Structure of the Lipophosphoglycan from *Leishmania major* 

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The major cell surface glycoconjugate of the parasitic protozoan *Leishmania major* is a heterogeneous lipophosphoglycan. It has a tripartite structure, consisting of a phosphoglycan (M, 5,000–40,000), a variably phosphorylated hexasaccharide glycan core, and a lysoalkylphosphatidylinositol (lysoalkyl-PI) lipid anchor. The structures of the phosphoglycan and the hexasaccharide core were determined by monosaccharide analysis, methylation analysis, fast atom bombardment-mass spectrometry, one- and two-dimensional 500-MHz (correlated spectroscopy (COSY), homonuclear Hartmann-Hahn spectroscopy (HOHAHA)) 1H NMR spectroscopy, and exoglycosidase digestions. The phosphoglycan consists of eight types of phosphorylated oligosaccharide repeats which have the general structure,

\[ [PO_4-6Galp(β1-4)Manp]_n \]

where \( R = H, Galp(β1-3), Galp(β1-3)Galp(β1-3), Arap(α1-2)Galp(β1-3), Glcp(β1-3)Galp(β1-3), Galp(β1-3)Galp(β1-3)Galp(β1-3), Arap(α1-2)Galp(β1-3)Galp(β1-3), \text{ or } Arap(α1-2)Galp(β1-3)Galp(β1-3)Galp(β1-3), \) and where all the monosaccharides, including arabinose, are in the \( D \)-configuration. The average number of repeat units/molecule (n) is 27. Data are presented which suggest that the nonreducing terminus of the phosphoglycan is capped exclusively with the neutral disaccharide Manp(α1-2)Manp. The structure of the glycan core was determined to be,

\[ PO_4-6Galp(α1-4)Galp(α1-3)Galp(β1-3)Manp(α1-3)Manp(α1-4)GlcNp(α1-6)mPS-inositol \]

where approximately 60% of the mannose residues distal to the glucosamine are phosphorylated and where the inositol is part of the lysoalkyl-PI lipid moiety containing predominantly 24:0 and 26:0 alkyl chains. The unusual galactofuranose residue is in the \( β \)-configuration, correcting a previous report where this residue was identified as \( αGalp \). Although most of the phosphorylated repeat units are attached to the terminal galactose 6-phosphate of the core to form a linear lipophosphoglycan (LPG) molecule, some of the mannose 6-phosphate residues may also be substituted to form a Y-shaped molecule. The *L. major* LPG is more complex than the previously characterized LPG from *Leishmania donovani*, although both LPGs have the same repeating backbone structure and glycolipid anchor. Finally we show that the LPG anchor is structurally related to the major glycolipid species of *L. major*, indicating that some of these glycolipids may have a function as precursors to LPG.

The protozoan parasite *Leishmania major* is the etiologic agent of human cutaneous leishmaniasis. It occurs as an extracellular promastigote in the alimentary canal of the sandfly vector and as an obligate intracellular amastigote in the phagolysosomal compartment of macrophages in the mammalian host. The cell surface of *L. major* promastigotes is coated by a complex glycocalyx which is rich in glycosylphosphatidylinositols (GPIs). Three distinct classes of GPI have been identified; those that are linked to polysaccharide to form the lipophosphoglycans (LPGs) (Handman and Godding, 1985; McConville et al., 1987), those that act as membrane anchors for cell surface glycoproteins (Bordier, 1987; Murray et al., 1989), and a family of low molecular weight glycoinositolphospholipids (GIPLs) that are not attached to either protein or polysaccharide (McConville and Racine, 1989, 1990; McConville et al., 1990).
LPG is the major cell surface macromolecule and plays a key role in determining parasite virulence and survival in the mammalian macrophage (Handman et al., 1986; McConville et al., 1987, McConville and Bacic, 1990; Elhay et al., 1990). It appears to be involved in facilitating the initial attachment of promastigotes to macrophages and their subsequent uptake into the phagolysosome. Uptake of the parasites may occur following direct binding of LPG to macrophage receptors (Handman and Goding, 1985; Russell and Wright, 1988) or after opsonization of surface LPG by complement components (C3b, C3bi) (Puente et al., 1988, da Silva et al., 1989). LPG-like molecules are also expressed on the cell surface of the amastigotes and may be necessary for parasite survival in the macrophage phagolysosome compartment (Handman et al., 1984, 1986; Chan et al., 1988; McNealy and Turco, 1990). In addition, LPG may be involved in the induction of a host protective response and has been used to vaccinate susceptible mice strains against cutaneous leishmaniasis (Handman and Mitchell, 1985, McConville et al., 1987). There is evidence that this protective response may be due to the specific recognition of LPG by T-cells (Moll et al., 1989).

The LPG from L. major has been partially characterized as a polymer (M, 5,000-40,000) of repeating phosphorylated di-, tri-, and tetrasaccharides that contain mannose, galactose, glucose, and arabinose (McConville et al., 1987). By contrast, the structurally similar LPG which has been characterized from Leishmania donovani, the etiologic agent of visceral leishmaniasis, contains the phosphorylated disaccharide PO4-GGal(1-4)Manα1- as the repeating units (Turco et al., 1987). However, both LPGs are anchored to the surface membrane by an unusual lysolipid-P1 containing 24:0 and 26:0 alkyl chains (Orlandi and Turco, 1987; McConville et al., 1987) and may have the same hexaglycosyl glycan core (Turco et al., 1989; McConville and Bacic, 1990). The site of attachment of the repeat units to the core glycan have not been determined in either structure.

In this study, we report the complete structure of L. major LPG. The structure of Leishmania mexicana LPG will be reported elsewhere.2 These results indicate that the LPGs of different species have common architectural elements. In particular, they all have the same backbone sequence of repeating PO4-GGal(1-4)Manα1- units which may either be unsubstituted (as in the L. donovani LPG) or substituted in a species-specific manner with saccharide residues. In this regard, the L. major LPG was found to be more complex than either the L. donovani or the L. mexicana LPGs, as the majority of the galactose residues in the backbone sequence were substituted with a diverse array of galactose-, arabinose-, and glucose-containing side chains. The results also show that the glycan core and lipid anchor are highly conserved in these molecules.

**EXPERIMENTAL PROCEDURES AND RESULTS**

LPG was extracted from delipitated promastigotes with 1-butanol-saturated water and purified to homogeneity by octyl-Sepharose chromatography as described previously (McConville et al., 1987). The scheme for the characterization of LPG is shown in Fig. 1. The purified LPG was depolymerized with mild acid, under conditions that hydrolyze phosphodies-2

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2 T. Ilg, R. Etges, P. Overath, J. Thomas-Oates, M. J. McConville, S. W. Homans, and M. A. J. Ferguson, manuscript in preparation.

3 Portions of this paper (including "Experimental Procedures," Table I-X, and Figs. 2, 4, E, 7, 9, and 11) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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24.5 ppm with a coupling constant of J1,2 = 7.8 Hz (Table V). Comparison of the COSY spectra of N3 and P3 showed the finding that β-galactosidase treatment of N3 converted it to a single peak that comigrated with hexoses on HPLC. The results of the NMR analyses were also consistent with the presence of phosphate on the C-6 position of the internal galactose residue (Gal-2, for numbering of residues see Table V).
FIG. 1. Scheme for the characterization of L. major LPG. Mild acid hydrolysis of LPG released a series of phosphorylated oligosaccharides (P2-P6) and a neutral oligosaccharide, N2' which no longer bound to octyl-Sepharose. Ion exchange HPLC was used to fractionate the phosphorylated oligosaccharides and their dephosphorylated derivatives (N2-N6). The glycolipid anchors were eluted from the octyl-Sepharose column with a gradient of 1-propanol. Treatment of the anchors with PI-specific phospholipase C released the phosphosaccharide-inositolphosphate moieties, whereas nitrous acid deamination and reduction released glycan moieties terminating in 2,5-anhydro-D-mannitol. Symbols: O, Gal, Man, or Ara; •, myo-inositol; ©, glucosamine; ◇, 2,5-anhydro-D-mannitol; P, phosphate; TFA, trifluoroacetic acid.

Fig. 3. Fractionation of the phosphorylated oligosaccharide repeats of L. major LPG. LPG was depolymerized with 40 mM trifluoroacetic acid (8 min, 100 °C) and fractionated by octyl-Sepharose chromatography. Oligosaccharides in the unbound fraction were chromatographed by ion exchange HPLC using gradient program b.

that the H-4 proton of Gal-2 was shifted downfield by 0.06 ppm in the phosphorylated trisaccharide (Table VI). By contrast, the chemical shifts of the protons in the terminal galactose residues of both N3 and P3 were identical. The phosphate was assigned to the C-6 position of Gal-2 from the absence of heteronuclear splittings in the resonances corresponding to H1-H4. From these results P3 has the structure, 

$$\text{PO}_4^{\text{6}} \text{Galp}\beta\text{Galp}\beta\text{Man}$$

Unexpectedly, the terminal galactose of this phosphorylated oligosaccharide was not removed by β-galactosidase.

Tetrasaccharide Repeats (P4a, P4b, P4c)—After alkaline phosphatase treatment, three of the oligosaccharide repeat units (P4a, P4b, and P4c) migrated as tetrasaccharides on Bio-Gel P4 chromatography. Positive ion FAB-MS of permethylated P4a gave an (M + H)^+ molecular ion at m/z 913 corresponding to Pent. Hex. - PO_4. (Fig. 5B). The presence of A^+ type fragment ions at m/z 1/3, 3/9, and 6/11 indicate the sequence Pent. Hex. - (PO_4)Hex. Hex. (Fig. 5B, Table II). Methylation analysis of the dephosphorylated oligosaccharide, N4a, indicated the structure Arap-(1-2/3)-Galp-(1-2/3)-Galp-(1-4)-Man (Table III). To resolve the ambiguity in the linkage assignments, N4a was treated with mild acid (40 mM trifluoroacetic acid, 1 h, 100 °C) to remove the terminal arabinose (confirmed by FAB-MS and HPLC (data not presented)). Methylation analysis of the hydrolysate showed that all the 2-O-substituted galactose was converted to terminal galactose, indicating that the arabinose was originally linked to the C-2 position of the subterminal galactose. NMR analysis showed that the galactose residues were in the β-configuration and that the arabinose residue was in the α-configuration (δ = 5.35, J_1,2 = 3.5) (Fig. 6, Table V). NMR analysis of P4a, showed that the chemical shift of the H-4 of Gal-2 was shifted downfield by 0.06 ppm, compared with the chemical shifts of the H-4 of Gal-2 in N4a (Table VI), consistent with the phosphate being located on Gal-2. The phosphate was assigned to the C-6 position from the absence of heteronuclear splitting on H1-H4 and the presence of galactose 6-phosphate in the compositional analyses (Table III). These results reveal that P4a has the structure, 

$$\text{PO}_4^{\text{6}} \text{Galp}\beta\text{Galp}\beta\text{Man}$$

Positive ion FAB-MS of permethylated P4b gave an (M + H)^+ molecular ion at m/z 957 and A^+ type fragment ions at m/z 219, 423, and 721 (Fig. 5C, Table II) corresponding to the sequence Hex$_{\alpha}$-(PO_4)Hex.-Hex. Methylation analysis of reduced and nonreduced N4b (Table IV) defined the sequence Gal-(1-3)-Gal-(1-3)-Gal-(1-4)-Man. This structure is consistent with the data obtained from the NMR COSY spectrum (Fig. 6, Table V), which also showed that all the galactose residues were in the β-configuration, and the finding that N4b was completely digested to hexoses with β-galactosidase. The location of the phosphate on the 6 position of Gal-2 was also
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As noted for P3, purified P4b was also resistant to calf intestine β-galactosidase.

The positive ion FAB mass spectrum of permethylated P4c contained an (M + H)⁺ molecular ion at m/z 957 and A⁺-type fragment ions at m/z 219, 423, and 721 corresponding to the sequence Hex⁻(PO₁)Hex-Hex (Table II). This is consistent with the methylation analysis of the dephosphorylated oligosaccharide, N₄c (Table IV) which defined the sequence Glc(1-3)-Gal(1-3)Gal(1-4)Man. The β-configuration for the glucosidic linkage was determined by one-dimensional 500 MHz ¹H NMR spectroscopy (not shown) from the presence of a doublet at 4.68 ppm with a coupling constant of J₁,₂ = 7.8 Hz. The coupling constants of the galactose residues were all 7.8 Hz, indicating that they were in the β-configuration. Together with the monosaccharide analysis (Table III), these results show that P4c has the structure,

\[
PentαGalβGalβGalβMan
\]

confirmed by comparison of the COSY spectrum of P₄b and N₄b (Table VI) and compositional analysis (Table III). These data define the structure of P₄b as,

\[
PentαGalβGalβGalβMan
\]

Pentasaccharide Repeats (P₅ₐ, P₅ₜ)—Two of the oligosaccharide repeat units (P₅ₐ and P₅ₜ) migrated as pentasaccharides on Bio-Gel P₄ chromatography after alkaline phosphatase treatment. The positive ion FAB mass spectrum of permethylated P₅ₐ contained an (M + H)⁺ molecular ion at m/z 1117 and A⁺-type fragment ions at m/z 175, 379, 583, and 881 consistent with the structure Pent-Hex⁻(PO₁)Hex-Hex, whereas the mass spectrum of P₅ₜ contained an (M + H)⁺ molecular ion at m/z 1161 and fragment ions at m/z 219, 423, 627, and 925 for the structure Hexα(PO₁)Hex-Hex (Table II). Methylation analysis (Table IV) and NMR analysis (Table V) showed that the dephosphorylated pentasaccharides, N₅ₐ and N₅ₜ, had the structures Arap(α1-2)GalβGalβGalβGalβMan and GalβGalβGalβGalβGalβGalβGalβGalβMan, respectively. Taken together with the monosaccharide analyses (Table III), these results indicate that the phosphorlyated pentasaccharides, P₅ₐ and P₅ₜ, have the structures,

\[
PentαGalβGalβGalβMan
\]

respectively.

Hexasaccharide Repeat (P₆)—Positive ion FAB-MS analysis of the permethylated and peracetylated derivates of P₆ afforded molecular ions and A⁺-type fragment ions for the structure Pent-Hex⁻(PO₁)Hex-Hex (Table II). Methylation (Table IV) and NMR (Table V) analyses of the dephosphorylated hexasaccharide indicated the structure; Arap(α1-2)GalβGalβGalβGalβGalβMan. Finally, monosaccharide analysis of P₆ indicated that the phosphate was located on the 6 position of the Gal-2 residue. The
combined results indicate that P6 has the structure,
\[
\text{PO}_4
\]
\[
\text{Arap(α-1,2)Galp(β-1,3)Galp(β-1,3)Galp(β-1,3)Galp(β-1,4)Man}
\]

Anomeric Configuration of the Mannose Residues—The finding that LPG was depolymerized after mild trifluoroacetic acid hydrolysis or hydrofluoric acid treatment (McConville et al., 1987) suggested that the oligosaccharide repeat units were linked together by phosphodiester bonds. The structural data described above are consistent with the phosphorylated oligosaccharide repeats being linked through C-1 of the mannose residue and C-6 of the Gal-2 residue, to form the repeating backbone structure -[6Galp(β-1,4)Man-PO4]-. The anomeric configuration of the mannose residue in this sequence was determined by one-dimensional 500-MHz NMR spectroscopy of the intact LPG. The spectrum (not depicted) showed a doublet of doublets for the anomeric proton of the major mannose resonance at δ = 5.44 ppm with a coupling constant J1,2 = ~2 Hz, JH-P = 7.3 Hz, consistent with these residues having the α-configuration.

Structure of the Glycolipid Anchor—Mild acid hydrolysis of LPG released two major inositol-containing glycolipid species (GPI-A and GPI-B) which had very slow HPTLC mobility (Fig. 2). Two additional minor glycolipid species (GPI-C and GPI-D) were also released which were probably hydrolysis products of the major species (see Supplemental Material). Negative ion FAB-MS of the underivitized glycolipids gave (M - H)- molecular ions at m/z 1800 and 1828 for (PO4)-Hex-HexN-lyso-PI with 24:0 and 26:0 alkyl chains, respectively, and at m/z 1720 and 1748 for (PO4)-Hex-Hex-N-lyso-PI with 24:0 and 26:0 alkyl chains, respectively (Fig. 8). Structurally informative fragment ions were formed by β-cleavage (Dell, 1987) as shown in Fig. 8. In particular, the presence of the ion at m/z 1478 indicated that the terminal hexose was phosphorylated in the monophosphorylated species, whereas those at m/z 992, 1234, and 1558 showed that the diphosphorylated species had the sequence (PO4)2Hex-Hex-PO, hex-PO, Hex-N-lyso-PI (Fig. 8). GPI-A and GPI-B were purified by HPTLC and subjected to monosaccharide analysis. Both glycolipids contained galactose, mannose, glucosamine, and galactose 6-phosphate, whereas GPI-A also contained mannose 6-phosphate (Table VII). Methylation analysis of dephosphorylated deaminated GPI-A and -B revealed that both glycans contained 3 galactose residues/mol (1 terminal galactopyranose, 1 6-O-substituted galactopyranose, and 1 3-O-substituted galactofuranose), 2 3-O-substituted mannose residues, and 1 4-O-substituted glucosamine (Table VIII). Treatment of the dephosphorylated glycans with coffee bean α-galactosidase removed the 2 galactopyranose residues and gave a major peak at 4.3 glucose units after Bio-Gel P4 chromatography. This product was resistant to jack bean α-mannosidase digestion, but was hydrolyzed with 40 mM trifluoroacetic acid (100 °C, 60 min) under conditions which hydrolyze hexofuranosidic but not hexopyranosidic bonds, to give a single peak eluting at 3.2 glucose units. Treatment with jack bean α-mannosidase converted this component to a peak at 1.7 glucose units on Bio-Gel P4 chromatography, corresponding to free 2,5-anhydro mannitol. These results indicate that the dephosphorylated glycans of GPI-A and -B contain the sequence Galp(α-1,6)Galp(α-1,3)Galp(β-1,3)Manp(α-1,3)Manp(α-1,4)GlcNp.

GPI-A and -B were treated with PI-specific phospholipase C and the released phosphoaccharide-inositolphosphate moieties analyzed by two-dimensional 500-MHz 1H NMR spectroscopy to identify the linkage between glucosamine and inositol and define the anomeric configuration of the galactofuranose residue. The 1H NMR chemical shifts for residues in these moieties are listed in Table IX and the spectrum for the GPI-A glycan shown in Fig. 9. Similar chemical shifts
were obtained for both glycans, although there were differences in the H-1 and H-2 resonances of some residues (e.g., GlcN, Man, and Gal residues), reflecting the presence of phosphate on the mannose residue distal to the glucosamine in the GPI-A glycan which was absent in the GPI-B glycan. From previous studies on the GPI-Ls of L. major (McConville et al., 1990), the cross-peak at $\omega_1 = 5.69$ ppm and $\omega_2 = 3.31$ ppm in the GPI-B glycan is diagnostic of non-N-acetylated glucosamine linked $\alpha$-6 to $m$-$n$-inositol 1,2-cyclic phosphate (Table IX). The $\beta$-anomeric configuration of Galf in GPI-A and -B was concluded from comparison of the coupling constants of H-1 ($J_{12} = 1.6$ Hz) with those of the anomeric signals of the synthetic disaccharide Galf($\beta$1-3)Manp($\alpha$1-3) (McConville et al., 1990) for methyl $\beta$-f-Galf ($J_{12} = 2.0$ Hz) and methyl $\alpha$-D-Galf ($J_{12} = 4.0$ Hz) (Table X). Galactofuranose residues also occur as constituents in the GPI-Ls of L. major and were tentatively assigned the $\alpha$-configuration in a recent study (McConville et al., 1990). However, comparison of the coupling constants of the Galf H-1 and H-2 in the GPI-Ls showed that they are identical to those in the LPG anchor, indicating that they are also in the $\beta$-configuration (Table X).

Homonuclear ($'H$) HOHAHA spectroscopy of the diphasphorylated glycan core was used to infer the location of the phosphate on the mannose residue distal to the glucosamine (Fig. 10). A cross-section parallel to $\omega_1 = 4.30$ ppm gave an essentially complete one-dimensional spectrum of Man-2 (Fig. 10B) from which two broad doublets corresponding to the C-6 protons at $\omega_1 \approx 3.81$ and 4.22 ppm could be observed. The broad nature of H6 and H6' due to multiplicity and anomalous downfield shifts of H5, H6, and H6' suggests that this residue is substituted at position 6 with phosphate. These results indicate that the glycolipid anchors of LPG have the following structures:

\[
\begin{align*}
\text{GPI-A} & : \text{PO}_4 \text{CPO}_{4}\text{Galp(\alpha1-6)}\text{Galp(\alpha1-3)}\text{Galp(\beta1-3)}\text{Manp(\alpha1-3)} \\
\text{GPI-B} & : \text{PO}_4 \text{CPO}_{4}\text{Galp(\alpha1-6)}\text{Galp(\alpha1-3)}\text{Galp(\beta1-3)}\text{Manp(\alpha1-3)}
\end{align*}
\]

The relative proportions of GPI-A and GPI-B in the intact LPG were approximately 62 and 38%, respectively (see Minirprint).

**Site of Attachment of the Oligosaccharide Repeat Units to the Core**—Cleavage of the oligosaccharide repeat units from the glycan core with mild acid suggests that they are attached to the core via a phosphodiester bridge. In the monophosphorylated core, the repeat units can only be attached to the terminal galactose 6-phosphate, whereas in the diphasphorylated core there are two possible sites of attachment, namely the terminal galactose 6-phosphate and/or the internal mannose 6-phosphate. The attachment of oligosaccharides to the core was probed by FAB-MS (see Supplemental Material) and by determining the susceptibility of the core phosphates in the intact chain to alkaline phosphatase treatment. Intact LPG was labeled at the reducing terminus, by nitrous acid deamination, and NaB$_3$H$_4$ reduction and digested with alkaline phosphatase. HPLC analysis of the mild acid-released cores showed that while the phosphate on the galactose was completely resistant to alkaline phosphatase, approximately 65% of the phosphate on the mannose was removed (Fig. 11). Extended digestion over 3 days with repeated addition of fresh enzyme did not remove any more phosphate. These results suggested that all the terminal galactose 6-phosphate residues in the core were substituted in the intact chain and that most of the mannose 6-phosphate residues in the diphasphorylated core were unsubstituted. However, the resistance of 35% of the mannose 6-phosphate residues to alkaline phosphatase suggests that some of them may also be substituted with saccharide residues.

**LPG Is Capped with Dimannoside**—To determine whether any of the phosphorylated oligosaccharides were present at the nonreducing terminus of the phosphoglycan chains, intact LPG was treated sequentially with alkaline phosphatase and then galactose oxidase/NaB$_3$H$_4$. The galactose oxidase/NaB$_3$H$_4$ treatment labels all the dephosphorylated oligosaccharide repeat units, which all contain either terminal, 2-, or 3-substituted galactose with a free 6 hydroxyl (results not shown). However, none of the repeat units in the intact LPG were dephosphorylated and labeled by this procedure, suggesting that the phosphoglycan chains were capped by other moieties. It is likely these chains are capped with the neutral oligosaccharide N2', which was released from LPG by mild acid and was present in approximately 1 mol/mol LPG. The positive ion FAB mass spectrum of permethylated N2' afforded (M + H)$^+$ and (M + NH$_4$)$^+$ molecular ions at $m/z$ 456 and 472, corresponding to Hex$_2$. Monosaccharide (Table III)
and methylation analysis (Table IV) identified N" as Manp(1-2)-Man. The mannose was present in the α-configuration from the coupling constant ($J_{1,2} = 1.9$) (Table V). These results define the structure of N" as Manp(α1-2)Man. N3' was present at a nonreducing terminus as jack bean α-mannosidase treatment of intact LPG released approximately 1.6 mol of mannose/mol of LPG. Moreover, after mannosidase treatment, N2' was not longer detected in the mild acid hydrolysate of LPG. Taken together, these results indicated that the nonreducing terminus of the phosphoglycan chains were capped with the sequence Manp(α1-2)Manα1-PO₄.

**Molecular Weight of L. major LPG**—The average molecular weight of the L. major LPG, obtained from stationary phase promastigotes, was estimated from the molar ratio of hexose/pentose to myo-inositol determined by GC-MS. Triplicate determinations revealed a ratio of 90:1, suggesting that the glycan moiety has an average molecular weight of approximately 15,000. This is in good agreement with the estimated number of repeats in LPG, determined from the one-dimensional 500-MHz $^1$H NMR spectrum of intact LPG. Integration of the anomeric proton peaks of the 4-O-substituted α-mannose from the oligosaccharide repeat units and the 6-O-substituted α-galactopyranose from the glycan core gave a ratio of 27:1. These results suggest that the mean number of repeat units per LPG is 27.

**DISCUSSION**

The lipophosphoglycan of L. major are a heterogeneous family of molecules which have a tripartite structure, consisting of a phosphoglycan ($M_r$ 5,000-40,000), a variably phosphorylated glycan core, and a lysoalkyl-PI lipid moiety. The proposed structure of L. major LPG is shown in Fig. 12. The lipophosphoglycan is built up of at least eight different oligosaccharide repeat units which are linked together in linear array by phosphodiester bonds. There are on average 27 repeat units/molecule. Analysis of the phosphorylated oligosaccharide suggests that these chains have a repeating backbone structure of -PO₄-6Galp(β1-4)Manpα1-, and that the 3 position of the galactose in this sequence is either unsubstituted or substituted with galactose or linear saccharide residues. Some of these side chains are capped by the highly unusual α-D-arabinopyranose.

As far as we are aware, this residue has not been reported previously in other eukaryotic glycoconjugates, although there is evidence that it may occur in a partially characterized arabinogalactan from the insect parasite *Crithidia fasciculata* (Gorin et al., 1970). Labeling with galactose oxidase/NaB₃H₄ indicates that none of the phosphorylated oligosaccharides are located at the nonreducing terminus of the phosphoglycan, and it is likely that this terminus is capped with the sequence Manp(α1-2)Manα1-PO₄. The phosphoglycan chain is attached to a linear phosphosaccharide core that has the sequence PO₄-6Galp(α1-6)Galp(α1-3)Galp(β1-3)Manp(α1-3)Manp(α1-4)GlcNp(α1-6)myo-inositol, where approximately 60% of the mannose residues distal to the glucosamine are phosphorylated on the 6 position. Furthermore, the site of attachment of the repeat units appears to be predominantly through the terminal galactose 6-phosphate residue, suggesting that the LPG is organized as a linear molecule. However the resistance of some of the mannose 6-phosphate residues to alkaline phosphatase digestion raises the possibility that a small proportion (<20%) of the LPG molecules have two saccharide chains branching from the core to form a Y-shaped LPG molecule. As shown previously, all these molecules are anchored to the membrane by a novel lysoalkylphosphatidylinositol lipid moiety that contains predominantly 24:0 and 26:0 alkyl chains (McConville et al., 1987).

It is now possible to compare the structures of LPG from L. major (this study), L. donovani (Orlandi and Turco, 1987; Turco et al., 1987, 1989), and L. mexicana.² These studies indicate that all LPGs have the same tripartite structure. A notable feature of these molecules is the presence of a common repeating backbone sequence, PO₄-6Galp(β1-4)Manpα1-, in all the phosphoglycan moieties, which may be variably substituted, on the 3 position of the galactose residues, with other sugars. In the L. donovani LPG, there is negligible substitution
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of the backbone sequence (Turco et al., 1987), whereas in the L. mexicana LPG, approximately 25% of the galactose residues are substituted with βGlc residues. The L. major LPG is the most complex LPG to be characterized as approximately 87% of the galactose residues in the backbone sequence are substituted with a diverse array of different side chains. The presence of a common backbone structure which is variably elaborated with species-specific side chains is consistent with serological studies which indicate the presence of both conserved and species-specific epitopes (Handman et al., 1984). The glycolipid anchor moiety is the most highly conserved region of Leishmania LPGs. All the LPGs contain the same hexasaccharide core sequence which is characterized by having the unusual 3-O-substituted galactofuranose residue. This residue was assigned the β-configuration in L. major LPG, from the results of NMR analysis and by comparison with an authentic standard. Although this residue was identified as αGalf in a recent study on the L. donovani LPG (Turco et al., 1989), reanalysis by NMR spectroscopy indicates that it is also in the β-configuration.

Another feature of the glycan core is that the terminal galactose residue is always phosphorylated on the 6 position. By contrast, phosphorylation of the internal mannose residue may either be partial, as in the L. major LPG, or complete, as in the L. donovani LPG (Turco et al., 1989). At present it is not known whether the LPGs of other Leishmania are also predominantly linear molecules or whether in some LPGs the mannose 6-phosphate residue of the core is more highly substituted or even the sole site of phosphoglycan attachment.

The phosphoglycan moieties of Leishmania LPGs are completely novel structures for eukaryote glycoconjugates. However, the glycolipid anchors of LPG show limited structural homology to the protein-linked GPI anchors containing in the sequence Manα(1-4)GlcNα(1-6)myo-inositol-1-PO3 (Ferguson et al., 1988, Homans et al., 1989, Schneider et al., 1990), suggesting that this motif may be conserved in all eukaryotic glycosylated phosphoinositides that function as membrane anchors for surface macromolecules. The glycolipid anchors of LPG also show structural homology to the major glycolipids of L. major (McConville and Bacic, 1989; McConville et al., 1990). In particular, all the GIPLo contain the same core sequence Galβ(1-3)Manα(1-3)Manα(1-4)GlcNα(1-6)myo-inositol (McConville et al., 1990). In this previous study we identified the galactofuranose as αGalf from comparison with the glycan core of L. donovani LPG. We now show that the galactofuranose residues in these glycolipids are also in the β-configuration. Some of these glycolipids have the same glycan structure as the LPG core and are selectively deacetylated in vivo, suggesting that they may function as biosynthetic precursors to LPG (McConville and Bacic, 1990; McConville et al., 1990). In L. major, the build up of phosphoglycan occurs predominantly or exclusively on these abundant glycolipids. However, recent studies suggest that in other species of Leishmania similar phosphoglycans may also occur on some parasite glycoproteins (Bates et al., 1990, Jaffe et al., 1990).

The heterogeneous nature of the L. major LPGs, compared with the LPGs of L. donovani and L. mexicana, raises the question of whether all the LPG chains contain a random selection of repeat units or whether they are a mixture of different chains that show restricted heterogeneity. The possibility that different cells may produce LPGs with different phosphoglycan compositions is indicated by recent studies on the LPG of L. major promastigotes as they undergo sequential development from an actively dividing noninfectious stage to a nondividing infectious "metacyclic" stage. The LPGs of metacyclic cells have a higher average molecular weight than those of logarithmic phase cells and no longer bind the lectin peanut agglutinin (Sacks and da Silva, 1987). They may also express epitopes not detectable in the LPGs from logarithmic cells (Sacks and da Silva, 1987). As the LPG characterized in this study was derived from cultures containing a mixed population of both actively dividing and metacyclic promastigotes, it is probable that it is a mixture of at least two chain types. One chain type, produced by actively dividing promastigotes, is likely to be enriched in oligosaccharide repeat units containing terminal βGlc residues (peanut agglutinin-negative), whereas the second type, produced by metacyclic promastigotes, is likely to be enriched in repeat units containing terminal αAra or βGlc residues (peanut agglutinin-positive). Whether changes in the composition of the repeat units of LPG are involved in increased infectivity of metacyclic promastigotes has not been established.

LPG probably forms a highly antigenic capsular network on the surface of L. major promastigotes. This is suggested by ultrastructural studies which show that the cell surface is coated by a glycoconjugate and that the thickness of this layer increases in metacyclic promastigotes, coincident with an increase in the average molecular weight of LPG (Fimenta et al., 1989). This is also consistent with estimates of the cellular copy number (approximately 5 × 105 molecules/cell), which indicate that LPG is the major macromolecule on the cell surface (McConville and Bacic, 1990). Furthermore, preliminary molecular modelling studies (Homans, 1990) of the phosphoglycan moiety indicate that the phosphorylated disaccharide backbone sequence exists in an extended configuration with a helical pitch (~6 repeats/turn) and that the oligosaccharide side chains are directed away from the main axis. These studies predict that the LPG will extend away from the plasma membrane for some distance and that it will cover a larger proportion of the cell surface than previously estimated (~25%) (McConville and Bacic, 1990) due to its large cross-sectional area (Homans, 1990). There is indirect evidence that this surface network may form a macromolecular diffusion barrier in metacyclic promastigotes, as there is a progressive decrease in the ability to detect the low molecular weight glycolipids on the cell surface by immunofluorescence as promastigotes progress from logarithmic to stationary growth (Elhay et al., 1988).

Cell surface LPG may be important for both infectivity and parasite survival in the sandfly vector and mammalian host. LPG appears to be the major acceptor for complement (predominantly C3b) and consequently may be involved in facilitating phagocytosis of opsonized promastigotes by macrophages via the CR1 receptor (Puentes et al., 1988; da Silva et al., 1989). The finding that L. major LPG is capped with a dimannoside may be significant in this regard as a mannose-binding protein has been found in various mammalian sera which activates complement through the classical pathway (Ohta et al., 1990). Activation of complement at the nonreducing terminus of the LPG chain, at some distance from the cell surface, would not only ensure that complement fragments are accessible to complement acceptors, but may also contribute to complement resistance by preventing stable insertion of the C5b-9 complex into the plasma membrane of the parasite. This is consistent with the observation that while both logarithmic growth phase promastigotes and metacyclic promastigotes bind the same amount of complement to their cell surface, metacyclic promastigotes produce larger LPG chains on average and are more resistant to complement mediated lysis (Franke et al., 1985; Sacks and da...
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Silva 1987). LPG may also bind directly to macrophage receptors (Handman and Goding, 1984, Russell and Wright, 1988), although the significance of this interaction in promastigote phagocytosis is unclear. It is also unknown whether structural differences in the LPGs of different species contribute to the pronounced tissue tropism of different Leishmania species. Once inside the macrophage, the LPG may protect the parasite cell surface from hydrolytic enzymes. In this regard it is of interest that the phosphorylated oligosaccharides are resistant to calf intestine \( \beta \)-galactosidase. LPG has been shown previously to inhibit \( \beta \)-galactosidase (El-On et al., 1980), and the resistance of the phosphorylated oligosaccharides suggests that they may be acting as competitive inhibitors of this enzyme. Finally, LPG may be important in protecting the parasite from the oxidative burst of the host macrophage, either by inhibiting the protein kinase C involved in the activation of the burst or by acting as an efficient scavenger of oxygen free radicals (McNealy et al., 1989; Chan et al., 1989; McNealy and Turco, 1990).

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SUPPLEMENTARY MATERIAL

To: Structure of the Lipophosphoglycan from Leishmania major

By: Melinda J. McCollum, Jane E. Thomas-Oates, Michael D. Ferguson and Steven H. Weiss

EXPERIMENTAL PROCEDURES

Materials: Materials were obtained as follows: Schneider’s Drosophila medium from NGF, commercial grade from BD Diagnostics, Clontech, and Sigma. Leishmania major from the University of Pennsylvania. The synthetic phosphodiester C (4-Phosphodiamidate) was obtained from Chir decreasing, and Sphingosine was kindly supplied by Dr. Philip A. Gouge and synthesized as described previously (Tang and Gouge, 1980). All other reagents were of the highest purity commercially available.

Parasite: The Leukocyte-isoform LIG1 was originally obtained from the World Health Organization (WHO) for Leishmaniasis. The Leukocyte-isoform LIG1 was grown in Schneider’s Drosophila medium supplemented with 10% fetal calf serum.

Isolation of LIG-1: LIG was isolated from V13 promastigotes in stationary growth phase purified and analyzed by erythrocyte as described previously. Leishmania promastigotes were harvested by centrifugation at 1,000 x g for 10 min and resuspended in Schneider’s medium containing 10% fetal calf serum. The acetone powder was suspended in 1 M acetate buffer, pH 5.0, and centrifuged at 10,000 x g for 10 min. The supernatant was then applied to a PTFE column of polyethyleneglycol (PEG) 6000 (Sephacryl S-200, Pharmacia) in 1 M acetate buffer, pH 5.0. The column was then washed with 1 M acetate buffer, pH 5.0, and eluted with a linear gradient of NaCl (0-1 M) in 1 M acetate buffer, pH 5.0. The fractions containing LIG were then pooled and dialyzed against 1 M acetate buffer, pH 5.0.

Preparation of the Oligosaccharide Repeat Unit and Glycylglycine Anchor. Purified LIG was dialyzed against 1 M acetate buffer, pH 5.0, and lyophilized. The dried material was then suspended in 0.1 M sodium acetate buffer, pH 5.0, and dialyzed against 1 M acetate buffer, pH 5.0. The dialyzed material was then suspended in 0.1 M sodium acetate buffer, pH 5.0, and dialyzed against 1 M acetate buffer, pH 5.0.

Monosaccharides Analysis: Samples containing 125-1,000 pmol of monosaccharides were digested with neuraminidase (100 I.U., 30 min, 37°C) and analyzed as described above.

Enzyme Treatment: Phosphorylated lipooligosaccharides were digested with 100 units of desialylase of L. major (100 I.U., 30 min, 37°C) and the digest was then used for the analysis of the monosaccharide composition.

Gel-Elution Chromatography: The material was applied to a Bio-Gel P1 column (1.5 x 100 cm) and eluted using a linear gradient of 0.15 M to 1 M sodium acetate buffer, pH 7.0 (500 ml per column). The fractions containing LIG were then pooled and dialyzed against 1 M acetate buffer, pH 5.0.

Biosynthetic Reactions: The material was applied to a Bio-Gel P1 column (1.5 x 100 cm) and eluted using a linear gradient of 0.15 M to 1 M sodium acetate buffer, pH 7.0 (500 ml per column). The fractions containing LIG were then pooled and dialyzed against 1 M acetate buffer, pH 5.0.

Fig. 2. Oligo-Specific chromatography of Leishmania major LIG. LIG was deglycosylated with 4 M HCl (5 min) and loaded onto a column of oligo-S-specific antibodies. The column was washed with 1 M sodium acetate buffer, pH 5.0, and eluted with 1 M sodium acetate buffer, pH 5.0.
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TABLE II

| Fraction | Assignment | M + Na⁺ |
|----------|------------|---------|
| P1       | P1         | 549     |
| P2       | P2         | 247     |
| P1-5     | P1-5       | 247     |
| P4       | P4         | 913     |
| P6       | P6         | 957     |
| P7       | P7         | 1117    |
| P8       | P8         | 1161    |
| P9       | P9         | 1321    |
| P10      | P10        | 1536    |

1 All the permethylated phosphates were directly identified as the phosphate group.
2 The permethylated Ph provided only a 3D molecular mass (m/z 557) and A type fragment ions at m/z 175, 199 and 215 due to the structure of Leishmania major.
3 The M + Na⁺ ± error were determined only by the fragment ions at m/z 175, 199 and 215 due to the structure of Leishmania major.

TABLE III

| Mass function | Yields of the Oligosaccharide Repeat Units from L. major LPG |
|---------------|-----------------------------------------------------------|
| Dose fraction | RF² (glucose units) | PMAP² (mg LPG) | Yield (mg LPG) |
| N1            | 1.00            | 1.00            | 1.00            |
| N2            | 2.00            | 2.00            | 2.00            |
| N3            | 3.65            | 0.90            | 3.65            |
| N4            | 5.35            | 1.00            | 5.35            |
| N5            | 6.34            | 0.40            | 6.34            |
| N6            | 4.80            | 1.10            | 4.80            |
| N7            | 7.91            | 0.50            | 7.91            |
| N8            | 6.31            | 0.50            | 6.31            |

1 Yields were determined from the peak area of the chromatographic pattern shown in Fig. 4. Similar ratios were determined when the isocratic eluent of the phosphorylated oligosaccharides were used. Values in parentheses were obtained by analysis of the antiprotic eluent system yield a valid quantitative mass estimate for these chemically related structures.
2 ND = Not determined

TABLE IV

| Mass function | Origin | N2 | N3 | N4 | N5 | N6 | N7 | N8 | NS | MS |
|---------------|--------|----|----|----|----|----|----|----|----|----|
| N1            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| N2            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| N3            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| N4            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| N5            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| N6            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| N7            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| N8            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| NS            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|
| MS            | 1.00   | 0.00| 1.00| 0.00| 0.00| 0.00| 1.00| 1.00| 1.00| 1.00|

The permethylated diastereomers (PMAPs) were isolated and quantified by GC-MS.
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Table VI

| Chemical Shift (ppm) | 3H-1 | H-2 | H-3 | H-4 | H-5 |
|---------------------|------|-----|-----|-----|-----|
| P5                  | 4.52 | 3.70 | 3.85 | 4.25 | 3.92 |
| N5                  | 4.61 | 5.71 | 5.84 | 4.19 | 3.76 |
| P6                  | 4.52 | 3.71 | 3.87 | 4.28 | 3.92 |
| N6                  | 4.66 | 3.69 | 5.93 | 4.18 | 3.76 |
| P7                  | 4.52 | 3.71 | 3.87 | 4.25 | 3.92 |
| N7                  | 4.50 | 3.71 | 5.85 | 4.19 | 3.77 |

Fig. 7. 

(a) Reverse phase HPLC of glycosylated lipids from L. major. The glycosylated lipids were purified by HPLC and identified by mass spectrometry. The data presented are typical for the fraction eluted at 40% acetonitrile and 60% water.

(b) High performance liquid chromatography (HPLC) of glycosylated lipids from L. major. The glycosylated lipids were purified by HPLC and identified by mass spectrometry. The data presented are typical for the fraction eluted at 40% acetonitrile and 60% water.

(c) Two-dimensional 1H-1H COSY spectrum of the glycosylated lipids from L. major. The glycosylated lipids were purified by HPLC and identified by mass spectrometry. The data presented are typical for the fraction eluted at 40% acetonitrile and 60% water.
### TABLE VII

| Monosaccharide          | Molar ratio |
|------------------------|-------------|
|                        | GPI-A       | GPI-B       |
| Gal                    | 2.3         | 2.2         |
| Man                    | 1.2         | 2.1         |
| GlcNAc                 | 1.0         | 1.0         |
| Gal-Galactosamine      | 0.7         | 0.6         |
| Man-6-phosphate        | 0.5         | 0.6         |
| GlcNAc-6-phosphate     | 0.3         | 0.2         |

1. All analyses relative to GlcNAc = 1.
2. Measured as TMS derivates of methyl 2-amino-2-deoxyglucose.

### TABLE VIII

**Methylated Analysis of the GPI-A and GPI-B.**

The aldonolactones, obtained by sodium periodate oxidation followed by NaHCO₃ reduction, were dephosphorylated and with alkaline phosphatase prior to methylation analysis.

| PMAA     | Source | GPI-A | GPI-B |
|----------|--------|-------|-------|
| Methyl   | 3,6-di-O-methyl | 3-O-substituted ManP | 2.0 | 2.0 |
| [D-Man](\alpha) 5,6-di-O-methyl | terminal GalP | 0.9 | 1.0 |
| [D-Man](\alpha) 6,6-di-O-methyl | 3-O-substituted GlcP | 0.8 | 0.8 |
| [L-Gal](\alpha) 6,6-di-O-methyl | 6-O-substituted GlcP | 0.9 | 0.8 |
| [L-Ido](\alpha) 5,5-di-O-methyl | 4-O-substituted AIB | 1.0 | 1.0 |

1. All analyses relative to ManP = 5.5.
2. [L-Gal](\alpha) 6,6-di-O-methyl 2,5-anhydro-D-mannitol was detected in low and variable yield due to its high polarity.

### TABLE IX

**Reporter Resonance Assignments for the Phosphatidyl-Phosphatidyl Phosphoryl-Phosphoryl Monomers of GPI-A and GPI-B.**

Chemical shifts (600.08 ppm) were reported relative to acetone (δ = 2.235 ppm).

| Residue | GPI-A | GPI-B |
|---------|-------|-------|
| H-1     | H-2   | J-1,2 |
| GalP(1-6) | 4.65  | 3.82  | 3.5 |
| GalP(1-3) | 5.06  | 3.81  | 3.5 |
| GalP(1-4) | 8.58  | 4.41  | 1.6 |
| ManP(1-3) | 5.06  | 6.20  | 1.9 |
| ManP(1-4) | 5.51  | 4.48  | 1.9 |
| GlcP(1-6) | 5.79  | 3.25  | 3.5 |

### Table X

**Chemical shifts (600.08 ppm) for H-1 and H-2 resonances and coupling constants (J-1,2) are shown for phosphatidyl monomers in the LPC core (GPI-A and GPI-B), in the major glycolipids (GPIA-1,2,3) of L. major and authentic saccharides.**

| Composed | H-1 (ppm) | J-1,2 (Hz) |
|----------|-----------|------------|
| GPI-A    | 5.22      | 4.41       |
| GPI-B    | 5.22      | 4.41       |
| GPI-1,A  | 5.11      | 4.40       |
| GPI-2,A  | 5.11      | 4.40       |
| GPI-3,A  | 5.11      | 4.40       |
| GlcP     | 5.13      | 4.32       |
| GalP(1-4) GlcP | 4.01 | 4.06       |
| GlcP(1-6) GlcP | 4.40 | 4.95       |

1. From McGee et al. (1990)
2. From Cerny et al. (1989)

Fig. 11. Susceptibility of the core phosphatidyl residues to alkaline phosphatase treatment of the intact skin. Amines and deamated, NaOH(10%) induced LPC was treated with alkaline phosphatase over three days with fresh additions of enzyme at 30th and 48th. Samples from each time point were hydrolyzed in 60% TFA (100%, 100%) and analyzed by HPLC. All samples were treated with peptidase and the recovery measured by RIA. The recovery of the glucosidic linkages of the glucosidic residue (Glcp(1-6) Galp, 10PC) gave only one labelled peak that covalently with ManP from exchange HPLC.

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Structure of the lipophosphoglycan from Leishmania major.
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