Early steps in glycosylphosphatidylinositol biosynthesis in *Leishmania major*

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A cell-free system based on washed *Leishmania major* membranes was labelled with GDP-[³H]Man in the presence of synthetic glucosaminyl-phosphatidylinositol (GlcN-PI) and *N*-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI). In both cases, the major radiolabelled products were Man α 1-4GlcN α 1-6*myo*-inositol-1-HPO₄- (*sn*-1,2-dipalmitoylglycerol) and Man α 1-4GlcN α 1-6*myo*-inositol-1-HPO₄- (*sn*-1-palmitoyl-2-*lyso*-glycerol), to which an additional D-mannose residue was added when a chase with an excess of GDP-Man was performed. The *L. major* cell-free system can therefore be used to observe the actions of four enzymes, namely

GlcNAc-PI de-N-acetylase, Dol-P-Man-GlcN-PI α 1-4-mannosyltransferase, a phospholipase A₂-like activity and a second α -mannosyltransferase activity. The substrate specificities of the first two of these enzymes were studied using a series of substrate analogues. GlcNAc-PI de-N-acetylase was tested against a variety of N-acylated GlcN-PI substrates and was able to cleave *N*-acetyl and *N*-propyl groups but not larger groups such as *N*-butyl, *N*-isobutyl, *N*-pentyl and *N*-hexyl. The Dol-P-Man-GlcN-PI α 1-4-mannosyltransferase activity required the amino group of the glucosamine residue and the D-configuration of the *myo*-inositol residue of the GlcN-PI acceptor substrate.

INTRODUCTION

The *Leishmania* are trypanosomatid protozoan parasites that cause a variety of diseases in humans, such as Kalazar (*Leishmania donovani*), Espundia (*Leishmania braziliensis*) and oriental sore (*Leishmania major*), collectively called the leishmaniases.

The dividing non-infectious procyclic promastigote form of the parasite is found attached to epithelial cells lining the midgut of the phlebotomine sandfly insect vector. During metacyclogenesis they develop into non-dividing infectious metacyclic promastigotes that detach from the midgut and migrate to the mouthparts of the sandfly. After transmission to a mammalian host during a blood meal, the parasites attach to and invade the macrophages through a receptor-mediated process and thereafter differentiate into round amastigote forms that proliferate inside the phagolysosome. Amastigotes are eventually released into the surrounding media where they invade other macrophages. The ingestion of infected macrophages by the sandfly completes the life cycle.

Leishmania express specialized cell-surface molecules that play key roles in parasite infectivity and survival in the insect vector and the mammalian host [1,2]. The major macromolecule on the surface of the procyclic promastigote is lipophosphoglycan (LPG), which is required for parasite attachment to the midgut of the insect vector [3–5] and which undergoes structural changes during metacyclogenesis [6,7]. Metacyclic promastigote LPG is the major complement acceptor on the parasite surface [8], and opsonization with C3b and C3bi allows the parasite to enter the macrophage by exploiting the host CR1 and CR3 complement receptors (reviewed recently in [9]). Once inside the macrophage, LPG is believed to play a role in suppressing the macrophage oxidative burst ([10] and references therein). The primary structure of LPG has been established for several *Leishmania* spp.

[1,2]. All LPGs contain a polydisperse phosphoglycan domain, composed of backbone structure of -6Gal β 1-4Man α 1-HPO₄- repeats, linked to the plasma membrane by way of a complex glycosylphosphatidylinositol (GPI) anchor that has the structure Gal α 1-6Gal α 1-3Gal β 1-3(Glc α 1-HPO₄-6)Man α 1-3Man α 1-4GlcN α 1-6*myo*-inositol-1-HPO₄- (*sn*-1-alkyl-2-*lyso*-glycerol). The phosphoglycan domain is attached to the 6-position of the non-reducing Gal residue of the LPG GPI anchor. This GPI structure is distinct from that of the widely distributed protein-linked GPI anchors, including that of *Leishmania* promastigote surface protease [10], which have a conserved core structure of NH₂CH₂CH₂-HPO₄-6Man α 1-2Man α 1-6Man α 1-4GlcN α 1-6*myo*-inositol-1-HPO₄-(lipid) [1]. Protein is attached by an amide linkage to the amino group of the ethanolamine residue. However, the aforementioned structures share the motif Man α 1-4GlcN α 1-6*myo*-inositol-1-HPO₄-(lipid) (Man α 1-4GlcN α 1-6PI) that defines the GPI family [1].

Leishmania also synthesize glycoinositol-phospholipids (GIPLs), a family of GPI-like glycolipids that can be divided into three types on the basis of their glycan structures. Type-1 GIPLs are homologous to protein-GPI anchors and contain the motif Man α 1-6Man α 1-4GlcN α 1-6-PI, whereas Type-2 GIPLs contain the motif Man α 1-3Man α 1-4GlcN α 1-6PI, and hybrid GIPLs contain both motifs [i.e. they contain the branched structure Man α 1-6(Man α 1-3)Man α 1-4GlcN α 1-6PI]. The LPG GPI anchor has a Type-2 GIPL structure, suggesting that the biosynthetic pathways of LPG and Type-2 GIPLs have a number of steps in common [11,12]. GIPLs are the predominant surface molecules of amastigote forms of *Leishmania* [13–15], which express little or no LPG [13,16] or surface proteins [16].

The biosynthesis of protein-GPI anchors has been studied in several parasites, including *Trypanosoma brucei*, *Trypanosoma cruzi*, *Plasmodium falciparum* and *Toxoplasma gondii* ([17–24] and references therein), as well as in mammalian cells and

Abbreviations used: AHM, 2,5-anhydromannitol; APAM, *Aspergillus phoenicis* α -mannosidase; GIPL, glycoinositol-phospholipid; GPI, glycosylphosphatidylinositol; GPI-PLD, glycosylphosphatidylinositol-specific phospholipase D; HPTLC, high-performance TLC; JBAM, jack-bean α -mannosidase; LPG, lipophosphoglycan; PI-PLC, phosphatidylinositol-specific phospholipase C; PI, phosphatidylinositol; PLA₂, phospholipase A₂.

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Saccharomyces cerevisiae ([25–30] and references therein). In all cases, GPI biosynthesis appears to involve the transfer of GlcNAc from UDP-GlcNAc to phosphatidylinositol (PI), to form GlcNAc-PI, followed by de-N-acetylation to produce GlcN-PI [31]. Three D-mannose residues and an ethanolamine phosphate group are subsequently transferred to GlcN-PI from Dol-P-Man [32] and phosphatidylethanolamine [33] respectively. In many organisms, some or all of the GPI intermediates also receive a fatty acid on the 2-position of the inositol ring [1,21,28].

Synthetic GPI compounds and analogues thereof have been used recently to establish assays for the GlcNAc-PI de-N-acetylase of *T. brucei* [34], the Dol-P-Man-GlcN-PI α 1-4-mannosyltransferase of *T. brucei* [35] and mammalian cells [36], and the UDP-Gal-GPI anchor α 1-3-galactosyltransferase of *T. brucei* [37].

In this report, synthetic GlcN-PI and its analogues [38,39] have been used with an *L. major* cell-free system to detect the GlcNAc-PI de-N-acetylase and Dol-P-Man-GlcN-PI α 1-4-mannosyltransferase activities and to study the substrate specificities of the enzymes.

EXPERIMENTAL

Materials

GDP-[2-³H]mannose (14.9–17.8 Ci/mmol) and En³Hance™ were purchased from Dupont-NEN. Jack-bean α -mannosidase (JBAM) and pig pancreas phospholipase A₂ (PLA₂) were purchased from Boehringer Mannheim and *Aspergillus phoenicis* α -mannosidase (APAM) and *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PI-PLC) from Oxford GlycoSystems. Whole human serum was used as a source of GPI-specific phospholipase D (GPI-PLD). Ion-exchange resins (AG50X12 and AG3X4) were obtained from Bio-Rad. Solvents were obtained from BDH-Merck and the other reagents from Sigma.

Substrates and substrate analogues

D-GlcNAc1-6D-*myo*-inositol-1-HPO₄-(sn-1,2-dipalmitoylglycerol) (GlcN-PI) and D-GlcNAc1-6L-*myo*-inositol-1-HPO₄-(sn-1,2-dipalmitoylglycerol) (GlcN-P[L]I) were synthesized as described by Cottaz et al. [38]. Compounds were N-acylated using the corresponding acid anhydride (acetic, propionic, butyric, isobutyric, pentanoic and hexanoic) under the conditions described below for N-acetylation. D-GlcNAc1-6D-*myo*-inositol-1-HPO₄-(sn-1,2-dipalmitoylglycerol) (Glc-PI) and D-2-deoxy-GlcNAc1-6D-*myo*-inositol-1-HPO₄-(sn-1,2-dipalmitoylglycerol) (2-deoxy-Glc-PI) were synthesized as described by Cottaz et al. [39]. The purity of the synthetic compounds was checked before use by negative-ion electrospray MS. The concentrations of stock solutions were measured by analysing the *myo*-inositol content by GC-MS, as described below.

Preparation of *L. major* membranes

L. major (V121) promastigotes were grown to 1.25×10^7 cells/ml in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal calf serum. The cells were pelleted, washed with ice-cold PBS and suspended in 0.1 mM Tos-Lys-CH₂Cl ('TLCK') containing 1 μ g/ml leupeptin to give a final density of 1×10^9 cells/ml. The cells were then disrupted twice at 2.8 MPa in a nitrogen-cavitation bomb. An equal volume of 0.1 M Hepes, pH 7.4, containing 50 mM KCl, 10 mM MgCl₂, 10 mM MnCl₂, 20% (v/v) glycerol, 0.1 mM Tos-Lys-CH₂Cl and 1 μ g/ml leupeptin was added, and aliquots (5×10^8 cells/ml) were snap-frozen in liquid N₂ and stored at -70°C [40].

Mannosyltransferase assays

L. major membranes were washed twice in 0.1 M Hepes, pH 7.4, containing 25 mM KCl, 5 mM MgCl₂, 0.1 mM TLCK and 2 μ g/ml leupeptin and suspended in $2 \times$ concentrated incorporation buffer [0.1 M Hepes, pH 7.4, 50 mM KCl, 10 mM MgCl₂, 10 mM MnCl₂, 20% (v/v) glycerol, 2.5 μ g/ml tunicamycin, 0.2 mM Tos-Lys-CH₂Cl, 2 μ g/ml leupeptin and, except for the incubations in Figure 1 (lanes 1, 2 and 4), 1 mM dithiothreitol.] The suspended lysate was vortex-mixed, sonicated briefly, added to a tube containing dried GDP-[³H]Man (0.3 μ Ci/ 10^7 cell equivalents) and sonicated. Aliquots of 20 μ l (equivalent to 3×10^7 cells) were added to the reaction tubes containing the various GlcN-PI analogues (to give a final concentration of 35 μ M) or to UDP-GlcNAc (to give a final concentration of 1 mM) in 20 μ l of water. The reaction tubes were incubated at 30°C for 1 h, and the labelled glycolipids were extracted by the addition of 270 μ l of chloroform/methanol (1:1, v/v), 16 h at 4°C . The glycolipid products were recovered in the chloroform/methanol/water fraction, which was concentrated to dryness and partitioned between butan-1-ol and water, as described previously [41]. Aliquots of the butan-1-ol phase containing the glycolipid products were subjected to HPTLC analysis (before and after enzyme digestions and chemical treatments).

For the experiments using amphomycin, the lysates were made 1 mg/ml with respect to amphomycin and 10 mM with respect to CaCl₂ and preincubated for 15 min at 0°C before being added to the donor and acceptor substrates, as described above.

De-N-acylation studies were performed under conditions identical with those described above, except that the washed *Leishmania* membranes were preincubated with 1 mM GDP-Man for 10 min at 30°C and washed again before use. This pretreatment mannosylates any endogenous mannose acceptors with non-radioactive Man, thereby precluding the labelling of endogenous glycolipids on subsequent addition of GDP-[³H]Man.

HPTLC

Samples and glycolipid standards were applied to 10 cm aluminium-backed silica-gel 60 HPTLC plates (Merck) and developed in solvent system A [chloroform/methanol/1 M ammonium acetate/13 M ammonium hydroxide/water (180:140:9:9:23, by vol.)]. Samples of glycan headgroups were applied to the same plates and developed with solvent system B [propan-1-ol/acetone/water (9:6:4, by vol.)]. Radiolabelled components were detected by fluorography at -70°C , after being sprayed with En³Hance™, using Kodak XAR-5 film and an intensifying screen. For preparative HPTLC, [³H]Man-labelled glycolipids were located using a Raytest RITA linear analyser and extracted as described previously [35].

Enzyme treatments of radiolabelled glycolipids

Digestions of glycolipids with APAM, JBAM, PI-PLC and GPI-PLD, and subsequent solvent-partitioning and HPTLC analysis, were performed as described by Güther et al. [41]. PLA₂ digestion was carried out at 37°C in 40 μ l of 25 mM Tris/HCl, pH 8.0, containing 2 mM CaCl₂ and 0.1% sodium deoxycholate, with the addition of 8 units of enzyme at hourly intervals over 3 h, followed by further incubation at 37°C for 12 h. Digestions of glycan headgroups with APAM and JBAM were performed as described previously [42].

Chemical treatments of radiolabelled glycolipids and glycan headgroups

Delipidation of glycolipids was carried out with 300 μ l of conc. $\text{NH}_4\text{OH}/50\%$ propan-1-ol (1:1, v/v) at 50 °C for 5 h.

Deamination of glycolipids was carried out in 20 μ l of 0.1 M sodium acetate, pH 4.0, containing 0.01% Zwittergent 3-16. Aliquots (10 μ l) of freshly prepared 0.5 M NaNO_2 were added at hourly intervals during incubation at 60 °C for 4 h. Lipid products were extracted with butan-1-ol for HPTLC analysis [41].

Glycans were N-acetylated at 0 °C in 100 μ l of saturated NaHCO_3 by the addition of three aliquots (2.5 μ l) of acetic anhydride over 20 min. The reaction mixture was warmed to room temperature, desalted by passage through AG50X12(H^+) ion-exchange resin and concentrated to dryness. Residual acetic acid was removed by coevaporation with toluene (2×50 μ l). N-Acetylated glycolipids were extracted into butan-1-ol and salts removed by washing the butan-1-ol phase with water [41].

Glycan headgroup analysis

Radiolabelled glycolipids were delipidated, deaminated and reduced with NaB^2H_4 , whereafter the glycan headgroups were desalted by passage through AG50(H^+) and AG3(OH^-) [43]. Radiolabelled neutral glycan headgroups from these procedures were dissolved in water and mixed with glucose oligomer internal standards. The solutions were filtered through a 0.22 μ m membrane and analysed by Bio-Gel P4 gel filtration using an Oxford GlycoSystems GlycoMap. Fractions (250 μ l) were collected and counted for radioactivity. The neutral glycan headgroups were also analysed by HPTLC and fluorography as described above.

Inositol analysis

GC-MS (Hewlett-Packard MSD 5970 series) was used to measure the *myo*-inositol content of the synthetic substrates. Aliquots of samples were mixed with an internal standard (*myo*-[1,2,3,4,5,6- ^3H]inositol (100 pmol)) and hydrolysed with 50 μ l of 6 M HCl at 110 °C for 16–18 h. The products were dried, converted into their trimethylsilyl derivatives and analysed by GC-MS, as described previously [43].

Electrospray MS

A Quattro (Micromass UK) spectrometer was used to acquire negative-ion electrospray mass spectra over the mass range m/z 150–1150. Samples (5–20 μ l at approx. 10 pmol/ μ l) were introduced in aq. 50% acetonitrile at a flow rate of 10 μ l/min. Several scans were averaged using MassLynx software.

Radiolabelled standards

Authentic standards of $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4[1\text{-}^3\text{H}]2,5\text{-anydromannitol}$ (Man_3AHM) and $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4[1\text{-}^3\text{H}]2,5\text{-anydromannitol}$ (Man_4AHM) were obtained from the GPI anchors of *T. brucei* variant surface glycoprotein MITat1.5 [44] and the GPI-protein fraction of *Saccharomyces cerevisiae* [45] respectively. The partial acid hydrolysate of Man_4AHM was prepared as described previously [42]. [^3H]Myristate-labelled $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{GlcN-}(\text{dimyristoyl})\text{PI}$ ($\text{Man}_3\text{GlcN-PI}$) was prepared by the method of Milne et al. [46].

RESULTS

Mannosyltransferase activity of *Leishmania* membranes

Washed *Leishmania* membranes (the *Leishmania* cell-free system) were incubated with GDP-[^3H]Man in the presence or absence of various compounds. With only GDP-[^3H]Man present (Figure 1, lane 1), endogenous dolichol-phosphate-[^3H]mannose (Dol-P-[^3H]Man) is formed, because of the presence of endogenous dolichol-phosphate-mannose synthetase, together with an endogenous glycolipid (glycolipid E). The addition of the exogenous acceptor GlcNAc-PI resulted in the formation of an additional radiolabelled glycolipid (glycolipid Z) (Figure 1, lane 2). The presence of a reducing agent, 1 mM dithiothreitol, increased the labelling of glycolipid Z and revealed the formation of another glycolipid (glycolipid Y) (Figure 1, lane 3) whereas ATP had no effect (Figure 1, lane 4). All further experiments were performed in the presence of 1 mM dithiothreitol.

Preincubation of the cell-free system with amphotycin and CaCl_2 prevented the formation of Dol-P-[^3H]Man from GDP-

Figure 1 Mannosylation of endogenous and exogenous acceptors by the *Leishmania* cell-free system

Washed *Leishmania* membranes were incubated with GDP-[^3H]Man without (lane 1) and with (lanes 2, 3 and 4) synthetic GlcNAc-PI and with 1 mM dithiothreitol (DTT) (lane 3) or 1 mM ATP (lane 4), as indicated. Labelled glycolipids were extracted and analysed by HPTLC using solvent system A and fluorography. The identities of the bands, including Dol-P-Man (DPM), are indicated on the left of the chromatogram. The positions of [^3H]myristate-labelled $\text{Man}_3\text{GlcN-}(\text{dimyristoyl})\text{PI}$ and glycolipid A ($\text{EtN-P-Man}_3\text{GlcN-}(\text{dimyristoyl})\text{PI}$) standards are indicated on the right of the chromatogram.

Table 1 Sensitivity of GPI intermediates to enzymic and chemical treatments

Summary of the glycolipid digestion data used to assign the general structure of each glycolipid species; + indicates a positive digestion, and – indicates resistance to the treatment. An authentic standard of [^3H]myristate-labelled $\text{Man}\alpha 1\text{-}2\text{Man}\alpha 1\text{-}6\text{Man}\alpha 1\text{-}4\text{GlcN-}6\text{PI}$ (STD) was used as a positive control for all of the digests. n.d., not determined.

Band	JBAM	PI-PLC	GPI-PLD	HONO	APAM	Base	PLA ₂	Assignment
DPM	—	—	—	—	—	—	—	Dol-P-Man
E	+	+	+	+	—	+	+	$\text{Man}_1\text{GlcN-}(\text{alkylacyl})\text{PI}$
E'	+	+	+	+	—	+	+	$\text{Man}_1\text{GlcN-}(\text{alkylacyl})\text{PI}$
Y	+	+	+	+	—	+	+	$\text{Man}_1\text{GlcN-}(\text{diacyl})\text{PI}$
E+	+	+	+	+	—	+	n.d.	$\text{Man}_2\text{GlcN-}(\text{alkylacyl})\text{PI}$
Y+	+	+	+	+	—	+	n.d.	$\text{Man}_2\text{GlcN-}(\text{diacyl})\text{PI}$
Z	+	+	+	+	—	+	—	$\text{Man}_1\text{GlcN-}(\text{lyso acyl})\text{PI}$
Z+	+	+	+	+	—	+	n.d.	$\text{Man}_2\text{GlcN-}(\text{lyso acyl})\text{PI}$
STD	+	+	+	+	+	+	+	$\text{Man}_3\text{GlcN-}(\text{diacyl})\text{PI}$

Figure 2 Characterization of the [³H]Man-labelled glycolipids

Washed *Leishmania* membranes were labelled with GDP-[³H]Man in the presence of synthetic GlcNAc-PI and the extracted [³H]mannosylated glycolipids were subjected to base hydrolysis (OH⁻), PI-PLC digestion, JBAM digestion, HNO₂ deamination (HONO) (top) or PLA₂ digestion (bottom), as indicated. The products that partitioned into butan-1-ol were analysed by HPTLC using solvent system A and fluorography. The identities of the bands are indicated on the left and the right of the chromatograms.

[³H]Man and dramatically reduced (> 95%) the labelling of glycolipids E, Y and Z (results not shown). This suggests that the relevant mannosyltransferase(s) are Dol-P-Man-dependent enzymes.

Analysis of glycolipids Y and Z (Table 1) showed that they were both sensitive to JBAM (Figure 2, top, lane 7), deamination (Figure 2, top, lane 9), base hydrolysis (Figure 2, top, lane 1) and PI-PLC (Figure 2, top, lane 5). This indicates that the mannosylated products contain α Man, non-N-acetylated glucosamine and base-labile (acyl-glycerol) lipids as part of a PI component. Therefore both glycolipids Y and Z are mannosylated GPI structures. The conversion of GlcNAc-PI into GlcN-PI before [³H]mannosylation (evident from the deamination result) shows that the *Leishmania* membranes contain an active GlcNAc-PI de-N-acetylase (see below). Both glycolipids Y and Z were resistant to APAM (data not shown), suggesting that neither terminates with a Man α 1-2Man structure. Digestion with PLA₂ (Figure 2, bottom) showed that glycolipid Z was insensitive to this enzyme, suggesting that it is a *lyso*- species, whereas glycolipid Y was sensitive to PLA₂ and was most likely transformed into a *lyso*- species identical with glycolipid Z.

Glycolipids Y and Z were characterized further by headgroup analysis. Samples of the [³H]Man-labelled glycolipids were purified by preparative HPTLC and subjected to mild base hydrolysis, HNO₂ deamination and NaB³H₄ reduction. This procedure generates glycan headgroups that terminate in AHM from GPI species [20,35]. Analysis of the glycan headgroup fractions of both glycolipids by Bio-Gel P4 chromatography showed that they co-chromatographed with an authentic standard of Man α 1-4AHM at 2.3 glucose units (results not shown). The glycolipid Y and Z glycan headgroups also co-migrated with

Figure 3 Characterization of the [³H]Man-labelled glycan headgroups

The [³H]Man-labelled glycan headgroups generated from HPTLC-purified glycolipid Y (Y) (a, lanes 2–4 and b, lanes 2 and 3) and glycolipid Z (Z) (a, lanes 5–7 and b, lanes 4 and 5), and an authentic standard of Man α 1-2Man α 1-6Man α 1-4[1-³H]AHM (M₂AHM) (a, lanes 8–9 and b, lanes 6 and 7), were digested with JBAM and APAM (a) or subjected to acetolysis (Ac₂O) (b), as indicated. The products were analysed by HPTLC using solvent system B and fluorography. A partial acid hydrolysate standard (STD) of Man α 1-2Man α 1-2Man α 1-6Man α 1-4[1-³H]AHM (Man₄AHM), which contains a mixture of labelled Man₄AHM, Man₃AHM, Man₂AHM, Man₁AHM and AHM, was run in (a, lanes 1 and 11) and (b, lanes 1 and 8). The position of free mannose monosaccharide is indicated on the left of the chromatogram in (a).

an authentic sample of Man α 1-4AHM on HPTLC (Figure 3a, compare lanes 4 and 7 with lanes 1 and 11) and were shown to be sensitive to JBAM, producing free [³H]Man (Figure 3a, lanes 2 and 5), whereas they were resistant to APAM (Figure 3a, lanes 3 and 6) and to acetolysis (Figure 3b) as expected for Man α 1-4AHM. The results of JBAM digestion (Figure 3a, lane 9), APAM digestion (Figure 3a, lane 10) and acetolysis (Figure 3b, lane 6) of a Man α 1-2Man α 1-6Man α 1-4[1-³H]AHM standard are shown as positive controls for these treatments.

These data are consistent with a Man α 1-4AHM structure for the glycan headgroups derived from both glycolipid Y and glycolipid Z. Since these headgroups are the products of HNO₂ deamination, it can be concluded that the original headgroup of both glycolipids is Man α 1-4GlcN.

From the results described above, glycolipids Y and Z can be identified as Man α 1-4GlcN α 1-6D-*myo*-inositol-1-HPO₄-(*sn*-1,2-dipalmitoylglycerol) and Man α 1-4GlcN α 1-6D-*myo*-inositol-1-HPO₄-(*sn*-1-palmitoyl-2-*lyso*-glycerol) respectively. The *sn*-1,2-dipalmitoylglycerol lipid structure may be assumed from the fact that these glycolipids are products of either the synthetic GlcN α 1-6D-*myo*-inositol-1-HPO₄-(*sn*-1,2-dipalmitoylglycerol)

Figure 4 Acceptor-substrate specificity of *Leishmania* Dol-P-Man-GlcN-PI α 1-4-mannosyltransferase activity

Washed *Leishmania* membranes were labelled with GDP-[3 H]Man without (lane 1) and with UDP-GlcNAc (lane 2) or synthetic GlcN-PI (lane 3) or analogues thereof (lanes 3–8). The products that partitioned into butan-1-ol were analysed by HPTLC using solvent system A and fluorography. The identities of the bands are indicated on the left and right of the chromatogram.

or GlcNAc α 1-6D-*myo*-inositol-1-HPO $_4$ - (*sn*-1,2-dipalmitoylglycerol) acceptors.

Acceptor substrate specificity of the Dol-P-Man-GlcN-PI α 1-4-mannosyltransferase activity

The cell-free system was incubated with GlcN-PI (the natural acceptor substrate) and various GlcN-PI analogues. In all cases, the membranes produced labelled Dol-P-[3 H]Man and low levels of endogenous GPI intermediates, i.e. glycolipid E (Figure 4, lane 1). The only substrates that produced additional labelled products were GlcN-PI (Figure 4, lane 3) and GlcNAc-PI (Figure 4, lane 4). Of these, GlcNAc-PI was superior in priming the pathway. The glycolipids labelled Y and Z in Figure 4 were identified as Man α 1-4GlcN-PI and Man α 1-4GlcN-(*lyso*)PI respectively on the basis of their R_f values and sensitivities to JBAM, PI-PLC, GPI-PLD, PLA $_2$, base hydrolysis and HNO $_2$ deamination (results not shown). The GlcN-PI analogues Glc-PI (Figure 4, lane 5), 2-deoxy-Glc-PI (Figure 4, lane 6), GlcN-P[L]I (Figure 4, lane 7) and GlcNAc-P[L]I (Figure 4, lane 8) showed no detectable mannose-acceptor activity, only labelled Dol-P-Man and glycolipid E being detected.

Analysis of the endogenous mannosylated products of the *L. major* cell-free system

Labelling of glycolipid E was independent of exogenous acceptor substrates (Figure 4, lane 1). Glycolipid E has an R_f value similar to that of glycolipid Y and is sensitive to JBAM and PI-PLC (Table 1), as expected for a Man $_1$ GlcN-PI structure. The PI moiety of glycolipid E is most likely an alkylacyl-PI since glycolipid E produced the same *lyso*- species on treatment both with PLA $_2$ and mild alkali (Table 1; results not shown). Thus glycolipid E is probably Man α 1-4GlcN α 1-D-*myo*-inositol-1-HPO $_4$ -(*sn*-1-alkyl-2-acylglycerol) [Man $_1$ GlcN-(alkylacyl)PI]. The formation of [3 H]Man-labelled glycolipid E in the absence of a glucosamine donor suggests that the *Leishmania* membranes contain some endogenous GlcN-(alkylacyl)PI. In the presence of GDP-[3 H]Man, the addition of UDP-GlcNAc to the cell-free system stimulated the labelling of glycolipid E and revealed a novel glycolipid (glycolipid E') having an R_f value slightly lower than that of glycolipid E (Figure 4, lane 2). Like glycolipid E, glycolipid E' is JBAM-sensitive and forms a *lyso*- species on

Figure 5 Pulse-chase labelling of the *Leishmania* cell-free system

Washed *Leishmania* membranes were labelled with GDP-[3 H]Man for 10 min (lane 1) and then chased with 1 mM unlabelled GDP-Man for 30 min (lane 2). The products that partitioned into butan-1-ol were analysed by HPTLC using solvent system A and fluorography. The identities of the bands are indicated on the left of the chromatogram.

treatment both with PLA $_2$ and mild alkali (Table 1; results not shown). Taken together with its R_f value, glycolipid E' is likely to be another Man $_1$ GlcN-(alkylacyl)PI species having an (alkylacyl)PI moiety slightly less hydrophobic than that of glycolipid E. The effects of UDP-GlcNAc suggest that the *Leishmania* membranes contain endogenous (alkylacyl)PI species that can act as substrates for the UDP-GlcNAc-PI GlcNAc-transferase/GlcNAc-PI de-N-acetylase system. The effects of UDP-GlcNAc were abolished on substituting dithiothreitol for *N*-ethylmaleimide in the reaction mixture (results not shown), suggesting that the *Leishmania* UDP-GlcNAc-PI GlcNAc-transferase, like that of *T. brucei* [46], is inhibited by thiol-alkylating reagents.

The bands with R_f values slightly lower than glycolipid Z (Figure 4, lanes 1 and 2) are probably the *lyso*- forms of glycolipids E and E', having only an *sn*-1-alkyl chain on the PI moiety.

Detection of a second mannosyltransferase activity

Evidence for a second mannosyltransferase activity was obtained when an unlabelled GDP-Man chase was applied 10 min after labelling the membranes with GDP-[3 H]Man in the presence of GlcNAc-PI (Figure 5, lane 2). In this experiment, three new mannosylated glycolipids (glycolipids E+, Y+ and Z+) were detected and characterized by enzyme and chemical treatments (see Table 1). Taking into account their R_f values, glycolipid E+ is most likely Man $_2$ GlcN-(alkylacyl)PI, and glycolipids Y+ and Z+ are most likely Man $_2$ GlcN-PI and Man $_2$ GlcN-(*lyso*)PI respectively, indicating that [3 H]Man $_1$ GlcN-PI and [3 H]Man $_1$ GlcN-(*lyso*)PI species formed in the presence of GDP-[3 H]Man (Figure 4, lane 1) can be further α -mannosylated when an excess of GDP-Man is present. The nature of the second mannosylation was not studied further but, given that glycolipids Y+ and Z+ are resistant to APAM, it seems likely that they have a Type-2 GIPL glycan structure, specifically Man α 1-3Man α 1-4GlcN (see the Discussion).

Substrate specificity of the *L. major* GlcNAc-PI de-N-acetylase

The requirement for de-N-acetylation of exogenous GlcNAc-PI before mannosylation by *T. brucei* membranes [35] also appears to hold true for *Leishmania* membranes, since all [3 H]mannosylated products are sensitive to deamination (see Figure 2a, lane 9). Thus de-N-acetylation of GlcNR-PI substrates (see below) by the *Leishmania* de-N-acetylase may be judged by

Figure 6 Substrate specificity of the *Leishmania* GlcNAc-PI de-N-acetylase

Washed *Leishmania* membranes were preincubated with GDP-Man, washed and labelled with GDP-[3 H]Man in the presence of a variety of synthetic GlcNR-PI substrates. The products that partitioned into butan-1-ol were analysed by HPTLC using solvent system A and fluorography. The identities of the bands are indicated on the left of the chromatogram.

their subsequent [3 H]mannosylation in the *Leishmania* cell-free system.

Synthetic GlcN-PI was N-acylated using a variety of acid anhydrides to yield GlcNR-PI, where R = acetyl (Ac), propyl (Pr), butyl (Bu), isobutyl (iBu), pentyl (Pent) and hexyl (Hex) groups. These compounds were incubated with the *Leishmania* cell-free system in the presence of GDP-[3 H]Man, and the glycolipid products were analysed by HPTLC (Figure 6). In this experiment, preincubation of the membranes with unlabelled GDP-Man prevented subsequent labelling of endogenous GlcN-(alkylacyl)PI by GDP-[3 H]Man. As expected, exogenous GlcN-PI was [3 H]mannosylated to yield glycolipids Y and Z (Figure 6, lane 1). De-N-acetylation of GlcNAc-PI before mannosylation was evident from the formation of the same products (Figure 6, lane 2). De-N-acylation of GlcNPr-PI was less efficient, as judged by the reduced levels of labelled glycolipids Y and Z (Figure 6, lane 3). The other synthetic GlcNR-PI substrates (R = Bu, iBu, Pent and Hex) did not yield detectable amounts of glycolipids Y and Z (Figure 6, lanes 4–7), indicating that they are poor substrates for the de-N-acetylase. These data point to the fact that the *Leishmania* de-N-acetylase can only remove short acyl chains (Ac and Pr) from GlcNR-PI.

DISCUSSION

In this paper, synthetic intermediates have been used to prime the GPI biosynthetic pathway in a cell-free system of washed *Leishmania* membranes, an approach similar to that used recently with *T. brucei* [35,36] and mammalian and yeast cells [36]. The results are summarized in Scheme 1. There are some clear differences between the results obtained with the *L. major* cell-free system and that of *T. brucei*.

(i) Mannosylation of exogenous GPI intermediates in the *Leishmania* system is stimulated by the reducing agent dithiothreitol. Whereas *N*-ethylmaleimide was included in buffers used with the trypanosome system to suppress the labelling of endogenous GPI intermediates (by selective inhibition of the UDP-GlcNAc-PI α 1-6-GlcNAc-transferase [46]), it was not included in the *Leishmania* cell-free system. A consequence of this is that labelled endogenous GPI intermediates (e.g. glycolipid

Scheme 1 Summary of the early steps in GPI biosynthesis observed using the *Leishmania* cell-free system

E) were observed in addition to those formed by the mannosylation of exogenous substrates. When necessary, the labelling of endogenous GPI intermediates in the *Leishmania* system can be suppressed by preincubating the membranes with unlabelled GDP-Man before the addition of GDP-[3 H]Man.

(ii) The range of [3 H]mannosylated products seen with the *Leishmania* cell-free system is limited when compared with those of the trypanosome system. The major products are Man $_{1,2}$ GlcN-PI and Man $_{1,2}$ GlcN-(lyso)PI, whereas the trypanosome system produces Man $_{1,3}$ GlcN-PI and EtN-P-Man $_3$ GlcN-PI [35]. Furthermore the production of Man $_2$ -containing species in the *Leishmania* system required a chase with an excess of unlabelled GDP-Man. These data suggest that the GPI biosynthetic enzymes are less tightly coupled in the *Leishmania* membranes and/or that the second mannosyltransferase, operating during the unlabelled GDP-Man chase, requires a relatively high concentration of GDP-Man. However, some coupling of the *Leishmania* GlcNAc-PI de-N-acetylase and Dol-P-Man-GlcN-

PI α 1-4-mannosyltransferase is evident from the greater labelling of glycolipids Y and Z when GlcNAc-PI is used to prime the pathway instead of GlcN-PI. The difference is similar to the 6-fold stimulation of mannosylation observed in the trypanosome system [35].

(iii) No inositol-acylated GPI intermediates are observed in the *Leishmania* system, whereas they are abundant in the trypanosome system [20,47]. In agreement with structural analyses [1,2] and labelling experiments with living cells [12], it appears that *Leishmania* do not perform this particular modification to their GPI intermediates.

(iv) The GPI intermediates observed in the *Leishmania* cell-free system most likely belong to the Type-2 GIPL/LPG pathways rather than the Type-1 GIPL/protein-GPI anchor pathways. This conclusion is based on the observations that: (a) *L. major* promastigotes produce about 10^7 Type-2 GIPLs and 5×10^6 LPGs per cell division, but few Type-1 GIPLs and only 5×10^5 protein-GPI anchors; (b) the largest intermediates produced were Man₂-containing species, as expected for the Type-2 GIPL/LPG pathways, which would require the presence of a galactofuranose donor to proceed further; (c) the formation of the *lyso*- species Man₁-GlcN-(*lyso*)PI (glycolipids Z and Z+) suggests the action of a PLA₂-like activity similar to the one postulated to generate the (*lyso*-alkyl)PI species of all mature LPGs.

Previous models of LPG biosynthesis have placed the putative PLA₂ event beyond GIPL-3 to reflect the observed steady-state levels of GIPL species [1,11]. However, the formation of glycolipids Z and Z+ in the cell-free system, as well as the relative abundance of *lyso*- species among the early GPI intermediates labelled with [³H]GlcN in living promastigotes [12], suggests that this view should be revised and that the PLA₂-like enzyme has access to the early GPI intermediate Man₁GlcN-PI. Whether this is a controlling step (i.e. whether removal of the *sn*-2-fatty acid from Man₁GlcN-PI directs GPI intermediates to the LPG biosynthetic pathway) or whether the PLA₂-like enzyme can act on all GPI intermediates up to and including GIPL-3 remains to be determined.

The substrate specificity of the *Leishmania* Dol-P-Man-GlcN-PI α 1-4-mannosyltransferase appears to be similar to that of the trypanosomal enzyme [35]. Neither Glc-PI nor 2-deoxy-Glc-PI were mannosylated by the *Leishmania* cell-free system, indicating that the amino group of GlcN-PI plays a key role in acceptor substrate recognition by the mannosyltransferase. Similarly, replacement of the D-*myo*-inositol residue with L-*myo*-inositol in GlcN-PI and GlcNAc-PI abolishes mannosylation. These data suggest that the orientation of one or more hydroxy groups and/or the spatial orientation of the phosphatidyl group (relative to GlcN) is/are crucial for acceptor-substrate recognition. The *Leishmania* and trypanosomal Dol-P-Man-GlcN-PI α 1-4-mannosyltransferases differ in one significant respect from those of yeast and mammalian cells. The latter enzymes appear to require acylation of the inositol ring of GlcN-PI before the addition of the first mannose residue [29,30,36], whereas this is not the case with the parasite enzymes ([20]; this paper). This apparent difference in substrate specificity between the trypanosomatid and mammalian enzymes suggests that the development of a trypanosomatid-specific inhibitor may be feasible.

The substrate specificity of *Leishmania* GlcNAc-PI de-N-acetylase was also investigated. In this case we exploited the requirement for the free amino group of GlcN-PI before mannosylation can occur (see above). Synthetic GlcNR-PI substrates were added to the cell-free system and their abilities to

undergo de-N-acylation and subsequent mannosylation were assessed. The results showed that the N-propyl derivative (GlcNPr-PI) was de-N-acylated less efficiently than GlcNAc-PI, whereas the larger N-acyl derivatives (N-butyl, N-isobutyl, N-pentyl and N-hexyl) were not de-N-acylated. This result is similar to that observed for both trypanosomal and mammalian GlcNAc-PI de-N-acetylase activities (D. K. Sharma, T. K. Smith, A. Crossman, J. S. Brimacombe and M. J. A. Ferguson, unpublished work), suggesting that there are no obvious differences in the substrate specificities of this activity in different species.

In summary, a convenient assay has been established for studying the substrate specificities of the enzymes involved in the early stages of GPI biosynthesis in *L. major* promastigotes. Thus far, the application of a range of synthetic GlcN-PI analogues [38,39] has shown that the *Leishmania* GlcNAc-PI de-N-acetylase and Dol-P-Man-GlcN-PI α 1-4-mannosyltransferase have similar specificities to the trypanosomal enzymes, and that the *Leishmania* mannosyltransferase has a different acceptor substrate specificity from that of mammalian cells. The assay also detected a PLA₂-like activity that is likely to be involved in LPG biosynthesis. It should be possible to probe the specificity of this enzyme using a cell-free system and GlcN-PI analogues with different lipid structures.

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