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Refined Structure of the Lipophosphoglycan of *Leishmania donovani**

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The primary structure of the major surface glycoconjugate of *Leishmania donovani* parasites, a lipophosphoglycan, has been further characterized. The repeating PO_4–6Gal$p\beta$1–4Man disaccharide units, which are a salient feature of the molecule, are shown to terminate with one of several neutral structures, the most abundant of which is the branched trisaccharide Gal$p\beta$1–4(Man$p\alpha$1–2)Man. The phosphosaccharide core of lipophosphoglycan, which links the disaccharide repeats to a lipid anchor, contains 2 phosphate residues. One of the core phosphates has previously been localized on *O*-6 of the galactosyl residue distal to the lipid anchor; the second phosphate is now shown to be on *O*-6 of the mannosyl residue distal to the anchor and to bear an α-linked glucopyranosyl residue. Also, the anomeric configuration of the unusual 3-substituted Gal*f* residue in the phosphosaccharide core is established as β. The complete structure of the core is thus PO_4–6Gal$p\alpha$1–6Gal$p\alpha$1–3Gal$f\beta$1–3[Glc$p\alpha$1-PO_4-6]Man$p\alpha$1–3Man$p\alpha$1–4GlcNα1–. This further clarification of the structure of lipophosphoglycan may prove beneficial in determining the structure-function relationships of this highly unusual glycoconjugate.

The protozoan parasite *Leishmania donovani* is the causative agent of the human disease kala azar, or visceral leishmaniasis. The major cell surface glycoconjugate of *L. donovani* promastigotes is a lipophosphoglycan (LPG).[1] LPG has been suggested to play a role in the successful infection of both insect and human hosts by the parasite (reviewed by Turco (1990). The parasite must survive harsh environments, both extracellularly as a promastigote in the alimentary tract of the sand fly vector and intracellularly as an amastigote in macrophages of infected mammalian hosts. Fragments of LPG are efficient inhibitors of protein kinase C (McNeely *et al.*, 1989) and may therefore prevent induction of the microbicidal oxidative burst within phagolysosomes of macrophages. LPG may also inhibit the oxidative burst by chelating intracellular calcium or other divalent cations and/or by scavenging oxygen free radicals (Chan *et al.*, 1989; McNeely and Turco, 1990; Turco 1990). Besides playing a role in protecting the parasite, LPG is also involved in attachment to and penetration of the host macrophage by *Leishmania major* (Handman and Goding, 1985; Puentes *et al.*, 1988; Talamas-Rohana *et al.*, 1990). Thus, it will be useful to know the detailed structure of LPG in order to understand at the molecular level the infectivity of *Leishmania* spp. and their ability to parasitize humans.

Many of the structural features of *L. donovani* LPG have previously been determined. The lipid anchor is a novel *lyso*-1-*O*-alkyl-PI (Orlandi and Turco, 1987). Linked directly to the PI moiety through *O*-6 of the *myo*-inositol residue is a phosphosaccharide core, with a proposed structure of PO_4–6Gal$p\alpha$1–6Gal$p\alpha$1–3Gal$f\alpha$1–3Man$p\alpha$1–3Man$p\alpha$1–4GlcNα1– (Turco *et al.*, 1989). In addition to the Gal-6-PO_4, one of the two mannosyl residues in the core also appears to bear a phosphate based on the results of methylation analysis before and after treatment with aqueous HF (Turco *et al.*, 1989). Finally, each molecule of LPG contains an average of 16 phosphorylated disaccharide repeats, PO_4–6Gal$p\beta$1–4Man$p\alpha$1–, arranged as a small linear polysaccharide (Turco *et al.*, 1989). Despite these detailed studies, several structural features of *L. donovani* LPG are not known, including (i) the identity and exact location of the substituent(s) on one of the

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This paper and the accompanying paper by Ilg *et al.* (1992) are dedicated to our good friend and colleague Dr. Wayne J. Masterson. Wayne was responsible for the delineation of the GPI biosynthetic pathway in African trypanosomes, which is pertinent to the work published herein. He died of cancer at the age of 31 on November 14th, 1991.

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[1] The abbreviations used are: LPG, lipophosphoglycan; PI, phosphatidylinositol; JBAM, jack bean α-mannosidase; PIPLC, phosphatidylinositol-specific phospholipase C; FAB-MS, fast atom bombardment mass spectrometry; GC-MS, gas chromatography mass spectrometry; GIPL, glycoinositolphospholipid; 1-D, one-dimensional; 2-D, two-dimensional; HOHAHA, homonuclear Hartmann-Hahn; COSY, correlated spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; PAD, pulsed amperometric detector; HPLC, high-performance liquid chromatography; Hex, hexose.

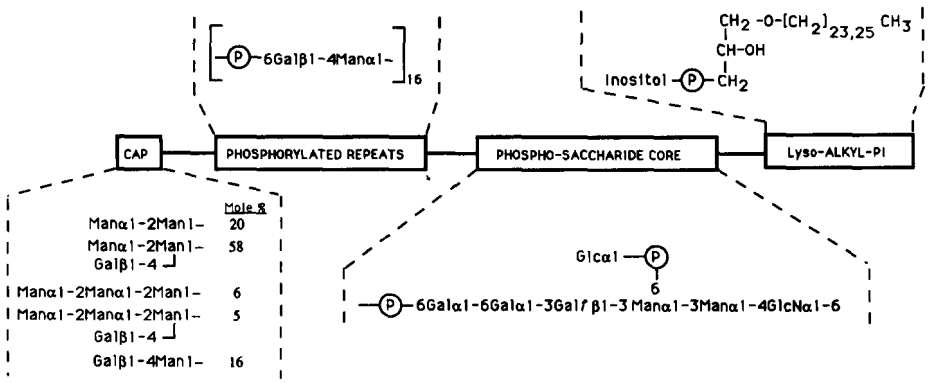

FIG. 1. **Complete structure of the LPG isolated from log-phase promastigotes of *Leishmania donovani*.** All molecules have a phosphosaccharide core substituted with the Glc$p\alpha$1–PO_4 moiety, and at least 90% have one of the oligosaccharide caps shown. The ratio of the oligosaccharide caps, Man$p\alpha$1–2Man, Man$p\alpha$1–2[Gal$p\beta$1–4]Man, Man$p\alpha$1–2Man$p\alpha$1–2Man, Man$p\alpha$1–2Man$p\alpha$1–2[Gal$p\beta$1–4]Man, and Gal$p\beta$1–4Man is 20:52:6:5:16. The structures of the lipid moiety and phosphorylated repeats, and a partial structure of the phosphosaccharide core, were determined previously (Orlandi and Turco, 1987; Turco *et al.*, 1987, 1989).

core mannosyl residues, (ii) the site(s) of attachment of the disaccharide repeats to the phosphosaccharide core, and (iii) the nature of the nonreducing terminal (capping) structures. We have completed the determination of the primary structure of *L. donovani* LPG to complement our studies of LPG isolated from *L. major* (McConville *et al.*, 1990b) and *Leishmania mexicana* (Ilg *et al.*, 1992).

EXPERIMENTAL PROCEDURES AND RESULTS[2]

DISCUSSION

The results presented here have completed our picture of the overall structure of the LPG isolated from promastigotes of *L. donovani* (Fig. 1). The Gal*f* residue in the phosphosaccharide core is shown to be in the β-anomeric configuration, correcting the previous assignment as the α-anomer (Turco *et al.*, 1989). The mannosyl residue distal to the GlcN in the phosphosaccharide core (Man-2) is shown to bear a phosphate residue at *O*-6, and this phosphate is substituted with an α-linked glucopyranosyl residue. This leaves the Gal-6-PO_4 of the core as the only other possible site of attachment of the disaccharide repeats to the core. Finally, the repeats are concluded to be capped with one of several neutral oligosaccharides, the most abundant of which is Gal$p\beta$1–4[Man$p\alpha$1–2]Man1–.

The overall structures of the LPGs isolated from *L. donovani* (Fig. 1), *L. major* (McConville *et al.*, 1990b), and *L. mexicana* (Ilg *et al.*, 1992) promastigotes are similar, with a linear arrangement of caps, repeats, glucosylated core, and *lyso*-alkylglycerol lipid moiety. *L. mexicana* LPG resembles that of *L. donovani* in having mostly phosphorylated disaccharide repeats, a variety of capping oligosaccharides (either Manα1–2Man, Manα1–2Manα1–2Man, or Galβ1–4(Manα1–2)Man), and a phosphosaccharide core in which Man-2 is always substituted with Glc$p\alpha$1–PO_4. They differ mainly in that some (30%) of the *L. mexicana* repeats are glucosylated to form Glcβ1–3[PO_4–6]Galβ1–4Manα1. In contrast, over 80% of the repeats in *L. major* LPG contain additional arabinosyl and/or galactosyl residues attached as side chains to the common PO_4–6Galβ1–4Manα1 disaccharide (McConville *et al.*, 1990b). In addition, *L. major* LPG is capped exclusively with the disaccharide, Manα1–2Man, and some of the phosphosaccharide cores are neither glucosylated nor phosphorylated at Man-2 (McConville *et al.*, 1990b). Thus, the LPGs of *L. mexicana* and *L. donovani* have more structural features in common with each other than with *L. major* LPG.

All but one of the capping oligosaccharides in the promastigote LPGs of the three Leishmania spp. contain within them a Manα1–2Man motif (see above). It is, therefore, tempting to speculate on the existence in the three *Leishmania* species of a Man(α1–2) mannosyltransferase. The activity of such a putative enzyme would result in the signal for cessation of LPG elongation with the formation of a chain-terminating, Manα1–2Man-containing oligosaccharide. One of the striking observations of LPG structure has been the recent finding that the LPG of *L. major* (Sacks *et al.*, 1990) and *L. donovani*[3] doubles in size during the process of metacyclogenesis, in which the promastigote form of the parasite differentiates from a logarithmically grown noninfective to a stationary phase infective organism. The doubling in size is mainly due to an approximate doubling in the number of repeat units in the metacyclic version of LPG. The increase in size of LPG has been hypothesized to enable the parasite to escape complement-mediated damage upon inoculation of the human host with the parasite (reviewed by Sacks, 1989). Thus, a chain-terminating mannosyltransferase may prove to be one of the key regulatory enzymes in LPG biosynthesis.

The refinement of LPG structure described herein may be useful in understanding structure-function relationships. LPG expression has been proposed to be important in a variety of functions, in addition to enabling the parasite to avoid complement-mediated damage as mentioned above. Other proposed functions include (i) attachment of parasites to the epithelial cells that line the midgut of the sand fly vector (Davies *et al.*, 1990), (ii) complement activation (Puentes *et al.*, 1988), (iii) attachment and entry in host macrophages (Handman and Goding, 1985; Talamas-Rohana *et al.*, 1990), and (iv) survival within the phagolysosomal compartment (Turco, 1990). Furthermore, elucidation of the complete structures of LPGs from related species and their subtle structural variations may lead to an understanding of the diverse tissue tropisms of the various *Leishmania* species and their consequent pathogenesis. Ultimately, *Leishmania*-specific cytotoxic agents might be developed with this surface glycoconjugate as the target.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 2–7, and Tables I and II) 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.

[3] S. J. Turco, unpublished observations.

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REFERENCES

Chan, J., Fujiwara, T., Brennan, P., McNeil, M., Turco, S. J., Sibille, J.-C., Snapper, M., Aisen, P. & Bloom, B. R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2453–2457

Ciucanu, I. & Kerek, F. (1984) *Carbohydr. Res.* **131,** 209–215

Davis, D. G. & Bax, A. (1985) *J. Am. Chem. Soc.* **107,** 2820–2821

Davies, C. R., Cooper, A. M., Peacock, C., Lane, R. P. & Blackwell, J. M. (1990) *Parasitology* **101,** 337–343

DeJongh, D. C., Radford, T., Hribar, J. D., Hanessian, S., Bieber, M., Dawson, G. & Sweeley, C. C. (1969) *J. Am. Chem. Soc.* **91,** 1728–1740

Dell, A. (1987) *Adv. Carbohydr. Chem. Biochem.* **45,** 19–72

Ferguson, M. A. J., Homans, S. W., Dwek, R. A. & Rademacher, T. W. (1988) *Science* **239,** 753–759

Handman, E. & Goding, J. W. (1985) *EMBO J.* **4,** 329–336

Homans, S. W., Dwek, R. A., Boyd, J., Soffe, N. & Rademacher, T. W. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 1202–1205

Ilg, T., Etges, R., Overath, P., McConville, M. J., Thomas-Oates, J., Thomas, J., Homans, S. W. & Ferguson, M. A. J. (1992) *J. Biol. Chem.* **267,** 6834–6840

Inagaki, F., Kodama, C., Suzuki, M. & Suzuki, A. (1987a) *FEBS Lett.* **219,** 445–450

Inagaki, F., Kohda, D., Kodama, C. & Suzuki, A. (1987b) *FEBS Lett.* **212,** 91–97

Lonngren, J. & Svensson, S. (1974) *Adv. Carbohydr. Chem. Biochem.* **29,** 41–106

McConville, M., Homans, S. W., Thomas-Oates, J. E., Dell, A. & Bacic, A. (1990a) *J. Biol. Chem.* **265,** 7385–7394

McConville, M., Thomas-Oates, J. E., Ferguson, M. A. J. & Homans, S. W. (1990b) *J. Biol. Chem.* **265,** 19611–19623

McNeely, T. B. & Turco, S. J. (1990) *J. Immunol.* **144,** 2745–2750

McNeely, T. B., Rosen, G., Londner, M. V. & Turco, S. J. (1989) *Biochem. J.* **259,** 601–604

Neuhaus, D., Wider, G., Wagner, G. & Wuttrich, K. (1984) *J. Magn. Reson.* **57,** 164–168

Orlandi, P. A., Jr. & Turco, S. J. (1987) *J. Biol. Chem.* **262,** 10384–10391

Puentes, S. M., Sacks, D. L., de Silva, R. P. & Joiner, K. A. (1988) *J. Exp. Med.* **167,** 887–902

Sacks, D. L. (1989) *Exp. Parasitol.* **69,** 100–103

Sacks, D. L., Brodin, T. & Turco, S. J. (1990) *Mol. Biochem. Parasitol.* **42,** 225–234

Sweeley, C. C., Bentley, R., Makita, M. & Wells, W. W. (1963) *J. Am. Chem. Soc.* **85,** 2497–2507

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975) *Carbohydr. Res.* **40,** 217–225

Talamas-Rohana, P., Wright, S. D., Lennartz, M. R. & Russell, D. G. (1990) *J. Immunol.* **144,** 4817–4824

Tsui, D. S., & Gorin, P. A. J. (1986) *Carbohydr. Res.* **156,** 1–8

Turco, S. J. (1990) *Exp. Parasitol.* **70,** 241–245

Turco, S. J., Hull, S. R., Orlandi, P. A., Jr., Shepherd, S. D., Homans, S. W., Dwek, R. A. & Rademacher, T. W. (1987) *Biochemistry* **26,** 6233–6238

Turco, S. J., Orlandi, P. A., Homans, S. W., Ferguson, M. A. J., Dwek, R. A. & Rademacher, T. W. (1989) *J. Biol. Chem.* **264,** 6711–6715

SUPPLEMENTAL MATERIAL

to

REFINED STRUCTURE OF THE LIPOPHOSPHOGLYCAN OF *LEISHMANIA DONOVANI*

by

Jerry R. Thomas, Malcolm. J. McConville, Jane E. Thomas-Oates, Steven W. Homans, M.A.J. Ferguson, Philip A.J. Gorin, Kenneth D. Greis, and Salvatore J. Turco

EXPERIMENTAL PROCEDURES

Preparation of LPG, hexaglycosyl-PI, phosphosaccharide core, and cap oligosaccharides. LPG was purified from *L. donovani* as described [Orlandi & Turco, 1987]. Disaccharide repeats and neutral mono- and oligosaccharides were released from intact LPG by mild-acid hydrolysis [40 mM trifluoroacetic acid for 8 min at 100°C (McConville *et al.*, 1990b)]. The phosphorylated disaccharide repeats and neutral mono- and oligosaccharides were separated from the $(PO_4)_2$-Gal$_3$Man$_2$GlcN-*lyso*-PI product of mild-acid hydrolysis by binding the glycolipid to a 2-ml column of octyl-Sepharose CL-4B (Pharmacia) equilibrated in 5% *n*-propanol and 100 mM NH_4-acetate. The non-lipidic oligosaccharides eluted in the void volume, and the hexaglycosyl-PI was eluted with 50% *n*-propanol. The neutral cap oligosaccharides were separated from the phosphorylated Galβ1-4Man repeats and fractionated by Dionex-HPLC (see below). Digestion of cap oligosaccharides with (JBAM) (Boehringer Mannheim) was carried out in in 50 mM Na-acetate, pH 5.0 at 37°C for 16 h at an enzyme concentration of 30 Units/ml.

Phosphosaccharide core (compound G, Table II) was released from the purified hexaglycosyl-PI by treatment with PIPLC from *Bacillus thuringiensis* (kindly provided by Dr. M. Low, Columbia University) in 25 mM Tris-acetate, pH 7.4, and 0.1% Triton X-100 at 37°C; after extracting the reaction mixture four times with toluene to remove lipid and detergent, the glycan product was desalted using a 1.5x20 cm column of Bio-Gel P-4 (Bio-Rad) eluted with water. Compound G (300 nmoles) was treated with 5 Units of alkaline phosphatase (Sigma Type VIII-N from bovine intestinal mucosa) in 100 μL of 100 mM NH_4HCO_3, pH 9.0, for 16 h at 37°C. After heating at 100°C for 5 min, the reaction mixture was lyophilized, and desalted as described above to give compound F. Compound F was treated with 1.8 Units of coffee bean α-galactosidase (Boehringer Mannheim) in 25 μl of 100 mM citrate-phosphate buffer, pH 6.0, for 40 h at 25°C and desalted using Bio-Gel P-4 (see above) to give compound E.

Mass Spectrometry. FAB mass spectra were obtained using a VG Analytical 70 250-SE mass spectrometer operated at an accelerating voltage of +8 kV (positive-ion mode) or -7 kV (negative-ion mode) and fitted with an Ion-Tech FAB gun (operated at 8 kV and 1 mA with xenon as the bombarding gas). Samples were dissolved in water (native) or methanol (per-*O*-methylated), loaded into a matrix of glycerol/thioglycerol (1:1, v/v), and 50-sec scans were acquired and averaged using a VG 11-250 data system. Per-*O*-methylation and methylation analysis were performed using the method of Ciucanu and Kerek [1984] as described by Ferguson *et al.* [1988]. For reduction prior to methylation, 20 mg/ml NaB[^{2}H]$_4$ in 0.1 M NH_4OH was used. Monosaccharides released from LPG by mild-acid hydrolysis (see above) were quantitated by GC-MS after trimethylsilylation with trimethylchlorosilane/hexamethyldisilazane/pyridine (1:3:10) [Sweeley *et al.*, 1963]. Phosphorylated sugars were analyzed by GC-MS after trimethylsilylation and treatment with diazomethane [see Ferguson *et al.*, 1988]. A Hewlett-Packard 5970 mass selective detector and 5890 gas chromatograph with a Econocap SE-54 capillary column (0.25 mm x 30 m; Alltech Associates) were used to analyze PMAAs [see Lonngren & Svensson, 1974] and trimethylsilyl ethers [DeJongh *et al.*, 1969].

NMR Spectroscopy. Samples were deuterium exchanged and dissolved in deuterium oxide (99.96%; Aldrich). ^{1}H-^{1}H HOHAHA spectra were recorded using a Bruker AM 500 spectrometer and the method of Davis and Bax [1985] as described by Homans *et al.* [1987]. 1-D ^{1}H NMR spectroscopy and 2-D ^{1}H-^{1}H COSY and NOESY were performed as previously described [Turco *et al.*, 1989]. The ^{1}H-^{31}P-^{1}H relay experiment was performed as described by Neuhaus *et al.* [1984].

Model compounds A, B, and C were prepared from *L. major* GIPLs-1, -2, and -3, respectively, by treatment with PIPLC [McConville *et al.*, 1990a]. Compound D was prepared from the fraction of *L. major* LPG that lacks the phosphate on Man-2 (distal to the GlcN) by treatment with mild acid and PIPLC [McConville *et al.*, 1990b]. Compound H was synthesized as previously described by Tsui and Gorin [1986].

Dionex HPLC. A Dionex BioLC Carbohydrate Analyzer equipped with PAD and a CarboPac PA-1 column (4x250 mm) was used. Oligosaccharides released from LPG by mild-acid hydrolysis and purified by octyl-Sepharose chromatography were chromatographed under high-salt conditions (column equilibrated in 150 mM NaOH and 150 mM Na-acetate and eluted with successive linear gradients of Na-acetate from 150 to 200 mM in 30 min and 200 to 500 mM in 10 min at 0.6 ml/min). Neutral oligosaccharides eluting in the void volume were desalted by passage through AG50-X12 (H^+ form), drying, and evaporation from toluene. They were then fractionated using the same column equilibrated in 150 mM NaOH and eluted with a gradient of Na-acetate increasing from 12.5 to 125 mM in 30 min at 0.6 ml/min (low-salt conditions) [McConville *et al.*, 1990b].

RESULTS

Substitution of Man-2 at *O*-6 with Glcα1-PO$_4$. Previous assignment of a sequence PO$_4$-6Galpα1-6Galpα1-3Galfα1-3Manpα1-3Manpα1- in the phosphosaccharide core of *L. donovani* LPG was accomplished using ^{1}H -^{1}H 500 MHz COSY and NOESY NMR spectroscopy [Turco *et al.*, 1989]. The presence of a phosphate on one of the mannosyl residues was indicated by the observation [Turco *et al.*, 1989] that one of the residues was cryptic to methylation analysis unless the core glycan had been treated with cold aqueous HF, a reagent which cleaves phosphate esters. After HF treatment, a 3-substituted and a terminal mannosyl residue were observed, the latter having been exposed by HF cleavage of the galactofuranosidic linkage; this result is consistent with either mannosyl residue being phosphorylated. Interestingly, alkaline phosphatase treatment failed to render the cryptic mannosyl residue amenable to methylation analysis [Turco *et al.*, 1989].

Several lines of evidence indicate that Man-2 of the phosphosaccharide core of *L. donovani* LPG is indeed phosphorylated at *O*-6, as previously demonstrated for a majority of the *L. major* LPG molecules [McConville, 1990b]. A component with the same mass spectrum and retention time as Man-6-PO$_4$ was detected in compound E (see Table II) by GC-MS (data not shown). Also, the phosphate could be removed with alkaline phosphatase (data not shown), but only after deamination of LPG with nitrous acid as previously demonstrated with *L. major* LPG [McConville, 1990b]. Finally, the results of FAB-MS of the hexaglycosylPI obtained after mild-acid depolymerization (Fig. 2) show that Man-2, but not Man-1, is phosphorylated in all *L. donovani* LPG molecules isolated from promastigotes.

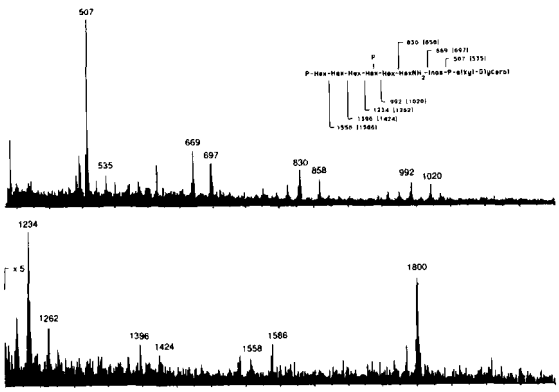

FIG. 2. **Negative-ion fast atom bombardment mass spectrum of the glycolipid derived from LPG by treatment with mild acid.** LPG was depolymerized by treatment with mild acid and the resulting diphosphorylated hexaglycosyl-*lyso*-alkylPI was separated from non-lipidic components by octyl-Sepharose chromatography (see "Experimental Procedures"). The spectrum contains pairs of fragment ions separated by 28 mass units, which in the proposed fragmentation scheme were formed by β-cleavage [Dell, 1987] and contain either 24:0 or 26:0 (brackets) alkyl chains [Orlandi & Turco, 1987]. The ion at m/z 1800 corresponds to the $(M-H)^-$ molecular ion of the $(PO_4)_2Hex_5HexN$-*lyso*-alkylPI species containing a C_{24} alkyl chain. Fragment ions at m/z 830, 992, and 1234 indicate that the hexosyl residue distal to the GlcN residue is phosphorylated. The intensity of the m/z 1234 signal is significantly higher than would be expected from a fragment ion alone; a portion of this signal was derived from the molecular ion corresponding to a truncated PO_4-Man_2GlcN-PI species generated during mild-acid hydrolysis by cleavage of the labile galactofuranosidic linkage (data not shown). The absence of a fragment ion at m/z 1072 indicates that there are no LPG molecules in which the hexosyl residue directly linked to the GlcN residue bears a phosphate. The absence of a molecular ion at m/z 1720 and a fragment ion at m/z 1154 indicates that all of the molecules bear a phosphate on Man-2.

The results of 2-D ^1H-^1H HOHAHA NMR spectroscopy of the phosphosaccharide core clearly indicate that Man-2 is substituted at *O*-6. This method allows the complex spectrum of an oligosaccharide to be decomposed into subspectra derived from the individual monosaccharide residues [Homans *et al.*, 1987; Inagaki *et al.*, 1987*a*,*b*], and was used to assign and compare the proton resonances of the two mannosyl residues in the phosphosaccharide core. A cross-section through the 2-D spectrum of the phosphosaccharide core at ω_1 = 4.46 p.p.m. (Fig. 3, panel A), corresponding to the H2 resonance of Man-1 [Turco *et al.*, 1989], reveals the individual resonances of this mannosyl residue. Although the resonances of the Man-1 H3-H6 protons are not well resolved, none of them are shifted downfield to the extent that would be expected in the presence of a phosphate. The cross section at ω_1 = 4.31 p.p.m. (panel B) corresponding to the H2 resonance of Man-2 contains multiplets at 3.85 and 4.23 p.p.m., which were assigned to the Man-2 H6 protons. The anomalous downfield shifts of the H5, H6, and H6' resonances is concluded to be due to deshielding in the presence of a phosphate group at *O*-6 of Man-2.

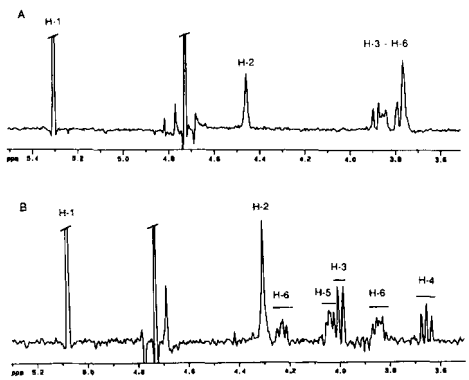

FIG. 3. **Cross sections of the 2-D ^1H-^1H HOHAHA NMR spectrum of the LPG phosphosaccharide core.** The phosphosaccharide core of LPG isolated from *L. donovani* promastigotes, PO_4-6Gal*p*α1-6Gal*p*α1-3Gal*f*β1-3[6-O-PO_4]Man*p*α1-3Man*p*α1-4GlcNα1-6inositol-1-PO_4 (compound G), was prepared and analyzed as described in "Experimental Procedures". Subspectra of the total spectrum (not shown) at ω_1=4.46 (panel A) and ω_1=4.31 ppm (panel B) contain the proton resonances of Man-1 (linked directly to the GlcN residue) and Man-2 (distal to the GlcN residue), respectively.

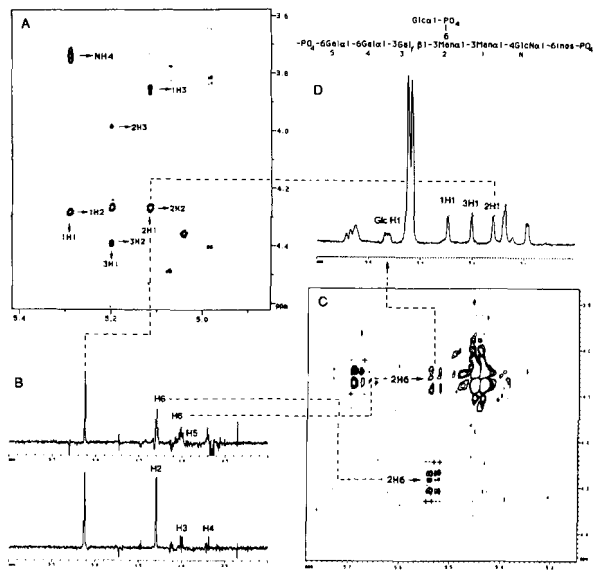

FIG. 4. **Determination of the sequence Glc*p*α1-PO_4-6Man*p* α1-3Man*p* α1-4GlcNα1- in intact LPG by NMR spectroscopy.** LPG was isolated and spectra recorded as described in "Experimental Procedures". A portion of the 2-D ^1H-^1H NOESY spectrum (panel A) contains cross-peaks that enable the resonance position of the Man-2 anomeric proton (2H1) signal to be assigned. A cross section through the 2-D ^1H-^1H HOHAHA spectrum through the 2H1 signal (panel B, lower trace) reveals the H2-4 resonance positions, and a cross section through 2H2 (panel B, upper trace) reveals H5 and the two H6s. These Man-2 H6 signals resonate in the same position as two sets of cross peaks in the ^1H-^{31}P. ^1H spectrum (panel C), which show the connectivity of the Man-2 H6s through a phosphate residue to the complex multiplet resonating at 5.53 p.p.m. in the 1-D ^1H spectrum (panel D). Residue numbering in the phosphosaccharide core is shown above panel D. The relative signs (+ or -) of certain crosspeaks are shown in panel C.

Structures of the caps. LPG was depolymerized by cleavage of phosphodiester linkages with mild acid, and the resulting oligosaccharides separated from the lipid-linked phosphosaccharide core by octyl-Sepharose chromatography (see "Experimental Procedures"). The neutral oligosaccharides, which were concluded to have been derived from the non-reducing termini of the LPG molecules [see McConville *et al.*, 1990*b*], were separated from the PO_4-6Galβ1-4Man repeats by Dionex-HPLC using high-salt conditions, and then fractionated using low-salt conditions (Fig. 5). The positive-ion FAB mass spectrum of the *per*-*O* methylated material in fraction **N4** contained signals at m/z 659 and 681 corresponding to the $(M+H)^+$ and $(M+Na)^+$ pseudomolecular ions, respectively, for a Hex_3 oligosaccharide. Methylation analysis of the material in fraction **N4** before and after treatment with JBAM (Table I) indicated that it contained the branched structure Galβ1-4[Manpα1-2]Man. The galactosyl residue was determined to be in the β-configuration by 1-D ^1H-NMR spectroscopy (H1 δ = 4.4 ppm, $J_{1,2}$ = 7.8 Hz). Taken together, these data suggest the following structure for the most abundant non-reducing terminal (capping) oligosaccharide of *L. donovani* LPG: Galpβ1-4[Manpα1-2]Man1-.

Table I

Methylation analysis of the neutral oligosaccharide fractions released from LPG by mild-acid hydrolysis and separated by Dionex HPLC

Partially methylated alditol acetates (PMAAs) were prepared and analyzed as described in "Experimental Procedures".

PMAA	Origin[a]	N2	N3	N4			N5
				Native	Reduced	JBAM	
		Relative moles[b]					
Galactitol							
2,3,4,6-Me_4	Terminal Gal*p*	0.6	-	0.7	1.0	1.0	0.8
Mannitol							
2,3,4,6-Me_4	Terminal Man*p*	1.0	1.0	1.0	1.0	-	1.0
3,4,6-Me_3	2-Linked Man*p*	0.7	1.4	-	-	-	1.0
2,3,6-Me_3	4-Linked Man*p*	0.8		-	-	-	-
3,6-Me_2	2,4-Linked Man*p*	-	-	0.7	-	-	1.1
1,2,3,5,6-Me_5	4-Linked Mannitol	-	-	-	-	0.3[c]	-
1,3,5,6-Me_4	2,4-Linked Mannitol	-	-	-	0.8	-	-

[a]Where ambiguous, mannosyl residues were assigned the pyranose ring form, which was confirmed by the results of digestion with JBAM.

[b]Determined from integration of total-ion chromatograms obtained by GC-MS, which, for the derivatives listed here, correspond to values obtained by integration of flame-ionization-detector responses and use of semi-empirical response factors (Sweet *et al.*, 1975; J.R. Thomas, unpublished data).

[c]The low yield of this compound was due to its relatively high volatility.

A series of 2-D NMR experiments was needed to determine the sequence Glcpα1-PO_4-6Manp α1-3Manp α1-4GlcNα1- in intact LPG (Fig. 4). First, ^1H-^1H NOESY was used to assign the H1 resonance of the Man-2 residue of LPG (Fig. 4A), as in the previous sequencing of the phosphosaccharide core itself [Turco *et al.*, 1989]. Next, a ^1H-^1H HOHAHA experiment was used to reveal the H6 resonances of the Man-2 residue (Fig. 4B). A heteronuclear ^1H-^{31}P-^1H relay experiment (Fig. 4C) was then used to demonstrate the connectivity between the Man-2 H6s, a phosphate residue, and Glc H1. The multiplet at 5.53 p.p.m. in the 1-D ^1H NMR spectrum of intact LPG (Fig. 4D) was concluded to be H1 of an α-linked glucopyranosyl residue. This conclusion derives from the ^1H-^{31}P-^1H relay experiment (Fig. 4C), in which the smaller of the two couplings (3.5 Hz) within the crosspeaks correlating Glc H1 with Man-2 H6 and H6' corresponds to the passive coupling (in-phase multiplets) $^3J_{1,2}$ for the glucosyl residue, and is diagnostic of an α configuration. The intense doublet at 5.44 p.p.m. in the 1-D spectrum (Fig. 4D) arises from the anomeric proton of the Manpα1-PO_4 residue in the disaccharide repeats. The complex signals at 5.63 p.p.m. probably arise from the GlcN H1 and the Man-1-PO_4 anomeric protons of the caps.

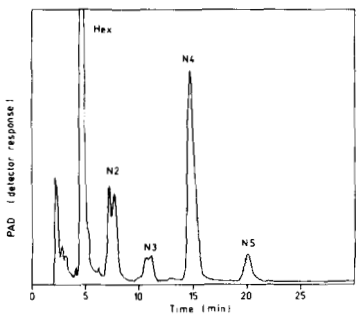

FIG. 5. **Dionex HPLC of the neutral mono- and oligosaccharides released from *L. donovani* LPG by mild-acid hydrolysis.** LPG was hydrolyzed and the non-lipidic products obtained by octyl-Sepharose chromatography (see "Experimental Procedures"). The neutral products that eluted in the void volume during Dionex HPLC using high-salt conditions (profile not shown) were separated using low-salt conditions as described in "Experimental Procedures". The peak labelled "Hex" had the retention time of hexose monosaccharides. The unresolved peaks designated as fraction **N2** eluted with the same retention times as Galpβ1-4Man (earlier peak) and Manpα1-2Man (later peak) isolated from *L. major* LPG [McConville *et al.*, 1990b]. The structures of the two oligosaccharides that eluted in fraction **N2**, as well as those eluting in fractions labelled **N3** (Manpα1-2Manpα1-2Man), **N4** [Manpα1-2(Galpβ1-4)Man], and **N5** [Manpα1-2Manpα1-2(Galpβ1-4)Man] were determined as described in "Results".

Methylation analysis of the minor species (Table I) indicated the presence of a linear trisaccharide (Manp1-2Manp1-2Man) in **N3** and a branched tetrasaccharide [Manp1-2Manp1-2(Galp1-4)Man] in **N5**. The α-anomeric configuration of the mannosyl residues in these fractions was demonstrated by their susceptibility to JBAM, as monitored by Dionex HPLC. Linkage of the terminal galactosyl residue in β-configuration directly to the reducing-terminal mannosyl residue in the tetrasaccharide was indicated by coelution of the material in fraction **N5** with Galpβ1-4Man after digestion with JBAM (profile not shown).

Methylation analysis (Table I) suggested that fraction **N2** contained two unresolved disaccharides, which were identified by their comigration with authentic Galpβ1-4Man and Manpα1-2Man, and the susceptibility of the later-eluting peak to JBAM. The Galpβ1-4Man disaccharides in the mild-acid hydrolysate of LPG were concluded not to have been derived from PO_4-6Galpβ1-4Man repeats, since rehydrolysis of isolated repeats does not lead to their dephosphorylation (data not shown). Furthermore, radiolabelling of terminal galactosyl residues in intact LPG by treatment with galactose oxidase and $NaB[^3H]_4$ [McConville *et al.*, 1990b] resulted in the formation of ^3H-labelled oligosaccharides coeluting from Dionex-HPLC with fractions **N2**, **N4**, and **N5** (profile not shown). Therefore, all of the Gal-containing neutral oligosaccharides are present as such in intact LPG prior to depolymerization.

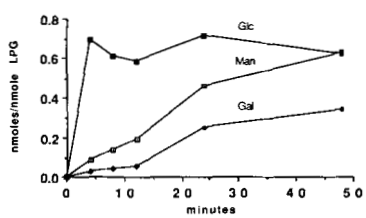

FIG. 6. **Time course of release of monosaccharides from LPG by mild-acid hydrolysis.** LPG was treated with 40 mM trifluoroacetic acid at 100° for varying lengths of time, and the released monosaccharides identified and quantified by GC-MS as described in "Experimental Procedures". The amount of LPG in the starting material was determined by GC-MS quantitation of *myo*-inositol as the per-*O*-trimethylsilyl esters after hydrolysis in 6 M HCl at 110° for 16 h, and is subject to an error of +/- 20%.

Neutral monosaccharides, in addition to the oligosaccharides described above, were released from LPG by mild-acid hydrolysis (labelled 'Hex' in Fig. 5). The kinetics of release were determined by GC-MS quantitation of each monosaccharide after varying times of hydrolysis (Fig. 6). The only monosaccharide released with the kinetics consistent with its attachment to LPG solely via a phosphodiester linkage was Glc. The steady release of Man was due to hydrolysis of the phosphorylated disaccharide repeats (data not shown). The relative amounts of the neutral mono- and oligo-saccharides were estimated from their PAD responses (Fig. 5), which are proportional to the mass of these oligosaccharides [McConville *et al.*, 1990b]. Fractions 'Hex' (with the Man and Gal contributions subtracted), **N2** (earlier eluting), **N2** (later eluting), **N3**, **N4**, and **N5** contained 57, 7, 9, 3, 22, and 2% by weight, respectively, of the total neutral products; the relative molar ratio of the five oligosaccharide caps is 16:20:6:52:5. [Different preparations of LPG have somewhat different ratios of the oligosaccharide caps, but with the galactosylated trisaccharide as the dominant component (data not shown).] Integration and comparison of the Glc and Galf H1 signals in the 1-D ^1H NMR spectrum of intact LPG (Fig. 4D) indicates that there is 1.2 Glc residue per molecule of LPG. Combining these results, it can be calculated that about 90% of the LPG molecules have an oligosaccharide cap at the non-reducing terminus of the repeat chain.

Anomeric configuration of the galactofuranosyl residue. Previous assignment of the anomeric configuration of the Galf residue in the phosphosaccharide core of *L. donovani* LPG was hampered by the lack of a suitable model compound [Turco *et al.*, 1989]. We have reevaluated this assignment using a variety of phosphorylated oligosaccharides derived from the LPG and GIPLs of *L. major* [McConville *et al.*, 1990a,b] and the LPG of *L. donovani* (see "Experimental Procedures"), and the chemically-synthesized compound, Galfβ1-3Manpα1-O-Me.

It is clear from inspection of Fig. 7 why it is difficult to assign the anomeric configuration of Galf solely from the spectrum of the phosphosaccharide core (panel A). The absence of detectable splitting of the H1 resonance makes a determination of the $J_{1,2}$ coupling constant impossible. Only in the spectrum of compound **E** (generated by removal of the terminal phosphate and two Galp residues) is the expected splitting of the H1 signal observable (Fig. 7, panel B). Comparison of all the H1 chemical shifts (Table II) suggests that it is the resonance at 5.18 ppm in panel B ($J_{1,2}$ = 1.5 Hz) that corresponds to the H1 of the Galf residue in compound **E** prepared from *L. donovani* LPG, and not the resonance at 5.11 ($J_{1,2}$=1.4 Hz) as might be expected by comparison with the spectrum of compound **H** (Fig. 7, panel C). The unexpected upfield shift of the Man-2 H1 signal upon phosphorylation of *O*-6 (compare A-D with E-G) is consistent with previous observations of yeast mannans [de Waard *et al.*, 1990]. Assignment of the Galf H1 signal was confirmed by examination of the ^1H-^1H COSY NMR spectrum of compound **E** (data not shown), and it is therefore concluded that the anomeric configuration of the Galf1-3Man glycosidic bond in the phosphosaccharide core of *L. donovani* LPG is β. The unexpectedly low value of $J_{1,2}$ for Galf in the phosphosaccharide core of LPG probably arises from distortion of the furanose ring on 3-*O*-substitution with the Gal-4 unit which changes the average dihedral angle of C1-H1 and C2-H2.

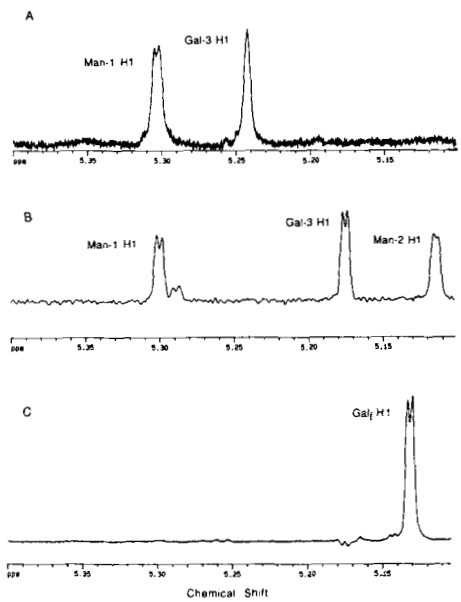

FIG. 7. **1-D ^1H NMR spectra of compounds E, G and H.** Preparation and recording of spectra of compounds **E** [Galfβ1-3(6-*O*PO_4)Manpα1-3Manpα1-4GlcNα1-6*myo*-Inositol1-PO_4], **G** [PO_4-6Galpα1-6Galpα1-3Galfβ1-3(6-*O*PO_4)Manpα1-3Manpα1-)GlcNα1-6*myo*-Inositol-1-PO_4], and **H** [Galfβ1-3Manpα1-O-Me] are described in "Experimental Procedures". Only the portions of the spectra of E (panel A), G (panel B), and H (panel C) that contain the Galf H1 resonance are shown.

Table II

Anomeric Proton Chemical Shifts for Phosphosaccharide Cores and Enzymatic Digestion Products Derived from LPGs and GIPLs[a]

Compound	Residue[b] Gal-5	Gal-4	Gal-3	Man-2	Man-1	Structure
			p.p.m.[c]			
A	·	·	5.17	5.17	5.27	Galf-Man-Man-GlcN-Inositol-P
B	·	5.07	5.19	5.17	5.27	Gal-Galf-Man-Man-GlcN-Inositol-P
C	4.99	5.07	5.19	5.17	5.27	Gal-Gal-Galf-Man-Man-GlcN-Inositol-P
D	4.99	5.07	5.22	5.16	5.25	P-Gal-Gal-Galf-Man-Man-GlcN-Inositol-P
E	·	·	5.18 (1.5)	5.11 (1.4)	5.30	P Galf-Man-Man-GlcN-Inositol-P
F	4.99	5.07	5.20	5.08	5.31	P Gal-Gal-Galf-Man-Man-GlcN-Inositol-P
G	4.98	5.07	5.24	5.08	5.31	P Gal-Gal-Galf-Man-Man-GlcN-Inositol-P (5 4 3 2 1)
H	·	·	5.13 (1.4)	·	·	Galf-β(1-3)-Man-α(1-O-Me)

[a]Compounds A-C were derived from *L. major* GIPLs (McConville *et al.*, 1990a). Compound D was derived from *L. major* LPG that lacks the phosphate on Man-2 (McConville *et al.*, 1990b). Compounds E-G were derived from *L. donovani* LPG. Compound H was chemically synthesized (Tsui and Gorin, 1986). See "Experimental Procedures" for details, and Fig. 1 for structural details.

[b]Residue numbering as for compound G.

[c]Chemical shifts are given relative to acetone, d=2.225 p.p.m. Values in parentheses are $J_{1,2}$ coupling constants in Hz +/- 0.2 Hz.