The Structure of *Leishmania major* Amastigote Lipophosphoglycan*

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Intracellular amastigotes of *Leishmania major* produce 6 × 10^5 copies/cell of a lipophosphoglycan (LPG) that is structurally distinct from the LPG produced by the extracellular promastigote form of *L. major*, *Leishmania donovani*, and *Leishmania mexicana* (reviewed by McConville, M. J. (1991) *Cell Biol. Int. Rep.*, 15, 779–798). *L. major* amastigote LPG is composed of a lysophosphatidylinositol lipid anchor that links via a diphasophorylated hexasaccharide core to a phosphoglycan (6–100 kDa). The structures of the anchor, the core, and the phosphoglycan were determined by monosaccharide and linkage analysis, fast atom bombardment-mass spectrometry, one-dimensional 1H NMR spectroscopy, and exoglycosidase microsequencing. The lipid anchor contains predominantly 1-O-alkylglycerols with 24:0 and 22:0 alkyl chains. The lipids are linked via a glycerol-β-myoinositol-P≤Po4 to a core glycan with the structure -PO4-6Galα(1-2)Galα(1-2) Galα(1-2)Glcα(1-PO4-)Manα(1-2)Manα(1-2)GlcNα(1-). The chromatographic characteristics of the core glycan suggest that the saccharide components are linked similarly in amastigote and promastigote LPG. The phosphoglycan attached to the core consists of -PO4-6Galα(1-4)Manα(1-2) repeats which are either unsubstituted (70%) or substituted (30%) at the 3-position of the Gal residues with oligosaccharide side chains containing primarily Gal and some Glc. Thirteen different types of side chains were identified with the structures [Galα(1-3)x], where x = 1–11, or Glc(1-3)Glc(1-3), or Glc(1-3)Galα(1-3), where glucose is probably in the β-configuration. All monosaccharides in the phosphoglycan domain are in the pyranose configuration. The average number of repeat units per molecule is 36. The nonreducing terminus of the phosphoglycan chains probably terminates predominantly in the neutral disaccharide Galα(1-4)Manα(1-). Comparison of the structure of *L. major* amastigote LPG to *L. major* promastigote procyclic and metacyclic LPG forms (McConville, M. J., Turco, S. J., Furguson, M. A. J., and Sacks, D. L. (1992) *Embo J.* 11, 3593–3600) indicates that this molecule is developmentally modified throughout the different stages of the parasites’ life cycle.

Protozoan parasites of the genus *Leishmania* annually afflict millions of people worldwide and induce a variety of disease forms. Leishmaniasis may develop as a cutaneous (due to injection with *L. major*, *L. tropica*, *L. mexicana*, *L. aethiopica*), mucocutaneous (*L. braziliensis*) and visceral (*L. donovani*, *L. infantum*) disease (Walton, 1989). *Leishmania* are dimorphic and survive as extracellular flagellated promastigotes in the alimentary tract of the sandfly vector and as obligate intracellular nonmotile amastigotes in the phagolysosome of the mammalian host macrophage. The glycoconjugates exposed at the cell surface of these parasites appear to determine their ability to survive in the harsh hydrolytic environments of the invertebrate and vertebrate hosts (reviewed by Turco and Descoteaux, 1992; McConville, 1991; Kweider et al., 1987). The dominant cell surface glycoconjugate of *Leishmania* promastigotes is a lipophosphoglycan (LPG)† (reviewed by McConville, 1991; Turco and Descoteaux, 1992). LPG is organized as a densely packed glycoalyx that covers a significant proportion, if not the entire promastigote surface, including the flagellum. In some *Leishmania* isolates LPG effectively masks exposure of other surface molecules, such as the major glycoprotein gp63 (Pimenta et al., 1991; Karp et al., 1991; Homans et al., 1992).

Promastigote LPG of *L. major* (McConville et al., 1990), *L. mexicana* (Ilg et al., 1992), and *L. donovani* (Orlandi and Turco, 1987; Turco et al., 1988; Thomas et al., 1992) is composed of four domains; LPG is anchored at the plasma membrane by a lys-1-O-alkyl phosphatidylinositol lipid moiety that is attached to a conserved di-phosphorylated hexasaccharide core, linked to a polymer of repeat units of -PO4-6[Galα(1-4)Manα(1-)], which are capped by a neutral oligosaccharide. Species-specific differences in LPG structure occur in the cap structures and the degree to which the disaccharide backbone is substituted with mono- or oligosaccharide side chains (reviewed by McConville, 1991). In addition, stage-specific differences in the size of the phosphoglycan chains and the relative abundance of different side chains were induced 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.

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Within host macrophages, since parasite variants lacking LPG to participate in macrophage binding and parasite infection by complement fragment C3bi apparently bound to LPG. The sandfly gut, which is an essential step for continued parasite development within the sandfly vector (Pimenta et al., 1991). The LPG trisaccharide repeat unit, \(-\text{PO}_4^-\)\(\text{Gal}(\alpha1-3)\text{Gal}(\beta1-4)\text{Man}\)\(_\alpha_1-\), capped with a-\text{arabinopyranose}. Masking of this epitope resulted in the release of metacyclic promastigotes from the midgut epithelium of the sandfly to establish a true infection. The LPG trisaccharide repeat unit, \(-\text{PO}_4^-\)\(\text{Gal}(\alpha1-3)\text{Gal}(\beta1-4)\text{Man}\)\(_\alpha_1-\), is the major structure involved in attachment of \(L.\) major procyclic promastigotes to the \text{Phlebotomus papatas}i gut epithelial cell microvilli (Pimenta et al., 1992). During metacyclogenesis, this epitope appears to be masked by an up-regulation of repeat units capped with a-\text{arabinopyranose}. Masking of this epitope results in the release of metacyclic promastigotes from the sandfly gut, which is an essential step for continued parasite transmission by the sandfly vector at a subsequent blood meal.

Receptor-mediated phagocytosis of promastigotes by macrophages appears to involve parasite binding to macrophage complement receptor 3 (CR3; Mosser et al., 1992), mediated by complement fragment C3bi apparently bound to LPG (Puentas et al., 1988), or possibly by LPG directly binding. The \(L.\) major LPG epitope, \(-\text{PO}_4^-\)\(\text{Gal}(\beta1-3)\text{Gal}(\beta1-4)\text{Man}\)\(_\alpha_1-\), has recently been identified to participate in macrophage binding and parasite infection (Kelleher et al., 1992). Exposure of LPG at the surface of \(L.\) major promastigotes has also been implicated in their survival within host macrophages, since parasite variants lacking LPG are avirulent (Handman et al., 1986; Elhay et al., 1990; McNeely and Turco, 1990).

The amastigote form of the parasite is responsible for disease manifestation in the vertebrate host. To gain an understanding of the mechanisms of survival utilized by the amastigote during its interaction with the macrophage, it is essential that functional molecules of the amastigote be identified. In contrast to \(L.\) major promastigote LPG, the structure and function of amastigote LPG has not been defined. Amastigote LPG is known to be antigenically distinct (Glaser et al., 1991) and have a molecular weight intermediate between procyclic and metacyclic promastigote LPG (Turco and Sacks, 1991). A preliminary compositional analysis of amastigote LPG found that the complete absence of \(\alpha\)-\text{arabinopyranose} is the most striking feature that distinguishes amastigote from promastigote LPG (Moody et al., 1991). In this paper we report the complete primary structure of \(L.\) major amastigote LPG and discuss the possible biological significance of the novel structural features of this molecule.

**EXPERIMENTAL PROCEDURES**

**Parasite**

The \(L.\) major isolate LRC-L137 was originally obtained from the World Health Organization Reference Center for Leishmaniasis, Jerusalem, Israel. The virulent cloned line V121 was selected by dilution cloning from this isolate (Handman et al., 1983) and maintained by passage in BALB/c mice. Amastigotes were harvested from 4-week-old lesions at the base of the tail. Before inoculation, amastigotes were stored at \(-70\) °C prior to LPG purification and analysis.

**Isolation of LPG**

LPG from the harvested amastigotes (in batches of \(5 \times 10^8\) cells) was purified by a modification on the procedure of McConville et al.
protein that co-purified with L. major amastigote LPG was suggested for purification. An alkylglycerol (with 24:0 alkyl chains) was added in 0.1 M ammonium acetate, pH 7, 16 h, 37 °C; enzyme/protein, 1:1, w/w, proteinase K (1 unit/ml aqueous, 37 °C, 3 h; Sambrook et al., 1989), Pronase digestion (Calbiochem, 0.1 M ammonium carbonate, 0.1 M calcium acetate, 16 h, 37 °C; 2%, w/w, solution), ion-exchange chromatography (sample was applied in 0.02 M ammonium acetate containing 5% 1-butanol onto a TSK-DEAE-650(s) Fractogel (Merck) column and eluted stepwise with ammonium acetate (0.05–0.32 M) containing 5% 1-butanol), and by hydrophobic interaction chromatography under denaturing conditions (sample was applied in 6 M urea onto an octyl-Sepharose washed with 6 M urea and eluted with a propanol gradient in 0.1 M ammonium acetate). Similarly, protein has been identified to co-purify with promastigote LPG prepared from L. major (McConville et al., 1990), L. donovani (Jardim et al., 1991; Russo et al., 1992), and L. mexicana (Ilg et al., 1992). Successful removal of protein has been reported for L. major (McConville et al., 1990) and L. mexicana (Ilg et al., 1992) by re-chromatographing over octyl-Sepharose and proteinase K digestion, respectively. In contrast, using similar approaches to those described in this study (Jardim et al., 1991), we were unsuccessful in purifying intact L. donovani promastigote LPG free of protein. Protein contamination was identified by (Jardim et al., 1991) at the levels similar to those reported in this study. The protein that co-purified with L. major amastigote LPG was suggested not to be a structural component of the LPG, because the LPG binding antibody WIC108.3 failed to immunoprecipitate radioactively labeled molecules with LPG from 32P-labeled amastigotes (modified (Handman et al., 1984); 106 purified amastigotes were incubated in 1 ml of methionine-free RPMI-Hepes buffer, pH 6, with 1% fetal calf serum, 37 °C. After 20 min, 500 μCi of 32P-methionine (specific activity, 800 Ci/mmol, Amersham Corp.) was added and incubated for 0, 1, 3, 5, or 7 h. Parasites were then washed four times in phosphate-buffered saline (mouse tonicity) with 2 mg/ml cold methionine and solubilized in 1 ml of 0.5% Triton X-100 in phosphate-buffered saline, containing a range of protease inhibitors (10 mM EDTA, 1 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide). Insoluble material was spun out at 27,000 × g and the supernatant used for immunoprecipitation with WIC108.3.

Although previously (Moody et al., 1991), we did not report a significant protein contamination in our amastigote LPG preparations, the presence of a protein contaminant was suggested by reactivity with the antibody L157 (Jardim et al., 1991). This antibody was initially proposed to react with LPG core but is now known to bind a polypeptide contaminant that co-purified with LPG (Jardim et al., 1991). Analysis of very small amounts of material in our preliminary analysis (Moody et al., 1991) resulted in the failure to quantify a protein contaminant.

Structural Characterization of Amastigote LPG

The strategy adopted for characterization of L. major amastigote LPG is outlined in Fig. 1. Amastigote LPG was either analyzed directly or after introduction of a tritium label.

LPG Depolymerization—Purified LPG was hydrolyzed at phosphodiester linkages with mild acid (40 mM trifluoroacetic acid, 8 min, 100 °C; McConville et al., 1990) to release a hexaglycosyl phosphatidylinositol (hexaglycosyl-PI) lipid anchor, phosphorylated saccharide repeats, and a neutral saccharide cap. Acid was removed from the samples under reduced pressure. Purification of Caps and Repeats from the Hexaglycosyl-PI Anchor—The lipidic hexaglycosyl-PI was separated from the neutral and phosphorylated saccharides by hydrophobic interaction chromatography over octyl-Sepharose (Thomas et al., 1992). FAB-MS analyses of LPG repeats and cap(s) were performed using both a phosphorylated mixture and dephosphorylated total mixture. Linkage analysis by methylation of amastigote LPG neutral cap(s) and oligosaccharide repeats was performed using a dephosphorylated total mixture. Samples were dephosphorylated using calf alkaline phosphatase (Boehringer Mannheim, 20 units/ml) in 0.1 M ammonium bicarbonate, pH 5.0 (16 h, 37 °C), boiled for 2 min to inactivate the enzyme, and deionized by passage through a tandem column of AG50X8(H+) over AG3X4(OH-−) prior to analysis.

![FIG. 2. Negative ion FAB-MS spectrum of native L. major amastigote LPG. Molecular ions were not observed as the LPG molecular species have masses (6–100 kDa) that are outside the operating range of the mass spectrometer. However, structurally informative fragment ions were generated by β-clavage to give a spectrum with the major signal corresponding to the di-phosphorylated hexasaccharide-Pl core (encompassing an alkylglycerol (species with 24:0 alkyl chains at m/z 1800 and 24:0 alkyl chains at m/z 1900) and MSn spectra of the 22:0 alkyl chains species at m/z 1772. A minor signal at m/z 1720 corresponding to the monophosphorylated 24:0 species was also observed. These data are consistent with the lipid anchor containing predominantly 24:0 alkyl chains (68%) with the next most abundant 22:0 alkyl chains (18%; Moody et al., 1991). Ions corresponding to the minor alkyl species 26:0 (m/z 1744) and 26:0 (m/z 1828) representing 8 and 6%, respectively, of the total alkyl chains (Moody et al., 1991) were not detected. The spectrum further confirms the presence of phosphate on Man-2 of the hexasaccharide core, with a prominent ion at m/z 1204 corresponding to the monophosphorylated disaccharide-Pl species presumably generated due to the highly labile nature of the adjacent hexofuranosyl residue.](image-url)
All samples were immediately neutralized with acetic acid, deionized, chromatographed under high salt conditions (program 1, detailed below).

The structures of co-eluting glycans derived from promastigote LPG core after the same treatment are shown.

Fractionation of Repeats — The neutral and phosphorylated saccharides were chromatographed using Dionex HPLC ion-exchange chromatography under high salt conditions (program 1, detailed below). All samples were immediately neutralized with acetic acid, deionized

by passage through AG50X8(H+ form) and lyophilized, prior to further analysis.

The structure of the LPG phosphorylated repeat units was elucidated using (i) enzymic microsequencing and (ii) linkage analysis by methylation.

(i) Phosphorylated saccharide repeat units were subjected to various combinations of exoglycosidase treatments either directly or following dephosphorylation using calf intestine alkaline phosphatase (Boehringer Mannheim), nitrous acid deaminated and tritium labeled upon reduction with NaB\['H\], and lyophilized, prior to further analysis.

(ii) Individual peaks collected from program 1 were subjected to linkage analysis by comparison with promastigote standards for identification.

NaB\['H\], Labeling of LPG — The hexaglycosyl-P1 released by mild acid from LPG was delipidated, using PI-specific phospholipase C from Bacillus thuringiensis (a kind gift of Dr. M. Low, Columbia University), nitrous acid deaminated and tritium labeled upon reduction with NaB\['H\], form [\(\text{PH}_{2}\)3,5-anhydromannitol, as described in McConville and Blackwell (1991). Enzymic and Chemical Microsequencing of the Core Glycan — Microsequencing of the core glycan was undertaken as described in McConville and Blackwell (1991). Phosphorylated deaminated \(\text{H})\] labeled glycans were fractionated by Dionex HPLC program 5, and identified by co-elution with L. major promastigote LPG core standards. The phosphorylated glycan core was collected and deionized over AG50X8(H+) and dephosphorylated repeats were deionized by passage through a tandem column of AG50X8(H+) over AG50X4(OH\(-\))

Phosphorylated repeats were chromatographed by Dionex HPLC using gradient program 1, and dephosphorylated repeats were chromatographed using gradient program 2. The retention times of the peaks were compared with promastigote standards for identification.

FIG. 3. Amastigote LPG was mild acid-depolymerized and the glycolipid anchor separated from the repeat unit and cap structures by octyl-Sepharose chromatography. The anchor was delipidated with PI-specific phospholipase C, then nitrous acid-deaminated and reduced with NaB\['H\]. The released radiolabeled glycan was analyzed after alkaline phosphatase treatment using Dionex HPLC program 2. The dephosphorylated glycan (Gal(α1-6)Gal(α1-3)Gal(β1-3)(Man(α1-3)Man(α1-4)AHM, 5.9 DU; A) was also analyzed at each step after the following sequential treatments: digestion with coffee bean α-galactosidase (CBG) (Gal(β1-3)(Man(α1-3)Man(α1-4)AHM, 6.1 DU; B), trifluoroacetic acid hydrolysis (40 mM trifluoroacetic acid, 60 min, 100 °C) (Man(α1-3)Man(α1-4)AHM, 2.0 DU; C), and jack bean α-mannosidase (JBoM) (AHM, 1.0 DU; D). The numbers at the top of the panel refer to the elution position of glucose oligomer internal standards. The structures of co-eluting glycans derived from promastigote LPG core after the same treatment are shown.

FIG. 4. Time course of glucose release from LPG by mild acid hydrolysis. L. major amastigote LPG was treated with 40 mM trifluoroacetic acid at 100 °C from 0 to 5 min. The released monosaccharides were analyzed directly as their TMS derivatives and identified and quantitated by GC-MS using selected ion monitoring. The amount of LPG in the starting material was determined assuming a single mole of inositol is released from a single mole of LPG. Low levels of Gal and Man were also detected (data not shown) after several minutes of hydrolysis as reported previously for L. major LPG (McConville and Homans, 1992).
mild acid hydrolysis release of monosaccharides from intact LPG (40 mM trifluoroacetic acid, 100 °C, 0–5 min; McConville and Homans, 1992). The released monosaccharides were directly trimethylsilylated and analyzed by gas chromatography-mass spectrometry (GC-MS).

Galactose Oxidase/NaB[\(\text{H}_4\)] Reduction of the Cap Structure

Introduction of a \(^1\text{H}\) label by Gal oxidase/NaB[\(\text{H}_4\)] reduction (McConville et al., 1990, 1992) provided information about the cap structure. This method introduces a \(^1\text{H}\) label into Gal residues that are not linked through C(0)6. The neutral saccharides capping the labeled LPG were subsequently fractionated by hydrolyzing with mild acid (40 mM trifluoroacetic acid, 8 min, 100 °C), deionizing over a column of AG50X8(H\(^+\)), and chromatographing using Dionex HPLC ion-exchange chromatography (program 2). Neutral saccharides were identified relative to promastigote standards. \(^1\text{H}\) labeling allowed discrimination between nonlabeled Gal-Man saccharides that may be generated by some dephosphorylation of repeat units (during trifluoroacetic acid hydrolysis) and authentic \(^1\text{H}\)-labeled cap structures.

NMR Analysis of the Anomeric Configuration of the Man Residue in the Repeat Backbone

NMR spectra were recorded at 500 MHz in D$_2$O, essentially as described previously (McConville et al., 1990).

**HPLC**

Saccharides generated by chemical or enzymic hydrolysis were chromatographed using a Dionex model BioLC Carbohydrate Analyzer equipped with a CarboPac PA-1 column and detected with a pulsed amperometric detector and Raytest Ramona radioactivity flow monitor. The column flow rate was 0.6 ml/min.

**Program 1**—Program 1 was used to separate phosphorylated oligosaccharides under high salt conditions. The column was equilibrated in 30% buffer A (150 mM NaOH) and 70% buffer B (150 mM NaOH, 250 mM NaOAc) and eluted with a linear gradient of NaOAc in 150 mM NaOH, from 175 mM (35% buffer C) to 250 mM (100% buffer B), over 60 min and held at 250 mM NaOAc for 10 min (modified McConville et al., 1990).

**Program 2**—Program 2 was used to separate dephosphorylated repeat unit oligosaccharides and neutral cap saccharides, under low salt conditions. The column was equilibrated in 96% buffer A and 4% buffer B and eluted with a linear gradient of NaOAc in 150 mM NaOH, from 12.5 mM (5% buffer B) to 72.5 mM (92% buffer B) over 80 min.

**Program 3**—Program 3 was used to separate under high salt conditions neutral and phosphorylated oligosaccharides generated from LPG by mild acid hydrolysis and NaB[\(\text{H}_4\)] reduction. The column was equilibrated in 65% buffer A and 35% buffer C (150 mM NaOH, 500 mM NaOAc) and eluted with two successive linear gradients of NaOAc in 150 mM NaOH, from 175 mM (35% buffer C) to 250 mM (100% buffer C) over 72 min and held isocratically at 500 mM NaOAc (100% buffer C) for 10 min (McConville and Blackwell, 1991).

**myo-Inositol and Glucose Analysis**

myo-Inositol was released by hydrolysis of LPG in 6 N HCl (110 °C, 16 h). Glc was released form the LPG core during the time course of hydrolysis (40 mM trifluoroacetic acid, 100 °C, 1–5 min; McConville and Homans, 1992). Acid released myo-inositol and Glc were derivatized to per-O-trimethylsilylated and measured by GC-MS using selected ion monitoring.

**Monosaccharide Determination**

Monosaccharides and alkylglycerols were released from LPG samples (30 μg) by solvolysis in methanolic HCl (1 M, 16 h, 80 °C), derivatized with pyridine/chloromethylsilane/N,O-bis(trimethylsilyl)acetamide (3:2:5, v/v) (Zinbo and Sherman 1970), and the Me$_3$Si derivatives of the 1-0-methyl glycosides in n-hexane were separated and quantified by GC-MS (McConville et al., 1987).

**Protein Determination**

Protein was quantitated using the BCA colorimetric assay (Pierce Chemical Co.).

**Gas Chromatography-Mass Spectrometry**

Linkage analysis by methylation of amastigote LPG neutral cap(s) and oligosaccharide repeats was performed using a dephosphorylated total mixture and individual fractions collected following Dionex HPLC (as described above). Oligosaccharides were methylated using the method of (Ciucanu and Kerek, 1984), with the modifications described by (McConville et al., 1990). Combined GC-MS was performed using a fully automated Finnigan MAT 1020B GC-MS (Sunnvale, CA), fitted with a WOOF fused silica 25 m × 0.3-mm CPSil-5 low polarity column (Chrompack, Middelburg, The Netherlands). Partially methylated alditol acetates were analyzed using the following temperature program; the oven was held for 2 min at 130 °C and then ramped to 220 °C at 3 °C/min and held at 220 °C for 5 min. GC-MS analyses were performed in the electron impact ionization mode using either the total ion current (reconstructed ion chromatogram) obtained by scanning from m/z 100–350 atomic mass units for 0.3 s,
Lipophosphglycan of Leishmania major amastigotes

Table 1

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Deduced glycosidic linkage</th>
<th>Total mix. cap + repeats</th>
<th>Molar ratios Purified dephosphorylated repeat units</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>Terminal 0.2</td>
<td>0.2</td>
<td>P3</td>
</tr>
<tr>
<td>B-Glc(p)</td>
<td>Terminal 0.2</td>
<td>0.2</td>
<td>P3</td>
</tr>
<tr>
<td>Man(p)</td>
<td>Terminal 1.0</td>
<td>1.0</td>
<td>P3</td>
</tr>
<tr>
<td>Gal(p)</td>
<td>Terminal 1.2</td>
<td>1.3</td>
<td>P5b + P4b</td>
</tr>
<tr>
<td>P4b</td>
<td>1.7</td>
<td>0.65</td>
<td>P5b + P4b</td>
</tr>
<tr>
<td>P4c</td>
<td>1.7</td>
<td>1.7</td>
<td>P5b + P4b</td>
</tr>
</tbody>
</table>

* For molar ratios, the value for 4-O-substituted Man is taken as 1.0.
* Methylation linkage analysis is consistent with coelution of the two structures: P6b, terminal-Gal3-Gal4-Man in the ratio 1:3:1 and P4d, terminal-Glc3-Glc3-Gal4-Man in the ratio 0.7:0.6:1.1.

or alternatively, using the selected ion mode and scanning for m/z 43, 101, 102, 113, 118, 129, 130, 131, 145, 161, 189, 201, 205, 233, and 234 atomic mass units with each ion scanned for 0.1 s.

TMS-derivatized monosaccharides and methyl-glycosides were analyzed using the same GC-MS and column as for methylation analysis, with the following temperature program; the oven was held at 110 °C for 2 min and then ramped to 320 °C at 6 °C/min and held at 320 °C for 10 min. Methanolyses were analyzed using GC-MS in the electron impact ionization mode using the total ion current (reconstructed ion chromatogram) obtained by scanning from m/z 70-650 atomic mass units for 0.5 s. TMS-derivatized Glc and myo-inositol were analyzed using GC-MS in the selected ion mode and scanning for m/z 191, 204, 305, and 318, with each ion scanned for 0.1 s.

Derivatives eluting from the GC were identified by their characteristic mass spectra and retention times relative to standard scylo-inositol hexacetate for methylation analyses and TMS-scyllo-inositol for monosaccharide, inositol, and Glc analyses.

**FAB-MS**

Liquid secondary ion mass spectrometry was performed on a Finnigan MAT 95 (Bremen, Germany) high resolution mass spectrometer operated at an accelerating voltage of +5 kV (positive ion mode) or -5 kV (negative ion mode) fitted with a cesium ion gun operated at 20 kV (positive ion mode) or 15 kV (negative ion mode) and 2 μA. Samples were dissolved in water (native) or methanol (permethylated), loaded into a matrix of thioglycerol, and scans (20 s/decade) acquired and averaged on the Finnigan MAT data system.

**RESULTS**

**Yield**—L. major amastigotes produced 1.25 μg of LPG (quantified with respect to carbohydrate)/10⁹ cells. This yield of LPG represents the harvest from a single mouse with two tail-base lesions. The abundance of L. major amastigote LPG was calculated to be 6 × 10⁴ copies/cell. This value was

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**Yield**—L. major amastigotes produced 1.25 μg of LPG (quantified with respect to carbohydrate)/10⁹ cells. This yield of LPG represents the harvest from a single mouse with two tail-base lesions. The abundance of L. major amastigote LPG was calculated to be 6 × 10⁴ copies/cell. This value was

TMS-derivatized Glc and myo-inositol were analyzed using GC-MS in the selected ion mode and scanning for m/z 191, 204, 305, and 318, with each ion scanned for 0.1 s.

Derivatives eluting from the GC were identified by their characteristic mass spectra and retention times relative to standard scylo-inositol hexacetate for methylation analyses and TMS-scyllo-inositol for monosaccharide, inositol, and Glc analyses.

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The developmental modification of LPG caps and repeat units from L. major procyclic through metacyclic promastigotes to amastigotes

<table>
<thead>
<tr>
<th>Structure (R)</th>
<th>Procyclic LPG</th>
<th>Metacyclic LPG</th>
<th>Amastigote LPG</th>
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<tbody>
<tr>
<td></td>
<td>mol %</td>
<td>mol %</td>
<td>mol %</td>
</tr>
<tr>
<td>(i) Caps</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gal(1→4)Man(α1-</td>
<td>8</td>
<td>6</td>
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<tr>
<td>Man(α1→2)Man(α1-</td>
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<td>20</td>
<td></td>
</tr>
<tr>
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<td>2</td>
<td></td>
</tr>
<tr>
<td>Ara(α1→2)Gal(1→3)Gal(1→4)Man(α1-</td>
<td>3</td>
<td>1</td>
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<tr>
<td>(ii) Repeat unit side branches</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>7</td>
<td>15</td>
<td>70</td>
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<tr>
<td>Ara(α1→2)Gal(1→3)-</td>
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<td>45</td>
<td></td>
</tr>
<tr>
<td>Ara(α1→2)Gal(1→3)Gal(1→3)-</td>
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<td>3</td>
<td></td>
</tr>
<tr>
<td>Glc(1→3)-</td>
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<td>7</td>
<td></td>
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<tr>
<td>Glc(1→3)Glc(1→3)-</td>
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<td>1</td>
<td></td>
</tr>
<tr>
<td>[Gal(1→3)]x</td>
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<td>x = 9</td>
<td></td>
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<td>x = 11</td>
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<tr>
<td>Average number of repeat units/molecule</td>
<td>14</td>
<td>30</td>
<td>36</td>
</tr>
</tbody>
</table>

^Low levels of this species can not be excluded based on current sequence analysis, but if present, enzymic microsequencing predicts levels less than 1%.
^Detected but not quantified. Based on pulsed amperometric detector response during enzymic microsequencing these species would be in the 1% range of the total number of repeats.
^Based upon FAB-MS analysis, detection by FAB-MS is not quantitative, but the intensity of the signal suggests that in aggregate they would not constitute more than 1% of the total.

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**Anchored Core**—Ions generated by negative ion FAB-MS of native LPG were diagnostic of a primarily di-phosphorylated core (Fig. 2) with a 24:0 alkyl chain in the lipid anchor. In addition, the amastigote 3H-labeled LPG core was identified to be predominantly di-phosphorylated by co-elution with L. major promastigote 3H-labeled di-phosphorylated LPG core standard, using Dionex HPLC (data not shown). Enzymic and chemical microsequencing of the dephosphorylated 3H-labeled LPG core (Fig. 3) is consistent with a hexasaccharide identical to the promastigote LPG cores of L. major, L. donovani and L. mexicana which have the structure: PO4-(6)Gal(c1-6)Gal(c1-3)Gal(c1-3)(Glcα1-PO4-6)Man(α1-3)Man(α1-4)GlcN. The presence of Glc linked via a phosphodiester to the Man-2 of the core was indicated by 1H NMR and by the kinetics of release of this residue over a time course of mild acid hydrolysis (Fig. 4).

**Repeats**—Ions generated by positive ion FAB-MS of a mixture of dephosphorylated oligosaccharide repeats and caps were diagnostic of a series of hexose oligosaccharides from 2 to 13 residues (Fig. 5 shows the spectrum of 2–11 hexoses; scanning beyond this region identified the 12 and 13 hexose oligosaccharides (data not shown).) The L. major amastigote repeat units released from the intact molecule by mild acid and separated by hydrophobic interaction chromatography from the hexasaccharide core-PI-anchor chromatographed as a series of phosphorylated oligosaccharides (Fig. 6A).

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**FIG. 7.** Anomeric (C-1) proton region of the 1H NMR spectrum of L. major amastigote LPG. The anemonic proton resonance of residues 1H1–3H1 are labeled according to the LPG core structure shown. The intense resonance marked "Man H1" corresponds to the repeating Man(α1-PO4- units. The resonance marked "Glc HI" identifies the Glc attached to the Man-2 of the core. Assignments were based on resonances determined for the core glycan of L. major promastigote LPG (McConville et al., 1990).
Amastigote LPG contained three major repeat units which accounted for approximately 90% of the phosphorylated glycans. These repeat units appear to contain the structures PO4-6)[Gal(1-3)]2Gal(1-4)Man (P3), and PO4-6)[Gal(1-3)]Gal(1-4)Man (P4b), based on their co-migration with the defined repeat units from promastigote LPG (McConville et al., 1990) on Dionex HPLC before and after alkaline phosphatase digestion (Fig. 6, Ai and Bi), 2) the susceptibility of putative P3 and P4b structures (and the corresponding dephosphorylated species, N3 and N4b) to BTβG (Fig. 6, Aii and Bi), and 3) methylation linkage analysis of the purified dephosphorylated glycans (Table I).

The Dionex HPLC profile contained a fourth peak, which co-eluted with the promastigote repeat unit, PO4-6)[Gal(1-3)]Gal(1-4)Man (P6b). The presence of this structure as well as a second structure (designated P4d) was suggested by methylation analysis (Table I) and the finding that only some of this peak was susceptible to BTβG digestion (Fig. 6 Aii). From the methylation linkage analysis it is likely that the second component contains 2 glucose residues (terminal and a 3-O-substituted Glc), a 3-O-substituted Gal and a 4-O-substituted Man (Table I). These residues probably all have a β-configuration as this species is completely digested by the combined action of BTβG and SAβG (Fig. 6 Aiii). Assuming this repeat unit has the same conserved disaccharide as the other repeat units, and the corresponding dephosphorylated species, N3 and N4b) to BTβG (Fig. 6, Aii and Bi), and 3) methylation linkage analysis of the purified dephosphorylated glycans (Table I).

A fifth peak, designated "•" also appeared to contain two structures. Based on elution characteristics on Dionex HPLC of promastigote repeat P4c,PO4-6)[Glc(1-3)]Gal(1-4)Man, and enzymic microsequencing (Fig. 6 Aii), we conclude this peak contains a mixture of P4c (resistant to BTβG (Fig. 6 Aii), but susceptible to SAβG + BTβG (Fig. 6 Aii) and P6b(PO4-6)[Gal(1-3)]Gal(1-4)Man (susceptible to BTβG).

Dionex HPLC revealed the presence of a spectrum of less abundant repeat units which were eluted over the entire NaOAc gradient. These species probably correspond to the range of phosphorylated repeat units detected by FAB-MS, which contain side chains of 5-13 hexose units. These longer side chains appear to contain exclusively Gal residues in (β1-3) linkage from the methylation analysis of the mixture and from the susceptibility of these peaks to BTβG digestion. Moreover, the product of the BTβG digestion was PO4-6)Gal(β1-4)Man (Fig. 6 Aii), indicating that in all these structures the phosphate residue is linked to the Gal residue of the conserved disaccharide repeat unit backbone.

The relative proportion of each of the repeat units to each other was calculated assuming a pulsed amperometric detection system response factor (RF) of 1.0 for N2, N3, N4b, N5b as described by (McConville et al., 1990), and an RF of 1.0 was assumed for the remainder of the series (Table II). Phosphate was identified by NMR to be linked Man(α1-P04, (Fig. 7). The amastigote LPG preparation was estimated to be composed of 36 repeat units from a calculation of the number of Man residues to inositol residues.

Caps—In addition to the phosphorylated oligosaccharides, low levels of a neutral oligosaccharide were also detected after depolymerization of LPG. This oligosaccharide co-migrated with authentic Gal(β1-4)Man on Dionex HPLC (2.0 DU, Fig. 8A) and was digested with BTβG (results not shown). It was also radiolabeled when intact LPG was treated sequentially with Gal oxidase and NaB[14H] (Fig. 8B). These results suggest that this disaccharide is at the nonreducing terminus of the phosphoglycan chains and that it did not result from dephosphorylation of the major repeat unit during mild acid depolymerization. Radiolabeled Gal was also detected (1.0 DU, Fig. 8). Previous analyses (McConville et al., 1992) have shown that similar levels of this monosaccharide are released from oligosaccharide side chains during the trifluoroacetic acid hydrolysis (representing approximately 1% hydrolysis), suggesting that it is not derived from a cap structure. The other labeled peaks (at 2.2, 2.3, and 6.0 DU) did not contain labeled Gal and were not further analyzed. Similarly, none of the other peaks in the pulsed amperometric detector profile contained detectable monosaccharides. These results suggest that the major neutral cap species at the nonreducing terminus of the phosphoglycan chains is the disaccharide Gal(β1-4)Man.

**DISCUSSION**

The complete primary structure of *L. major* amastigote LPG is the first reported for *Leishmania* amastigotes. *L. major* amastigote LPG, like promastigote LPG of all *Leishmania* species studied, is composed of a PI lipid anchor, a phosphorylated hexaascaride core, phosphorylated repeats, and a neutral cap (Fig. 9). The structures of the PI-lipid anchor (with minor lipid chain length variation; Moody et al., 1991) and the core appear to be essentially conserved between amastigote and promastigote *L. major* LPG. In contrast, there is clear developmental regulation of the repeat units and the cap of LPG throughout the parasite life cycle (Table II). Amastigotes and promastigotes of *L. major* share the same lyso-1-O-alkyl lipid glycerol anchor with only slight variation in both the length and the frequency of the component alkyl chains. Amastigote LPG is anchored by the alkyl chains 24:0, 22:0, 26:0, and 26:0 in the molar ratio 68:18:8:6 (Moody et al., 1991) and promastigote LPG with 24:0, 26:0, 22:0, 25:0, 23:0 in the molar ratio 74:19:3:3:1.5 (McConville et al., 1987). The 24:0 alkyl chains are the predominant lipid species throughout the parasite life cycle, a feature common to promastigote LPG from all *Leishmania* species.
FIG. 9. The complete primary structure of \textit{L. major} amastigote LPG. A PI-lipid moiety anchors a glucosylated phosphohexasaccharide core, which is linked to a series of phosphorylated oligosaccharides that are capped by a neutral disaccharide.

The glycan core-PI structure of LPG appears to be conserved between amastigote and promastigote LPG with variation only at the level of glucosylation. Like the \textit{L. major} amastigote core, the procyclic LPG core is fully substituted with a Glc(\alpha1-PO_4-6)-[R-3]-Gal(\beta1-4)Man(\alpha1- repeat units (Table II). The majority (70\%) of repeats are unsubstituted (R = H). The remaining repeats contain side chains which comprise exclusively galactopyranose and glucopyranose. Ara is completely absent, highlighting a major structural difference to \textit{L. major} promastigote LPG. Repeat side chains containing 1–3 Gal residues linked through a \beta1-3 linkage are common to \textit{L. major} amastigote and promastigote LPG. In contrast the side chains of 4–11 Gal residues linked through a \beta1-3 linkage are unique to \textit{L. major} amastigote and promastigote LPG. In contrast the side chains of 4–11 Gal residues linked through a \beta1-3 linkage are unique to \textit{L. major} amastigote and promastigote LPG. In contrast the side chains of 4–11 Gal residues linked through a \beta1-3 linkage are unique to amastigote LPG. The presence of a single Glc side chain at less than 1\% levels cannot be ruled out.

The structure and frequency of the LPG repeats is distinct between the three developmental forms (Table II). Most of the repeat units in procyclic LPG contain side chains that terminate in galactose. These repeat units are down-regulated during metacyclogenesis in favor of repeat units containing side chains with terminal \alpha-arabinopyranose. The largest repeat side chain identified for promastigotes is Ara(\alpha1-2)[Gal(\beta1-3)]_n (McConville et al., 1990). Promastigote and amastigote LPG share the repeat unit side chains containing 1–3 Gal residues, but longer side chains of 4–11 Gal residues are unique to amastigote LPG. The side chain containing Glc(\beta1-3)Gal(\beta1-3) is produced throughout the life cycle.

The molecular mass of amastigote LPG is heterogeneous ranging from 6 to 100 kDa (Moody et al., 1991). Amastigote LPG is intermediate in molecular mass between \textit{L. major} procyclic and metacyclic promastigote LPG (Turco and Sacks, 1991), which have an estimated 14 and 30 repeats, respectively (McConville et al., 1992; Table II). The number and the composition of the repeat units of these molecules is largely responsible for the molecular mass variation within and between these populations of molecules. From these studies it is apparent that considerable regulation of \textit{L. major} LPG repeat units accompanies transformation of the parasites during their maturation in the sandfly and in preparation for their intracellular life in the mammalian macrophage.

The degree of substitution and the complexity of the repeat side branches of \textit{L. major} LPG is a feature unique to the species characterized to date. \textit{L. donovani} promastigote LPG is not substituted with side branches, and only 30\% of \textit{L. mexicana} repeats are substituted with single Glc(\beta1-3)-. Although \textit{L. major} amastigote LPG shares with these structures a significant proportion of unsubstituted backbone repeats (and the presence of the Glc(\beta1-3)- repeat unit found in \textit{L. mexicana} cannot be ruled out), there are numerous species-specific and stage-specific epitopes not shared between these molecules. The biological significance of these species- and stage-specific epitopes is a subject of intense research in numerous laboratories, including our own.

\textit{L. major} amastigote LPG is predominantly capped at the nonreducing terminus by the disaccharide Gal(\beta1-4)Man(\alpha1-1). In contrast, this structure caps only a minor population of \textit{L. major} promastigote molecules (Table II). The dominant cap
of *L. major* promastigote LPG Man(α1-2)Man(α1- (McConville et al., 1990, 1992) was not detected in amastigote LPG. The cap structure of *L. major* amastigote LPG has not been detected in *L. mexicana* promastigote LPG (Ilg et al., 1992), and although present in *L. donovani*, it represents only 15% of the promastigote LPG cap structures in that species (Thomas et al., 1992). From these studies we conclude that the cap structures not only vary throughout the parasite life cycle but are also distinct in different species.

Amastigote LPG is expressed at much lower levels (6 × 10⁴ copies/cell) than promastigote LPG (5 × 10⁶ copies/cell; McConville and Bacic, 1989). However, these levels are still higher than those found in *L. donovani* amastigotes, where LPG was below detectable levels (McConville and Blackwell, 1985). LPG was also not detected in amastigote LPG (McConville and Bacic, 1989). However, these levels are still below 5% of the promastigote LPG cap structures in that species (McConville et al., 1990, 1992). From these studies we conclude that amastigotes during macrophage infection have yet to be identified. It is conceivable, however, that *L. major* amastigotes bind to macrophage receptors that are distinct from those engaged by promastigotes (as suggested to be the case for *L. donovani*; Blackwell, 1985), possibly using LPG epitopes that are unique to amastigotes or using other surface molecules.

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REFERENCES


S. F. Moody, E. Handman, M. J. McConville, and A. Bacic, unpublished data.