Glycoinositol-phospholipid profiles of four serotypically distinct Old World Leishmania strains

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Glycoinositol-phospholipids (GIPLs) are the major glycolipid class and prominent surface antigens of leishmanial parasites. The GIPLs from four serologically distinct Old World strains of *Leishmania* were characterized to determine inter- and intraspecific differences in these glycolipids. These studies showed that: (1) the major GIPLs of *Leishmania tropica* (LRC-L36) and *L. aethiopica* (LRC-L495) belong to the α-mannose-terminating GIPL series (iM2, iM3 and iM4) that are structurally related to the glycosyl-phosphatidylinositol anchor of both the surface proteins and the abundant lipophosphoglycan (LPG). In contrast, the GIPLs from two *Leishmania major* strains (LRC-L456 and LRC-L580) belong to the α-galactose-terminating GIPL series (GIPL-1, -2 and -3) that are more structurally related to the LPG anchor; (2) the GIPL profiles of the *L. major* strains differed in that a significant proportion of the GIPL-2 and -3 species (approximately 40% and 80%, respectively) in LRC-L580 are substituted with a glucose-1-PO₄ residue, while this type of substitution was not detected in LRC-L456; and (3) all the GIPLs contained either an alkyl- or a lysoalkyl-phosphatidylinositol moiety. However, the alkyl chain compositions of different GIPLs within the same strain were variable. In *L. major*, the major GIPL species contained alkylacylglycerols with predominantly C₁₈:₀ and C₂₄:₀ alkyl chains, whereas the glucose-1-PO₄-substituted GIPLs contained exclusively lysoalkylacylglycerols with C₂₄:₀ alkyl chains. In *L. tropica*, the major GIPL, iM2, contained predominately C₂₄:₀ alkyl chains whereas the structurally related iM3 and iM4 GIPLs in this strain contained predominantly C₁₈:₀ alkyl chains. In *L. aethiopica* all the GIPLs (iM2, iM3, iM4) contained C₂₄:₀ alkyl chains. These data suggest that the synthesis of the GIPLs may occur in more than one subcellular compartment. The possibility that species-specific differences in the predominant surface glycan structures may modulate the interaction of the parasite with the insect and mammalian hosts is discussed.

INTRODUCTION

Protozoan parasites of the genus *Leishmania* are the causative agents of a group of diseases called the leishmaniasis, which affect over 12 million people worldwide (WHO, 1990; Ashford et al., 1992). These parasites have a digenetic life-cycle, alternating between a flagellated, promastigote stage that lives extracellularly in the digestive tract of its sandfly vector and a non-motile amastigote stage that proliferates within the acidified phagolysosomes of the macrophages of the mammalian host. Although all *Leishmania* have very similar morphologies, physiologies and life-cycles, the different species can be distinguished by their selective association with particular sandfly vector species, the overall clinical spectrum with which each is associated and the various intrinsic biochemical and serological differences they display (Peters and Killick-Kendrick, 1987).

There is considerable evidence that glycoconjugates on the parasite surface are involved in mediating specific host–parasite interactions as well as being highly antigenic in the mammalian host. The major glycoconjugates on the promastigote surface include a number of glycosyl-phosphatidylinositol (GPI)-anchored proteins, a complex lipophosphoglycan (LPG) and a family of low-molecular mass glycosphingolipids (GIPLs) (reviewed in Turco and Descoteaux, 1992; McConville and Ferguson, 1993). Together, these molecules form a continuous glycoalyx network over the promastigote surface (Pimenta et al., 1991). The thickness of this glycoalyx is considerably reduced in the amastigote stage due to the reduced surface expression of the major promastigote proteins and the LPG (McConville and Blackwell, 1991; Schneider et al., 1992; Bahr et al., 1993; Medina-Acosta et al., 1993). The residual amastigote glycoalyx is composed principally of parasite-derived GIPLs and glycosphingolipids which mainly appear to have been acquired from the mammalian host (McConville and Blackwell, 1991; Schneider et al., 1993b; Straus et al., 1993).

The structures of GIPLs have been determined from a single virulent strain each of the species *Leishmania major* (McConville et al., 1990), *L. mexicana* (McConville et al., 1993) and *L. donovani* (McConville and Blackwell, 1991; Sevlever et al., 1991). These studies indicate the presence of marked species-specific differences in the respective GIPL profiles. In this study we have characterized the GIPLs from four strains representing three Old World species of *Leishmania* to determine, further, the nature and extent of this inter-species and intra-specific polymorphism. These species are associated with human cutaneous (CL) and diffuse cutaneous (DCL) leishmaniasis and display defined selectivities for particular sandfly vector species. They also showed distinct serological profiles, as evidenced by the binding of specific polyclonal and monoclonal antibodies to cell-surface antigens and the extracellular pool of "excreted factors" (EFs) (Schnur et al., 1972; Schnur, 1982; Jaffe et al., 1984; Jaffe and Sarnfield, 1987; Schnur et al., 1990).

The results obtained here indicate species- and strain-specific differences and suggest that most species express predominantly

Abbreviations used: DU, Dionex unit; EF, excreted factor; LPG, lipophosphoglycan; GIPL, glycoinositol-phospholipid; h.p.l.c., high-performance thin layer chromatography; PI, phosphatidylinositol; AHM, 2,5-anhydro-mannitol; iM2, Manα1-3Manα1-4GlcN-Pi; iM3, Manα1-3[Manα1-2Manα1-6]Manα1-4GlcN-Pi; iM4, Manα1-3[Manα1-2Manα1-6]Manα1-4GlcN-Pi; GIPL-1, Galβ1-3Manα1-3Manα1-4GlcN-Pi; GIPL-2, Galα1-6Galα1-3Galβ1-3Manα1-3Manα1-4GlcN-Pi; GIPL-3, Galα1-3Galβ1-3Manα1-3Manα1-4GlcN-Pi; GIPL with either Glc-1-PO₄ or PO₄ located on the mannose residue distal to the core GlcN.

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mannose-terminating GIPLs, with the exception of *L. major* which synthesizes high levels of galactose-terminating GIPLs. Differences in the terminal hexose residues of these abundant surface glycolipids may reflect species-specific adaptations to different sandfly vectors (Schlein et al., 1990) and modulate the interaction between the parasite and the macrophages of the mammalian host.

**MATERIALS AND METHODS**

**Materials**

NaB³H₄ (5–15 G/mm) and EnHance spray were obtained from New England Nuclear (Little Chalfont, U.K.), alkaline phosphatase was from Sigma, coffee-bean α-galactosidase and jack-bean α-mannosidase were from Boehringer (Mannheim, Germany) and *Aspergillus saitoi* α-mannosidase was purchased from Oxford Glycosystems (Oxford, U.K.). Phosphatidylglycositol (PG)-specific phospholipase C from *Bacillus thuringiensis* was a gift from Dr. M. G. Low (Columbia University, NY, U.S.A.). Other reagents were of analytical grade.

**Parasites**

The following strains were selected because they were of distinct EF serotypes: *L. major* LRC-L456 (serotype B2), *L. major* LRC-L580 (serotype A1B2), *Leishmania tropica* LRC-L36 (serotype A2), and *Leishmania aethiopica* LRC-L495 (serotype B1). Promastigotes were grown in Schneider’s *Drosophila* medium containing 10% fetal calf serum, and harvested at the beginning of the stationary phase of growth.

**Extraction and isolation of GIPLs**

GIPLs were extracted as previously described (McConville and Bacic, 1990). Briefly, cells were extracted twice by sonication in chloroform/methanol/water (5:10:4, by volume). Water was added to obtain chloroform/methanol/water (5:10:7, by volume) and the two phases were separated by centrifugation. The organic phase was dried under a stream of nitrogen and redissolved in chloroform. The aqueous phase was dried, suspended in 0.1 M ammonium acetate, pH 7.0, containing 5% propan-1-ol and loaded onto a column of octyl-Sepharose pre-equilibrated in the same buffer and eluted with a 5%–60% propan-1-ol gradient. GIPL-containing fractions were identified by staining an aliquot with orcinol/H₂SO₄, and pooled. GIPLs were separated by h.p.t.l.c. in solvent A or B (see below), localized by staining the edges of the plate with orcinol/H₂SO₄, scraped individually from the plate and eluted from the silica with three washes in chloroform/methanol/water (10:10:3, by volume).

**Compositional analyses**

Carbohydrate content was determined by the phenol–sulphuric acid assay, as described (Rao and Pattabiraman, 1989). For monosaccharide and alkylglycerol analyses, purified GIPL samples were subjected to solvolysis in 0.5 M methanolic-HCl (4 h, 80 °C), followed by derivatization in pyridine/trichloroacetic acid (10:3:1, by volume) and analysis by g.c.–m.s. as previously described (McConville et al., 1990).

**Analysis of glycan head groups**

The mixture of GIPLs in the chloroform and methanol/water phases, or purified individual GIPL species, were treated with PI-specific phospholipase C (30 munits in 5 μl of 20 mM Tris-acetate, pH 7.5, 0.1% Triton X-100, 2 h, 37 °C). The delipitated GIPLs were deamminated by addition of 0.4 M sodium acetate, pH 4, (15 μl) and 1 M sodium nitrite (7.5 μl) and incubation at 40 °C for 2.5 h. The reaction mixture was adjusted to pH 9–10 (5 μl 0.4 M boric acid, 5 M NaOH) and immediately reduced with NaB³H₄ (30 mM in 0.1 M NaOH, 10 μl, 2 h, room temperature). Reaction was stopped by acidification with acetic acid and the sample passed through 200 μl AG50 X12 (H⁺), freeze dried, redried twice from methanol and submitted to descending paper chromatography for 48 h in butan-1-ol/ethanol/water (4:1:0.6, by volume). The origin was eluted with water. Samples were desalted through 200 μl each of AG50 X12 (H⁺) over AG3 X4 (OH⁻) before analysis by Dionex h.p.l.c. Phosphorylated glycans were not desalted, but purified by h.p.t.l.c. in solvent system C (see below) and the glycans eluted from the silica with three extractions in 40% propan-1-ol.

**H.p.t.l.c.**

The deaminated and reduced glycan headgroups of the GIPLs were analysed by Dionex h.p.t.l.c. using a CarboPac PA1 column (4 × 250 mm) equilibrated in 0.15 M NaOH (Buffer A) and eluted at 0.6 ml/min with 0.25 M NaOAc in 0.15 M NaOH (Buffer B). Gradient was as follows: 0%–22% B over 1 h, 22%–100% B over 20 min, then held at 100% B for 10 min before re-equilibration. Radioactivity in fractions was measured by liquid scintillation counting and unlabelled dextran oligomer internal standards were detected by pulsed amperometric detection (McConville et al., 1990). The relative retention times of labelled glycans were calculated by linear interpolation of their elution position between adjacent dextran oligomer standards and expressed as Dionex Units (DU).

**Enzymic and chemical treatments**

Jack-bean α-mannosidase (30 μl, 25 units/ml, pH 5, 16 h, 37 °C), coffee-bean α-galactosidase (30 μl, 25 units/ml, pH 6, 16 h, 37 °C) and *A. saitoi* α-mannosidase (10 μl, 0.5 munits/ml, pH 5, 24 h, 37 °C) and acetylation (peracetylation for 30 min at 100 °C in acetic anhydride/pyridine (1:1, v/v), acetylation for 7.5 h at 37 °C in acetic anhydride/acetic acid/conc. sulphuric acid (10:10:1, by volume), de-O-acetylation for 48 h at 37 °C in methanol/conc. ammonia (1:1, v/v)) treatments were as described (Schneider et al., 1993a). Mild acid hydrolysis (40 mM trifluoroacetic acid, 1 h, 100 °C) and strong acid hydrolysis (2 M trifluoroacetic acid, 2 h, 100 °C) were used to cleave preferentially hexofuranosidic linkages or all glycosidic linkages, respectively (McConville et al., 1990). Alkaline phosphatase treatment was performed in 20 μl of 10 mM NH₄HCO₃ with 3 units of enzyme for 16 h at 37 °C. When successive enzyme treatments were performed on the same sample, the previous enzyme was inactivated by heating for 10 min at 100 °C and the appropriate pH for the next enzyme obtained by addition of 30 μl of the next buffer.

**H.p.t.l.c.**

Glycolipids were separated on silica 60 h.p.t.l.c. using chloroform/methanol/1 M NH₄OH (10:10:3, by volume, solvent A) or methanol/chloroform/conc. ammonia/1 M ammonium acetate/water (180:140:9:9:23, by volume, solvent B). Water-soluble glycan head groups were separated on silica 60 h.p.t.l.c., together with α-H-reduced dextran hydrolysate standards. The h.p.t.l.c. was developed twice in propan-1-ol/acetone/water (9:6:5, by volume, for the first and 5:4:1, by volume, for the
second development; solvent system C) (Schneider et al., 1993). Glycolipids were stained by spraying with orcinol/H$_2$SO$_4$ reagent and heating for 5 min at 110 °C. Radioactivity was detected by fluorography after spraying with EnHance spray.

RESULTS

The total glycolipid pools of the four strains were extracted in chloroform/methanol/water and partially fractionated in a two-phase separation. In this system, GIPLs with up to four hexose residues partition mainly into the chloroform-enriched phase, while GIPLs with more than four hexose residues partition mainly into the aqueous phase. Yields of glycolipid in the combined phases ranged from 0.5 to 1.5 mg of carbohydrate per 10$^{10}$ cells. All these glycolipids were shown to be susceptible to PI-specific phospholipase C, indicating that they contained a PI lipid moiety (results not shown). The structures of the GIPLs from each species were determined from their migration behaviour on t.l.c. relative to defined standards, compositional analyses and chemical and enzymic sequencing of the labelled glycan headgroups released by nitrous acid deamination and NaB$^3$H$_4$ reduction as described below.

L. major LRC-L456 and LRC-L580

The yields of GIPLs from L456 and L580 were 0.8 mg and 1.3 mg of carbohydrate per 10$^{10}$ cells, respectively. H.p.t.l.c. analysis showed that the GIPL profiles of these strains were qualitatively similar but quantitatively distinct. Two doublets of orcinol-staining bands (bands 1/2 and 3/4) were detected in both strains, which co-migrated with GIPL-1 and GIPL-2 standards respectively (Figure 1A and 1B). The appearance of doublets is characteristic of these glycolipids and reflects heterogeneity in the alkyl-chain composition of the lipid moieties. The glycan moieties of these GIPLs were obtained by nitrous acid deamination and NaB$^3$H$_4$ reduction of the combined chloroform and aqueous fractions and analysed by Dionex h.p.l.c. and h.p.t.l.c. The major labelled glycans of both species eluted around 6 DU on Dionex h.p.l.c. (Figure 5A and 5B), the expected elution position of the deaminated/reduced glycans of GIPLs-1-3 (McConville et al., 1990). When the 6 DU peak was analysed by h.p.t.l.c., two major glycans were resolved which comigrated with Gal$_\alpha$1-3Man$_\alpha$1-3Man$_\alpha$1-4AHM (GIPL-1 glycan) and Gal$_\alpha$1-3Gal$_\alpha$1-1-3Man$_\alpha$1-3Man$_\alpha$1-4AHM (GIPL-2 glycan) (Figure 2A and B), where AHM is 2,5-anhydrogalactomannitol. A minor band which comigrated with Gal$_\alpha$1-6Gal$_\alpha$1-1-3Man$_\alpha$1-3Man$_\alpha$1-4AHM (GIPL-3 glycan) was also present in the L456-derived GIPLs (Figure 2A). The structures of each of these glycans was confirmed by: (1) the sensitivity of the putative GIPL-3 and GIPL-2 glycans to coffee-bean $\alpha$-galactosidase to generate the GIPL-1 glycan (Figure 2A and B, lanes 5); (2) the resistance of the three glycans to jack-bean $\alpha$-mannosidase (Figures 2A and B, lanes 4); (3) the sensitivity of all three bands to mild acid treatment, which selectively hydrolyses hexofuranosidic and not hexopyranosidic bonds, to yield a glycan that migrated with Man$_\alpha$1-2Man$_\alpha$1-4AHM (Figures 2A and B, lanes 6); (4) the sensitivity of the mild acid hydrolysis product to jack-bean $\alpha$-mannosidase to yield AHM (Figures 2A and B, lanes 7). LRC-L580 contained two additional glycolipids (bands 5 and

Figure 2 Microsequencing of purified glycan head groups

The total GIPL pools of each species were deaminated and NaB$^3$H$_4$-reduced and the labelled glycan head-groups separated on Dionex h.p.l.c. (see Figure 5). Labelled glycans which eluted at 2.1 DU (iM2), 2.9 DU (iM3), 3.3 DU (iM4) and between 6.0 DU and 6.4 DU (GIPL-1-3) were delabelled and analysed by h.p.t.l.c. using solvent system C, before and after various enzymic and chemical treatments. A. L. major LRC-L456; B. L. major LRC-L580; C. L. tropica LRC-L36; D. L. aethiopica LRC-L495. Treatments were: JIAM, jack-bean $\alpha$-mannosidase; ASAM, A. salitri Man$_\alpha$2Man-specific mannosidase; CBAG, coffee-bean $\alpha$-galactosidase; Acetol, partial acetylation which preferentially cleaves Man$_\alpha$1-6Man linkage; TFA, mild trifluoroacetic acid hydrolysis which preferentially cleaves hexofuranosidic linkages. The migration position of defined glycan standards is indicated on the left. Dex: NaB$^3$H$_4$-reduced dextran hydrolysat standards. The 2.1 DU peaks in the two L. major Dionex profiles were indistinguishable from the iM2 glycan, although iM2 was not detectable when the total GIPLs were analysed. It is thus likely that this glycan was generated during the deamination/reduction procedure which is known to result in some hydrolysis of acid-labile galactofuranosidic linkage of the Type-2 GIPL glycans.
Figure 3  Characterization of the P-GIPL-2 and P-GIPL-3 from L. major LRC-L580

A, L. major LRC-L580 band 5 (lanes 1 and 2) and band 6 (lanes 3 and 4) were deaminated and NaB\textsubscript{3}H\textsubscript{4}-reduced. Glycan head groups were analysed by h.p.t.l.c. before (lanes 1 and 3) and after (lanes 2 and 4) treatment with alkaline phosphatase (APase) and desalting (using AG50/AG3 resins). Migration position of defined glycan standards is indicated on the left. The radioactive band migrating at about 2.9 D, just below GIPL-3 glycan (A, lane 1) was purified and submitted to enzymic and chemical treatments as indicated in the diagram. Samples were always desalted before h.p.t.l.c. analysis. Abbreviations for each treatment are as described in Figure 2. The major product of each treatment is shown schematically in the diagram. Numbers beside the structures (B') correlate with lane numbers. Phosphorylated species (1 and 4) are lost during final desalting step and thus appear as empty lanes. (\(\Delta\)) [\(\text{[H]}\text{AHM}\); (O) Man; (O) Gal; (O) Gal. Dex: NaB\textsubscript{3}H\textsubscript{4}-reduced dextran hydrolysate standards. Chromatograms were developed in solvent system C, and radioactivity was detected by fluorography.

6) which had a slow mobility on h.p.t.l.c. (Figure 1B). Following h.p.t.l.c. purification, two fractions containing only band 5 and a mixture of bands 5 and 6, respectively, were obtained. These fractions were deaminated and NaB\textsubscript{3}H\textsubscript{4}-reduced, and analysed directly by h.p.t.l.c., without desalting on anion-exchange resins, to detect phosphorylated species. Both fractions contained labelled species with a slow h.p.t.l.c. mobility that were converted into neutral glycans after alkaline phosphatase treatment (Figure 3A, lanes 1–4). A single neutral glycan was obtained from the band 5 fraction which comigrated with the GIPL-2 glycan standard, whereas two neutral glycans were obtained from the band 5/6 mixture which comigrated with the GIPL-2 and -3 glycans (Figure 3A, lanes 2 and 4). Other radiolabelled species, which were also present in the blank reduction, were not affected by the alkaline phosphatase treatment and were removed after desalting. These data suggest that bands 5 and 6 contained GIPL-2 and GIPL-3 species, respectively, with an extra phosphate(s) (P-GIPL-2 and P-GIPL-3). The location of the phosphate group on P-GIPL-2 was identified by enzyme and acid treatments of the h.p.t.l.c.-purified glycan. Sequential treatments with coffee-bean \(\alpha\)-galactosidase followed by alkaline phosphatase generated a glycan that comigrated with GIPL-1 glycan (Figure 3B, lane 3), indicating that the terminal galactose residue was not phosphorylated. Moreover, the product of mild acid hydrolysis, which removes both terminal galactose residues, was removed by desalting (Figure 3B, lane 4) indicating that the phosphate was retained on one of the core mannose residues. This product was resistant to jack-bean \(\alpha\)-mannosidase digestion unless the glycan was first treated with alkaline phosphatase (Figure 3B, lanes 5–7) suggesting that the phosphate residue was located on the mannose distal to the AHM residue. A similar set of treatments indicated that the phosphate residue of P-GIPL-3 was also located to the distal Man residue (data not shown).

From previous analyses of both the LPG glycan core and the polar GIPLs of other \textit{Leishmania} strains, it is likely that this phosphate residue is substituted with a single \(\alpha\)Glc residue (McConville and Homans, 1992; Thomas et al., 1992; McConville et al., 1993). The Glc-1-P\textsubscript{4}O\textsubscript{4} bond is extremely labile and almost completely removed during deamination and reduction. The presence of this residue is consistent with the identification of 1 mol Glc per mol Man in the monosaccharide analyses of bands 5 and 6, compared with trace amounts of Glc in the other h.p.t.l.c.-purified GIPLs. These data suggest that the GIPLs in bands 5 and 6 have the glycan structures: Galz-3Gal\textsubscript{r}/\beta\textsubscript{1}3[Glc-1-P\textsubscript{4}O\textsubscript{4}][Manz-1-3Manz-1-4GlcN and Galz-1-6Galz-1-3Gal\textsubscript{r}/\beta\textsubscript{1}3Glc-1-P\textsubscript{4}O\textsubscript{4}Manz-1-3Manz-1-4GlcN, respectively.

The GIPL-1 and -2 doublets of LRC-L546 and LRC-L580 showed a reduced t.l.c. mobility after mild base hydrolysis, consistent with the loss of a single ester-linked fatty acid (Figure 4A and B). G.c.m.s. of the h.p.t.l.c.-purified bands indicated the presence of stochiometric amounts of 1-O-alkylglycerols (Table 1), suggesting that all these GIPL contained an alkylacylglycerol lipid moiety. In each case, the upper band of the doublets contained predominantly \(C_{24:0}\) alkyl chains while the lower band in the doublet was enriched for \(C_{24:0}\) alkyl chains (Table 1). In contrast, the phosphorylated GIPLs from LRC-L580 were resistant to mild base hydrolysis (Figure 4B\textsuperscript{'} and contained 1-O-alkylglycerols with exclusively \(C_{24:0}\) and \(C_{24:0}\) alkyl chains (Table 1).

Taken together, these results suggest that \textit{L. major} LRC-L546 contains GIPL-1 (bands 1/2), GIPL-2 (bands 3/4) and GIPL-3, in the ratio 25:70:5, and that all the major species contain a heterogeneous alkylacylglycerol lipid moiety. In contrast, LRC-L580 contains GIPL-1 (bands 1/2), GIPL-2 (bands 3/4), GIPL-3 (not detected by orcinol stain) and the Glc-1-P\textsubscript{4}O\textsubscript{4} substituted species, P-lysoGIPL-2 (band 5) and P-lysoGIPL-3 (band 6), in the ratio 75:15:0.5:10:2. While the GIPL-1 and GIPL-2 species contain a heterogeneous alkylacylglycerol lipid moiety, P-lysoGIPL-2 and P-lysoGIPL-3 contain a lysoalkylglycerol moiety with long alkyl chains.

\textit{L. tropica} LRC-L36

This strain contained approximately 0.5 mg of GIPL per 10\textsuperscript{8} cells. H.p.t.l.c. resolved a major glycolipid doublet and three poorly defined minor bands (Figure 1C). Deamination/NaB\textsubscript{3}H\textsubscript{4} reduction of the mixture yielded three peaks
which migrated at 2.1 DU, 2.9 DU and 3.3 DU on Dionex h.p.l.c (Figure 5C). These glycans were shown to correspond to Manα1-3Manα1-4AHM (iM2 glycan), Manα1-6[Manα1-3]Manα1-4AHM (iM3 glycan) and Manα1-2Manα1-6[Manα1-3]Manα1-4AHM (iM4 glycan), respectively, based on their comigration with standards on h.p.l.c. and h.p.t.l.c. and sequential α-mannosidases and acetylation treatments (Figure 2, 3C). The ratio of iM2, iM3 and iM4 glycans was 90:3:7. In order to determine the lipid moieties of each of these species, bands 1–5 were purified by h.p.t.l.c. and submitted individually to the deamination/NaB3H4 procedure (to assign glycan head group) and compositional analysis. Bands 1 and 2 contained only the iM2 glycan head group and were sensitive to mild base hydrolysis. The base-hydrolysed species comigrated with bands 3 and 4. These results suggest that bands 1 and 2 correspond to different molecular species of iM2 with distinct alkylacyl-PI lipid moieties. This was consistent with the g.c.-m.s. analysis which showed that band 1 was enriched for C24:0 and C26:0 alkyl chains (iM2 C24:0/C26:0, while band 2 was enriched for C18:0 alkyl chains (iM2 C18:0) (Table 1). Band 3 comigrated with iM3 C18:0 but contained both iM3 and iM2 glycan head groups in the ratio 1:2. Taken together with the g.c.-m.s. lipid analysis, the data indicate that this band probably contains a mixture of iM3 C18:0 and lyso iM2 C24:0/C26:0. Band 4 was a diffuse orcinol-staining region between bands 3 and 5 and contained a mixture of iM2, iM3 and iM4 glycan head groups in the ratio 3:1:3. This band contained predominantly 1-O-alkylglycerols with C18:0 alkyl chains. It is not possible to unambiguously assign the lipid composition of each of these species. However, it is likely to comprise lyso iM2 C18:0 (comigrates with standard) and contaminating species from band 3 (iM3 C18:0 and lyso iM2 C24:0/C26:0) and band 5 (iM4 C18:0). Band 5 yielded only iM4 glycan head group, was susceptible to mild base hydrolysis and contained predominantly C18:0 alkyl chains, suggesting that it comprised primarily iM4 C18:0.

**L. aethiopica LRC-L495**

This strain contained ~ 1 mg of G1PL per 10⁶ cells. The h.p.t.l.c. profiles of the chloroform and aqueous phases contained one predominant G1PL that comigrated with iM4 C18:0 and two minor species which comigrated with iM3 C18:0 and iM2 C18:0 (Figure 1D). The h.p.t.l.c. profiles of this strain were also distinctive in containing a smear of phospholipids which migrated faster than the GIPLs. The nature of these lipids was not investigated. The presence of the above GIPL species was confirmed by deamination/NaB3H4 reduction of the total mixture, which generated three labelled glycans that migrated at 2.0 DU, 2.9 DU and 3.3 DU (Figure 5D). The structures of these glycans were confirmed by sequential treatments with α-mannosidases and acetylation (Figure 2D). The h.p.t.l.c. analyses revealed an additional jack-bean α-mannosidase-sensitive com-

### Table 1 Alkyl chain composition of h.p.t.l.c.-purified GIPL bands

<table>
<thead>
<tr>
<th>Major GIPL species</th>
<th>Alkyl chain</th>
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<td>Band 7</td>
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</table>

**Figure 5 Dionex h.p.l.c. analysis of deaminated/reduced glycan head groups**

Combined chloroform and methanol/water phases from each species were submitted to the deamination/NaB3H4 reduction procedure. Neutral labelled glycans were analysed by Dionex h.p.l.c. The elution position relative to co-injected dextran hydrolysate is indicated beside the peaks in DU. Peaks labelled with an asterix did not yield AHM after strong acid hydrolysis. Some of these peaks (e.g. the peak eluting at ~ 30 min in A and B) were tentatively identified as alternative, minor products of the deamination/reduction procedure (results not shown). A. L. major LRC-L456; B. L. major LRC-L580; C. L. tropica LRC-L36; D. L. aethiopica LRC-L495.
ponent in the 2.0 DU peak, which did not contain AHM. This component is thought to be a minor deamination and reduction product of iM4 which elutes more rapidly from the Dionex column (with glycan headgroup of iM2) but has a similar polarity on h.p.l.c. (see Figure 5 legend for evidence of similar minor deamination/reduction products of GPls-1-3). Bands 1–3 were all susceptible to mild base hydrolysis, which generated species that comigrated with the corresponding lyso derivatives (Figure 4), and contained exclusively C₁₈₀ or C₁₇₀ alkyl chains (Table 1). These data indicate that the L. aethiopica GPl profile consists of iM2 C₁₈₀ (band 1), iM3 C₁₇₀ (band 2) and iM4 C₁₈₀ (band 3) in the ratio 18:7:75.

DISCUSSION

This study significantly expands the information available on the distribution of different GPl classes in the promastigote stage of Old World strains of Leishmania. In all cases, they were the only detectable class of glycolipid and were present in comparable yields to previous estimates, suggesting the presence of at least 10⁷ copies of GPl per cell (McConville and Bacic, 1990). An important conclusion of these data is that most Leishmania species appear to synthesize the mannose-terminating GPls, iM2, iM3 and iM4 as major species (Table 2). The last two GPls belong to the hybrid series because they have branched glycan structures that share sequence similarity with the GPl glycolipid anchors of both the proteins and LPG (McConville and Blackwell, 1991). In addition to the Leishmania species studied here, mannose-terminating GPls are also the major glycolipids in L. mexicana (McConville et al., 1993) and L. donovani (McConville and Blackwell, 1991; Sevlever et al., 1991) (Table 2). L. major is therefore unusual in expressing high levels of galactose-terminating GPls. These GPls belong to the type-2 series which show structural similarity to the GPl anchor of LPG. Although there are quantitative differences in the GPl profiles of different L. major strains, all strains contain GPl-1 and -2 as major components (this study, McConville and Bacic, 1989; McConville et al., 1990).

The function of the GPls and the significance of these species-specific differences in the GPl profiles is not known. It is likely that the mannose-terminating GPls will act as ligands for a number of host receptors. For example there is evidence that L. donovani promastigotes bind to the midgut of its sandfly vector, Phlebotomus argentipes, via a mannos-binding receptor (D. L. Sacks, P. F. P. Pimenta, M. J. McConville, P. Schneider and S. J. Turco, unpublished work) and that both promastigotes and amastigotes bind to mammalian macrophages to some extent via the well-characterized mannose receptor (Blackwell et al., 1985; Wilson and Pearson, 1986). In addition, these GPls appear to be ligands for the mannan-binding protein in mammalian serum (Green et al., 1994) which has been shown to enhance the uptake of mannan-rich pathogens by circulating macrophages. The distinctive GPl profile of the L. major strains may be a specific adaptation which has allowed this species to colonize the sandfly vector Phlebotomus papatasii and other members of this Phlebotomus species group. This is suggested by recent studies which show that the binding of L. major promastigotes to the midgut epithelial cells of these sandflies is mediated by a galactose receptor that recognizes terminal β- and α-galactose residues that occur on the LPG and GPls, respectively (Pimenta et al., 1992; McConville et al., 1992). These data raise the possibility that species-specific differences in the terminal galactan sequences of the major surface glycoconjugates may influence the vector tropism of different Leishmania species.

All species of Leishmania appear to contain at least two pathways of GPl biosynthesis, which lead to the formation of the protein and LPG glycolipid anchors, respectively (McConville and Ferguson, 1993). Our data suggest that the GPls may have arisen as a result of the up-regulation of one or more steps in these biosynthetic pathways. There is evidence that over-expression of GPl anchor precursors also occurs in the African trypanosomes where less than 5% of the GPl anchor precursors are required to maintain normal levels of GPl-anchored protein (Masterson and Ferguson, 1991). The characterization of these overexpressed species has provided important insights into the likely steps in these biosynthetic pathways. In particular, the identification of the new structure, P-lysoGPl-2, in L. major LRC L580 suggests that the synthesis of the LPG anchor may involve the sequence of intermediates: GPl-1 → GPl-2 → P-lysoGPl-2 → P-lysoGPl-3 → LPG. However, substitution of the glycana core with Glc-1–PO₄ is probably not essential for addition of the LPG repeat units as some LPG species retain a non-substituted core (McConville et al., 1992).

A striking feature of the GPls is the presence of distinct alkyl-chain compositions in the different GPl series. In particular, the type-2 GPls all contain a heterogeneous alkyl chain composition which includes a high proportion of long (C₂₄-₀/C₁₈-₀) alkyl chains, whereas the hybrid type GPls of most species are highly enriched for short C₁₈₀ alkyl chains (this study, McConville et al., 1990, 1993; McConville and Blackwell, 1991).

Table 2 Distribution of GPls in seven Old and New World strains of Leishmania.

<table>
<thead>
<tr>
<th>GPl</th>
<th>Structure</th>
<th>L. Major</th>
<th>L. tropica</th>
<th>L. aethiopica</th>
<th>L. mexicana</th>
<th>L. donovani</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM2</td>
<td>Man₃, GlcN-PI</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IM3</td>
<td>Man₃ [Man] Man, GlcN-PI</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IM4</td>
<td>Man₃, [Man] Man, GlcN-PI</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GPl-1</td>
<td>Gal₃, Man₃, GlcN-PI</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GPl-2</td>
<td>Gal₂, Gal⁺, Man, GlcN-PI</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GPl-3</td>
<td>Gal₂, Gal⁺, Man, GlcN-PI</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P-GPl-2</td>
<td>Gal₂, Gal⁺, GlcN-PI</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P-GPl-3</td>
<td>Gal₂, Gal⁺, GlcN-PI</td>
<td>++</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
1991). These differences in alkyl chain composition are evident even when both type-2 and hybrid GIPLs are expressed within the same strain, as in L. tropica (this study) or L. mexicana (McConville et al., 1993), suggesting that there is some requirement for a specific alkyl chain composition in the different GIPL series. As there is no precedent for exchange of ether-linked alkyl groups, these results suggest either that some of the glycosyltransferases involved in GIPL biosynthesis select for precursor species with defined alkyl-chain compositions, or alternatively that the pathways of type-2 GIPL and hybrid GIPL biosynthesis occur in distinct subcellular compartments. Evidence for the compartmentalization of distinct GPI biosynthetic pathways in Leishmania has recently been obtained by transfecting L. major promastigotes with a cytoplastically disposed GPI-specific phospholipase C (Mensa-Wilmot et al., 1994). This study suggests that type-2 GIPLs and LPG are synthesized in a different subcellular compartment from the precursors of the protein anchors. Based on the finding that the hybrid series GIPLs have a distinct alkyl-chain composition from either the protein anchors or LPG it is possible that these GIPLs may be synthesized in yet another compartment. Interestingly, the proportion of long alkyl chains in the type-2 GIPLs increases with increasing size of the glyc an head group. In particular, the phosphorylated GIPLs have a similar enrichment for C_{24:0} and C_{26:0} alkyl chains as the LPG anchor. Although the mechanism by which this enrichment occurs is not known, the finding that it occurs in all strains and species of Leishmania suggests that it may be critical for LPG function. In this regard it is possible that long alkyl chains are required to prevent LPG monomers from partitioning out of the plasma membrane too rapidly and thus regulate the turnover of surface LPG.

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REFERENCES


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