by the membranes. For labeling with [3H]GlcNAc, 2 µCi of 0.1 M GDP-mannose (Sigma) was substituted for the UDP-GlcNAc. Preliminary experiments using 100,000 x g pelleted membranes indicated that incorporation of [3H]GDP-mannose 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 [3H]GPI was made dependent on [3H]Dol-P-Man, radioiodinated Dol-P-Man was synthesized in situ as described by Menon et al. (1990b). Amphomycin (a gift from Dr. R. T. Schwarz, Philips-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, system 1). Chromatogram was 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 En3Hance (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 from *Crotalus adamanteus* (Sigmas) 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 system 2; chromatograms were scanned in presaturated tanks with chloroform/methanol/water (9:9:2, v/v, system 1), chloroform/methanol/water (10:10:2.5, v/v, system 2) or chloroform/methanol/1 M NH4OH (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 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 NH4OH (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.

**Results**

**Table I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Radiolabel*</th>
<th>Chloroform/ methanol</th>
<th>CMW</th>
<th>CMW*</th>
<th>PP1</th>
<th>PP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GDP-Man</td>
<td>0.95</td>
<td>16.0</td>
<td>-d</td>
<td>6.3</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>UDP-GlcNAc</td>
<td>4.5</td>
<td>0.65</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>UDP-GlcNAc</td>
<td>4.5</td>
<td>0.65</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>GDP-Man</td>
<td>50.0</td>
<td>20.0</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>GDP-Man (+ 0.2 µg/ml amphomycin)</td>
<td>5.6</td>
<td>2.4</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>GDP-Man (+ 0.8 µg/ml amphomycin)</td>
<td>0.68</td>
<td>0.05</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*H-labeled sugar nucleotide is indicated. Additions or deletions from the standard reaction supplements are given in parentheses.

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

Radioactivity incorporated is expressed as cpm (counts per minute) per 10⁶ cell equivalents.

Not determined.

**Figure 1**

Thin layer chromatograms of *in vitro*-labeled glycolipids. **A**, species labeled with [3H]DGG-mannose in a reaction supplemented with UDP-GlcNAc. **B**, species labeled with [3H]UDP-GlcNAc in the absence of GDP-mannose. TLC plates were developed using system 2. Arrows indicate the migration positions of PP1 and PP3 (procyclic form GPs) (these study and Field et al., 1991a), P2, P3, I (ManGlcNPI), II (MAN-GlcNPI), III (ManGlcNPI), and GlcN-P1 (bloodstream form trypanosome GPs) (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 GPs corresponding to Man₂GlcNPI species.
TABLE II  
Structural analysis of the two major [3H]mannose-labeled GPI lipids synthesized by procyclic membranes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cleavage/result</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP1 ( (R_e = 0.14) )</td>
<td>PP3 ( (R_e = 0.29) )</td>
</tr>
<tr>
<td>B. thuringiensis PI-PLC (^a)</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit serum GPI-PLD (^b)</td>
<td>+</td>
</tr>
<tr>
<td>Nitrous acid (^c)</td>
<td>58.2</td>
</tr>
<tr>
<td>C. adamanteus PLA (^d)_2</td>
<td>EPM-AHM</td>
</tr>
</tbody>
</table>

\(^a\) 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. Chromatography with the headgroup from P2 and P5 (EPM-AHM, ethanalamine-P-[Man\(\alpha\)]-anhymannositol) obtained.

RESULTS

Incorporation of \(^3\)H-Labeled Sugars into Lipids—The simple hypotonic lyase employed by Masterton et al. (1989) was more efficient at incorporating \(^3\)H-labeled sugars into glycolipid than the 100,000 X g membranes, 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 material (Menon et al., 1990b). Addition of amphotericin to the reaction caused almost complete inhibition of the synthesis of GPIs at 0.8 \(\mu\)g/ml (Table I) consistent with Dol-P-Man as the direct mannosyl-donor, as shown previously for the BSF (data not shown). Labeling of membranes with \(^3\)H UDP-GlcNAc in the presence of GDP-Mann 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 metabolically radiolabeled glycolipids contains GPls, acyl-inositol GPls, and non-GPI glycolipids (e.g. Dol-P-P-GlcNAc, sphingolipids). Headgroup glycans can be cleaved from the GPls by PI-PLC, and recovered by a phase-separation between water and butanol. The acyl-inositol GPls remain untreated, and their headgroups can be released by a deamination reaction, and similarly recovered by phase partitioning. Lipids that are not GPls are unaffected by these two procedures. The respective headgroups from the GPls and acyl-inositol GPls are separately worked up and analyzed by Dionex anion-exchange HPLC.](image-url)
those lacking ethanalamine. 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 (RF = 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% 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 GlcNAc-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 mannosamine substitution have been identified in BSF trypanosomes; GlcNac-Pi and GlcN-Pi (Doering et al., 1989; Menon et al., 1990a). GlcNac-Pi is deacylated 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 1 and Fig. 1B). A single predominant species was seen (RF = 0.71 on system 2), which cochromatographed with GlcN-Pi from BSF on TLC (systems 2 and 3), and two minor, more polar, species (RF\(_{\text{M1}} = 0.54\) 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 GlcNacinositol-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. 1B) 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 mannosse-containing GPls. Membranes were first preincubated with a small amount of GDP-Man to complete synthesis of any endogenous GPI species, and then pulsed with 2 \(\mu\)Ci 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 GPls (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, RF = 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 GPls rises rapidly (the Man\(_{3}\) 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;

\[
\text{[Man\(_{1}\),GlcN-PI}] \to E-P-[Man\(_{1}\),GlcN-PI*] \to E-P-[Man\(_{3}\),GlcN-lysoPI*] \to [PP3]
\]

where PI* is inositol-acyl-P, 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-
by inclusion of amphomycin in the reaction. We cannot draw
labeling, i.e. if the inhibition of incorporation of radiolabel is
strong conclusions about the effect of amphomycin on protein
nously synthesized D~l-p-[~H]Man, and synthesis is inhibited
all GPI-anchored proteins in procyclics are insensitive to PI-
tion is consistent with our previous data demonstrating that
the complete headgroup, EthN-P-Man3GlcN. This observa-
possibilities in the normal physiological system. The latter
present it is not possible to discriminate between these two
omitting GDP-Man from the labeling reaction. We failed to
the inositol, and is therefore not seen by our analysis. At
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 Man1GlcN-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
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 acylhy-
drolase 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 manno-
sylated 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 deter-
mine 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
do effects on the GPI, because our attempts to release the
GPI glycan from the in uitro-labeled PARP were unsuccessful.
However, we did note that the in uitro-labeled PARP was
significantly smaller and less heterogeneous than the tunic-
mycin-treated PARP labeled with [3H]mannose in vivo. In
addition, we saw no evidence of a large heterogeneous glycan
as seen for the PARP anchor, and therefore it is possible that
the in uitro 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 acylhy-
drolase decreases with increased headgroup size, but from the
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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
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, that acts on PP3, is unable to act on the earlier GPI intermediates, as it is not restricted to expression in the procyclic stage is clearly ruled out by the presence of this GPI. A recent observation, 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 further processing of the sn-1 fatty acid does not occur. The phosphate moiety may be a structural requirement for recognition by the PLA 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 PI-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 synthesize PI-PLC-resistant GPI-anchored proteins, and to compare these with the procyclic and the T cell lipids.

Acknowledgments—M. C. F. wishes to thank H. Field, B. Spiner, R. Strauss, and R. Wagner for stimulation.

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