Intra-Species and Stage-Specific Polymorphisms in Lipophosphoglycan Structure Control *Leishmania donovani*−Sand Fly Interactions†

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Received March 30, 1999

ABSTRACT: The *Leishmania* lipophosphoglycan conveys the ability for the parasites to avoid destruction in diverse host environments. During its life cycle within the sand fly vector, the parasite differentiates from a dividing procyclic promastigote stage that avoids expulsion from the midgut by attaching to the gut wall, to a nondividing metacyclic promastigote stage that is unable to attach to the midgut and migrates to the mouth parts for reinfection of a mammalian host. Lipophosphoglycan plays an integral role during this transition. Structurally, lipophosphoglycan is a multidomain glycoconjugate whose polymorphisms among species lie in the backbone Gal(β1,4)Man(α1)-PO_4 repeating units and the oligosaccharide cap. We have characterized the lipophosphoglycan from an Indian *L. donovani* isolate. Unlike East African isolates, which express unsubstituted repeats and a galactose- and mannose-terminating cap, procyclic lipophosphoglycan from the Indian isolate consists of β1,3-linked glucose residues that branch off the backbone repeats ($n \sim 17$) and also terminate the cap. Of biological significance, metacyclic lipophosphoglycan lacks the glucose residues while doubling the number of repeats. The importance of these developmental modifications in lipophosphoglycan structure was determined using binding experiments to *Phlebotomus argentipes* midguts. Procyclic promastigotes and procyclic LPG were able to bind to sand fly midguts in vitro whereas metacyclic parasites and LPG lost this capacity. These results demonstrate that the *Leishmania* adapts the synthesis of terminally exposed sugars of its LPG to manipulate parasite−sand fly interactions.

Leishmania donovani is the causative agent of kala-azar, an often fatal form of visceral leishmaniasis that is characterized by fever, weight loss, hepatosplenomegaly, anemia, leukopenia, and thrombocytopenia. The disease is currently epidemic in regions of the Sudan and India as well as selected foci in other parts of the world. In eastern India (Bihar state), the situation is becoming more and more severe with an official estimate of 430 000 cases over the past 11 years, although the actual number is believed to be at least 5 times as great (*1*). As such, Bihar state is estimated to carry the burden of about half of the world's annual cases of visceral leishmaniasis. The vast majority of experimental studies involving *L. donovani* have been confined to a small number of 'prototypic' strains derived from East Africa, and the possibility that Indian isolates might possess unique biological characteristics has not been seriously addressed.

Leishmania parasites have a digenetic life cycle alternating between a flagellated promastigote stage in the sand fly and an aflagellated amastigote stage within the mammalian macrophages (*2*). The life cycle of the parasite within the sand fly also includes development of promastigotes into a stage that is uniquely adapted for life in the vertebrate. Sequential development of promastigotes from a dividing, noninfective or procyclic stage to a nondividing, infective or metacyclic stage has been observed for promastigotes growing both within the sand fly midgut and in axenic culture. Metacyclic promastigotes display increased resistance to certain microbicidal mechanisms, including complement-mediated lysis, and the oxygen-dependent and oxygen-independent leishmanicidal activities of their host macrophages. In addition, studies in *L. major* and *L. donovani* have demonstrated that the procyclic and metacyclic promastigote stages behave differently within the sand fly (*3, 4*). Procyclic promastigotes attach to the epithelial cells lining the midgut, and this allows the parasite to avoid excretion along with the digested blood meal (*3, 4*). In contrast, metacyclic promastigotes lose this capacity, thereby permitting the detachment and anterior migration of infective forms so that they can be transmitted when the fly takes another bloodmeal (*5*). Metacyclic promastigotes are thus well adapted for both arrival at and survival in the vertebrate.

Lipophosphoglycan (LPG),[1] the major surface glycoconjugate of promastigotes, plays key roles in mediating specific host−parasite interactions, such as protecting the different

† This investigation received financial support from National Institutes of Health Grant AI20941.

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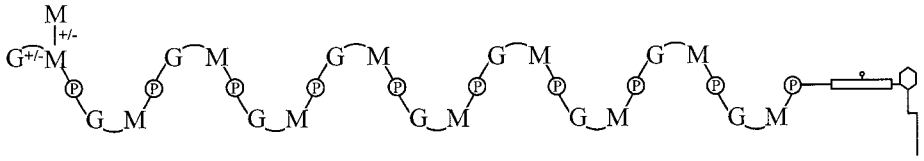
[1] Abbreviations: LPG, lipophosphoglycan; PG, phosphoglycan; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; MALDI, matrix-assisted laser desorption ionization; HPAEC, high-pH anion exchange chromatography; PMAA, partially methylated alditol acetate; GC-MS, gas liquid chromatography−mass spectrometry; FACE, fluorophore-assisted carbohydrate electrophoresis.

10.1021/bi990741g CCC: $18.00 © 1999 American Chemical Society
Published on Web 07/16/1999

LPG from procyclic Sudanese *L. donovani*



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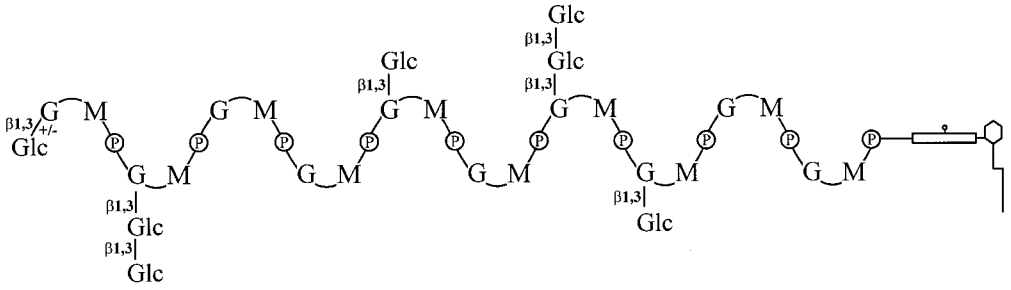

LPG from metacyclic Indian *L. Donovani*

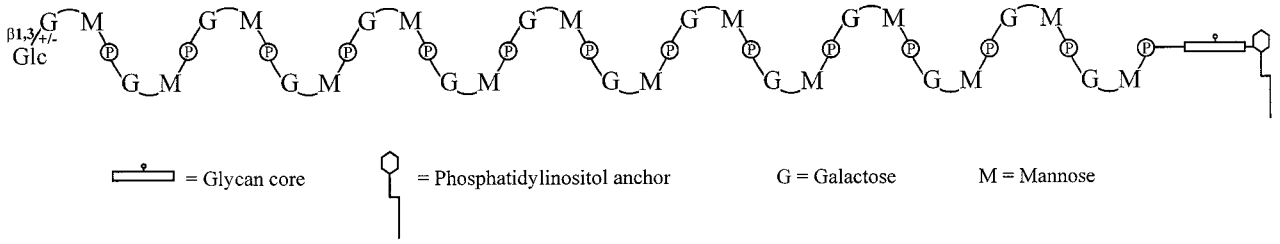

FIGURE 1: Schematic diagram of LPG from procyclic Sudanese *L. donovani* and procyclic and metacyclic Indian *L. donovani*. These structures are modified from Butcher et al. (1996) (*7*). The structure of the glycan core is Gal(α1,6)Gal(α1,3)Gal$_f$(*β*1,3)[Glc(α1-PO$_4$)-6]-Man(α1,3)Man(α1,4)GlcN(α1,6) linked to 1-*O*-alkyl-2-lysophosphatidylinositol anchor. The repeating units contain -6-Gal(*β*1,4)Man(α1)-PO$_4$. The precise location of the Glc side chains in the repeating unit domain is not known.

developmental stages of the parasite in the hostile host environments (*2*). LPG forms a dense glycocalyx that covers the entire cell surface and contains the carbohydrate motif (specific for the particular species of *Leishmania*) necessary for attachment to the sand fly midgut (*4, 6*). *Leishmania* strains and mutants deficient in LPG biosynthesis are unable to successfully infect sand flies (*6, 7*).

The structure of LPG has been described for numerous species (*8*−*12*). All LPGs contain a conserved glycan core region of Gal(α1,6)Gal(α1,3)Gal$_f$(*β*1,3)Man(α1,3)Man(α1,4)-GlcN(α1) linked to a 1-*O*-alkyl-2-lysophosphatidylinositol anchor. The polymorphisms among the species lie in the repeating units and cap structures. The backbone of LPG consist of multiple repeat phosphodisaccharide units of -6Gal(*β*1,4)Man(α1)-PO$_4$- that are either unsubstituted in Sudanese *L. donovani* (Figure 1) and *L. braziliensis* (*9, 12*), substituted at the C-3 position of the Gal in *L. major* and *L. mexicana* (*8, 10*), or substituted at the C-2 position of the Man in *L. aethiopica* (*11*). The nonreducing terminus of LPG is capped with mannose-containing structures which vary quantitatively and qualitatively among species (*8*−*12*).

By controlling the extent of binding to lectins or lectin-like receptors present on the midguts of different *Phlebotomine* vectors, the inter-species variations in LPG structure have been implicated in the species specificity of vectorial competence that is observed in nature (*11*). For example, binding of promastigotes to midguts of *P. papatasi*, which is a natural vector of *L. major* but a nonpermissive vector for all other species of *Leishmania*, requires expression of LPG containing Gal-terminating side chains, which are abundantly expressed by the procyclic repeating units of *L. major* but not of other *Leishmania* species (*3*). Furthermore, developmentally regulated polymorphisms in LPG structure appear to control the stage-specificity of midgut adhesion. In the transition of *L. major* promastigotes from procyclic to metacyclic forms, LPG repeating units approximately double in number and terminate with Ara, which masks the Gal-binding moiety (*13*). In contrast to *P. papatasi*, the receptors in *P. argentipes* for Sudanese *L. donovani* recognize the terminal α-Man- and *β*-Gal-containing cap structures (*4*). During metacyclogenesis, an increased number of repeating units causes the cap to become cryptic, thus losing the epitope for binding to the midgut epithelium of this vector species (*4*).

In this study, we characterize for the first time the LPG structure of a *L. donovani* strain from India. In contrast to the previously described Sudanese strain (*9*), the Indian LPG contains Glc side chains which branch off the repeating unit backbone and terminate the cap structure. In addition to these intraspecific polymorphisms, metacyclogenesis is associated with a novel form of structural modification involving down-regulation of side chain biosynthesis. The function of these

structural modifications is investigated in the context of stage-specific midgut attachment.

EXPERIMENTAL PROCEDURES

Materials. Materials were obtained as follows: Dulbecco's modified Eagle's medium from Gibco Life Technologies; Bacto-Brain Heart Infusion from Difco Laboratories (Mauston, WI); fetal bovine serum from Atlanta Biologicals; AG50W-X12 cation-exchange resin, AG1-X8 anion-exchange resin, and Bio-Gel P2 resin from Bio-Rad (Hercules, CA); phenyl-Sepharose CL-4B, octyl-Sepharose, adenosine, PI-specific phospholipase C from *Bacillus cereus*, alkaline phosphatase (*Escherichia coli*), β-glucosidase (sweet almond), β-galactosidase (*E. coli*), α-glucosidase (brewers' yeast), and α-mannosidase (jack beans) from Sigma; Sephadex G-150 from Pharmacia Biotech, Inc.; C-18 Sep-Pak columns from Millipore; and [^{3}H]galactose from American Radiolabeled Chemicals (St. Louis, MO).

Cell Culture. L. donovani strain 1S from Sudan (MHOM/SD/00/1S-2D) and *L. donovani* Mongi strain from India (MHOM/IN/83/Mongi-142) were originally isolated from bone marrow biopsies of patients with visceral leishmaniasis. Starter cultures of promastigotes were grown in Dulbecco's modified Eagle's medium supplemented as described (*14*) with 10% fetal bovine serum at 25 °C. For isolation and purification of large amounts of LPG, 1 L cultures of Brain Heart Infusion supplemented with adenosine (27 mg/L) and hemin (5 mg/L) were seeded with 100 mL of starter cultures. Cells were grown at room temperature in an incubator shaker to a density of $(7-8) \times 10^7$ cells/mL (*15*).

Purification of Metacyclic Promastigotes. The monoclonal antibody MG1, which recognizes an epitope on LPG that is specifically expressed by logarithmic phase promastigotes of Indian *L. donovani* strains (Saraiva et al., manuscript in preparation), was used for purification of metacyclic promastigotes by negative selection. The Indian strain promastigotes were grown in medium 199 supplemented with 20% heat-inactivated fetal calf serum, penicillin (100 units/mL), streptomycin (50 μg/mL), and 12.5 mM glutamine, 40 mM Hepes, pH 7.4, 0.1 mM adenine, and 0.0005% hemin, a medium which favors the generation of a higher number of metacyclic promastigotes during growth. Promastigotes from 5−6 day stationary cultures were washed in DMEM and resuspended to $(1-2) \times 10^8$/mL, and MG1 ascites was added to a final dilution of 1:200. After a 30 min incubation at room temperature, agglutinated parasites were removed by low-speed centrifugation (150*g*, 5 min), and the metacyclic cells remaining in the supernatant were washed 2 times by centrifugation at 2100*g* for 15 min at 4 °C with DMEM.

Metabolic Labeling of LPG. Promastigotes were radiolabeled during the appropriate phase of growth at 1.5×10^9 cells/mL with 90 μCi/mL D-[6-^{3}H]Gal at 27 °C for 6 h; the pH was maintained with the addition of sodium bicarbonate (*15*).

Extraction and Purification of LPG. LPG was organically extracted from other lipids and glycosylated products and solubilized in solvent E (H_2O/ethanol/ethyl ether/pyridine/NH_4OH; 15:15:5:1:0.017) as previously described (*15*). The solvent E extract was dried by N_2 evaporation, resuspended in 0.1 N acetic acid/0.1 M NaCl, and applied to a column of phenyl-Sepharose (2 mL), equilibrated in the same buffer. LPG was eluted using solvent E (*15*). Large preparations of LPG were extracted as described (*8*) with modifications. Cells were extracted with $CHCl_3$/MeOH (1:2) followed by three extractions with 9% 1-propanol. LPG was purified on successive columns of octyl-Sepharose with a 5-60% 1-propanol gradient followed by a 20−60% gradient as described (*8*).

Size Selection of Phosphoglycan. [^{3}H]Gal-radiolabeled LPG was purified and delipidated by nitrous acid deamination (300 μL of 0.25 M sodium acetate and 300 μL of 0.5 M $NaNO_2$) for 16 h at 37 °C (*16*). Samples were resuspended in 40 mM NH_4OH and 1 mM EDTA and applied to a Sephadex G150 column (1 cm × 88 cm) which was equilibrated in the same buffer (*4*). The eluent was collected in 0.5 mL fractions. To prepare PG for binding studies, high molecular weight PG was pooled from the largest 40% of the size-fractionated PG, whereas low molecular weight PG was the smallest 40%.

Preparation of Repeating Units and Caps. Purified LPG was subjected to mild acid hydrolysis (0.02 N HCl, 5 min, 100 °C) to depolymerize the repeating units and cap structures. Water-soluble fractions were partitioned using 1-butanol (*17*), diluted to 1 mL in water, and applied to a reverse-phase Sep-Pak C18 column equilibrated in water to remove any unhydrolyzed LPG which bound to the column (*18*). Caps were separated from repeating units by applying the sample to a column of AG1-X8 (acetate) in water. Repeating units were collected with 0.3 M NaCl and desalted over a column of Bio-Gel P2 resin (1 cm × 27 cm) run in water.

Isolation of Core. Following mild acid hydrolysis, fractionated LPG was resuspended in 0.1 M NaCl/0.1 N acetic acid and applied to a column of phenyl-Sepharose (2 mL) (*15*). Core-PI was eluted with solvent E (*15*) and delipidated by nitrous acid for 16 h at 37 °C (*16*), and the lipid was removed by partitioning with 1-butanol (*17*).

Enzymatic Treatments. Neutral oligosaccharides were treated with sweet almond β-glucosidase in 200 mM ammonium acetate buffer, pH 5.0 (1 unit, 16 h, 37 °C), brewer's yeast α-glucosidase in 1 M Hepes, pH 6.8 (1 unit, 16 h, 37 °C), *E. coli* β-galactosidase in 80 mM Na_3PO_4, pH 7.3 (4 units, 16 h, 37 °C), or jack bean α-mannosidase in 0.1 M sodium acetate, pH 4.5 (1 unit, 16 h, 37 °C, followed by 1 unit, 16 h, 37 °C). Phosphorylated oligosaccharides were treated with alkaline phosphatase in 15 mM Tris buffer, pH 9.0 (1 unit, 16 h, 37 °C) (*14*). Purified LPG was treated with phospholipase C from *B. cereus* for 16 h at 37 °C (*15*).

FACE Analysis. Repeating units were purified as described above and dephosphorylated using alkaline phosphatase (*19*). Both caps and repeating units were treated with various exoglycosidases, and salt was removed by passage through a two-layered column of AG50W-X12 over AG1-X8 (acetate) (*4*). Samples were fluorescently labeled and subjected to electrophoresis according to the manufacturer's specifications (GLYKO-FACE electrophoresis products) (*7*). The GLYKO UV imager was used for visualization.

Descending Paper Chromatography. Purified, dephosphorylated repeating units radiolabeled with [^{3}H]Gal were desalted and applied to Whatman 3-mm chromatography paper. The samples were subjected to descending chromatography equilibrated with 1-butanol/pyridine/H_2O (6:4:3) for 40 h and analyzed as described previously (*7*).

Methylation Analysis. Neutral oligosaccharides were methylated following the method as described (*20*) with modifications (*21*). The partially methylated samples were further derivatized as described (*22*). The partially methylated alditol acetates were analyzed on a Hewlett-Packard 5980-MSD system with a Supelco 2380 column (0.25 mm × 30 m). The oven temperature was held at 80 °C for 1 min and then raised from 80 to 140 °C at 15 °C/min. The temperature was then raised from 140 to 250 °C at 5 °C/min.

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. Neutral glycans were subjected to methylation. 2,5-Dihydroxybenzoic acid matrix was prepared and mixed with the sample (*23*) with the addition of KCl. Samples were run on an Ionspec 4.7 T FT/MS with an external MALDI source. The samples were irradiated with UV light (337 nm) from an N_2 laser. An external calibration of PEG was used for mass assignments.

Lipid Analysis. The lipid anchor of LPG was cleaved with phospholipase C and extracted as described previously (*15*). The product was analyzed by thin-layer chromatography on Silica Gel 60 plates using $CHCl_3/CH_3OH$ (19:1.5 v/v) and visualized in an iodine chamber (*15*).

Quantification of Repeating Units. The number of repeating units per LPG molecule was determined as described previously (*7*). The samples were analyzed using GC-MS. The oven temperature was held at 80 °C for 1 min and then raised from 80 to 215 °C at 15 °C/min. The temperature was then raised from 215 to 250 °C at 5 °C/min and held for 5 min.

High-Performance Liquid Chromatography. Both phosphorylated and neutral oligosaccharides were separated using a DX-500 HPLC from Dionex Corp. with ED40 electrochemical detection. The samples were run on a CarboPac PA1 column (4 × 250 mm). For neutral oligosaccharides, program A was used. Program A started with 100% buffer A (150 mM NaOH) for 20 min followed by a linear gradient for 35 minutes to 60% buffer A and 40% buffer B (500 mM sodium acetate/150 mM NaOH). Phosphorylated samples were isolated using program B. The program started with 75% buffer A and 25% buffer B for 20 min followed by a linear gradient for 10 min to 65% buffer A and 35% buffer B. Next, a slower gradient was used for 20 min ending with 60% buffer A and 40% buffer B. The column was rinsed with 100% buffer B. The flow rate was 1 mL/min.

Midgut Binding Studies. Phlebotomus argentipes sand flies were reared and maintained in the Department of Entomology at Walter Reed Army Institute of Research. Binding of promastigotes to sand fly midguts was quantitated by a modification of an in vitro technique previously described (*6*). Three to five day old nonfed female sand flies, maintained on 30% sucrose solution, were dissected in PBS. Heads, crops, hindguts, and Malpighian tubules were removed, and the isolated midguts were opened along the length of the abdominal segment with a fine needle. Midguts were placed in the concave wells of a microscope chamber slide. Promastigotes (2.0×10^6 or 10^7 cells/mL) in a total volume of 50 μL were added to the guts and incubated for 45 min at room temperature. The guts were then individually washed by placing them in successive drops of PBS. Guts were homogenized and released promastigotes counted as described above. Statistics (*p* values) were obtained from Student's *t*-test for paired samples. For binding of purified PG to midguts in vitro, opened, dissected midguts were fixed with 2% formaldehyde in PBS, at 4 °C for 20 min. After several washes in PBS, they were incubated for 45 min with either high molecular weight PG (50 μg/mL) or low molecular weight PG (10 μg/mL). After several washes, the guts were incubated in a 1:200 dilution of ascites containing the anti-LPG monoclonal antibody 45D3 (*6*) followed by incubation with fluorescein anti-mouse IgG. Stained guts were examined microscopically under ultraviolet and bright field exposures.

Table 1: Mass Determinations of Caps and Repeating Units Using MALDI-MS[a]

Dionex peak	observed *m/z* $(M+Na)^+$ ions	calculated *m/z* $(M+Na)^+$ ions	observed *m/z* $(M+K)^+$ ions	calculated *m/z* $(M+K)^+$ ions
N1	477.24	477.23	493.21	493.21
N2	681.33	681.33	697.31	697.31
P1	477.24	477.23	493.21	493.21
P2	681.34	681.33	697.31	697.31
P3	885.44	885.43	901.41	901.40

[a] The peaks designated in Figure 3 were analyzed by MALDI-MS and resulted in the observed *m/z* values. The *m/z* values are compared to calculated *m/z* values of methylated saccharides.

Table 2: PMAA Analysis of Caps and Repeating Units Using GC-MS

Dionex peak	PMAA derivative	origin
N1	2,3,4,6-Me_4Gal	terminal Gal
	2,3,6-Me_3Man	4-O-substituted Man
N2	2,3,4,6-Me_4Glc	terminal Glc
	2,4,6-Me_3Gal	3-O-substituted Gal
	2,3,6-Me_3Man	4-O-substituted Man
P1	2,3,4,6-Me_4Gal	terminal Gal
	2,3,6-Me_3Man	4-O-substituted Man
P2	2,3,4,6-Me_4Glc	terminal Glc
	2,4,6-Me_3Gal	3-O-substituted Gal
	2,3,6-Me_3Man	4-O-substituted Man
P3	2,3,4,6-Me_4Glc	terminal Glc
	2,4,6-Me_3Glc	3-O-substituted Glc
	2,4,6-Me_3Gal	3-O-substituted Gal
	2,3,6-Me_3Man	4-O-substituted Man

RESULTS

Purification of LPG from an Indian isolate of *L. donovani* generated approximately 1 mg of LPG per 10^{10} cells. The schematic for isolation of the LPG fragments is shown in Figure 2. Depolymerization of LPG with mild acid resulted in two pools: a core-PI fraction and a mixture of repeating units and caps.

Neutral Caps. Individual caps were isolated by chromatographing the cap mixture on HPAEC using program A (Figure 3A). The first peak (N1) comigrated with standard Gal(β1,4)Man, and the second peak (N2) did not migrate with any previously characterized cap structure. Individual caps were methylated and mixed with 2,5-dihydroxybenzoic acid and KCl for MALDI analysis. The smaller cap N1 gave an $(M+Na)^+$ molecular ion at *m/z* 477.24 and an $(M+K)^+$ peak at 493.21 which correlated to the calculated mass for a methylated disaccharide (Table 1). PMAA derivatization of N1 followed by analysis on GC-MS indicated a structure of Gal*p*(1,4)Man (Table 2). FACE analysis of N1 also demonstrated that it comigrated with standard Gal(β1,4)-

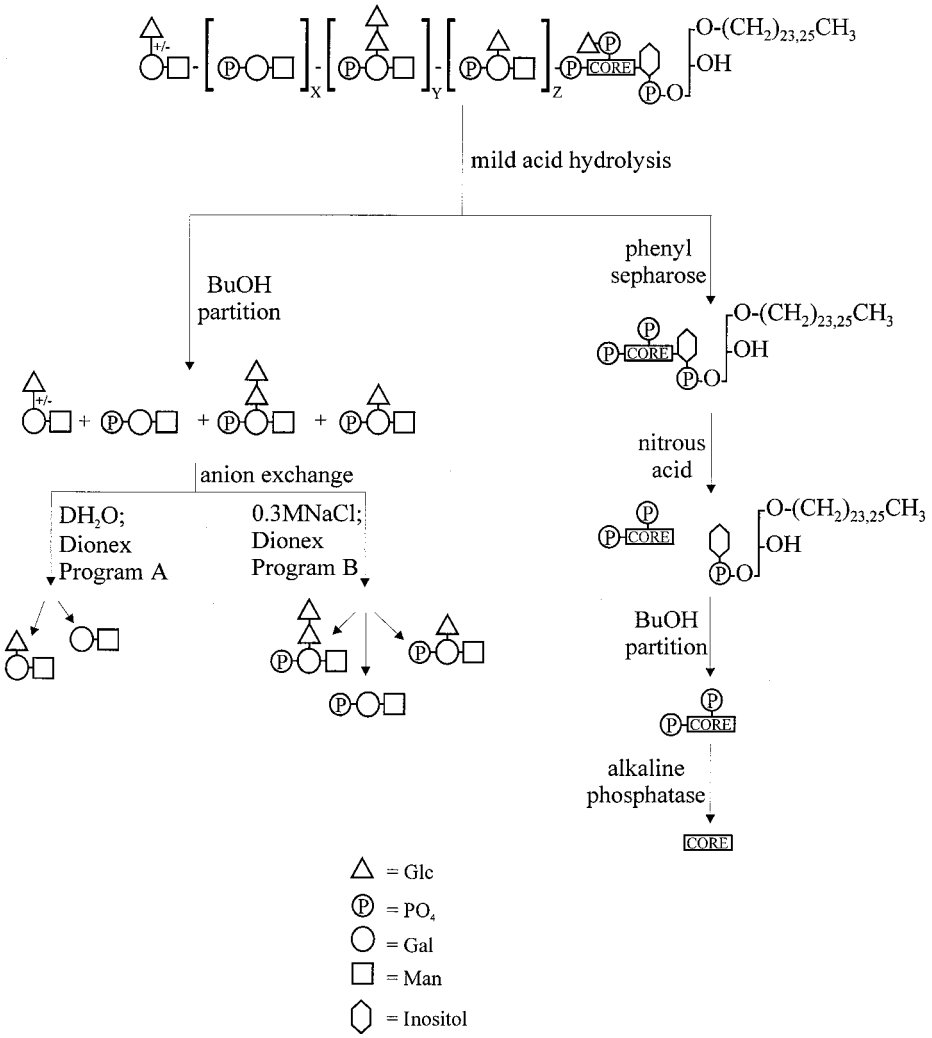

FIGURE 2: LPG fractionation scheme. Mild acid hydrolysis (0.02 N HCl, 5 min, 100 °C) releases neutral and phosphorylated glycans in addition to the core-PI anchor. The latter was separated from the sample with butanol extraction, and the remaining fragments were resolved into neutral and phosphorylated pools using an anion exchange column. Each of these pools was further resolved using HPAE chromatography. The core-PI was purified over a phenyl-Sepharose column followed by cleavage of the lipid with nitrous acid. The core was isolated in the aqueous phase of butanol extraction and analyzed following alkaline phosphatase treatment.

Man (Figure 4A, lanes 2 and 6). N1 is susceptible to β-galactosidase but not α-mannosidase or β-glucosidase (Figure 4A, lanes 3−5). These results demonstrated that N1 has the structure Gal(β1,4)Man.

MALDI analysis of N2 gave an $(M+Na)^+$ molecular ion at *m*/*z* 681.33 and an $(M+K)^+$ peak at 697.31 which precisely agree with the calculated values for a methylated trisaccharide (Table 1). PMAA analysis demonstrated that N2 had a structure of Glc*p*(1,3)Gal*p*(1,4)Man (Table 2). Glucose has not previously been reported in a cap structure of LPG. These observations were confirmed by enzymatic digestions using α-mannosidase, β-galactosidase, and α- and β-glucosidase with FACE analysis (Figure 4B). N2 was susceptible only to β-glucosidase (Figure 4B, lane 6). Sequential digestions demonstrated that following β-glucosidase treatment, the product was further cleaved by β-galactosidase (Figure 4B, lane 7). These results indicated a structure of Glc(β1,3)Gal-(β1,4)Man for N2. A possible additional cap (Figure 3A, retention time of 16.98 min) was present in this LPG but was not fully characterized due to its low abundance (<5% of total caps). Nevertheless, MALDI analysis suggested that this additional cap was a tetrasaccharide (data not shown).

Phosphorylated Repeating Units. Purified, phosphorylated repeating units were resolved into three different units on HPAEC using program B (Figure 3B). The first peak (P1) comigrated with standard PO_4-Gal(β1,4)Man. The other two peaks (P2 and P3) were larger in size. For further characterization, the repeating units were dephosphorylated with alkaline phosphatase and then desalted via passage over a column of AG1-X8 (acetate) and AG50W-X12. The smallest repeating unit, dephosphorylated P1, was analyzed with MALDI and yielded a $(M+Na)^+$ molecular ion at *m*/*z* 477.24 and an $(M+K)^+$ peak at 493.21 characteristic of a methylated disaccharide (Table 1). PMAA analysis demonstrated that dephosphorylated P1 contained Gal*p*(1,4)Man (Table 2). Further, dephosphorylated P1 comigrated with standard Gal-(β1,4)Man on FACE analysis and was cleaved by β-galactosidase (Figure 5, lanes 3, 6 and 7). These results confirmed the structure of P1 as PO_4-Gal(β1,4)Man.

Dephosphorylated P2 was deduced as a trisaccharide by MALDI analysis which yielded a $(M+Na)^+$ molecular ion at *m*/*z* 681.34 and a $(M+K)^+$ peak at 697.31 (Table 1). PMAA analysis demonstrated that dephosphorylated P2 contained [Glc*p*(1,3)]Gal*p*(1,4)Man (Table 2). Enzymatic

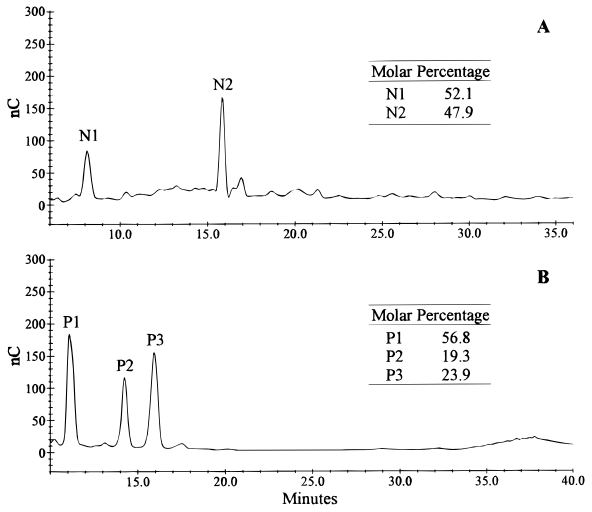

FIGURE 3: HPAEC fractionation of neutral caps and phosphorylated repeating units. LPG (3 mg) was depolymerized by mild acid hydrolysis, and the core-PI portion was removed. After isolation of neutral and phosphorylated oligosaccharides over an anion exchange column, the neutral fractions were chromatographed on HPAEC using gradient program A (panel A), while the phosphorylated sample was analyzed with gradient program B (panel B). Neutral (N) and phosphorylated (P) fractions designated by the marked peaks were pooled for further analysis. Molar percentages were calculated based on comparing peak areas with Gal(β1,4)-Man and Glc standards.

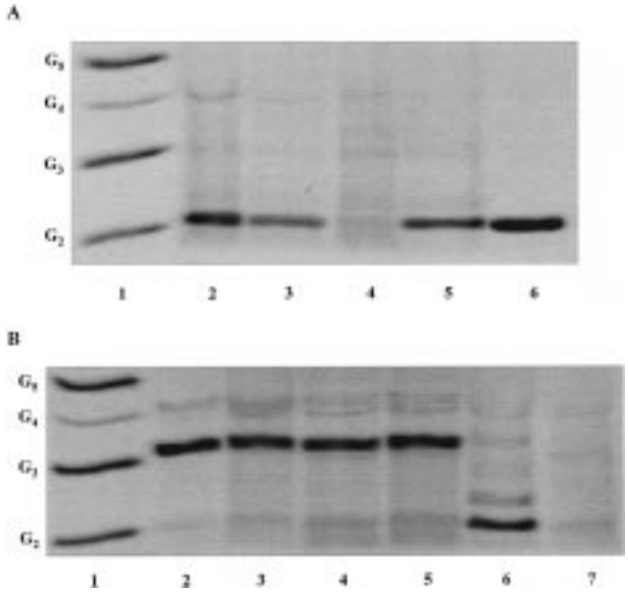

FIGURE 4: FACE analysis of exoglycosidase-treated neutral fragments. LPG (1 mg) was depolymerized with mild acid, and caps were isolated following anion exchange chromatography and HPAEC. Panel A: lane 1, oligoglucose ladder represented by G_2-G_5; lane 2, untreated N1; lanes 3−5, N1 treated with α-mannosidase, β-galactosidase, and β-glucosidase, respectively; lane 6, standard Gal(β1,4)Man. Panel B: lane 1, oligoglucose ladder represented by G_2-G_5; lane 2, untreated N2; lanes 3−6, N2 treated with α-mannosidase, β-galactosidase, α-glucosidase, and β-glucosidase, respectively; lane 7, N2 treated with β-glucosidase followed by β-galactosidase.

digestions with β-galactosidase and α- and β-glucosidase showed that dephosphorylated P2 contains a terminal β-glucose at its nonreducing end (Figure 5, lanes 3−7). These data indicated a branched trisaccharide structure of PO_4-[Glc(β1,3)]Gal(β1,4)Man for P2.

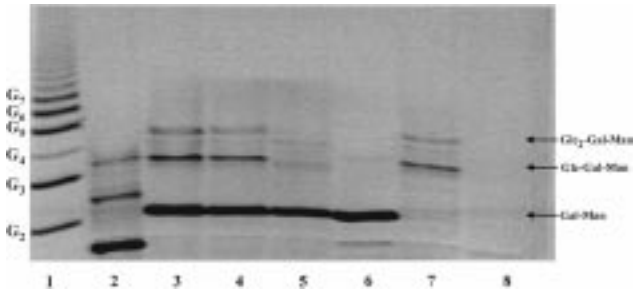

FIGURE 5: FACE analysis of exoglycosidase-treated phosphorylated fragments. LPG (1 mg) was depolymerized with mild acid, and the repeating units were collected from an anion exchange column by batch elution with 0.3 M NaCl. Lane 1, oligoglucose ladder represented by G_2-G_7; lane 2, untreated repeating units; lane 3, repeating units dephosphorylated with alkaline phosphatase; lanes 4, 5, and 7, repeating units treated first with alkaline phosphatase and then with α-glucosidase, β-glucosidase, or β-galactosidase, respectively; lane 6, standard Gal(β1,4)Man lane 8, standard Gal-(β1,4)Man treated with β-galactosidase.

The third repeating unit, P3, was the largest in size as shown by MALDI analysis. Dephosphorylated P3 had a $(M+Na)^+$ molecular ion at *m/z* 885.44 and an $(M+K)^+$ peak at 901.41 (Table 1), indicating that P3 was a tetrasaccharide. PMAA analysis demonstrated that this repeating unit contained Glc*p*(1−3)Glc*p*(1−3)Gal*p*(1−4)Man (Table 2). Treatment of dephosphorylated P3 with β-glucosidase resulted in the generation of Gal-Man (Figure 5, lane 5). These results established a branched tetrasaccharide structure of P3 as PO_4-[Glc(β1,3)Glc(β1,3)]Gal(β1,4)Man.

Core-PI Anchor. Following depolymerization of purified LPG with mild acid, the core-PI was isolated by hydrophobic chromatography on phenyl-Sepharose. The core-PI was then delipidated with nitrous acid and dephosphorylated with alkaline phosphatase (Figure 2), and analyzed using FACE. The resulting band migrated identically on the gel with nitrous acid-treated Gal(α1,6)Gal(α1,3)Gal_f(β1,3)Man(α1,3)-Man(α1,4)GlcN−PI isolated from Sudanese *L. donovani* LPG (data not shown). Consistent with the conserved glycan core region of all LPGs (*8*−*12*), this result indicated that the core region of LPG did not vary in the Indian strain of *L. donovani*. For analysis of the lipid anchor, LPG was treated with PI-specific phospholipase C (*B. cereus*). The lipid anchor was extracted with $CHCl_3/CH_3OH$ (19:1.5, v/v) followed by addition of $CHCl_3$ and 1 N HCl. This product was compared to the Sudanese *L. donovani* lipid anchor extracted in the same manner. Both samples were loaded on a Silica TLC plate and chromatographed with a solvent of $CHCl_3/CH_3OH$ (19:1.5 v/v). Each sample yielded two spots indicative of the previously characterized 1-*O*-alkyl-2-lysoglycerol (*8*−*11*) containing either C_{24} or C_{26} as the aliphatic substituent (data not shown).

To summarize the above structural information, the Indian *L. donovani* LPG structure differed from the previously characterized Sudanese *L. donovani* LPG by β1,3-glucosylation of almost half of the repeating Gal(β1,4)Man units and caps, while the core and PI anchor were conserved (Figure 1).

Modifications of LPG Structure during Metacyclogenesis. The extracellular growth of *Leishmania* within their sand fly vectors and within in vitro culture is accompanied by their differentiation to a stage which is uniquely adapted for

survival within the vertebrate host. During this process of metacyclogenesis, in which *Leishmania* promastigotes convert from a noninfectious, procyclic form to an infectious, metacyclic form, the LPG from other species or strains (such as in *L. donovani* from Sudan) undergoes modifications in size and structure (*4*). To examine the relative size of both procyclic and metacyclic Indian *L. donovani* LPG, the LPGs were extracted from logarithmic phase promastigotes and metacylic promastigotes purified from stationary phase cultures of parasites growing within the M199 differentiation medium. The number of metacyclic cells that are generated during the growth of this species is typically fewer than 5% of the total number of cells in a stationary phase culture. After delipidation with nitrous acid followed by reduction, the resultant phosphoglycans possess a single 2,5-anhydromannitol at the reducing end due to deamination of the glucosamine residue. Since each repeating unit of LPG contains one Man residue, the number of repeating units per LPG molecule can be deduced by comparing the molar ratio of Man to 2,5-anhydromannitol. Thus, the phosphoglycans were subjected to strong acid hydrolysis, and the molar ratio of Man to 2,5-anhydromannitol was determined by GC-MS. The ratio for procyclic Indian LPG was 17 while the metacyclic LPG was 36. By comparison, earlier work demonstrated the number of repeating units in Sudanese *L. donovani* LPG to be approximately 16 for procyclic and 30 for metacyclic molecules (*19, 24*).

An additional developmental modification of the structure of the Indian LPG was elucidated upon analysis of the repeating units. The HPAEC and FACE analyses of the procyclic LPG repeating structures could not be performed on metacyclic LPG due to the difficulty in obtaining a sufficient amount for chemical analysis. Therefore, for these comparisons, [^{3}H]Gal-labeled LPG was analyzed. The radioactive LPG was prepared from unselected stationary growth phase promastigotes. The samples were delipidated by nitrous acid and applied to a column of Sephadex G150 (Figure 6). Although the stationary phase profile (Figure 6B) contained mostly PG of a similar size to the PG extracted from logarithmic phase promastigotes (Figure 6A), approximately 15% of the material was of a higher molecular weight that eluted near the column void volume. To investigate the composition of this material, the indicated fractions were combined into three pools (Pool A, fractions 43−58; Pool B, fractions 60−68; and Pool C, fractions 70−90). Each pool was treated with mild acid and alkaline phosphatase to isolate the repeating units, applied to a descending paper chromatogram, and developed for 30 h in butanol/pyridine/water (6:4:3). The largest molecular weight pool consisted of only Gal-Man (Figure 7, Pool A, peak b) and residual Glc (Figure 7, Pool A, peak a). The latter monosaccharide arose from mild acid cleavage of the Glc-(α-1)PO_4 present in the core region and a small percentage of cleavage of O-glycosidically linked Glc bonds in the side chains. Pool B contained two larger peaks corresponding to Glc-Gal-Man (Figure 7, Pool B, peak c) and Glc-Glc-Gal-Man (Figure 7, Pool B, peak d). Pool C had an even larger percentage of the two glucosylated repeating units (Figure 7, Pool C, peaks c and d). Thus, the largest size phosphoglycan chains are associated with down-regulation of glucose side chains in their biosynthesis.

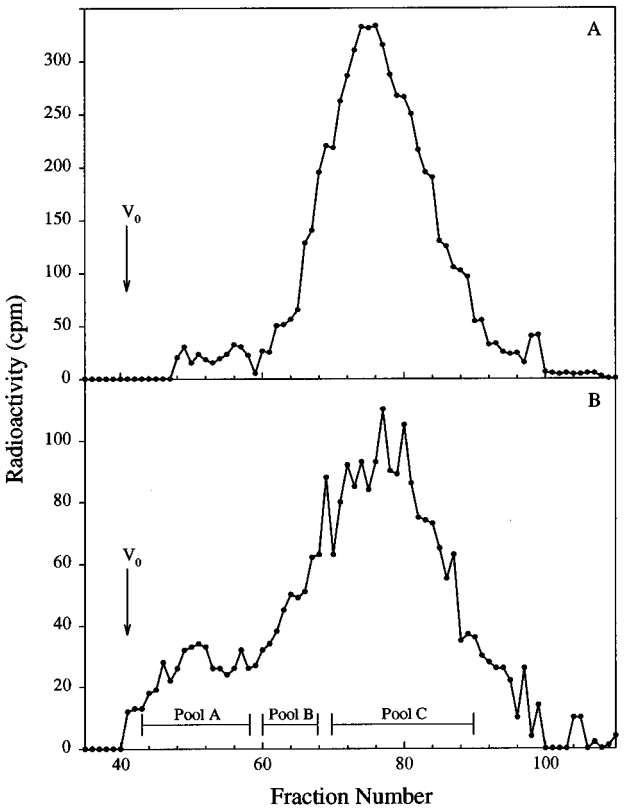

FIGURE 6: Sephadex G150 gel filtration chromatography of log and stationary phase LPG. log (panel A) and stationary (panel B) phase LPGs were radiolabeled with [^{3}H]Gal and delipidated with nitrous acid. The resulting PGs were applied to a G150 column. The eluent was collected, and an aliquot was counted before the indicated fractions were pooled.

To confirm that the repeating unit structures of the high and low molecular weight PGs prepared from unselected stationary phase promastigotes reflected the respective structures of metacyclic and procyclic PGs, the paper chromatographic analysis was repeated on [^{3}H]Gal-labeled PG prepared from logarithmic phase promastigotes and from metacyclics purified from stationary phase cultures using the MG1 antibody. As shown in Figure 8, the major ^{3}H-labeled fragments of procyclic promastigotes comigrated with the disaccharide Gal-Man (closed circles, fractions 34−44), the trisaccharide Glc-Gal-Man (fractions 23−30), and the tetrasaccharide Glc-Glc-Gal-Man (fractions 16−22). In contrast, the only significant radiolabeled fragment of the metacyclic promastigotes was the disaccharide Gal-Man (Figure 8, open circles). These results confirm a lack of glucosylated repeating units in the metacyclic form of LPG.

Binding of Promastigotes and PG to Sand Fly Midgut in Vitro. The relationship between stage differentiation and midgut adhesion has been previously addressed for *L. major* and for *L. donovani* from Sudan using a quantitative in vitro assay for the attachment of living promastigotes to whole midguts (*3*). Dissected midguts from *Phlebotomus argentipes*, which is the natural vector of *L. donovani* transmission in India, were opened by an incision extending from the posterior gut to the middle of the anterior region and incubated with high and low concentrations of culture-derived procyclic or metacyclic promastigotes of the Indian *L. donovani* strain. During the incubation period, there was extensive interaction between each developmental stage and the external and luminal surfaces of the midgut epithelium.

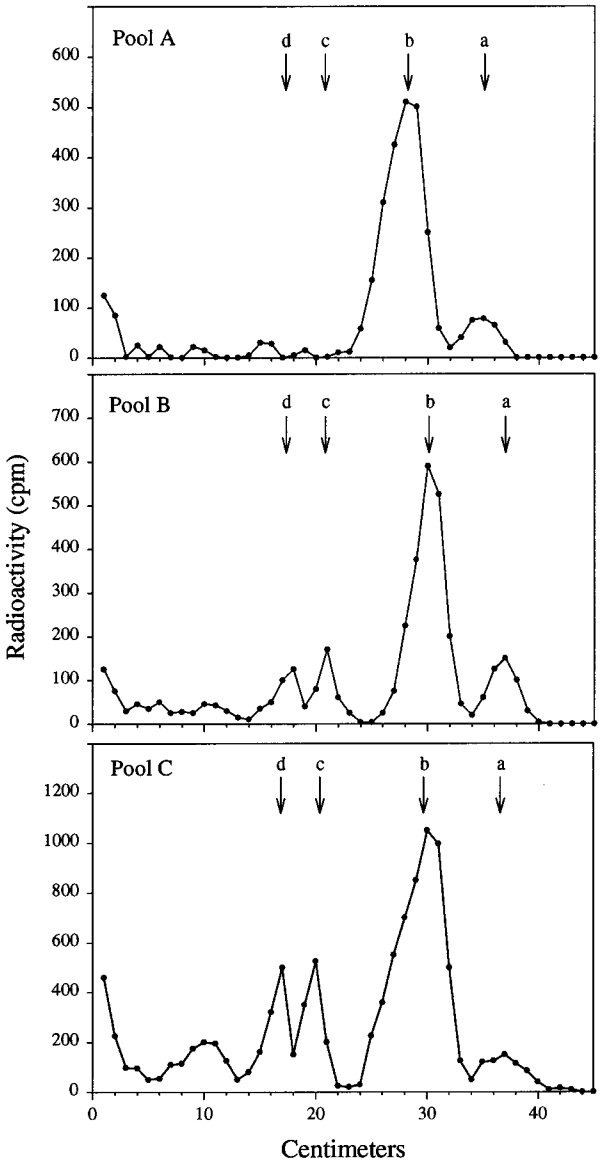

FIGURE 7: Descending paper chromatography of repeating units pooled from Sephadex G150. The pooled fractions (Figure 6B) were treated with mild acid and alkaline phosphatase, applied to a descending paper chromatogram, and developed for 30 h in butanol/pyridine/water (6:4:3). Standards: a, Glc; b, Gal-Man; c, Glc-Gal-Man; d, Glc-Glc-Gal-Man.

After washing, however, the number of procyclic promastigotes that remained bound per midgut was approximately 5-fold greater than the metacyclics for each of the two experiments shown in Figure 9. An identical pattern of stage-specific binding was observed using high (metacyclic) and low (procyclic) molecular weight PG prepared from nitrous acid delipidated LPG extracted from stationary phase promastigotes and size-fractionated over a G150 column. The PGs were incubated with dissected, opened *P. argentipes* midguts, followed by washing and immunofluorescent staining with monoclonal antibody 45D3, which recognizes an epitope common to all LPGs. Midguts incubated with procyclic PG (10 μg/mL) were intensely stained throughout the abdominal and thoracic regions of the gut (Figure 10). Midguts incubated with metacyclic PG (50 μg/mL) were stained at the above background levels in every region of the gut, though in each case the staining was poor relative to midguts incubated with procyclic PG.

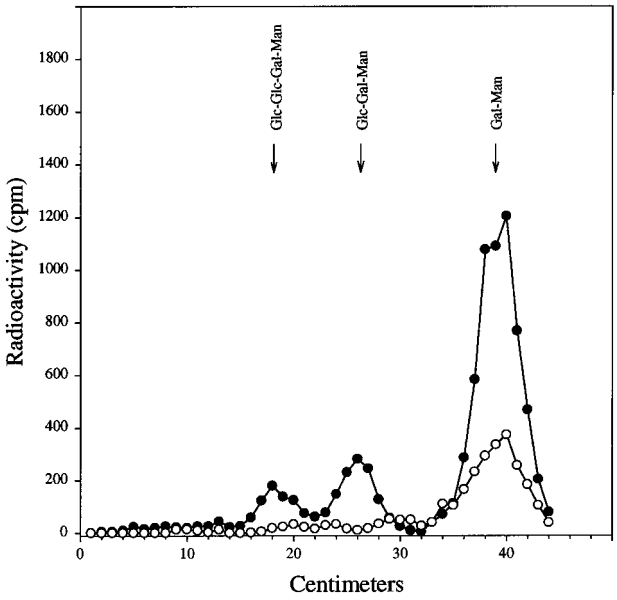

FIGURE 8: Descending paper chromatography of repeating units from procyclic and metacyclic LPGs. Procyclic (closed circles) and metacyclic (open circles) promastigotes were incubated with [^{3}H]-Gal, and the radiolabeled LPGs were extracted, purified, and depolymerized with mild acid. The [^{3}H]Gal repeating units were treated with alkaline phosphatase, and the dephosphorylated samples were chromatographed on paper using butanol/pyridine/water (6:4:3) for 40 h.

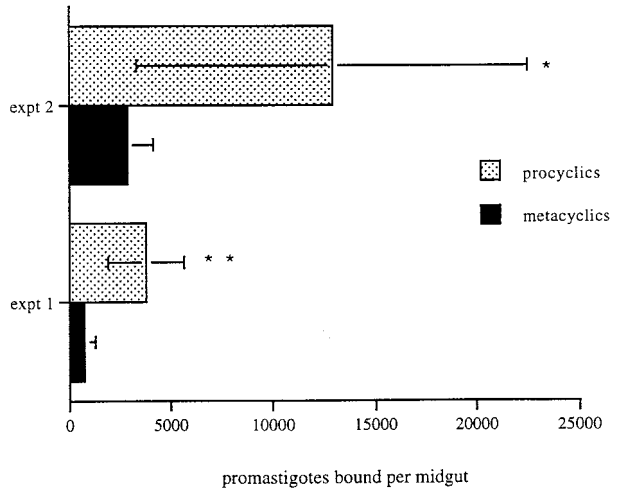

FIGURE 9: Attachment of Indian *L. donovani* to *P. papatasi* midguts in vitro. In vitro binding of the Indian *L. donovani* strain promastigotes to midguts of *P. argentipes* following 45 min incubation with either 2 million (expt 1) or 10 million (expt 2) promastigotes per milliliter. Data show the mean number of promastigotes bound ± 1 sd, 7−10 guts per group. An asterisk indicates a significant difference between procyclic and metacyclic binding (*$p < 0.05$; **$p < 0.01$).

DISCUSSION

LPG is a heterogeneous, multifunctional molecule essential for survival of *Leishmania* parasites. We have characterized both procyclic and metacyclic promastigote LPG structures for Indian *L. donovani* (Figure 1), which is responsible for one of the most severe and epidemic forms of leishmanial disease in the world today. The phosphoglycan core, the 1-*O*-alkyl-2-lysophosphatidylinositol anchor, and the -6Gal(β1,4)-Man(α1)-PO_4 repeating unit backbone are conserved with previously characterized LPG structures from many species (*8*−*12*). The heterogeneity of the multidomain molecule

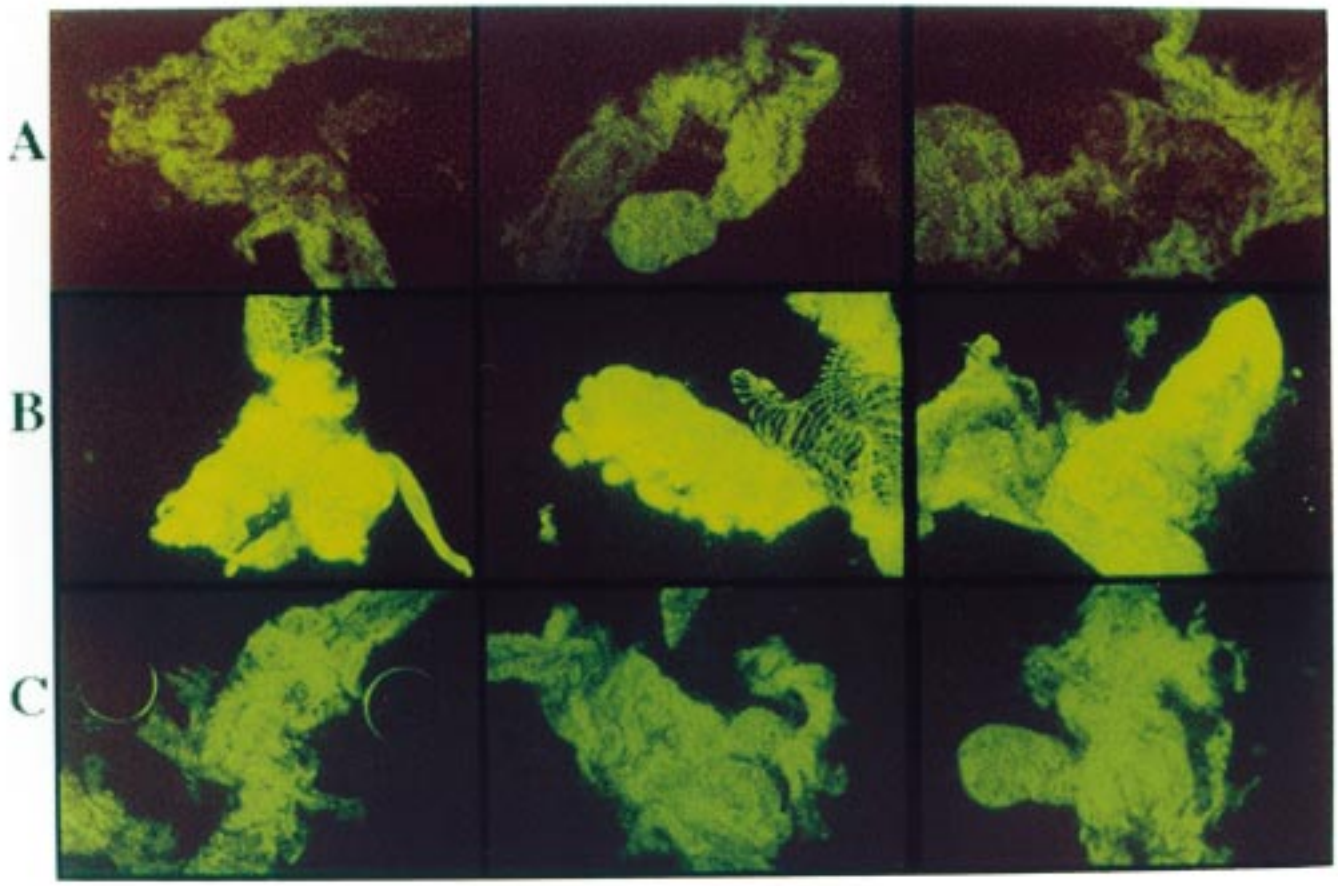

FIGURE 10: Fluorescent staining of *P. papatasi* midguts incubated with PGs from Indian *L. donovani*. Fluorescent staining of individual midguts of *P. argentipes* incubated with PG purified from (panel B) procyclic promastigotes (10 μg/mL) or from (panel C) metacyclic promastigotes (50 μg/mL). Control guts (panel A) were incubated with primary and secondary antibodies only.

resides in branching from the repeating unit backbone and the composition of the cap. The Indian repeating units either can be unsubstituted or have one or two β1,3-Glc branching from the Gal C-3 position. In contrast, the previously characterized Sudanese *L. donovani* repeating units contain no side chain branching (*19*). Most other characterized species have side chain branching, including *L. tropica* which also contains Glc in the repeating units, but they are further substituted with Ara or Gal in multiple arrangements (*11*). *L. mexicana* and *L. major* have been characterized as having a singular β-linked Glc from the repeating units with approximately 25% and 1% substitution, respectively (*8, 10*). We have demonstrated that the procyclic promastigotes of the Indian strain not only have approximately 19% singular substitution but also contain 24% of repeating units with two side chain Glc (Figure 3), not previously demonstrated in another strain. The cap of Indian *L. donovani* consists of two structures, of which the Gal(β1,4)Man structure has been reported for all species with the exception of *L. mexicana* (*8*−*12, 19*). Previously, Man and Gal were the only monosaccharides reported in cap structures. Here, we have demonstrated that the Indian strain contains a novel cap structure of Glc(β1,3)Gal(β1,4)Man. This result was not caused by contamination from repeating unit analysis because caps and repeating units were isolated over an anion exchange column with the neutral caps eluted with water.

The differences between the LPG structures of *L. donovani* strains from India and Sudan are the first, so far as we are aware, intra-species molecular differences that have been identified for these strains. It has not been possible, for example, to distinguish these strains by comparing other commonly used polymorphic markers, such as isoenzymes and kinetoplast DNA. The existence of these intra-specific LPG polymorphisms may not be so surprising given the role that LPG is thought to play in the survival of the parasite within its sand fly vector. Because of its critical role in mediating midgut adhesion, we have proposed that LPG polymorphisms are driven by the heterogeneity in parasite recognition sites expressed on midgut epithelial cells of different sand fly species. For *P. papatasi*, which transmits only *L. major*, these molecules appear to have specificity for terminal Gal-containing oligosaccharides, and only *Leishmania* species that display an abundance of Gal side chains on their surface LPG (e.g., *L. major*) will maintain infection in the *P. papatasi* midgut (*6, 7*). According to this view then, the expression of terminal Glc residues on both the capping and side chain structures potentiates the binding of Indian *L. donovani* to the midguts of a sympatric sand fly species, e.g., *P. argentipes*. In contrast, Glc-containing caps and side chains were presumably not needed to promote midgut attachment in the phlebotomine species available to *L. donovani* strains in East Africa, which rely on Man- and Gal-containing capping oligosaccharides to mediate binding (*4*). A significant difference in the binding of procyclic promastigotes of Indian and Sudanese *L. donovani* strains to *P. argentipes* midguts was not observed in our studies

(data not shown). It should be noted that the colony of *P. argentipes* used in these studies has been maintained under laboratory conditions for over 12 years, and certain characteristics of natural field isolates may have been lost.

In addition to inter- and intra-species differences in LPG structure, stage-specific modifications in LPG structure have also been described (*4*, *13*, *25*). The differentiation of dividing, noninfectious procyclic promastigotes to nonproliferative, infectious metacyclic promastigotes has consistently been associated with an approximate doubling in the number of repeating units expressed by LPG, resulting in a substantial thickening in the LPG coat. The elongation of LPG imparts a state of relative complement resistance to metacyclic promastigotes by hindering the access of the membrane attack complex to the cell membrane (*5*). In these studies, we have demonstrated that during metacyclogenesis of Indian *L. donovani*, the number of repeating units approximately doubles from 17 to 36. The ED_{50} of fresh normal human serum required for complement-mediated lysis was approximately 8 times higher for metacyclic promastigotes of this strain compared to procyclic promastigotes (data not shown). The data reinforce the notion that the elongation of LPG during development of promastigotes in the sand fly pre-adapts the parasite for survival within the vertebrate host.

Along with the number of repeating units expressed, previous studies have also demonstrated that the display of specific terminally exposed sugars changes during metacyclogenesis and that these changes control the stage-specific attachment and release to and from the sand fly midgut that is required for development of transmissible infections (*3*, *4*). Our studies have formally demonstrated that the binding of an Indian *L. donovani* strain to its vector midgut is highly stage-specific. In the in vitro assays, the binding of procyclic forms was 4−5-fold greater than metacyclics, and purified PG from procyclics bound with much higher affinity than did metacyclic PG. In previous studies, the structural modifications in LPG that account for stage-specific midgut binding were shown to be quite different depending on the species of *Leishmania*. For *L. major*, the loss of binding to *P. paptasi* midguts during metacyclogenesis is controlled by the down-regulation of LPG side chain sugars terminating in Gal in favor of side chains terminating in Ara (*3*). In contrast, the ability of procyclic promastigotes of the 1S *L. donovani* strain from Sudan, which bears no LPG side chain structures, to attach to midguts of *P. argentipes* is lost during metacyclogenesis as a consequence of a conformational masking of the capping sugars associated with LPG chain elongation (*4*). There is thus the likelihood, not formally addressed in these studies, that the elongation of LPG on metacyclics of Indian *L. donovani* strains similarly leads to a masking of capping sugars that would otherwise remain available to mediate midgut attachment. However, since a unique aspect of the Indian *L. donovani* strain is that it also synthesizes potential ligands in the form of Glc side chains, the loss of binding by metacyclics might require modification of both the capping and side chain domains. Our studies reveal a novel alteration in LPG biosynthesis that accomplishes this result. Unlike *L. major* LPG, for which a different set of nonbinding side chain oligosaccharides are synthesized on metacyclic LPG, metacyclics of the Indian *L. donovani* strain down-regulate the synthesis of side chain sugars altogether, leaving predominantly an elongated disaccharide backbone.

The structural data suggest that the Indian strain contains at least one and most likely two active glucosyltransferases that attach the side chain Glc residues during LPG synthesis. As the metacyclic LPG does not bear Glc side chains, developmental regulation of Glc addition must occur. This down-regulation of Glc addition could be accomplished at several points, such as transcriptional or translational inhibition of the enzyme in metacyclic promastigotes. Also, the nucleotide−sugar transporter for translocating UDP-Glc into the Golgi for its utilization could be regulated during this growth phase. Another issue that warrants investigation is the difference in glucosyltransferase activities between the Indian and Sudanese strains. Because the Sudanese strain lacks Glc side chains, this strain may simply lack the glucosyltransferase genes. However, other possibilities exist such as the genes are present but the UDP-Glc transporter or the transferases themselves are not active. Thus, a detailed examination of the glucosyltransferases can extend our understanding of both developmental and intra-species regulation of the LPG biosynthetic pathway.

ACKNOWLEDGMENT

We thank Drs. Charles Waechter, Jeffery Rush, and Brenda Jo Mengeling for their thoughtful suggestions during the course of this work. We thank the members of the Mass Spectrometry facility at the University of Kentucky for their help with the MALDI analysis.

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BI990741G