Identification of the Defect in Lipophosphoglycan Biosynthesis in a Non-pathogenic Strain of Leishmania major*

Malcolm J. McConville§ and Steven W. Homans

From the Department of Biochemistry, University of Dundee, Dundee DD1 4HN, United Kingdom

The major macromolecule on the surface of the protozoan parasite, Leishmania major, is a complex lipophosphoglycan (LPG), which is anchored to the plasma membrane by an inositol-containing phospholipid. A defect in LPG biosynthesis is thought to be responsible for the avirulence of the L. major strain LRC L119 in mice. In order to identify the nature of this defect we have characterized two truncated forms of LPG, which are accumulated in this strain, by one- and two-dimensional 500-MHz 1H NMR spectroscopy, two-dimensional heteronuclear 1H-13C NMR spectroscopy, methylation analysis, and exoglycosidase digestions. The structures of these glycoinositolphospholipids, termed GIPL-4 and -6, are as follows:

Man(1-PO)2-Gal(1-PO)2-Man(1-PO)2-GlcN(1-PO)2-lyso-alkylglycerol

The glyccan moieties of GIPL-4 and -6 are identical to the anchor region of LPG, which is also substituted with a Glc-1-PO residue in approximately 60% of the structures. However, instead of being capped with chains of phosphorylated oligosaccharide repeat units, both glycans terminate in Manα1-PO₂, suggesting that the defect in LPG biosynthesis is in the transfer of galactose to this residue to form the disaccharide backbone of the first repeat unit. These results indicate that the phosphoglycan moiety of LPG is essential for intracellular survival of the parasite and have implications for LPG biosynthesis.

The etiological agent of human cutaneous leishmaniasis, Leishmania major, is a parasitic protozoan that alternates between an extracellular, flagellated promastigote stage in the digestive tract of its sandfly vector and an intracellular amastigote stage within the phagolysosome compartment of mammalian macrophages. The cell surface of the promastigote stage is coated by a complex glycoalkalx which contains two novel classes of glycoconjuate; the heterogeneous lipophosphoglycans (LPGs) (Handman and Goding, 1985; McConville et al., 1987, 1990a) and the low molecular weight glycoinositol-phospholipids (GIPLs) (McConville and Bacic, 1989; McConville et al., 1990b). LPG consists of a polymer of repeating phosphorylated oligosaccharides, containing the backbone sequence PO₄-6Gal-4Manα1-3Manα1-4GlcNα1-6-myo-inositol-1-PO₄-lyso-alkylglycerol.

The glycan moieties of GIPL-4 and -6 are structurally similar to the LPG anchors in having the same glycán core sequence, but differ from these anchors in containing predominantly alkylacyl-PI lipid moieties (McConville and Bacic, 1989; McConville et al., 1990b).

Studies with LPG-deficient strains of Leishmania have provided evidence that LPG is essential for promastigote infectivity in the mammalian host (Handman et al., 1986; Elhay et al., 1990; McNeely and Turco, 1990). These strains are non-pathogenic in mice and are rapidly killed in the phagolysosome of in vitro infected macrophages. Importantly, intracellular survival of these strains can be prolonged if exogenous LPG is inserted into the promastigote plasma membrane (Handman et al., 1986; McNeely and Turco, 1990). In this regard, LPG is thought to prevent complement-mediated lysis of promastigote in the bloodstream of the host (Puentes et al., 1988), to be involved in mediating the initial attachment of promastigotes to the macrophage (Handman and Goding, 1985; Puentes et al., 1988; da Silva et al., 1989; Talamas-Rohana et al., 1990), and to protect the parasite membrane from hydrolytic enzymes and the oxidative burst in the phagolysosome (El-On et al., 1980; Chan et al., 1989; McNeely and Turco, 1990). We have previously shown that the LPG-deficient strain of L. major, LRC-L119 (L119) accumulates a number of polar GIPLs which may be truncated forms of LPG (McConville and Bacic, 1989, 1990). We have now characterized these glycolipids in order to identify the defect in LPG biosynthesis and to determine which portions of LPG are still expressed in this strain. These studies indicate that L119 has a mutation effecting the galactosyltransferase which is involved in forming the backbone sequence of the repeat units. They also refine the structure of the L. major LPG anchor and have implications for LPG biosynthesis.

* This work was supported by the Wellcome Trust and the Medical Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of an Australian National Health and Medical Research Council C. J. Martin Fellowship.

1 The abbreviations used are: LPG, lipophosphoglycan; GIPL, glycoinositolphospholipid; PI, phosphatidylinositol; AHM, 2,5-anhydromannitol; GC-MS, gas liquid chromatography-mass spectrometry; COSY, correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HPTLC, high performance thin layer chromatography.
The GIPL profiles of the virulent, LPG-producing \textit{L. major} strain V121 and the avirulent, LPG-deficient strain L119 are shown in Fig. 1. As reported earlier (McConville and Bacic, 1990; McConville \textit{et al}., 1990a), both strains contain similar levels of GIPL-1, -2, and -3. However, L119 differs notably from V121 in containing three additional species of GIPL (GIPL-4, -5, and -6) which have a slow HPTLC mobility. These species account for approximately 50% of the L119 GIPLs and were not detectable in the V121 profiles. Two of these species (GIPL-4 and -6) were purified by a combination of octyl-Sepharose chromatography and HPTLC. From the yields of myo-inositol in each fraction, and assuming 1 inositol residue/molecule, there are approximately $5 \times 10^6$ GIPL-6 and $2 \times 10^6$ GIPL-4 molecules per cell. The glycan head groups of these GIPLs were characterized as described below.

**Structure of the GIPL-6 Glycan**—The glycan moiety of GIPL-6 was released with PI-specific phospholipase C and analyzed by one-dimensional $^1$H 500-MHz NMR and by two-dimensional $^1$H-$^1$H correlated spectroscopy (COSY). Eight anomic protons of unit intensity were observed in the low field region of the spectrum (Fig. 2a), indicating the presence of eight monosaccharide residues in GIPL-6, consistent with the monosaccharide analysis (Table I). Six of these proton resonances bear a strong resemblance in both splittings and shifts (Table II) to those observed in the $^1$H NMR spectrum of the hexasaccharide-inositol phosphate moiety derived from GIPL-3 (McConville \textit{et al}., 1990b). In particular, the through-bond connectivities in the $^1$H COSY spectrum of GIPL-6 (Fig. 3), together with knowledge of spin-coupling constants between endocyclic protons measurable from cross-peak multiplicities, suggest the presence of two Manp, two Galp, one Glcp, one GlcNp, and one myo-inositol residue. The sequential arrangement of these residues and myo-inositol was obtained from $^1$H NOESY measurements (Fig. 4) as described previously (McConville \textit{et al}., 1990b). By use of inter-residue through-space NOE connectivities across the glycosidic linkages, together with methylation analysis of the neutral, deamidated glycan core (Table III), the following partial sequence could be unambigously defined: Galp$\alpha$-1$\rightarrow$6Galp$\alpha$-1$\rightarrow$3Galp$\alpha$-1$\rightarrow$3Manp$\alpha$-1$\rightarrow$4GlcNp$\alpha$-1$\rightarrow$6-myoinositol, the anomic configurations were defined from the magnitude of the scalar coupling ($J_{i,j}$) between the C-1 and C-2 protons of each residue, with the exception of the Galp residue. The very small splitting (below the linewidth) of the C-1 proton of the latter did not reliably reflect the anomeric configuration. However, the near identity of the chemical shifts of the C-1 proton of Galp in GIPL-6 (Table II) and GIPL-3 (McConville \textit{et al}., 1990a, 1990b) strongly suggested that this residue was in the $\beta$-anomeric configuration in GIPL-6.

The remaining two proton resonances in Fig. 2a were composed of a doublet of doublets, and had a large spin-coupling constant (~8 Hz), suggesting that they corresponded to the anomic protons of two hexose-1-PO$_4$ residues. To confirm this postulate, a one-dimensional $^1$H NMR spectrum of GIPL-6 was recorded with broadband $^3$P decoupling. Under these conditions, each doublet of doublets collapsed into a single doublet (Fig. 2b). From the spin coupling constants and the chemical shifts measured in the $^1$H COSY experiment, the two residues phosphorylated at C-1 were identified as $\alpha$Manp and $\alpha$Glcp. Furthermore, mild acid hydrolysis of native GIPL-6 released the equivalent of 1 nmol each of Man and Glc/ Gln mole of myo-inositol (see Fig. 5 in the Miniprint Section), consistent with these residues being linked to the hexasaccharide core via phosphodiester bonds. The site of attachment of these residues to the core was determined by a two-step heteronuclear relayed correlation experiment ($^1$H-$^1$P-H). From the spectrum in Fig. 6 it can be seen that the C-1 protons of both the Glco-1-PO$_4$ and Mano-1-PO$_4$ residues exhibit relayed connectivities via phosphorus to a pair of resonances.

---

8 Portions of this paper (including "Experimental Procedures," part of "Results," Tables I–III, and Figs. 5, 7, and 8) 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.
Leishmania Lipophosphoglycan Biosynthesis

Fig. 3. $^1$H-$^1$H COSY spectrum (region 3.25-4.45 ppm) of the GIPL-6 glycan. The resonance assignment pathway is illustrated for Man-2. Assignment of C1-C4 proton resonances is straightforward from this spectrum. Assignment of the C-6 proton can be made from the $^1$H-$^3$P-$^1$H relay experiment (see Fig. 6). A weak cross-peak can be observed between these proton resonances in the COSY spectrum, together with a cross-peak correlating one of the C-6 proton resonances with the C-5 proton resonance (see inset). The latter has a chemical shift virtually identical to the C-3 proton resonance.

Fig. 4. Reporter region of the $^1$H-$^1$H NOESY spectrum of the GIPL-6 glycan. Relevant through-space connectivities were labeled using the same notation as in Fig. 2a.

These resonances were readily assigned to C-6 protons from the large observable geminal splittings ($\sim -12$ Hz) in the cross-peaks. From these data, it was concluded that the Man-1-PO$_4$ was attached to the 6-position of the terminal Gal residue in the hexasaccharide core, since the C-6 protons of the Gal residue could be determined unambiguously from the COSY spectrum. The assignment of the Glc-1-PO$_4$ linkage position to the 6-position of the Man residue distal to the glucosamine was similarly obtained, although the cross-peaks to, from, and between the C-6 protons of this latter residue were of lower intensity in the COSY spectrum (Fig. 3). The site of attachment of the Man-1-PO$_4$ and the Glc-1-PO$_4$ residues to the core was also deduced by assessing the susceptibility of the phosphate residues to alkaline phosphatase before and after digestion of the deaminated glycan with jack bean $\alpha$-mannosidase (see Miniprint Section, Fig. 7). Taken together, these data indicated that the structure of the G4 glycan was as shown in Structure 1, where the myo-inositol residue formed part of the lyso-1-O-alkyl-PI lipid moiety which contained predominantly C$_{26}$o and C$_{26}$o alkyl chains (McConville and Bacic, 1989).

Fig. 6. Reporter region of the $^1$H-$^3$P-$^1$H relayed correlation spectrum of the GIPL-6 glycan. This spectrum displays relayed connectivities via phosphorus between the anomeric protons of Glc-1-PO$_4$ and Man-1-PO$_4$ to the C-6 protons of the corresponding glycan. These connectivities were labeled using the same notation as in Fig. 2a.

Glc-1-PO$_4$
| 6 |
Man-1-PO$_4$-6Gal-1-3Gal|β1-3Man-1-
3Man-1-4GlcNac-1-6-myoinositol

Structure 1.

Structure of the GIPL-6 Glycan—The PI-specific phospholipase C-released glycan of GIPL-4 contained seven anomeric protons of unit intensity in the low field region of the $^1$H NMR spectrum (Fig. 2c). These resonances were present in essentially identical positions to the anomeric resonances in the GIPL-6 spectrum (Table II), suggesting that GIPL-4 had the same sequence as GIPL-6, minus the Glc-1-PO$_4$ residue. This was confirmed from the $^1$H NOESY spectrum of GIPL-4 (not shown) which gave identical through-bond connectivi-
ties to GIPL-6 and by methylation analysis which indicated that both GIPLs contained the same glycan core (Table III). The location of the Manα1-P04 residue on the terminal Gal residue of the core was inferred from the near identity of the chemical shifts of the C-1 proton of this residue in LPG-4 and in GIPL-6, together with the near identity of the shifts of the C-6 protons of the terminal Galp residue. This postulate was confirmed by Dionex HPLC analysis of the products of sequential jack bean α-mannosidase and alkaline phosphatase digestion (see Miniprint Section, Fig. 8). Taken together, these data suggest that the glycan moiety of GIPL-4 is as shown in Structure 2.

\[
\text{Manα1-P04-Galpα1-6Galpα1-3Galβ1-3Manα1-4GlcNα1-6-myo-inositol}
\]

**Structure 2.**

In contrast to GIPL-6, GIPL-4 contained an 1-O-alkyl-2-acyl-PI moiety with a mixture of unsaturated fatty acids (mainly C14:0, C16:0, and C18:2; C20:4, C22:0, and C24:0) (McConville and Bacic, 1989).

**Location of Glc-1-P04 on the Inner Mannose of the L. major LPG Anchor**—We have previously shown that the oligosaccharide repeat units of *L. major* LPG are attached via phosphodiester linkages to the 6-position of the terminal Gal residue of the core (McConville et al., 1991a). In addition, approximately 60% of the LPG molecules contain a second phosphate residue on the mannose residue in the core which is distal to the glucosamine. This phosphate was partially resistant to alkaline phosphatase suggesting that it was also substituted, although the nature of this substituent was not determined. Mild acid hydrolysis of intact *L. major* LPG released approximately 0.6 nmol of Glc/nmol of inositol (Fig. 5, panel C), raising the possibility that this mannose residue was substituted with Glc-1-P04 in the native molecule. This was confirmed by analysis of the one dimensional \(^1\)H NMR spectrum of *L. major* LPG (not shown) which exhibited a pair of doublets corresponding to the C-1 proton resonance of the Glcα1-P04 moiety, at essentially identical chemical shifts to that found in the GIPL-6 glycans.

**DISCUSSION**

The highly polar GIPLs of *L. major* L119 appear to be truncated forms of LPG which are accumulated due to a defect in LPG biosynthesis in this strain. This is suggested by the finding that 1) these glycolipids are not detectable in wild type strains of *L. major* which express LPG, 2) the levels of expression of these glycolipids in L119 are comparable with the levels of expression of LPG in virulent strains of *L. major* (McConville and Bacic, 1990) and 3) they show a remarkable degree of structural homology to the anchor region of LPG. The structures of these glycolipids were determined by two-dimensional \(^1\)H and \(^1\)H-\(^3\)P 500 MHz NMR, methylation analysis and chemical and enzymatic digestions and are summarized in Fig. 9. The structure of the GIPL-5 glycans was not determined in this study, but previous results suggest that this species is the lyso- derivative of GIPL-4 (McConville and Bacic, 1989). All these glycolipids contain a hexasaccharide core with a terminal Galp residue. In LPG, this residue is the site of attachment for the linear chains of phosphorylated oligosaccharide repeat units which have the backbone structure \(-6Galβ1-4Manα1-P04\) (McConville et al., 1991a, see Fig. 9). A striking feature of the polar L119 GIPLs is that they all terminate with Manα1-P04, suggesting that the biosynthetic defect is in the transfer of galactose to this residue to form the first repeat unit. The identical location of the unusual

\[
\text{Glcα1-P04}
\]

**Glcα1-P04** residue on both GIPL-6 and on more than 60% of the LPG molecules further demonstrates the similarity between the polar GIPLs and LPG. The presence of this substituent on the LPG core was previously indicated by the apparent resistance of the core Man-6-P04 to alkaline phosphatase treatment (McConville et al., 1990a), although the nature of the substituent was not identified. Subsequent analysis of the LPGs from *L. donovani* and *L. mexicana* has revealed that nearly 100% of these molecules are substituted on the core with Glcα1-PO4.\(^3\) This substitution appears to be unique to LPG in wild type strains, as none of the GIPLs are similarly glucosylated (Fig. 9). GIPL-5 and -6 are also homologous to the LPG anchor in having the same lyso-alkyl-PI moiety with predominantly C14:0 and C16:0 alkyl chains. By contrast, GIPL-4 has an alkylseryl-PI which is identical to the lipid moieties of GIPLs 1–3 in containing C16:0 and C18:0 alkyl chains. McConville and Bacic, 1989; McConville et al., 1990b).

The most likely explanation to account for the accumulation of these glycolipids is that L119 has a mutation effecting the β1–4 galactosyl transferase which is involved in forming the first repeat unit of LPG. Alternative possibilities, involving an impairment in intracellular vesicular transport or a defect in UDP-Gal-GIPL-6 synthesis or transport are unlikely, as all these Gal-containing GIPLs are still expressed in high copy number at the cell surface (McConville and Bacic, 1989).

Several other LPG-deficient clones of *L. major* have been prepared by mutagenesis of the LPG-producing strain V121 (Elhay et al., 1990). Analysis of the GIPL profiles of these clones suggests that a similar defect in the enzyme(s) involved in the assembly of the repeat units is responsible for the failure of these clones to synthesize mature LPG.\(^5\) It is of interest that none of these mutants have a defect in GIPL

\[^{3}\]J. Thomas, M. J. McConville, J. Thomas-Oates, K. Greiss, S. J. Turco, S. W. Homans, and M. A. J. Ferguson, manuscript in preparation.

\[^{4}\]T. Hg, R. Eiges, P. Overath, M. J. McConville, J. Thomas-Oates, S. W. Homans, and M. A. J. Ferguson, manuscript in preparation.

\[^{5}\]M. J. Elhay, M. J. McConville, E. Handman, and A. Bacic, unpublished results.
biosynthesis, raising the possibility that such mutations may be lethal.

This study defines precisely which portion of the LPG has been deleted in L119 and supports the notion that the phosphoglycan moiety of LPG is essential for intracellular survival of the promastigote in macrophages. These phosphoglycan chains may be involved in mediating the phagocytosis of promastigotes by binding to the appropriate macrophage receptor (Handman and Goding, 1984; Russell and Wright, 1988; da Silva et al., 1989; Talamas-Rohana et al., 1990) and in protecting the surface of the promastigote from hydrolytic enzymes and oxygen radicals in the phagosomal compartment (El-On et al., 1980; Chan et al., 1989). Less is known about the role of the anchor domain of LPG. This portion of the LPG provides the sole mechanism of attachment for the phosphoglycan moiety of LPG in macrophages. These phosphoglycan chains to the plasma membrane in L. major. It may also prevent the induction of the macrophage oxidative burst by inhibiting the protein kinase C of the host cell (McNeely and Turco, 1987; McNeely and Turco, 1990). However, GIPL-6 is an equally effective inhibitor of purified protein kinase C* and is also expressed in high levels in L119, suggesting that this latter property of LPG is not sufficient, by itself, to protect the intracellular promastigotes.

These results have several implications for LPG biosynthesis. First, the finding that, in L119, only the Manα-1-PO₄ residue is transferred to the glycan core strongly suggests that the repeat units of LPG are built up by the sequential addition of monosaccharide units, rather than by the en bloc transfer of preformed repeat units from a lipid carrier, as occurs in the synthesis of many prokaryote glycoconjugates (reviewed by Sutherland (1985)). This proposal is consistent with the results of Carver and Turco (1991) on the cell-free synthesis of L. donovani LPG, which also indicated that lipid-linked precursors were not involved in the formation of the repeat units. Second, GIPL-4 has the same alkylacyl-PI lipid moiety as GIPL-3, suggesting that LPG biosynthesis may be initiated by the transfer of a Man-1-PO₄ residue to GIPL-3, without the prior deacylation of this species to lysyl GIPL-3. It is not known whether the GIPL-3 lysyl derivatives, which are present in significant levels in some strains (Fig. 1) (McConville et al., 1990b), are also LPG precursors. Third, in addition to the transfer of Man-1-PO₄, it is likely that there are two other early steps in LPG biosynthesis, involving the deacylation of the PI moiety and the glycosylation of the glycan core. It is intriguing that, while the alkylacyl-PI lipid moiety of GIPL-4 has the same heterogeneous alkyl chain composition as GIPL-3, the deacylated PI lipid moiety of GIPL-6 is highly enriched for C₄₋₅ and C₆₋₇ alkyl chains, as found in the LPG lipid moiety. These results indicate either that the alkyl chains of these glycolipids are being remodeled, or alternatively, that one or more of the enzymes involved in the deacetylation and glycosylation of repeat units from other species of Leishmania (Orlandi and Turco, 1987)* and may be necessary for the stable association of LPG molecules with the plasma membrane.

Acknowledgments—We thank Dr. M. A. J. Ferguson for stimulating discussions and for critical review of the manuscript, Dr. M. Low for providing the PI-specific phospholipase C and Dr. E. Handman for providing the L. major LRC-L119 clone.

REFERENCES

Carver, M. A., and Turco, S. J. (1991) J. Biol. Chem. 266, 10974-10981
Chan, J., Fujiwara, T., Brennan, P., McNeil, M., Turco, S. J., Sibille, J. C., Snapper, M., Aisen, P., and Bloom, B. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2453-2457
da Silva, R. P., Hall, B. F., Joiner, K. A., and Sacks, D. L. (1989) J. Immunol. 143, 617-622
Elhay, M. J., Kelleher, M., Bacic, A., McConville, M. J., Tolson, D. L., Pearson, T. W., and Handman, E. (1990) Mol. Biochem. Parasitol. 40, 151-300
El-On, J., Bradley, D. J., and Freeman, J. C. (1980) Exp. Parasitol. 49, 167-174
Ferguson, M. A. J., Homans, S. W., Dwek, R. A., and Rademacher, T. W. (1988) Science 239, 753-759
Handman, E., and Goding, J. W. (1986) EMBO J. 4, 329-336
Handman, E., Schnur, L., Spithill, T. W., and Mitchell, G. F. (1986) J. Immunol. 137, 3608-3614
McConville, M. J., and Bacic, A. (1989) J. Biol. Chem. 264, 757-766
McConville, M. J., and Bacic, A. (1990) Mol. Biochem. Parasitol. 38, 57-68
McConville, M. J., Bacic, A., Mitchell, G. F., and Handman, E. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8941-8945
McConville, M. J., Thomas-Oates, J. E., Ferguson, M. A. J., and Homans, S. W. (1990a) J. Biol. Chem. 265, 19611-19623
McConville, M. J., Homans, S. W., Thomas-Oates, J. E., Dell, A., and Bacic, A. (1990b) J. Biol. Chem. 265, 7385-7394
McNeely, T. B., and Turco, S. J. (1987) Biochem. Biophys. Res. Commun. 148, 653-657
McNeely, T. B., and Turco, S. J. (1990) J. Immunol. 144, 2745-2750
Neuhaus, D., Wider, G., Wagner, G., and Wuthrich, K. (1984) J. Magn. Reson. 57, 164-168
Orlandi, P. A., Jr., and Turco, S. J. (1987) J. Biol. Chem. 262, 10384-10391
Puentes, S. M., da Silva, R. P., Sacks, D. L., Hammer, C. H., and Joiner, K. A. (1988) J. Immunol. 145, 4311-4316
Russell, D. G., and Wright, S. D. (1988) J. Exp. Med. 168, 279-292
Sutherland, I. W. (1985) Annu. Rev. Microbiol. 39, 243-270
Talamas-Rohana, P., Wright, S. D., Lennartz, M. R., and Russell, D. G. (1990) J. Immunol. 144, 4817-4824

* M. J. McConville and P. Robinson, unpublished results.

Continued on next page.
Leishmania Lipophosphoglycan Biosynthesis

EXPERIMENTAL PROCEDURES

Parasites - The L. major strain LRC 119 was originally isolated from a teased preparation in Kenya and a cloned line derived by limit dilution cloning. It was shown to be L. major by genetic characterization and by enzyme analysis (McConville and Horns 1989). Promastigotes were cultured in Schneider's Drosophila medium (Gibco) supplemented with 10% fetal bovine serum.

Isolation of GPls - The GPls were isolated from promastigotes (10^10) in ice-cold exponential growth, purified by acryl-Sepharose chromatography and fractionation on a column as previously described (McConville and Horns 1989).

Enzyme Treatments and Lipid Acid Hydrolysis - Phospholipid glycosytes were treated with 10% aqueous KOH, 70% aqueous HCl (9 M NaCl, 0.5 M NaCl) or a combination of both reagents followed by passage through a column (0.5 M NaCl) over (0.1 M NaCl) (Smith and Horns 1990).

ISO-MRS for NMR Spectroscopy - The glycans released from the purified GPls were released by treatment with N-acetylglucosamine (5.5 mg/ml) in DMSO/ether (1:1) for 18 h. After acidification of the mixture with acetic acid and removal of the precipitate, the glycans were further purified by gel filtration on a column of BioGel P-4 (5 x 30 cm) (BioRad) eluted with water. The glycans, which eluted in the void volume, were desalted by incubation overnight with repeated resuspension in water and lyophilized.

Nitrous acid deamination - N4H2O(2H)O, reduction of the purified GPls was catalyzed by 0.01 M nitrous acid (pH 6.5) containing 0.1 M NaCl, 0.2 M KCl, 0.1 M glycerol, 0.1 M Hepes, 0.1 M KCl, 0.01 M EDTA, and 0.1 M S-2238. The deaminated glycans were treated with 4N HCl/1 N NaOH (20:1) and lyophilized.

Other analyses - Monosaccharide analysis, methylation analysis, and gas-liquid chromatography-mass spectrometry analysis were performed as described elsewhere (McConville and Horns 1990).

RESULTS

Monosaccharide composition of GPls 1-4

| Monosaccharide      | GIPL-4 | GIPL-6 |
|---------------------|--------|--------|
| Mannon               | 5.1    | 3.9    |
| Galactose            | 2.0    | 1.8    |
| Glucose              | 0.9    | 0.9    |
| Mannon-6-phosphate   | 0.8    | 0.8    |
| Galactose-6-phosphate| 0.8    | 0.7    |
| Glucosamine         | 0.7    | 0.9    |
| Nacetyl             | 1.0    | 1.0    |

1 N-acetylated and dodecylated monosaccharides were analyzed after TMS derivatization by gas-liquid chromatography-mass spectrometry analysis performed as described elsewhere (McConville and Horns 1990).

2 Glucosamine was determined as the TMS derivative of N-acetylgalactosamine after acetylation and TMS derivatization of the GPls.

3 Monosaccharides were analyzed after TMS derivatization by gas-liquid chromatography-mass spectrometry analysis performed as described elsewhere (McConville and Horns 1990).

4 The results are expressed as molar ratio.

TABLE II

| Monosaccharide | GIPL-4 | GIPL-6 |
|----------------|--------|--------|
| Mannose        | 5.1    | 3.9    |
| Galactose      | 2.0    | 1.8    |
| Glucose        | 0.9    | 0.9    |
| Mannon-6-phosphate | 0.8    | 0.8    |
| Galactose-6-phosphate | 0.8    | 0.7    |
| Glucosamine   | 0.7    | 0.9    |
| N-acetyl      | 1.0    | 1.0    |

1 The molar ratio for this molar is 9.0:1.0.
2 The larger of these two coupling constants corresponds to the D-glucosamine (1H,2H)-coupling.

TABLE III

| Monosaccharide | GIPL-4 | GIPL-6 |
|----------------|--------|--------|
| Mannose        | 5.1    | 3.9    |
| Galactose      | 2.0    | 1.8    |
| Glucose        | 0.9    | 0.9    |
| Mannon-6-phosphate | 0.8    | 0.8    |
| Galactose-6-phosphate | 0.8    | 0.7    |
| Glucosamine   | 0.7    | 0.9    |
| N-acetyl      | 1.0    | 1.0    |

1 All analyses relative to Man = 1.0
2 The larger of these two coupling constants corresponds to the D-glucosamine (1H,2H)-coupling.

3 The results are expressed as molar ratio.

4 The molar ratio for this molar is 9.0:1.0.
5 The larger of these two coupling constants corresponds to the D-glucosamine (1H,2H)-coupling.

Mild acid hydrolysis of GIPL-4 and 6 liberated approximately 1% of Man per molar of GIPL. The kinetics of release, as determined by GC-MS analysis, were consistent with this monosaccharide being linked to the GPl by glycosidic linkages. Approximately 1% of Gal was also released from GIPL-4, with similar kinetics, suggesting that GIPL-4 was also substituted with Glc-1-P. The only monosaccharide to be released from the L. major LPG with the kinetics consistent with a glycosidic linkage was Glc. All other derivatives (approximately 0.6% of molar of Glc per molar of LPG) were released after alkaline hydrolysis. The total release of Man and Gal under these conditions was due to the hydrolysis of glycosidic linkages in the repeat units (data not shown).
Leishmania Lipophosphoglycan Biosynthesis

To identify the site of attachment of Man-1-P, Gal and the Ca-1-P,Glc on GPI-6, the truncated GPI-6 glycon was treated with 100 mM ethylmercaptan at 40°C for varying time periods, and the released monosaccharides were identified and quantified by GC-MS as described in "Experimental Procedures". The levels of GPI-6 and GPI-4 in each sample were determined by GC-MS quantitation of theeryl ether derivative of methyl 6-deoxy-4-azido-4-deoxyglucose 4-azidocyclodecyl-4-deoxyglucosyl-methyl glycoside after hydrolysis in 0.1 M HCl at 100°C for 16 h.

The site of attachment of Man-1-P, Gal and the Ca-1-P,Glc was confirmed by treatment of the precursor with 100 mM ethylmercaptan at 40°C for varying time periods. The released monosaccharides were identified and quantified by GC-MS as described in "Experimental Procedures". The levels of GPI-6 and GPI-4 in each sample were determined by GC-MS quantitation of theeryl ether derivative of methyl 6-deoxy-4-azido-4-deoxyglucose 4-azidocyclodecyl-4-deoxyglucosyl-methyl glycoside after hydrolysis in 0.1 M HCl at 100°C for 16 h.

Fig. 5. Substitution of GPI-6, GPI-4 and the LPG core with hexose-1-phosphate residues. GPI-4 (Panel A), GPI-6 (Panel B) and LPG (Panel C) were treated with 100 mM ethylmercaptan at 40°C for varying time periods, and the released monosaccharides were identified and quantified by GC-MS as described in "Experimental Procedures". The levels of GPI-6 and GPI-4 in each sample were determined by GC-MS quantitation of theeryl ether derivative of methyl 6-deoxy-4-azido-4-deoxyglucose 4-azidocyclodecyl-4-deoxyglucosyl-methyl glycoside after hydrolysis in 0.1 M HCl at 100°C for 16 h.

Fig. 6. HPLC analysis of the GPI-6 glycon. The glycon was prepared as described in "Experimental Procedures". The glycon was treated with 100 mM ethylmercaptan at 40°C for varying time periods, and the released monosaccharides were identified and quantified by GC-MS as described in "Experimental Procedures". The levels of GPI-6 and GPI-4 in each sample were determined by GC-MS quantitation of theeryl ether derivative of methyl 6-deoxy-4-azido-4-deoxyglucose 4-azidocyclodecyl-4-deoxyglucosyl-methyl glycoside after hydrolysis in 0.1 M HCl at 100°C for 16 h.

Fig. 7. HPLC analysis of the GPI-4 glycon. The glycon was prepared as described in "Experimental Procedures". The glycon was treated with 100 mM ethylmercaptan at 40°C for varying time periods, and the released monosaccharides were identified and quantified by GC-MS as described in "Experimental Procedures". The levels of GPI-6 and GPI-4 in each sample were determined by GC-MS quantitation of theeryl ether derivative of methyl 6-deoxy-4-azido-4-deoxyglucose 4-azidocyclodecyl-4-deoxyglucosyl-methyl glycoside after hydrolysis in 0.1 M HCl at 100°C for 16 h.

Fig. 8. RFLP analysis of the GPI-6 glycon. The glycon was prepared as described in "Experimental Procedures". The glycon was treated with 100 mM ethylmercaptan at 40°C for varying time periods, and the released monosaccharides were identified and quantified by GC-MS as described in "Experimental Procedures". The levels of GPI-6 and GPI-4 in each sample were determined by GC-MS quantitation of theeryl ether derivative of methyl 6-deoxy-4-azido-4-deoxyglucose 4-azidocyclodecyl-4-deoxyglucosyl-methyl glycoside after hydrolysis in 0.1 M HCl at 100°C for 16 h.

Fig. 9. RFLP analysis of the GPI-4 glycon. The glycon was prepared as described in "Experimental Procedures". The glycon was treated with 100 mM ethylmercaptan at 40°C for varying time periods, and the released monosaccharides were identified and quantified by GC-MS as described in "Experimental Procedures". The levels of GPI-6 and GPI-4 in each sample were determined by GC-MS quantitation of theeryl ether derivative of methyl 6-deoxy-4-azido-4-deoxyglucose 4-azidocyclodecyl-4-deoxyglucosyl-methyl glycoside after hydrolysis in 0.1 M HCl at 100°C for 16 h.

Fig. 10. RFLP analysis of the GPI-4 glycon. The glycon was prepared as described in "Experimental Procedures". The glycon was treated with 100 mM ethylmercaptan at 40°C for varying time periods, and the released monosaccharides were identified and quantified by GC-MS as described in "Experimental Procedures". The levels of GPI-6 and GPI-4 in each sample were determined by GC-MS quantitation of theeryl ether derivative of methyl 6-deoxy-4-azido-4-deoxyglucose 4-azidocyclodecyl-4-deoxyglucosyl-methyl glycoside after hydrolysis in 0.1 M HCl at 100°C for 16 h.