Structure of Leishmania lipophosphoglycan: inter- and intra-specific polymorphism in Old World species

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The most abundant surface macromolecule on the promastigote stage of leishmanial parasites is a polymorphic lipophosphoglycan (LPG). We have elucidated the structures of two new LPGs, from Leishmania tropica (LRC-L36) and L. aethiopica (LRC-L495), and investigated the nature of intra-specific polymorphism in the previously characterized LPG of L. major (LRC-L456 and -L580). These molecules contain a phosphoglycan chain, made up of repeating PO₄-6Gal/J1-4Man units and a conserved hexaglycosyl-phosphatidylinositol membrane anchor. Extensive polymorphism occurs in the extent to which the LPG repeat units are substituted with different glycan side chains. The L. tropica LPG is the most complex LPG characterized to date, as most of the repeat units are substituted with more than 19 different glycan side chains. All of these side chains, including the novel major glycans, Arapβ1-3Glcβ1- and ± Arapβ1-2Glcβ1-4± Arapβ1-2]Glcβ1-, are linked to the C-3 position of the backbone disaccharide galactose. In contrast, the L. aethiopica LPG repeat units are partially substituted (35%) with single α-mannose residues that are linked, unusually, to the C-2 position of the mannose in the backbone disaccharide. Polymorphism is also evident in the spectrum of α-mannose-containing oligosaccharides that cap the non-reducing terminus of the phosphoglycan chains of these LPGs. Finally, analysis of the L. major LPGs showed that, while some strains contain LPGs which are highly substituted with side chains of βGal, Galβ1-3Galβ1- and Arapβ1-2Galβ1-3Galβ1-, the LPGs of other strains (i.e. L. major LRC-L456) are essentially unsubstituted. Recent studies have shown that the LPG side chains and cap structures can mediate promastigote attachment to a number of different receptors along the midgut of the sandfly vector. The possible significance of LPG polymorphism on the ability of these parasites to infect a number of different sandfly vectors is discussed.

INTRODUCTION

Leishmania spp. are digenetic protozoal parasites that alternate between a promastigote stage in the sandfly vector and an obligate intracellular amastigote stage that resides in phagolysosomes of mammalian macrophages. The most abundant macromolecule on the surface of the promastigote stage is a complex lipophosphoglycan (LPG) which forms a continuous coat over the parasite plasma membrane (reviewed in [1,2]). Many studies have focused on the role of LPG in facilitating the survival of promastigotes in the mammalian host. In this regard it is thought to protect promastigotes from complement-mediated lysis and also facilitate their attachment to, and survival within, the host macrophage [1,2]. However, there is accumulating evidence that LPG is also required for parasite development within the sandfly vector. LPG-deficient strains of Leishmania are unable to establish an infection in sandflies [3,4], and it is thought that one function of LPG may be to protect the plasma membrane and surface proteins from hydrolyses in the digestive tract and complement components taken up with the bloodmeal [5–7]. Recent studies suggest that LPG may also mediate the binding of rapidly dividing procyclic promastigotes to epithelial cells along the sandfly midgut [4,8,9]. Promastigote adherence to the midgut wall appears to be essential for successful infection, as unattached parasites are apparently lost during excretion of the digested bloodmeal early in the infection.

It has been known for some time that the LPGs are highly polymorphic molecules, as shown by their reactivity to species- and strain-specific antibodies [10–12]. Antibodies directed against extracellular factors (EFs) in spent culture medium, which is now known to contain shed surface LPG and a number of LPG-related molecules as major components (reviewed in [13]), provided an early method for serotyping different species and strains of Leishmania (reviewed in [14]). Inter-specific polymorphism in LPG structure has been confirmed by studies on the structure of LPG from L. major, L. mexicana and L. donovani [15–17]. These LPGs have a conserved backbone which comprises a linear phosphoglycan chain made up of PO₄-6Gal/J1-4Man repeat units that is linked to a glycosyl-phosphatidylinositol (GPI) membrane anchor. Interspecific differences in LPG structure occur in the extent to which the disaccharide repeat units are substituted with saccharide side chains and the nature of these side chains. While the phosphoglycan chain of L. donovani LPG is essentially unsubstituted, approx. 30 % of the repeat units in L. mexicana, and nearly all the repeat units in L. major, are substituted with glucose or a complex array of galactose- and arabinose-containing side chains respectively [15–17]. In all these LPGs, the side chains are linked exclusively to the C-3 position of the galactose in the disaccharide backbone. The phosphoglycan chains of the LPGs are generally capped with a range of small mannose-containing oligosaccharides, which also vary quantitatively and qualitatively in different species [15–17].

There is evidence that inter-specific LPG polymorphism reflects, at least in part, the presence of different lectin-like receptors in the midguts of different Phlebotominae sandfly vectors which recognize epitopes on the LPG coat and mediate procyclic

Abbreviations used are: EF, excreted factor; LPG, lipophosphoglycan; GPI, glycosyl-phosphatidylinositol; HPAEC, high performance anion-exchange chromatography; HPAGE-PAD, HPAGEC with pulsed amperometric detection; HPTLC, high-performance TLC; PI, phosphatidylinositol; TMS, trimethylsilyl.

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binding to the midgut wall. For example, binding of *L. major* procyclic promastigotes to the midgut of its natural vector, *Phlebotomus papatasii*, is mediated by a receptor(s) that recognizes the clustered βGal-terminating side chains that predominate on the procyclic LPG of this species [8]. These side chains appear to be essential for infection of *P. papatasii* as other species of *Leishmania* which lack these side chains on their LPGs (i.e. *L. donovani*) are unable to establish an infection in this sandfly [4,18]. In contrast, *L. donovani* promastigotes bind to the midgut of its normal vector, *P. argentipes*, via a different class of receptors that probably recognize the terminal cap structures of LPG [9]. Similar cap structures are present on the three LPGs that have been characterized to date, consistent with the finding that this sandfly is a permissive vector for a number of different *Leishmania* spp. [8]. Information on the structure of the LPGs from other species should provide further insights into the relationship between LPG polymorphism and vectorial competence and the nature of the midgut receptors in other Phlebotominae sandflies.

In the present study we have elucidated the structures of the LPGs from *L. tropica*, *L. aethiopica* and two new strains of *L. major* to determine the extent of inter- and intra-specific LPG polymorphism. These strains have overlapping geographic distributions, but belong to different EF serotypes and are transmitted by a number of different sandfly vectors. These analyses show that the LPG side chains may be considerably more complex than previously thought and also that they may be attached to both the Gal and Man residues in the disaccharide repeat units. The elucidation of these structures defines potential epitopes that are recognized by the serotyping antibodies and provides a basis for understanding the tropism these *Leishmania* species display for particular sandfly vectors.

**MATERIALS AND METHODS**

**Materials**

NaB³H₄ (5–15 Ci/mmol) and Enhance spray were obtained from New England Nuclear (Little Chalfont, Bucks., U.K.), alkaline phosphatase from Sigma and sweet-almond β-glucosidase, coffee-bean α-galactosidase and jack bean α-mannosidase from Boehringer-Mannheim (Mannheim, Germany). Phosphatidylinositol (PI)-specific phospholipase C from *Bacillus thuringiensis* was a gift from Dr. M. G. Low (Columbia University, NY, U.S.A.). Other reagents were of analytical grade.

**Parasites**

The source and EF serotype of the parasite strains were as follows; *L. major* LRC-L545 (MTAT/KE/??/NLB-089), serotype B2, was isolated from the spleen of a gerbil, *Tatera robusta*, caught in Kenya; *L. major* LRC-L380 (MHOM/IL/85/P241), serotype A1B2, was isolated from a patient with cutaneous leishmaniasis acquired in Israel; *L. tropica* LRC-L36 (MHOM/IO/66/L75), serotype A2, from a patient with cutaneous leishmaniasis acquired in Iraq; and *L. aethiopica* LRC-L495 (MHOM/ET/85/T-VA5), serotype B1, from a patient with cutaneous leishmaniasis acquired in Ethiopia. Promastigotes were grown in Schneider's *Drosophila* medium containing 10% fetal-calf serum and harvested at the beginning of the stationary phase of growth.

**Extraction and purification of LPG**

LPG was extracted from delipidated promastigotes (1 × 10⁶) in 9% butan-1-ol as described previously [15]. The extracted LPG was purified on a column of octyl-Sepharose (10 cm × 0.5 cm) pre-equilibrated in 0.1 M sodium acetate, pH 7.0, containing 5% propan-1-ol. The extract was loaded onto the column at a flow rate of 5 ml/h and the column was eluted with 0.1 M sodium acetate (pH 7.0) at 5% propan-1-ol (20 ml) followed by a gradient of propan-1-ol (5–50%) in water (60 ml). The elution of carbohydrate was monitored by spotting 1 µl aliquots of each fraction onto a high-performance TLC (HPTLC) sheet and staining with orcinol/H₂SO₄. All the LPGs were eluted as a sharp peak at 29% propan-1-ol in this gradient. Fractions containing LPG were pooled and freeze-dried to remove volatile salts.

**Preparation of phosphorylated repeat units, cap oligosaccharides and GPI anchor from depolymerized LPG**

The purified LPG was processed as shown in Scheme 1. Hydrolysis in 40 mM trifluoroacetic acid (100°C, 10 min), which quantitatively cleaves hexose-1-PO₄ linkages, but not the inositol-1-PO₄ linkage, was used to depolymerize the LPG [15]. The GPI anchor was separated from the oligosaccharide repeat units and caps by chromatography on a column of octyl-Sepharose (5 ml), pre-equilibrated and loaded as described above, and then eluted with 40% propan-1-ol (5 ml). The bound and unbound fractions were freeze-dried to remove volatile salts and the unbound fraction further fractionated on a Dionex HPLC apparatus equipped with pulsed amperometric detector (HPAE-PAD) and a CarboPac PA1 column (4 mm × 250 mm). The following conditions were used to obtain the neutral cap structures (which were eluted in the unbound fraction) and partially fractionate the phosphorylated repeat units. The column was eluted at 0.6 ml/min with 60% buffer A (0.15 M NaOH) and 40% buffer B (0.15 M NaOH/0.25 M sodium acetate) for 1 min after sample injection, followed by a linear gradient to 100% buffer B over 40 min, which was maintained for 20 min (gradient program a). Peaks detected by PAD were desalted by passage down a column (0.5 ml) of AG 50 X12 (H⁺) and evaporation in the presence of toluene. Prior to further purification, fractions containing the phosphorylated repeat units were reduced with either NaB³H₄ (5 mCi, 30 mM) or NaB²H₄ (1 mg/ml) in 0.1 M NaOH (2 h,
26 °C). Reduction was stopped by the addition of acetic acid, and samples were passed down a column (400 μl) of AG50 X12 (H+), evaporated to dryness and washed four times with methanol to remove borate ions. Radiolabeled phosphorylated glycans were purified from radiochemical impurities by descending paper chromatography on Whatman no. 3 paper developed in butanol-1-ol/ethanol/water (8:2:1, by vol.) over 48 h and eluted from the origin with water. Both radiolabelled and unlabelled reduced glycans were depolymerized with alkaline phosphatase in 0.1 M NH4HCO3 (37 °C, 4 h), and desalted by passage down a column of AG50 X12 (H+) over AG3 X4 (OH-). The neutral glycans were analysed on the CarboPac column, which was eluted at 0.6 ml/min with 95 % buffer A/5 % buffer B for 1 min after injection, followed by a linear gradient to 28 % buffer A/2 % buffer B over 60 min, then a second gradient to 100 % buffer B over 20 min (gradient program b). Radioactivity in fractions was measured by liquid-scintillation counting. Unlabelled dextran oligomer internal standards were detected by pulse amperometric detection, and the relative retention times of labelled glycans calculated by linear interpolation of their elution position between adjacent unlabelled dextran oligomer standards and expressed as Dionex Units (Du). Samples were desalted by passage down a column of AG50 X12 (H+) over AG3 X4 (OH-).

The purified GPI anchor was treated with PI-specific phospholipase C [30 min in 20 mM Tris/acetate (pH 7.5)/0.1 % Triton X-100 for 2 h at 37 °C] and the delipidated glycan deaminated by incubation in 0.5 M NaNO2 in 0.4 M sodium acetate, pH 4 (20 μl, 50 °C, 3 h). The reaction mixture was adjusted to pH 9–10 with 5 M NaOH and immediately reduced with NaB3H4 (5 μCi, 30 mM in 0.1 M NaOH, 10 μl, 2 h, room temperature). The reaction was stopped, and radiolabelled glycans purified from radiochemical contaminants as described above. After alkaline phosphatase treatment, labelled glycans were desalted by passage down a column of AG50 X12 (H+) over AG3 X4 (OH-) before analysis by HPAEC.

Compositional and methylation analyses
Neutral monosaccharide and alklyglycerols were analysed by GC–MS as their trimethylsilyl (TMS) derivatives, following solvolysis in methanolic 0.5 M HCl (4 h, 80 °C) and derivatization in pyridine/trichloromethylsilane/hexamethyldisilazane (10:3:1, by vol) as previously described [15,19]. In order to detect phosphorylated monosaccharides, TMS-derivatized samples were methylated with diazomethane and then re-TMS-derivatized prior to GC–MS analysis [20]. Neutral glycan fractions were methylated using the method of Cluican and Kerek [21] with the modifications as previously described [15] and the partially methylated alditol acetates analysed by GC–MS [15].

Enzymic and chemical treatments
Neutral glycans were treated with jack-bean α-mannosidase in 0.1 M sodium acetate buffer, pH 5.0 (30 μl, 25 units/ml, 16 h, 37 °C), coffee-bean α-galactosidase in 0.1 M sodium phosphate/citrate buffer, pH 6.0 (30 μl, 25 units/ml, 16 h, 37 °C), or sweet-almond β-glucosidase in 0.1 M citrate buffer, pH 4.5 (30 μl, 20 units/ml, 16 h, 37 °C). Alkaline phosphatase treatment was performed in 20 μl of 10 mM NH4HCO3 with 3 units of enzyme for 16 h at 37 °C. When successive enzyme treatments were performed on the same sample, the previous enzyme was inactivated by heating for 5 min at 100 °C and the appropriate pH for the next enzyme obtained by addition of 30 μl of the next buffer. Mild acid hydrolysis (40 mM trifluoroacetic acid, 1 h, 100 °C) and strong acid hydrolysis (2 M trifluoroacetic acid, 2 h, 100 °C) were used to cleave preferentially arabinopyranosidic linkages or all glycosidic linkages respectively [15].

HPTLC
The dephosphorylated 3H-labelled glycans derived from the repeat units were analysed by silica-gel 60 HPTLC. The HPTLC process was developed twice in propan-1-ol/acetone/water (9:6:5, by vol. for the first and 5:4:1, by vol. for the second development, solvent system C) [22]. Radioactivity was detected by fluorography after spraying with En3Hance spray. The migration positions of the labelled glycans are given relative to a series of NaBH4 reduced dextran polymers (TLC units or Tu).

NMR spectroscopy
Intact L. tropica LPG was exchanged with 2H2O (99.96 %3H, Aldrich) by repeated evaporation. One and two-dimensional spectra were acquired on a Bruker AM-500 spectrometer as previously described [16].

RESULTS
The LPGs were purified from L. tropica (LRC-L36), L. aethiopica (LRC-L495) and two strains of L. major (LRC-L456 and -L580), using a previously established protocol. The yields of LPG from each strain were similar (approx. 1.5 mg of LPG from 109 cells). The LPGs were depolymerized by very mild acid hydrolysis and the various domains fractionated as described in Scheme 1. This procedure allowed the purification of the phosphorylated oligosaccharide repeat units, the neutral capping oligosaccharides and GPI anchor of each LPG. The results of the characterization of these components is described below.

Phosphorylated repeat units of L. tropica LPG
The fraction containing the phosphorylated repeat and neutral cap oligosaccharides of L. tropica LPG was analysed by HPAE-PAD using gradient program a. This chromatography revealed the presence of at least ten major phosphorylated oligosaccharide peaks which were pooled into eight fractions (P1–P8; Figure 1a) and an unbound fraction (N), which contained the neutral cap oligosaccharides (described below). Each of the fractions containing the phosphorylated oligosaccharides were reduced (with either NaBH4 or NaB3H4) and dephosphorylated with alkaline phosphatase, then rechromatographed on the CarboPac column using gradient program b (Figure 2). This procedure resolved 15 glycans species, most of which appeared to be pure when analysed by HPTLC with several different solvent systems (Figure 3a). However, two of these fractions (1b and 6c) contained a minor contaminating glycan species which were resolved from the major species by HPTLC (these minor species are denoted by an asterisk; i.e., see Figure 3c) to give a total of 17 glycans species. The structures of the dephosphorylated glycans (containing approx. 80 % NaBH4- and 20 % NaB3H4-reduced material) were deduced by GC–MS compositional analysis, HPTLC analysis of the products of exoglycosidase and mild acid hydrolysis treatments, and where sufficient material was available, by methylation analysis. These analyses indicated that all the dephosphorylated repeat units of L. tropica LPG contained the sequence R-3Galβ1-4Man and that they could be grouped into six classes based on the structure of R.

The smallest repeat unit was the minor species, P4, which co-migrated exactly with authentic PO3-6Galβ1-4Man on HPAE-PAD and, after dephosphorylation and reduction, co-migrated
The purified LPG was acid-depolymerized and the non-lipidic fraction (containing cap and repeat unit structures) chromatographed by HPAE-PAD using gradient program A (a). Fractions containing the marked peaks were pooled for further analysis. The unbound fraction (N) contained the neutral oligosaccharides (cap structures) which were subsequently resolved by HPAE-PAD using gradient program B (b).

The second class of dephosphorylated repeat units comprised 6a, 2a and 5a. These glycans were indistinguishable on both HPAE and HPTLC from the dephosphorylated repeat units, Galβ1-3Galβ1-4Man, Araβ1-2Galβ1-3Galβ1-4Man and Araβ1-2Galβ1-3Galβ1-3Galβ1-4Man, respectively, derived from *L. major* LPG [15]. The structures of these glycans were confirmed by methylation analysis (Table 1), and HPTLC analysis of the products of mild acid hydrolysis (to cleave arabinopyranosidic linkages) and β-galactosidase treatments (Figure 4).

The third series of dephosphorylated repeat units comprised 7a and the main repeat unit, 3a. The glycan 7a had the same HPAE retention time as the dephosphorylated repeat unit, Glcβ1-3Galβ1-4Man, derived from *L. mexicana* LPG [16], consistent with the methylation analysis (Table 1), and its susceptibility to sequential digestion with sweet-almond β-glucosidase, followed by β-galactosidase digestion to give mannitol (Figure 4). In contrast, the glycan 3a was not eluted with any of the previously characterized LPG repeat units. Methylation analysis suggested that 3a comprised a linear tetrasaccharide with a terminal...
arabinopyranose residue, a 2-substituted glucose, a 3-substituted galactose and a 4-substituted mannose (detected as 4-substituted mannitol in the reduced glycan) (Table 1). The terminal arabinose could be released by mild acid hydrolysis to give a product that co-migrated with Glc/β1-3Gal/β1-4mannitol on HPTLC. This product was susceptible to sweet-almond β-glucosidase, but not to bovine testicular β-galactosidase, and could be converted into mannitol after digestion with both alcohol β-glucosidase and β-galactosidase (Figure 4). These data suggest that the repeat unit 3a has the structure: Arap1-2Glc/β1-3Gal/β1-4Man.

The fourth series comprised the abundant repeat units, 8, 3b and 2b, and the minor species 7b. None of these glycans were co-eluted with previously characterized leishmanial LPG repeat units on HPAE (Figure 2). Methylation analysis indicated that fraction 8 contained a linear tetrasaccharide with a terminal glucose, a 4-substituted galactose, a 3-substituted mannose and a 4-substituted mannose at the reducing terminus. Digestion of the labelled glycan with β-glucosidase generated a product which co-migrated with Gal/β1-4mannitol on HPTLC, suggesting that the terminal and penultimate sugars were β-Glc (Figure 3b, lanes 1–4; Figure 4). These data suggest that the dephosphorylated repeat unit 8 has the structure Glc/β1-4Glc/β1-3Gal/β1-4Man. Fractions 3b and 2b contained the same monosaccharide composition as fraction 8, with an additional one or two arabinose residues respectively (results not shown). In both cases, mild acid hydrolysis of the labelled glycans generated a product that had the same HPTLC mobility as glycan 8, which could be converted into mannitol after β-glucosidase and β-galactosidase digestion (Figure 3b, lanes 5–11 and lanes 12–18; Figure 4). Methylation analysis suggested that the arabinose branch in the 3b glycan was located on the subterminal Glc residue (Table 1). After mild acid hydrolysis to remove this arabinose residue and a second methylation analysis, the 2,4-di-substituted Glc was replaced by a 4-substituted Glc (results not shown), confirming that the
Table 1  Methylation analysis of purified, dephosphorylated repeat units of *L. tropica* LPG

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*The dephosphorylated repeat units of *L. tropica* LPG were reduced with NaB\(_3\)H\(_4\) prior to methylation analysis, whereas the dephosphorylated repeat units of *L. aethiopica* LPG were analysed without prior reduction; the *L. aethiopica* LPG fractions 2 and 2* are the results of the methylation analysis of the dephosphorylated repeat unit P2 before and after jack-bean a-mannosidase digestion respectively.*

†The PMMA derivatives were detected from the extracted ion profile of these low-abundance species and were not quantified.

‡The PMMA of 4-substituted Glc was found in all samples, including the blank sample, and was not quantified, except for fraction 8, where it was present in stoichiometric amounts relative to the PMMA of 3-substituted Gal in three different preparations of this glycan. The presence of this residue was also suggested by the sequence analysis (Figures 3b and 4).

The permethylated alditol acetates (PMMAS) were identified and quantified by GC–MS.

subterminal Glc was substituted with a Glc residue on the C-4 position and an arabinose residue on the C-2 position. These data suggest that the 3b glycan has the structure Glcβ1-4[Arapβ1-2]Glcβ1-3Galβ1-4Man. Unexpectedly, this glycan was found to be resistant to β-glucosidase (Figure 3b, lanes 5 and 7), suggesting that this enzyme is unable to cleave terminal β-Glc residues when the glycerone residue is branched. Methylation analysis of the 2b glycan, before (Table 1) and after mild acid hydrolysis (results not shown) indicated that both arabinose residues were attached to the two glucose residues in this core in 1-2 linkage. Taken together with the sequence data, these results suggest that the 2b glycan has the structure: Arapβ1-2Glcβ1-4[Arapβ1-2]Glcβ1-3Galβ1-4Man. A minor glycan species, 7b, which also appears to belong to this series, was partially characterized. From its monosaccharide composition (results not shown) and HPTLC mobility (Figure 3b, lanes 15–21), it appeared to correspond to an isomer of 3b in which the arabinose residue was linked to the terminal rather than the subterminal Glc residue.

The fifth series was represented by the 1a glycan, which contained both terminal galactose and arabinose residues as well as a 2,4-di-substituted glucose residue (Table 1). It was susceptible to both mild acid hydrolysis and β-galactosidase digestion, with the latter treatment generating a product that co-migrated on HPTLC with the previously characterized 3a glycan (Figure 4). These data suggest that 1a has the structure: Galβ1-4[Arapβ1-2]Glcβ1-3Galβ1-4Man. The minor repeat unit, 6b, appeared to correspond to 1a without the arabinose branch. This species co-migrated with the de-arabinosylated 1a glycan on HPTLC. After β-galactosidase treatment it co-migrated with authentic Glcβ1-3Galβ1-4Man on HPTLC (Figure 4), suggesting that it had the structure: Galβ1-4Glcβ1-3Galβ1-4Man.

The final class of repeat units was represented by the 1b glycan (Figure 2a). Compositional and methylation analyses indicated that this glycan had two terminal arabinose residues, two 2-substituted Glc residues, a 2,4-di-substituted glucose, as well as the core galactose and mannose residues (Table 1). After mild acid hydrolysis there was a significant increase in the HPTLC mobility of this glycan consistent with the loss of two arabinose residues (Figure 3c, lanes 10 and 11). An aliquot of the acid-treated glycan was subjected to a second methylation analysis. Removal of the arabinose residues was confirmed by the absence of the 2,4-di-substituted Glc and the appearance of a terminal Glc in this analysis (results not shown). However, at least one of the 2-substituted Glc residues remained after removal of the arabinose residues, indicating that it was substituted with another Glc residue. Unexpectedly, the acid-treated glycan was still resistant to β-glucosidase digestion (Figure 3c, lane 13; Figure 4), suggesting either that the terminal Glc in this mild-acid-resistant core was in the α-configuration or that β1-2-linked glucose is cleaved very poorly relative to 1-4-linked Glc by the β-gluco- side. This feature has not been resolved, although attempts to cleave this residue with yeast α-glucosidase, which cleaves terminal α1-2-linked Glc, were unsuccessful, supporting the β-configuration for this Glc. These data suggest that the 1b glycan has the structure: Arapβ1-2Glcβ1-4[Arapβ1-2]Glcβ1-3Galβ1-4Man. The glycans 5b and 6b appeared to have the same glycan backbone as 1b, but contained less arabinose according to the monosaccharide analysis (approx. 1 mol/mol of mannitol). After mild acid hydrolysis they both co-migrated with the de-arabinosylated core of 1b on HPTLC, and, like the 1b core, were resistant to further glycosidase digestion (Figure 3c, lanes 4 and 11; Figure 4). Methylation analysis suggested that the arabinose in 5b was linked to the Glc residue proximal to the disaccharide core, as this species contained both a terminal and a 2,4-di-substituted Glc residue (Table 1). These data suggest that the arabinose in 6c is linked to either the terminal or penultimate Glc.
Polymorphism in *Leishmania* lipophosphoglycan

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**Figure 4** Summary of the properties of the oligosaccharide repeat units from *L. tropica* LPG

The elution time on HPAEC (Du) and migration position on HPTLC (Tu) of the dephosphorylated, 3H-labelled repeat units from *L. tropica* LPG are summarized, together with the results of the exoglycosidase and acid treatments of these glycans. These glycans were treated with β-glucosidase (βGlc), β-galactosidase (βGal), trifluoroacetic acid hydrolysis (40 mM, 60 min, 100 °C (TFA) or 2 M, 2 h, 100 °C (2M TFA)). Resistance (R) or susceptibility (S) to these treatments was determined by an increase in the HPTLC mobility of each species. Co-migration of these products with other glycans or mannitol is indicated in parentheses or by the mannitol symbol. The proposed structures are based on these data, and methylation analyses. Numbers in these structures indicate the linkage type; key to symbols: ■ mannitol; △ galactose; △ arabinose; □ glucose; ○ mannose. --, not tested. Two of the HPAEC-purified glycans (1b* and 6c*) contained minor contaminating glycans 1b* and 6c* respectively.

Residue, although insufficient material was available for methylation analysis.

Fractions 1b and 6c each contained a minor contaminating species (1b* and 6c* respectively) which was detected by HPTLC analysis (Figure 3c, lane 8). Information on the structures of these glycans was deduced from their susceptibility to the exoglycosidase and acid treatments. 1b* and 6c* appeared to contain a common core, as mild trifluoroacetic acid hydrolysis
generated a unique product with the same HPTLC mobility. They were also susceptible to β-galactosidase digestion, which generated a product that co-migrated with the previously defined glycans, Ara-Glc-[Ara-]Glc-Gal-mannitol in the case of \(1^β\) and Ara-Glc-Glc-Gal-mannitol in the case of \(6^ε\) (Figure 4). Combined treatment with trifluoroacetic acid followed by β-galactosidase generated a product that co-migrated with Glc-Glc-Gal-mannitol in each case (Figure 4). These data provide evidence that \(1^β\) and \(6^ε\) have the common core, Gal-Glc-Glc-Gal-Mann, with arabinose residues on one \(6^ε\) or both \(1^β\) of the Glc residues (Figure 4).

GC–MS analysis of the phosphorylated oligosaccharide fractions P1–8 revealed that they all contained approximately stoichiometric amounts of galactose 6-phosphate relative to mannose (results not shown). This result unequivocally locates the phosphate residue on the core galactose of most of the above structures in the native LPG. In those repeat units which contain more than one galactose residue (i.e. 2a, 5a, 6a), the location of the phosphate on the core Gal was suggested by the co-elution of these glycans on HPAE-PAD with previously defined LPG repeat units under conditions that are capable of resolving structures that differ only in the location of the phosphate group [23]. These data strongly suggest that all the repeat units have the conserved core sequence: \(P04-6[R-3]Galβ1-4Man\), where \(R\) represents monosaccharide or oligosaccharide side chains that branch off the backbone of phosphorylated disaccharide repeat units in the intact phosphoglycan chain.

The anomic configurations of the mannose residues in the disaccharide backbone and of the terminal arabinose residues were assigned from the one-dimensional \(^1\)H-NMR spectrum and two-dimensional COSY spectrum of the intact \(L.\ tropica\) LPG (results not shown). The anomic-proton region of the one-dimensional spectrum contained a doublet of doublets at \(δ = 5.44 \text{ppm with a coupling constant } (J_{1,2} = 2 \text{ Hz, } J_{3,4} = 7.3 \text{ Hz})\) which was identical with that of the 4-substituted α-Man-1-P\(_4\) sequence observed in \(L.\ major\) LPG [15], confirming that the mannose residues in the disaccharide backbone of the repeat units are in the \(α\)-configuration. This region of the one-dimensional spectrum also contained a major resonance at \(δ = 5.35 (J_{1,2} = 3.5)\), which is identical with the resonance for the terminal arabinopyranose residue in \(L.\ major\) LPG [15]. This residue was initially reported to be in the \(α\)-configuration, but has now been assigned the \(β\)-configuration (C. Weller and S. W. Homans, unpublished work). The reassignment is based on two-dimensional COSY, which shows that the coupling constant for \(J_{1,2}\) is large \((10.5 \pm 0.7 \text{ Hz})\), which, together with the small \(J_{3,4}\) coupling, indicates that this residue is in the \(^1\)C\(_4\) conformation [24] and that H1–H2 are in an equatorial–axial configuration (i.e. \(β\)-configuration) [25].

**Phosphorylated repeat units of \(L.\ aethiopica\) LPG**

The \(L.\ aethiopica\) LPG contained two major repeat units, P1 and P2 (Figure 5). P1 corresponded to the basic disaccharide repeat, \(PO4-Galβ1-4Man\), on the basis of its co-migration with authentic standards before and after dephosphorylation, from the methylation analysis and from the susceptibility of the neutral glycan to β-galactosidase digestion (Table 1 and Figure 6). P2 was not co-eluted with any of the previously characterized phosphorylated repeat units on HPAEC. Monosaccharide analysis revealed the presence of Man and galactose 6-phosphate in the molar ratio 1:0.3. Methylation analysis indicated that dephosphorylated P2 had the structure: Gal1-4/2[Man1-2/4]Man (Table 1). This glycan co-migrated with the neutral cap structure, Gal1-4[Man1-2]Man on HPAEC (result not shown) and HPTLC (Figure 4d, lanes 1 and 4) and was susceptible to both β-galactosidase and α-mannosidase (Figure 4d, lanes 5 and 6). A second methylation analysis of the dephosphorylated glycan after jack-bean α-mannosidase digestion confirmed that the mannose residue was linked to the C-2 position (Table 1). Taken together, these data suggest that P2 has the structure PO4-6Galβ1-4[Man1-2]Man, and is thus the first LPG repeat unit to have a side chain on the mannose residue of the disaccharide core.

The \(L.\ aethiopica\) LPG also contained two minor repeat units, P3 and P4 (Figure 5a), which were identified as Gal1-3[PO4-6]Galβ1-4Man and Gal1-3Galβ1-3[PO4-6]Galβ1-4Man on the basis of their co-migration with authentic standards on HPAEC and HPTLC sequence analysis (Figure 6).

**Phosphorylated repeat units of \(L.\ major\) LPG**

The \(L.\ major\) strains LRC-L580 and LRC-L456 belong to the A1B2 and B2 subtypes respectively and were shown to distinctly different repeat unit profiles (Figure 5b and 5c). These
The phosphorylated oligosaccharide repeat units from each LPG were purified by HPAE-PAD (gradient program a), then reduced with NaB₃H₄/NaB₂H₄ and dephosphorylated. The HPAE elution time (Du) and HPTLC migration position (Tu) of each glycan, and their resistance and susceptibility to exoglycosidase digestion and acid hydrolysis are summarized. The symbols are the same as for Figure 4.

Repeat units were identified by their co-migration with authentic standards, before and after dephosphorylation and by HPTLC sequence analyses (Figure 6). The LRC-L456 LPG contained mainly PO₄-6Galβ1-4Man (Figure 5c and Figure 6), suggesting that less than 5% of the repeat units were substituted with side chains. In contrast, more than 85% of the repeat units in the LRC-L456 LPG were substituted on the C-3 position of the Gal, primarily with the monosaccharide βGal (70%), but also with the disaccharide branches, Galβ1-3Galβ1 and Arapβ1-2Galβ1 (13 and 12% respectively) (Figure 5b and Figure 6).

Capping oligosaccharides of LPG

The phosphorylcan chains of LPG are capped at the non-reducing terminus with phosphodiester-linked glycans. After acid-depolymerization of the LPG, these glycans were released as neutral components and subsequently resolved by the phosphorylated oligosaccharides by HPAE-PAD using gradient program a, and then from each other using gradient program b (Figures 1a and 1b). HPAE analysis of neutral glycans released from L. tropica LPG yielded five monosaccharide-containing fractions (Figure 1b). From the retention time and GC-MS analysis, fractions 1 and 2 were shown to contain exclusively arabinose and a mixture of Glc, Gal and Man (in molar ratio 10:3:1) respectively. The presence of these monosaccharides probably reflects the release of some arabinose (corresponding to approx. 5% of the total arabinose) and glucose (approx. 100% of the total) residues from the repeat units and the GPI anchor (see below) respectively. Fractions N1 and N2 were co-eluted with the previously defined cap structures Manβ1-2Man and Manβ1-2Manβ1-2Man (Figure 1b), consistent with the monosaccharide and methylation analyses of these species (results not shown). Fraction N3 was co-eluted with the dephosphorylated, non-reduced repeat unit, 3a. Methylation analysis showed that this species contained a terminal arabinopyranose, a 2-substituted Glc, a 3-substituted Gal and a 4-substituted Man (results not shown). These data suggest that N3 has the sequence Araβ1-2Glcα1-3Galβ1-4Man and that it corresponds to the non-phosphorylated form of the major phosphorylated repeat unit.

The neutral oligosaccharide fraction of the L. aethiopica LPG comprised monomeric hexose (90%, Glc) and six oligosaccharides that were identified as Galβ1-4Man (N1), Manβ1-2Man (N2), Manβ1-2Manβ1-2Man (N3), Manβ1-2[Galα1-4]Man (N4), Manβ1-2Manβ1-2Manβ1-2Man (N5) and Manβ1-2Manβ1-2Manβ1-2Manβ1-2Man (N6) from their co-migration with authentic standards on HPAE (Figure 5d), monosaccharide composition and methylation analysis and their susceptibility to jack-bean α-mannosidase digestion (results not shown).

The LPGs of both L. major strains contained a limited profile of neutral cap oligosaccharides. In both preparations, the predominant neutral oligosaccharides were co-eluted with Galβ1-4Man and Manβ1-2Man (Figures 5e and 5f), consistent with their monosaccharide composition and their susceptibility to α-mannosidase digestion.

The GPI anchor of the LPGs

The GPI anchors of the purified LPGs were separated from the cap and repeat-unit oligosaccharides by octyl-Sepharose chromatography and shown to comprise a single glycolipid species by HPTLC (results not shown). These glycoprotein anchors were shown to be indistinguishable from those in previously characterized LPGs [15–17,23]. The GPI anchor of L. tropica LPG was characterized by (1) GC-MS monosaccharide and lipid analysis, (2) methylation analysis of the dephosphorylated glycan released after HNO₃ deamination and (3) enzymic sequencing of the deaminated/NaB₃H₄-reduced core by HPTLC (results not shown). These data indicate a GPI anchor with the structure PO₄-6Galα1-6Galα1-3Galβ1-3Glcβ1-PO₂-6Manβ1-3Manβ1-4Glcν-lysOalkyl-P, where the main alkyl chains are predominantly C₃₄₋₅₀ and C₃₆₋₅₀ (60–40 mol %). The structures of the L. aethiopica and L. major LPG anchors were deduced from compositional analysis and enzymic sequencing of the deaminated/NaB₃H₄-reduced core. These analyses indicated that the anchors of these LPGs were indistinguishable from the previously characterized LPGs (results not shown).

Discussion

The structures of the LPG from L. tropica, L. aethiopica and L. major are summarized in Figure 7. These results show that, while there is extensive polymorphism in the structure of LPG between and within different species, this polymorphism is largely restricted to differences in the branching saccharide moieties of the phosphorylated disaccharide repeat units. Some differences in the range of mannose-containing cap structures are also evident (Figure 7). In contrast, the backbone structure, comprising the glycolipycan chain, [-6Galβ1-4Manα1-P°n]ₙ, where n can vary from 1 to > 40, and the GPI anchors are highly conserved in all these LPGs.

On the basis of the nature of the side-chain substitutions on the repeat unit, we have classified the leishmanial LPGs into...
Figure 7  Summary of the structures of leishmanial LPG

The structures of LPG from L. tropica, L. aethiopica and L. major (strains L456 and L580) were determined in the present study. The structures of LPG from L. major strain LRC-L137 [15], L. donovani S-1 or LV9 [17,26] are shown for comparison. Values beside each of the repeat units refer to their relative abundance in the phosphoglycan chain.
three classes. The type-1 LPGs contain no side chain substitutions, as exemplified by the LPGs from the East African L. donovani strains, S-1 and LV9 [17,26] and L. peruwiana (A. Skinner, J. M. Blackwell and M. J. McConville, unpublished work). It is noteworthy that Leishmania with type-1 LPGs all belong to the EF serotype, B2, suggesting that the antibodies which define this serotype recognize epitopes in unsubstituted phosphoglycan chains. This is supported by the finding that the anti-B2 antibodies also recognize Leishmania strains with minimally substituted or partially substituted LPGs such as those which occur in the L. major strains LRC-L456 and -L580 (B2 and A1B2 serotype respectively) (Figure 7).

Type 2 LPGs contain a variety of saccharide side chains, all of which are linked to the C-3 position of the Gal residue in the disaccharide repeat unit. These side chains may vary from single βGlc residues, as in L. mexicana LPG, to the more complex side chains of L. tropica and L. major LPG (Figure 7). The L. tropica LPG contains the most complex side-chain profile of any of the LPGs examined to date, with as many as 19 distinct structures being detected (Figure 7). Most of these side chains contained one or two terminal arabinopyranose residues which were attached to either βGlc or a novel cellobiose core (Glcβ1-4Glcβ1-) (Figure 7). Arabinopyranose-terminating side chains with the same βGal core as the L. major LPG side chains [15] were also identified in L. tropica LPG. This is consistent with the EF serotyping data, which show that antibodies that react with the L. tropica A2 serotype recognize the majority of L. major strains which belong to the A subtype [11,27]. Whether the addition of arabinopyranose residues to both Gal- and Glc-containing side chains requires several linkage-specific arabinotransferases or a single less-specific transferase is not known.

The Type-3 LPGs, exemplified by the L. aethiopica LPG, contain side chains on both the C-2 position of the mannose residue, as well as on the C-3 position of the galactose residue, in the disaccharide repeats (Figure 7). In the L. aethiopica LPG, approx. 35% of the disaccharide repeat units were substituted with single αMan residues. This is the first time that this branch type has been found within the phosphoglycan chain, although it occurs in the terminal cap structures of most leishmanial LPGs (Figure 7). This type of side chain may have a profound effect on the three-dimensional conformation of the phosphoglycan chain. In this regard, recent studies using NMR and molecular dynamics modelling [28] suggest that a chain of unsubstituted phosphorylated disaccharide repeat units assumes an extended helical conformation in which the C-3 position of the Gal residues is exposed on the outer face of the helix, while the C-2 position of the Man residues are directed toward the inside of the helix. These studies predict that addition of side chains to the Gal could occur without a major change in the conformation of the backbone, while the opposite would be true for additions to the Man residue. Interestingly, although the L. aethiopica LPG contains many unsubstituted repeat units, it is not recognized by the anti-B2 antibodies [29], supporting the notion that the mannose-substituted repeat units have a different conformation from those in the type-1 LPGs.

This is the first study to define the nature of intraspecific variation in LPG structure, which was predicted by the EF serotyping system. These analyses indicate that, in the case of L. major, strain-specific variation may occur in the extent to which the phosphoglycan chains are substituted with the Gal- and Ara-containing side chains. While the LPGs from two L. major strains so far examined (LRC-L137 and -L580) are heavily substituted with these side chains, the LPG from L. major strain LRC-L456 is essentially unsubstituted (Figure 7). Further polymorphism in the structure of L. major LPG is predicted by the EF serotyping system, which has defined at least seven different subtypes (A1-7) of L. major [11,14,30]. Interestingly, L. major may be unusual in this regard, as most other Old World species (i.e. L. tropica, L. aethiopica and L. donovani) each belong to a single serotype. While this suggests that the LPGs from L. major are more heterogeneous than other species, it might also reflect the fact that in this species, the structures of the repeat unit side chains are developmentally regulated [31] and that the types of structures in the EF fraction will vary depending on the physiological state of the parasite.

L. major is the most common form of zoonotic cutaneous disease in the Old World and is normally transmitted by the sandfly vector Phlebotomus papatasi. The relationship between L. major and this vector appears to be highly specific, as there are no reports of other Leishmania species, including L. tropica and L. aethiopica, which have overlapping geographic distributions with L. major, being transmitted by P. papatasi [18]. This specificity has been confirmed by laboratory studies which show that after experimental infection of P. papatasi with a variety of Leishmania species, only L. major is able to establish a full infection. Pimenta and colleagues [4] have recently shown that there is a correlation between the vectorial competence of a sandfly vector for a particular Leishmania species and the ability of procyclic promastigotes to bind to the midgut via receptors that recognize procyclic LPG. These data suggest that midgut binding might be crucial for successful infection by preventing the parasites from being excreted with the remains of the bloodmeal [32]. Only the LPG of L. major promastigotes bound to the isolated midguts of P. papatasi, while those of several other species, including L. donovani and L. tropica, did not [4]. The midgut receptor(s) for L. major appears to recognize the abundant βGal-terminating side chains, which to date have only been found on procyclic LPG [8,31]. The requirement for polyvalent or clustered Gal epitopes is suggested by the finding that the L. donovani LPG, which contains a single terminal Gal residue in the cap structure, is not bound by this receptor [4]. From the present study, it is not surprising that L. tropica promastigotes are unable to adhere to the midgut of P. papatasi, as the LPG of this species contains few terminal Gal residues (Figure 7). Similarly, our results suggest that L. aethiopica promastigotes should also be unable to establish an infection in this sandfly, as the L. aethiopica LPG contains even fewer terminal Gal residues. With regard to vectorial competence of P. papatasi, it is noteworthy that we have shown that not all L. major promastigotes produce a highly galactosylated LPG (Figure 7). It will be of interest to determine whether strains such as L. major LRC-L456 are able to infect P. papatasi and whether intra-specific polymorphism in LPG structures contribute to variability in the vectorial competence and geographic range of these strains. Alternatively, it is possible that LRC-L456 promastigotes can still bind to the P. papatasi midgut by virtue of containing an extremely abundant family of glycoinositol phospholipids on their cell surface [19,33]. The glycoinositol phospholipids of L. major differ from those found on most other Leishmania species in containing terminal Gal residues [2] and their high density on the cell surface may make them suitable ligands for the midgut receptor. In contrast to P. papatasi, some other sandfly vectors are permissive vectors for a wide variety of Leishmania species. This includes P. argintipes, the natural vector of L. donovani, and a permissive host for a number of Old and New World Leishmania species [4]. Attachment of L. donovani promastigotes to P. argintipes midguts appears to be mediated by a different class of lectin receptor(s) that recognizes the terminal Gal and Man residues in the LPG capping oligosaccharides [9]. From the present study it appears that these, or
structurally related, cap structures are present on all LPGs, providing an explanation for the uniform binding properties of different LPGs to the *P. argentipes* midguts [4]. These data support the notion that differences in the specificity of midgut receptors in the insect vector of these parasites may provide a selective force for greater inter- and intra-specific polymorphism in LPG structure.

We thank Dr. T. Rutherford for acquiring the NMR spectra, Dr. M. G. Low for generously providing PI-PLC, and Dr. M. A. J. Ferguson for his support. This work was supported by the Wellcome Trust, the Australian National Health and Medical Research Council and the World Health Organisation. P. S. is an EMBO Long Term Fellow and M. J. M. is a Wellcome Trust Senior Research Fellow.

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Received 7 February 1995/5 May 1995; accepted 12 May 1995