ELSEVIER

Molecular and Biochemical Parasitology 94 (1998) 27–40

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Leishmania donovani has distinct mannosylphosphoryltransferases for the initiation and elongation phases of lipophosphoglycan repeating unit biosynthesis

Albert Descoteaux [1,a], Brenda Jo Mengeling [a], Stephen M. Beverley [b], Salvatore J. Turco [a,*]

[a] *Department of Biochemistry, University of Kentucky Medical Center, Lexington, 40536 KY, USA*
[b] *Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, 63110 MO, USA*

Received 12 January 1998; received in revised form 16 March 1998; accepted 18 March 1998

Abstract

Lipophosphoglycan (LPG) is the predominant surface glycoconjugate of *Leishmania* promastigotes and plays several roles in the infectious cycle of this protozoan parasite. The salient feature of LPG is the presence of 15–30 copies of a disaccharide-phosphate repeating unit Gal(β1,4)Man(α1-PO_4), which is also found on many other secreted molecules (secretory acid phosphatase, phosphoglycan, proteophosphoglycan). This structural diversity suggests that a multiplicity of enzymes mediating repeating unit addition may exist, especially for the mannosylphosphoryltransferases (MPTs), which initiate repeating unit synthesis. This work has taken a combined biochemical-genetic approach to resolve this issue. An *lpg*$^-$ mutant of *Leishmania donovani*, JEDI, was obtained by antibody selection against cells expressing a repeating unit epitope of LPG. Metabolic and surface labeling experiments revealed that JEDI cells accumulated a truncated form of LPG bearing only a single repeating unit: [Gal(β1,4)Man(α1-PO_4)]-[Gal(α1,6)Gal(α1,3)Gal_f(β1,3)[Glc(α1-PO_4)]Man(α1,3)Man(α1,4)GlcN(α1,6)]-PI. Enzymatic assays of microsomal preparations showed that JEDI lacked MPT activity when tested with a repeating unit acceptor but retained wild-type

Abbreviations: AnhMan, 2,5-anhydromannose; CHAPS, 3-(3-chloramidopropyldimethyl-ammonio)-1-propanesulfonic acid; eMPT, elongation LPG mannosylphosphoryltransferase; GIPL, glycosylinositolphospholipid; HBSS, Hank's balanced salt solution; HPLC, high pressure liquid chromatography; iMPT, initiating LPG mannosylphosphoryltransferase; LPG, lipophosphoglycan; MPT, mannosylphosphoryltransferase; PI-PLC, phosphatidylinositol-specific phospholipase C; TLCK, 1-chloro-3-tosylamido-7amino-2-heptone; WT, wild-type.

* Corresponding author. Tel.: +1 606 3236693; fax: +1 606 3231037; e-mail: turco@pop.uky.edu

[1] Present address: Centre de recherché en immunologie, Institut Armand-Frappier, CP 100, 531 boul. des Prairies Laval, H7N 4Z3 Quebec, Canada.

PII S0166-6851(98)00047-4

levels of the MPT activity with an LPG glycan core acceptor. These data indicate that at least two distinct MPT activities are required for LPG repeating unit synthesis: one involved in the 'initiation' of repeating unit synthesis on the LPG core (iMPT), and a second (lacking in JEDI) participating in the 'elongation' phase of repeating unit addition (eMPT), leading to the mature full-length LPG.

Keywords: Lipophosphoglycan; *Leishmania*; Mannosylphosphoryltransferases; Glycoconjugates

1. Introduction

Infection by protozoan parasites of the genus *Leishmania* results in a spectrum of clinical diseases, termed leishmaniasis, that afflict millions of people world-wide. Following inoculation into a mammalian host by the sand fly vector, infectious promastigote forms successfully evade immune defenses to differentiate and proliferate as amastigotes within a phagolysosomal compartment of mammalian macrophages. Specialized cell surface molecules play important roles in the infectious cycle [1]. One such molecule is lipophosphoglycan (LPG), the dominant surface glycoconjugate of *Leishmania* promastigotes [2,3]. In *L. donovani*, LPG consists of a polymer of the disaccharide-phosphate repeating unit Gal(β1,4)Man(α1-PO_4) attached via a heptasaccharide glycan core to a 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositiol anchor (Fig. 1). The non-reducing end of LPG is capped with one of several small oligosaccharide-phosphates containing mannose and/or galactose. LPG acts in various aspects of the parasite's life cycle: in survival and transmission in the sand fly, resistance to complement, entry and survival in the macrophage and modulation of host signal transduction pathways [2,3].

Many components that make up LPG occur on other parasite molecules [2–4]. For example, LPG repeating units occur on several glycoconjugates that potentially play important roles in the infectious cycle, such as the secreted acid phosphatase (sAP), proteophosphoglycan (PPG) and phosphoglycan (PG) [5–7]. The presence of these repeating units is a general characteristic of all *Leishmania* species, although the repeating unit can be further modified with additional sugar residues in *L. major*, *L. tropica*, *L. mexicana*, and others. The ubiquitous nature of repeating units, found on the parasite surface (LPG) or secreted into the fly midgut or macrophage parasitophorous vacuole (sAP, PPG, PG), has caused many workers to suggest a functional involvement in the parasite's infectious cycle. Correspondingly, mutants defective in repeating unit biosynthesis such as C3PO [8,9] show an inability to survive within the mammalian macrophage [10] or sand fly vector (D. Sacks, S. Turco, S. Beverley, unpublished observations). Purified repeating units have been shown to mediate some of the activities attributed to LPG, such as inhibition of PKC activity [11], inhibition of monocyte chemotaxis [12] and down-regulation of *c-fos* expression [13]. Thus, repeating units are important to *Leishmania*

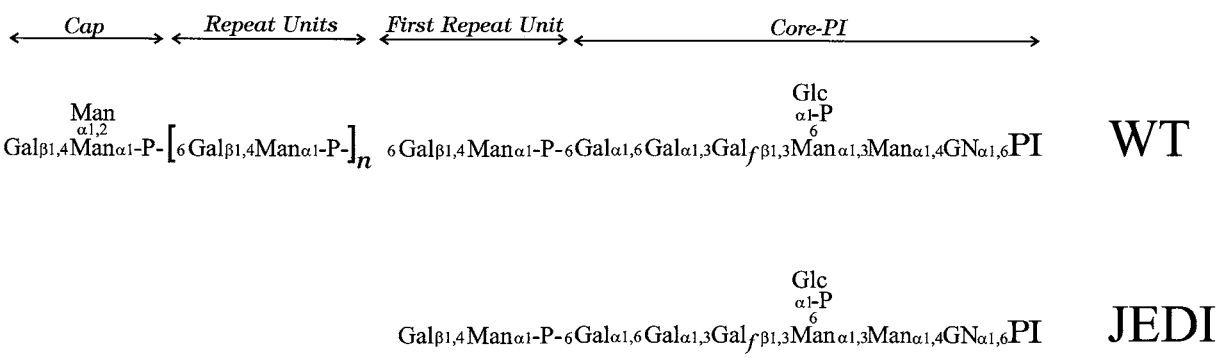

Fig. 1. Structure of the LPG from *L. donovani* and the truncated LPG accumulated in JEDI.

virulence, and thus they represent an attractive target for chemotherapeutic intervention.

Current data suggest that repeating unit addition occurs by the sequential activities of a mannosylphosphoryltransferase followed by a galactosyltransferase [14,15]. Given the structural diversity of the repeating unit-bearing molecules above, it seems likely that a number of independent MPTs will be required for repeating unit biosynthesis. For LPG, there may be at least two MPTs: an 'initiating' MPT (iMPT) that recognizes the Gal(α1,6)Gal of the glycan core and one or more 'elongating' MPTs (eMPT) that recognize the Gal(β1,4)Man(α1)-PO_4 repeating units as acceptors to expand the number of repeating units to 15–30 per LPG. It is possible that these 'LPG' MPTs participate in the synthesis of PG, which structurally resembles an LPG molecule minus the core and GPI anchor [16], or alternatively, separate activities may carry out PG synthesis. Moreover, another class of MPTs specific for proteins may exist to add Man(α1-PO_4) units to serine residues of secreted acid phosphatase [6] and the proteophosphoglycan [7]. The complexities of the MPT family within *Leishmania* thus pose interesting questions of specificity and regulation. Due to their role in the synthesis of molecules implicated in the parasite's infectious cycle and their absence in the mammalian host, several MPTs may offer attractive targets for anti-parasitic chemotherapy.

We have embarked on a program to characterize LPG repeating unit MPT activities, using a combination of genetic and biochemical strategies. Genetic approaches could prove especially useful to discriminate among MPT enzymes of similar or overlapping specificities. Fortunately, LPG is particularly amenable to the application of genetic methods. With lectins such as ricin agglutinin (ricin 120), which recognize β-linked galactosyl residues at the nonreducing end of LPG (Fig. 1), it is possible to recover readily LPG-deficient (*lpg*$^-$) parasites from mutagenized populations [8,17,18]. With these parasites, functional genetic rescue can then recover genes that restore the LPG phenotype [19]. In this manner, *LPG1* was identified, implicated in the synthesis of the LPG glycan core [19] and *LPG2*, a gene implicated in the biosynthesis of LPG repeating units [9]. *lpg2*$^-$ mutants such as C3PO [8] or ones obtained by homologous gene targeting [9] lack repeating units on all molecules, due to the lack of a Golgi GDP-Man translocation activity requiring the LPG2 protein [20].

A mutant of *L. donovani* lacking the postulated LPG eMPT would yield a truncated LPG that would terminate with a β-linked galactose (see Fig. 1, 'JEDI'). Since cells expressing such a molecule on their surface should be reactive with ricin agglutinin, it is reasoned that potential *lpg*$^-$ mutants defective in this step probably would not be recovered in ricin agglutinin-based screens. Therefore, reactivity with the monoclonal antibody CA7AE was used, which recognizes the LPG repeating units specifically, to develop a more general selection for isolating *lpg*$^-$ mutants. Here we describe one such mutant, JEDI, and demonstrate that it is deficient in the predicted LPG eMPT activity but is normal in iMPT activity. This provides the first combined biochemical and genetic evidence for two distinct MPTs.

2. Materials and methods

2.1. Parasites

Promastigotes of *Leishmania donovani*, Sudanese strain 1S2D, were grown in either modified MEM (Gibco) supplemented with 10% fetal calf serum (Gibco) as described previously [17] or M199 with 10% fetal calf serum [21].

2.2. Mutagenesis of parasites

Mutagenesis of promastigotes and selection of *lpg*$^-$ mutants were carried out as previously described [17] with the following modifications. A 300 ml culture was grown to a density of 5–10×10^6 cells ml^{-1}, harvested by centrifugation, resuspended to a density of 10^7 cells ml^{-1}, and incubated for 4 h with shaking in the presence of *N*-methyl-*N*-nitroso-*N*′-nitroguanidine (Sigma) (6.0 μg ml^{-1}). Parasites were washed, re-

suspended in fresh medium, and grown for several generations. Prospective *lpg*$^-$ mutants were recovered as free cells remaining after three consecutive rounds of agglutination with the IgM monoclonal antibody CA7AE (1:500 dilution of ascites fluid) [22]. The non-agglutinated cells were plated on medium containing 1% agar, and individual colonies were picked and grown in liquid medium for subsequent tests.

JEDI cells were not agglutinated by a 1:500 dilution of CA7AE ascites, a dilution that readily agglutinates WT cells. When examined for their reactivity with ricin agglutinin, JEDI cells optimally agglutinated at 20 μg ml^{-1}, while as little as 1 μg ml^{-1} was sufficient to agglutinate WT cells.

2.3. Metabolic labeling

Cells (2×10^9 ml^{-1}) were incubated in the presence of 200 μCi [^{3}H]galactose (20 mCi mmol^{-1}, American Radiolabeled Chemicals) or [^{3}H]mannose (15 mCi mmol^{-1}, American Radiolabeled Chemicals) for 18 h and LPG was extracted in Solvent E, water:ethanol:diethyl ether: pyridine:NH_4OH (15:15:5:1:0.017) [23]. Alternatively, GIPLs were extracted in chloroform:methanol:water (4:8:3) [24]. Extracted glycolipids were further purified by chromatography on phenyl-coupled Sepharose.

2.4. Cell surface labeling

Promastigotes (4×10^8) were centrifuged and washed twice with 10 ml HBSS buffered with 15 mM HEPES, pH 7.3. The cells were resuspended in 0.2 ml buffered HBSS containing 10 units of galactose oxidase (*Dactylium dendroides*, Boehringer Mannheim Biochemica), incubated for 40 min at 25°C and washed once with 10 ml buffered HBSS. Radiolabeling of cell surface carbohydrates was achieved by incubating the galactose oxidase-treated promastigotes in 0.2 ml buffered HBSS containing 2 mCi NaB[^{3}H]$_4$ (718 mCi mmol^{-1}, ICN) for 90 min at 25°C. Labeled parasites were then washed four times with 10 ml buffered HBSS and GIPLs were extracted in chloroform:methanol:water (4:8:3).

2.5. Phenyl-coupled sepharose chromatography

Samples were resuspended in 0.1 M NaCl/0.1 M acetic acid and applied to a column (1.5 ml) of phenyl-coupled Sepharose equilibrated in 0.1 M NaCl/0.1 M acetic acid. The column was washed sequentially with 3.0 ml of 0.1 M NaCl/0.1 M acetic acid, 1.2 ml of 0.1 M acetic acid, 0.6 ml of H_2O, and 3.0 ml of Solvent E. Lipid-linked glycoconjugates, such as the GIPLs and LPG, were retained by the hydrophobic support and eluted with Solvent E.

2.6. DE52-cellulose chromatography

Desalted samples were applied to a column (0.5×3 cm) of DE52-cellulose (Whatman) equilibrated in 1.0 mM Tris–HCl, pH 8.0. After the fifth fraction was collected, a gradient of NaCl (0–0.1 M) in 1.0 mM Tris–HCl, pH 8.0 was applied to the column. Fractions of 0.6 ml were collected and radioactivity was determined by liquid scintillation.

2.7. Enzyme digestions and chemical hydrolyses

Alkaline phosphatase (*E. coli*, Sigma) digestions were carried out in 1 mM Tris–HCl, pH 8.0 for 16 h at 37°C; α-galactosidase (green coffee beans, Sigma) digestions in 50 mM sodium citrate, pH 5.0 for 16 h at 37°C; β-galactosidase (Jack beans, Sigma) digestions in 50 mM sodium acetate, pH 5.8 for 48 h at 37°C. Phosphatidylinositol-specific phospholipase C was purified from *Bacillus thuringiensis* as described [25] and digestions were carried out in water for 16 h at 37°C. Mild acid hydrolysis (0.02 N HCl, 5 min, 100°C) was used to cleave sugar 1-phosphate bonds. After hydrolysis, samples were dried under a stream of N_2 and redried three to four times with toluene to remove traces of acid. To deaminate by nitrous acid, glycolipids were resuspended in 0.3 ml of 0.25 M sodium acetate, pH 4 and 0.3 ml 0.5 M sodium nitrite and incubated for 16 h at 42°C. After nitrous acid treatment, 30 μl of 2 M NaCl/2 M acetic acid were added to the samples and applied to a column of phenyl-coupled Sepharose. Glycans were recovered in the salt fractions.

2.8. HPLC

Radiolabeled glycans were analyzed by anion exchange chromatography on a Dionex Model BioLC System with a CarboPac PA1 anion exchange column (4 × 250 mm). Monosaccharides and oligosaccharides were separated using programs A and B at a flow rate of 0.5 and 1.0 ml min^{-1}, respectively. Program A was an isocratic elution over 25 min with 10.5 mM NaOH. Program B was an isocratic elution over 10 min with solution 1 (150 mM NaOH), followed by a linear gradient from 0 to 50% solution 2 (150 mM NaOH, 500 mM sodium acetate) over the next 20 min. A linear gradient from 50 to 100% solution 2 was applied for the next 3 min. Isocratic elution with 100% solution 2 was continued for 3 min, followed by equilibration back to initial conditions over the next 8 min. Fractions were collected every 0.5 min, neutralized with 4 M acetic acid, and radioactivity was measured by liquid scintillation.

2.9. Preparation of exogenous acceptor substrate for the eMPT assay

To radiolabel the truncated LPG from JEDI cells, the GIPL fraction (1.3 mg) from JEDI cells was incubated at 26°C for 16 h with 25 units of galactose oxidase in 0.1 ml of the manufacturers' buffer (25 mM KPO_4, pH 6.0, 0.25 mM $CuSO_4$). After oxidation, the GIPLs were desalted on a column (1 ml) of phenyl-coupled Sepharose as described above and dried under a N_2 stream. The oxidized GIPLs were resuspended in 88 μl of 400 mM boric acid/NaOH, pH 11 and 6 μl of 100 mM $NaB[^3H]_4$ (11 Ci $mmol^{-1}$) and incubated at room temperature for 16 h. The reaction was terminated by the addition of 25 μl of glacial acetic acid. The sample was applied to a column (2 ml) of phenyl-coupled Sepharose as described above. The reduced GIPLs were resuspended in methanol and redried five times before resuspension in a minimal volume of methanol:water (1:1) and application to a Whatmann 3MM descending paper chromatogram. The chromatogram was developed for 48 h in 1-butanol:ethanol:water (4:1:1). The GIPLs were eluted from the origin with solvent E, dried under a N_2 stream, resuspended in water and stored at 4 °C. Mild acid hydrolysis to cleave sugar 1-phosphate bonds of the reduced GIPLs generated a labeled disaccharide (Gal-Man) as determined by a descending paper chromatogram developed in 1-butanol:pyridine:water (6:4:3).

Table 1
Incorporation of [^{3}H]mannose into glycoconjugates

	WT (cpm)	JEDI (cpm)
LPG extract	354 000	0
GIPL extract	84 000	545 000
Glycoproteins	155 000	65 000

Parasites were metabolically labeled with 100 μCi of [^{3}H]mannose for 16 h, extracted with organic solvents, and radioactivity (cpm) incorporated into the various glycoconjugates was measured as described in Section 2. Values shown are an average of two separate experiments.

2.10. Preparation of microsomes and MPT assays

Microsomal fractions were prepared from wild-type and JEDI cells as described [14,26]. eMPT assay mixtures (100 μl final volume) contained microsomes (0.5 mg protein), 50 mM HEPES, pH 7.4, 25 mM KCl, 10 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM ATP, 0.4 mM dithiothreitol, 1.0 μg ml^{-1} leupeptin, 0.1 mM TLCK, 0.7 μg ml^{-1} pepstatin A, 100 μM UDP-Gal (Sigma), 800 μM GDP-Man (Sigma) and radioactive exogenous acceptor (5×10^5 cpm JEDI GIPLs). After 60 min incubation at 30°C, the membranes were extracted with Solvent E [14]. Radiolabeled LPG was dried under N_2, resuspended in 0.02 N HCl, and incubated at 100°C for 5 min to cleave sugar 1-phosphate bonds. Following removal of lipid-linked material by butanol partitioning, the released radiolabeled glycans were resuspended in 300 μl 1 mM Tris–HCl, pH 8.0, and analyzed on a column (2 ml) of AG1-X2 anion exchange resin (BioRad, Hercules, CA) with a linear gradient of 0–0.3 M NaCl in 1mM Tris–HCl, pH 8.0. The amount of radioactivity in each fraction was determined by liquid scintillation. MPT assays using stachyose as an exogenous acceptor substrate were done as described [26].

3. Results

3.1. Isolation of mutants resistant to agglutination with CA7AE

The monoclonal antibody CA7AE recognizes a series of Gal(β1,4)Man(α1-PO_4) repeating units from *L. donovani* LPG [22,27] and readily agglutinates WT promastigotes [8]. We exploited this observation to select LPG-defective mutants of *L. donovani*. Mutagenized promastigotes were subjected to three consecutive rounds of agglutination with CA7AE, and then non-agglutinated cells were plated on semi-solid medium. Ten clonal lines failed to agglutinate with CA7AE but were agglutinated by 20 μg ml^{-1} ricin agglutinin, which binds terminal, β-linked galactose residues. The reactivity of these clonal lines with ricin agglutinin was clear, but diminished relative to WT *L. donovani*, which agglutinate readily in 5 μg ml^{-1}. Since all mutant lines were phenotypically identical, but not necessarily genetically independent, we chose one clone for further characterization, designated JEDI.

3.2. JEDI lacks LPG and is an lpg^- mutant

The ability of JEDI to synthesize glycoconjugates was examined by metabolic labeling, followed by extraction with organic solvents. JEDI incorporated no detectable [^{3}H]mannose into LPG (Table 1), confirming that it represented a bona fide lpg^- mutant. Radiolabeling of glycoproteins was 42% of that observed in WT *L. donovani*, whereas incorporation of [^{3}H]mannose into GIPLs was 6-fold higher in JEDI. Similar results were obtained with [^{3}H]galactose labeling (not shown). Since some GIPLs are precursors of LPG [28], and other lpg^- mutation(s) can lead to accumulation of these [9,29], we inferred that a similar process was occurring in JEDI.

3.3. Analysis of the [^{3}H]galactose- and [^{3}H]mannose-labeled GIPLs from JEDI cells

To identify the GIPLs that accumulated in the mutant, parental and JEDI cells were metabolically radiolabeled for 16 h with [^{3}H]galactose or [^{3}H]mannose. The GIPLs were extracted, purified by affinity chromatography on phenyl-coupled Sepharose and delipidated by nitrous acid treatment. The [^{3}H]mannose-labeled (Fig. 2A and Fig. 2B) and the [^{3}H]galactose-labeled (Fig. 2C and Fig. 2D) glycans were separated by chromatography on DEAE-cellulose. Previous studies [29] indicated that the anionic glycan core Gal-Gal-Gal_f-[Glc-PO_4]-Man-Man-AnhMan (one net negative charge) elutes in fractions 14–19 (Fig. 2), whereas fractions greater than 21 contain glycan core substituted with one or more Gal(β1,4)Man(α1-PO_4) repeating units. *L. donovani* also synthesize a series of GIPLs consisting of two to four α-linked mannose residues linked to the GlcN-PI [28]; their nitrous acid generated glycans are neutral and do not bind to the anionic support (fractions 3–5, Fig. 2).

In contrast to WT cells, JEDI cells accumulated a [^{3}H]mannose- and [^{3}H]galactose-labeled GIPL in which the glycan possesses a (-2) net charge (Fig. 2B and D), fractions 21–28). To characterize this (-2) charged product from JEDI cells, it was subjected to mild acid hydrolysis to cleave sugar 1-phosphate bonds and then it was reapplied to a column of DEAE-cellulose. The [^{3}H]galactose-labeled material chromatographed primarily as a neutral product and a product with a (-4) net charge (Fig. 3). Analysis of the neutral fraction by Dionex HPLC indicated that most of this material co-eluted with authentic glucose and Gal(β1,4)Man (data not shown). The occurrence of the radiolabeled glucose is attributed to the metabolic epimerization of galactose, its subsequent attachment to the glycan core region as Glc(α1-PO_4) (Fig. 1) and its release upon mild acid hydrolysis.

The [^{3}H]galactose-labeled fragment from JEDI with a (-4) net charge after mild acid hydrolysis (fractions 32–35, Fig. 3) was dephosphorylated with alkaline phosphatase, and the resulting neutral material was analyzed by Dionex HPLC. The major portion of this material co-eluted with authentic, nitrous acid-

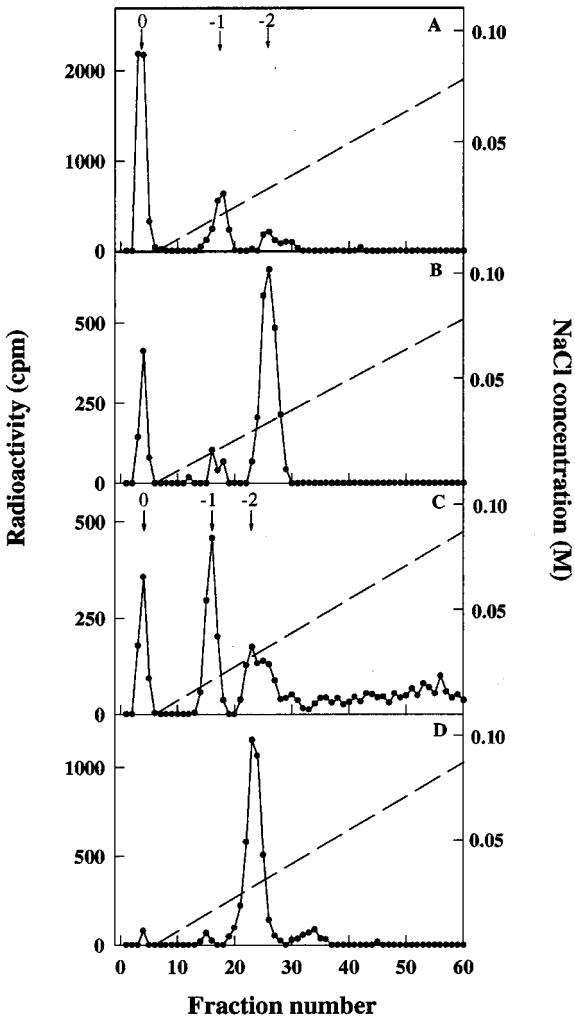

Fig. 2. DEAE-cellulose chromatography of the water-soluble fragments released by HONO deamination of [^{3}H]Man- and [^{3}H]Gal-labeled GIPLs from wild-type and JEDI cells. Radiolabeled GIPLs from WT and JEDI cells were pre-treated with nitrous acid. The deaminated water-soluble portion was collected by chromatography on phenyl-coupled Sepharose, desalted on a column of Sephadex G10 equilibrated in water, resuspended in 1.0 mM Tris–HCl, pH 8 and applied to a column of DE-52-cellulose. Panel A [^{3}H]Man-labeled wild-type glycans; Panel B [^{3}H]Man-labeled JEDI glycans: Panel C [^{3}H]Gal-labeled wild-type glycans; Panel D [^{3}H]Gal-labeled JEDI glycans. Calibration of the ion exchange support with anionic oligosaccharide standards indicated that samples with one net negative charge (-1) elute in fractions 14–19, and those with two net negative charges (-2) elute in fractions 21–28.

treated, dephosphorylated glycan core (GalαGalαGal$_f$-Man-Man-AnhMan) (Fig. 4A). Digestion with α-galactosidase prior to chromatography resulted in elution of all [^{3}H]galactose-label in the monosaccharide fraction (Fig. 4B). It should be pointed out that radiolabeling of parasites with [^{3}H]galactose does not result in label associated with the galactofuranose residue. Collectively, these data are consistent with the identification of the fragment with a (-4) net charge as the glycan core of LPG, and further indicate that JEDI cells accumulate Gal(β1,4)Man(α1-PO$_4$)-[Glycan Core]-PI. Its occurrence in wild-type cells, but in lesser abundance, is consistent with it being a LPG precursor.

3.4. Analysis of cell-surface GIPLs

As noted earlier, ricin agglutinin agglutinates JEDI cells, suggesting the expression of a surface glycoconjugate that terminates in a β-linked galactose residue. To determine whether this was due to surface expression of the truncated LPG molecule, the surface of JEDI promastigotes was labeled by sequential treatment with galactose oxidase and tritiated sodium borohydride [30] and then examined incorporation of the radiolabel into GIPLs.

The surface-labeled GIPLs were delipidated by nitrous acid and analyzed by anion exchange chromatography. The dominant [^{3}H]glycan contained a (-2) charge and the radiolabel was converted to a neutral product following mild acid hydrolysis (data not shown). The neutral species co-eluted with [^{3}H]Gal(β1,4)Man following Dionex HPLC (Fig. 5A) and was not susceptible to α-galactosidase digestion (Fig. 5B). The fragment was hydrolyzed by β-galactosidase (Fig. 5C), resulting in the release of a labeled monosaccharide co-eluting with the galactose standard (Fig. 5C, inset). These results indicate that JEDI cells express the Gal(β1,4)Man(α1-PO$_4$)-[Glycan Core]-PI truncated LPG molecule at the cell surface and probably accounts for the reactivity of this mutant with ricin agglutinin.

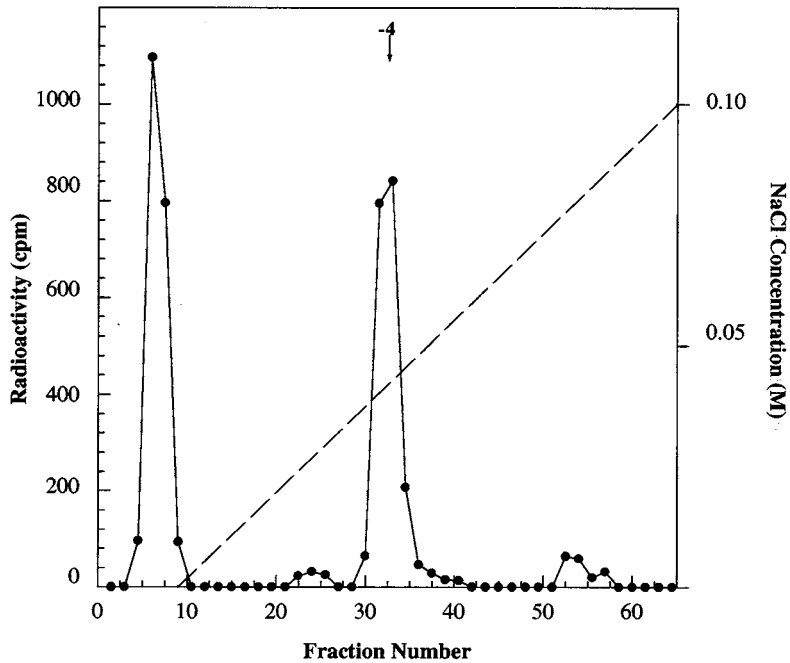

Fig. 3. DEAE-cellulose chromatography of the fragments generated by mild acid hydrolysis of the (−2) charged, [^{3}H]Gal-labeled GIPLs from JEDI cells. The (−2) charged, [^{3}H]Gal-labeled product from JEDI cells (Fig. 2(D), fractions 21–28) was subjected to mild acid hydrolysis, and the fragments were applied to a column of DE-52-cellulose. Calibration of the ion exchange support with standards indicated that samples with four net negative charges (−4) elute in fractions 32–35.

3.5. Analysis of MPT activities of JEDI membranes

Previous studies suggested that LPG repeating unit synthesis in an in vitro system occurs by the alternating transfer of Man(α1-PO$_4$) and Gal residues from GDP-Man and UDP-Gal, respectively [14,15]. Since JEDI completes the synthesis of only one Gal(β1,4)Man(α1-PO$_4$) repeating unit to the glycan core of LPG, we inferred that it lacked the MPT activity responsible for the transfer of Man(α1-PO$_4$) to the single Gal(β1,4)Man(α1-PO$_4$) of the underlying repeating unit. In contrast, we predicted that it would retain the MPT activity responsible for the addition of the first Man(α1-PO$_4$) to the LPG glycan core, which terminates in an α-linked galactose residue (Fig. 1). These two activities were termed 'elongating' and 'initiating' MPT (eMPT and iMPT) activities, respectively.

To assay each of these activities using microsomes from JEDI cells, we generated exogenous acceptor substrates for both the initiating and elongating MPT activities. Recently, we have shown that [^{3}H]stachyose, like the glycan core of LPG, can function as an acceptor substrate for the iMPT [26]. Stachyose is a tetrasaccharide that terminates in the same Gal(α1,6)Gal(α) motif as does the glycan core of LPG. [^{3}H]Stachyose does not bind to an anion exchange support, but the resulting product of the iMPT reaction [Man(α1-PO$_4$)- [^{3}H]stachyose] does bind and can be eluted with salt. After incubation of [^{3}H]stachyose with GDP-Man and microsomes, substrates and products were separated by anion exchange chromatography, and the radioactivity that bound and then salt-eluted was quantitated by scintillation counting. Microsomes from both WT and JEDI cells possessed equivalent rates of transfer of Man(α1-PO$_4$) to stachyose (Fig. 6). This result confirmed that JEDI cells retain wild-type levels of iMPT, as expected.

To assay for the eMPT, a [^{3}H]Gal(β1,4) Man(α1-PO$_4$)-[Glycan Core]-PI acceptor was prepared, which had the important feature of radiolabel in the terminal galactose residue. The strategy of the assay was that upon

mild acid hydrolysis of this particular acceptor substrate, the neutral [^{3}H]Gal(β1,4)Man that is released does not bind to an anion exchange resin. Transfer of a Man(α1-PO_4) from GDP-Man to the exogenous acceptor at the radiolabeled galactose residue generates PO_4-[^{3}H]Gal(β1,4)Man upon mild acid hydrolysis; this moiety is retained by anion exchange. Thus, the amount of transferase activity can be assessed by determining the radioactivity that is retained on the anion exchange column.

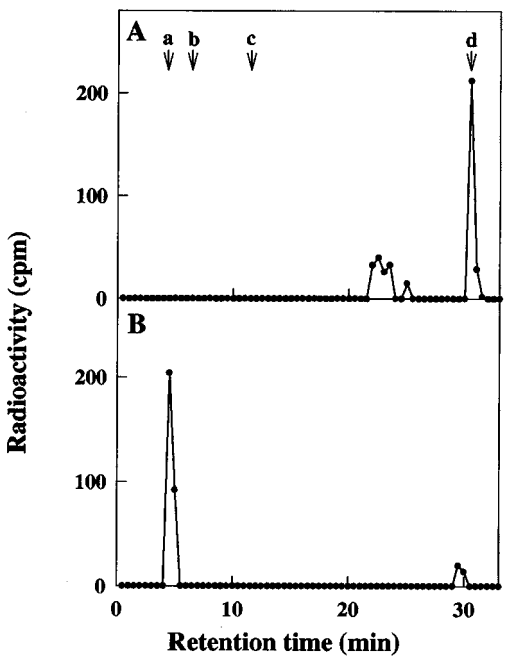

Fig. 4. Dionex HPLC analysis of the (−4) charged mild acid generated glycan fragment from JEDI cells. The [^{3}H]Gal-labeled fragment with two net negative charges (Fig. 2D, fractions 21–28) was subjected to mild acid hydrolysis and reapplied to a column of DE-52-cellulose. The [^{3}H]Gal-labeled material eluting with fractions 32–35 (−4 charged) was desalted on a column of Sephadex G10 equilibrated in water and treated with alkaline phosphatase. The resulting neutral [^{3}H]Gal-labeled material was recovered after chromatography on a column of DE-52-cellulose, and an aliquot was applied to Dionex HPLC using program B for oligosaccharides (panel A). Another aliquot was treated with α-galactosidase prior to Dionex HPLC analysis (panel B). Standards: a, glucose; b, Gal(β1,4)Man; c, maltose; d, Gal-Gal-Gal_f-Man-Man-AnhMan. The radioactive material eluting in fractions 24–27 is likely [^{3}H]Gal-Gal-Gal obtained my partial mild acid cleavage of the galactofuranosidic bond in the glycan core.

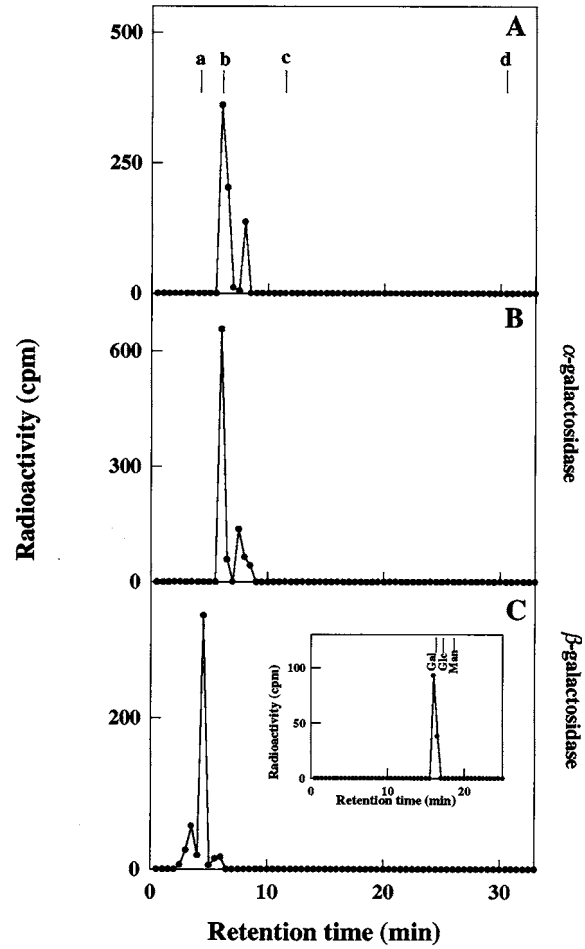

Fig. 5. Dionex HPLC analysis of the galactose oxidase/NaB[^{3}H]$_4$-labeled GIPLs from JEDI cells. Galactose oxidase/NaB[^{3}H]$_4$-labeled GIPLs from JEDI cells was subjected to mild acid hydrolysis. An aliquot of the neutral [^{3}H]-labeled material released by mild acid hydrolysis was applied to Dionex HPLC using program B for oligosaccharides (Panel A). Other aliquots were treated with α-galactosidase (Panel B) or β-galactosidase (Panel C) prior to Dionex HPLC chromatography using program B. Another aliquot of the β-galactosidase-treated [^{3}H]-labeled material was applied to Dionex HPLC using program A for monosaccharides (Panel C, inset). Standards: a, glucose; b, Gal(β1,4)Man; c, maltose; d, Gal-Gal-Gal_f-Man-Man-AnhMan and inset, galactose, glucose, and mannose.

With this strategy, we compared microsomes from both WT and JEDI cells for eMPT activity. After incubation with radiolabeled acceptor and GDP-Man, the microsomes were extracted for LPG. The LPG extract was subjected to mild acid

hydrolysis and applied to an anion exchange column as described in Section 2. Microsomes from WT produced a product that was retained by the column indicating the presence of elongating MPT activity (Fig. 7A). No significant radioactive peak was observed from JEDI microsomes, indicating a loss of eMPT activity in this mutant. Furthermore, addition of detergent to the enzyme reaction (Fig. 7B) enhanced the activity in the WT membranes, presumably by either stimulating the transferase or increasing the solubility of the acceptor. Microsomes from JEDI cells still lacked detectable activity. These data suggest that JEDI cells are completely defective in eMPT activity and have not mislocalized an otherwise active enzyme to the wrong cellular compartment. Transferase assays that utilized endogenous acceptor substrates present within the microsomes gave identical results (data not shown). Thus, a summary of the data from both of the transferase assays indicates that JEDI cells express only the iMPT and lack significant levels of the eMPT, accounting for the accumulation of Gal(β1,4)Man(α1-PO_4)-[Glycan Core]-PI found on the cell surface.

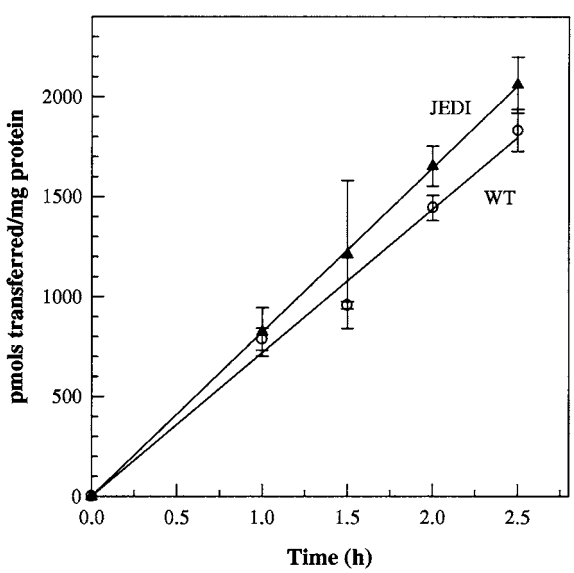

Fig. 6. Comparison of iMPT activities in WT and JEDI microsomes. Microsomes were prepared and incubated with 4 mM [^{3}H]stachyose (3.9 dpm pmol^{-1}), 800 μM GDP-Man and 7 mM Mn^{2+} for the indicated times as described in Section 2. The lines represent the average of two separate experiments.

4. Discussion

Leishmania donovani synthesizes a number of glycoconjugates bearing distinctive [Gal(β1,4) Man(α1-PO_4)] repeating units that include LPG, phosphoglycan (PG) and secretory proteins (acid phosphatase (sAP), proteophosphoglycan (PPG). This manuscript presents the identification and characterization of JEDI, an *L. donovani lpg*$^-$ mutant defective in the 'elongation phase' of LPG repeating unit synthesis. Our results with JEDI provide evidence that repeating unit biosynthesis requires at least two distinct MPTs, as expected given the variety of acceptor substrates encountered in repeating unit biosynthesis of both LPG and other repeating unit-bearing molecules.

Agglutination-based approaches permit the recovery of mutants occurring at very low frequencies, which is normally the situation in Leishmania (even after heavy mutagenesis) since it is a laboratory, asexual diploid. Previously, *lpg*$^-$ mutants were obtained on the basis of their lack of reactivity with ricin agglutinin and this approach yielded a number of different mutants with alterations in different steps of LPG biosynthesis [8,17,18] (unpublished data). However, LPG contains internal β-linked galactose residues, and mutants expressing truncated LPG fragments that exposed this monosaccharide would not be expected to emerge from a ricin-agglutinin-based screen. Thus far, this has been the case experimentally.

We sought a more general *lpg*$^-$ mutant selection protocol based upon resistance to agglutination with the monoclonal antibody CA7AE, which recognizes the LPG repeating units [22,27]. This procedure has yielded a number of independent *lpg*$^-$ mutants (this work, and unpublished data), as defined by a complete absence of LPG following biosynthetic labeling studies (Table 1). Remarkably, the first mutant characterized here, JEDI, exhibits exactly the structure that would not have emerged from ricin-agglutinin based selections. Thus, selection against CA7AE binding provides a more general screen for *lpg*$^-$ mutants. Antibody selection strategies have been used previously in *Leishmania major* to generate LPG

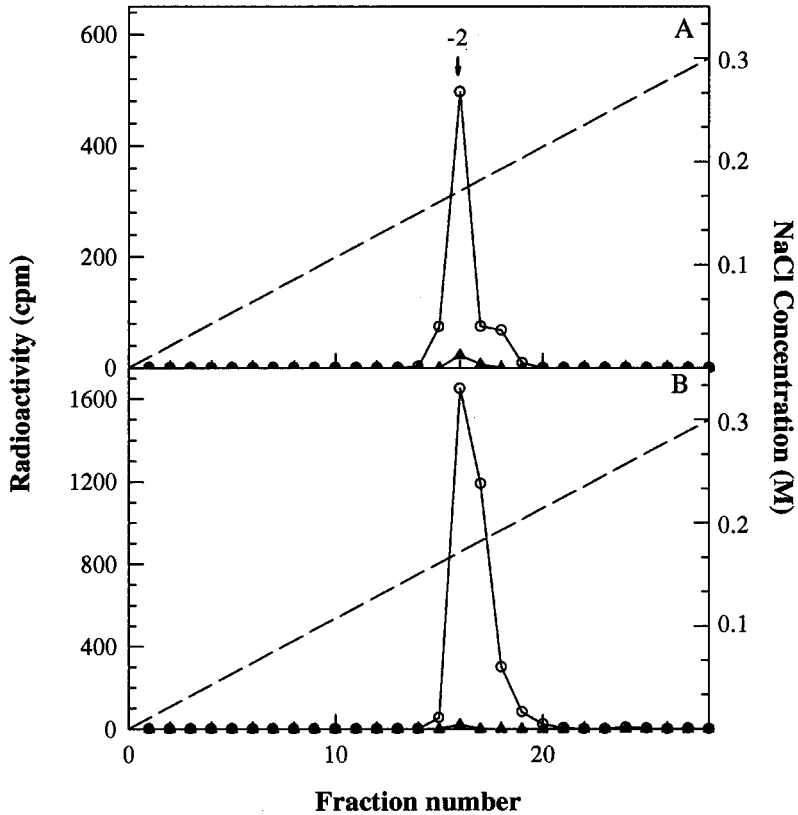

Fig. 7. Analysis of eMPT activities in WT and JEDI microsomes. Microsomes from WT (open circles) or JEDI (closed triangles) cells prepared as described in the Section 2 were incubated with GDP-Man and [^{3}H]-Gal(β1,4)Man(α1-PO$_4$)-[Glycan Core]-PI for 1 h at 30°C in the absence (Panel A) or presence (Panel B) of 0.2% Genapol X-100. The LPG extract was isolated, subjected to mild acid hydrolysis, and the released glycans, purified by butanol partitioning, were separated on a column of AG1-X2 anion exchange resin using a linear NaCl gradient.

mutants [31]. However, these often were not truly *lpg*$^-$, as they synthesized variant forms of LPG.

WT *Leishmania* contain high levels of a family of GIPLs on the cell surface [3]. Included amongst these are a minor population of GIPLs which appear structurally to be intermediates in the LPG biosynthetic pathway [3]. The truncated LPG found in JEDI, consisting of a GIPL bearing a single repeating-unit attached to the glycan-PI anchor (Fig. 1B), appears structurally to be an expected intermediate in the LPG biosynthetic pathway. Correspondingly, the levels of this GIPL were much higher in JEDI than in WT cells. We presume that the genetic defect in JEDI leads to the accumulation of this normal metabolic intermediate. Similarly, related metabolic intermediates accumulate in several other LPG mutants [19,29,32–34]. Surface labeling studies show that the accumulated JEDI GIPL is expressed on the parasite surface, thereby accounting for the ricin reactivity of JEDI cells.

Current data suggest that the LPG repeating units are formed by the sequential action of a mannosylphosphoryltransferase (MPT), followed by a galactosyltransferase [14,15]. Examination of the LPG structure suggests that at least two different MPTs could be required: one with a specificity for the LPG glycan core-PI, and a second with specificity for the Gal(β1,4)Man(α1-PO$_4$) repeating unit (Fig. 1). These have been termed LPG 'initiating' and 'elongation' phase MPTs respectively (iMPT or eMPT). Enzymatic assays of

WT *L. donovani* showed the presence of both activities, whereas only the elongation phase activity was deficient in the JEDI mutant (Figs. 6 and 7). Loss of this activity would be predicted to result in the accumulation of a GIPL bearing a single repeating unit, as observed with the JEDI GIPL (Fig. 1B). In addition, the defective eMPT activity could account for the relatively low metabolic radiolabeling of glycoproteins in JEDI compared to WT (Table 1). Since some specific *Leishmania* proteins bear phosphoglycan chains (e.g. secreted acid phosphatase, proteophosphoglycan), a defective eMPT activity could conceivably affect their assembly as well. Thus, the studies presented in this paper provide biochemical and genetic evidence for the existence of the two types of MPTs predicted above. Recently, an MPT activity in *L. major* cell extracts was reported, assayed using synthetic phospho-oligosaccharide fragments as acceptors [35] and defined as a GDP-Man:Gal(β1,4)Man(α1-PO_4) MPT. This *L. major* activity resembles the eMPT described here in *L. donovani*.

Our data show that the eMPT defective in the JEDI mutant mediates elongation of 'short' nascent LPGs, bearing one repeating unit. Possibly, this eMPT also carries out mannosylphorylation of nascent LPGs bearing higher numbers of repeating units. Furthermore, while our data strongly suggest that JEDI is defective in the eMPT, they do not exclude the possibility that the mutation is in an accessory protein required for eMPT activity. Resolution of these issues will require extensive characterization of the activity encoded by the JEDI-defective gene. This is underway, making use of functional complementation approaches used successfully for other LPG biosynthetic genes [9,19,20]. Alternatively, *Leishmania* may contain a number of different eMPTs, each with distinct affinities for nascent LPGs bearing different numbers of repeating units, as suggested by Brown et al. [35]. Interestingly, the *L. donovani* mutant RT5 has been described previously, which possesses an LPG bearing of only a few repeating units, instead of the normal 15–30 [8]. Characterization of the gene(s) defective in this mutant may help in resolving the question of the number of eMPTs required for full-length LPG synthesis.

Significantly, the control of LPG length is critical to the survival and development of *Leishmania* parasites in their sand fly host [36–38]. Log-phase 'procyclic' promastigotes are non-infective and attached to the fly midgut wall through binding by LPG bearing an average of approximately 15 repeating units [39]. In contrast, stationary-phase 'metacyclic' promastigotes are highly infective, and synthesize an LPG bearing many more repeating units (an average of approximately 30) that, in the case of *L. donovani*, causes the glycoconjugate to undergo a conformational change precluding attachment [39].

The control of the number of repeating units in LPG also has major implications in promoting survival of the parasite within the vertebrate host. Procyclic *Leishmania* are serum sensitive due to complement-mediated lysis while metacyclic forms are serum resistant (reviewed in [40]). The larger LPG on metacyclic parasites protects the cells from complement-mediated damage by sterically hindering access of macromolecules to the cell membrane, thereby preventing channel formation and lysis by the C5–9 membrane attack complex [41]. Formation and release of C5–9 by metacyclic *L. major* has been demonstrated [42]. Inside the macrophage, LPG repeating units enable freshly phagocytized promastigotes to inhibit phagosome–endosome fusion, possibly by modifying membrane fusogenic properties [43,44]. This inhibition may protect promastigotes from hydrolytic degradation and provide an environment propitious for their differentiation into amastigotes. The number of repeating units appears to be critical, as mutant promastigotes expressing truncated forms of LPG with few repeating units are unable to inhibit phagosome–endosome fusion [43]. Thus, it seems very likely that regulation of the activity and/or specificity of eMPTs are likely to play key roles in the parasite's infectious cycle.

Acknowledgements

This investigation received financial support from National Institutes of Health Grant AI31078. A.D. was supported by a postdoctoral fellowship from the Medical Research Council of

Canada. S.M.B. and S.J.T. are Burroughs Wellcome Scholars in Molecular Parasitology.

References

[1] Chang K-P, Chaudhuri G. Molecular determinants of *Leishmania* virulence. Annu Rev Microbiol 1990;44:499–529.

[2] Turco SJ, Descoteaux A. The lipophosphoglycan of *Leishmania* parasites. Annu Rev Microbiol 1992;46:65–94.

[3] McConville MJ, Ferguson MAJ. The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J 1993;294:305–24.

[4] Mengeling BJ, Beverley SM, Turco SJ. Designing glycoconjugate biosynthesis for an insidious intent: phosphoglycan assembly in *Leishmania* parasites. Glycobiology 1997;7:873–80.

[5] Ilg T, Stierhof Y-D, Wiese M, McConville MJ, Overath P. Characterization of phosphoglycan-containing secretory products of *Leishmania*. Parasitology 1994;108:63–71.

[6] Wiese M, Ilg T, Lottspeich F, Overath P. Ser/Thr-rich repetitive motifs as targets for phosphoglycan modifications in *Leishmania mexicana* secreted acid phosphatase. EMBO J 1995;14:1067–74.

[7] Ilg T, Stierhof YD, Craik D, Simpson R, Handman E, Bacic A. Purification and structural characterization of a filamentous, mucin-like proteophosphoglycan secreted by *Leishmania* parasites. J Biol Chem 1996;271:21583–96.

[8] McNeely TB, Tolson DL, Pearson TW, Turco SJ. Characterization of *Leishmania donovani* variant clones using anti-lipophosphoglycan monoclonal antibodies. Glycobiology 1990;1:63–9.

[9] Descoteaux A, Luo Y, Turco SJ, Beverley SM. A specialized pathway affecting virulence glycoconjugates of *Leishmania*. Science 1995;269:1869–72.

[10] McNeely TB, Turco SJ. Requirement of lipophosphoglycan for intracellular survival of *Leishmania donovani* with human monocytes. J Immunol 1990;144:2745–50.

[11] Giorgione JR, Turco SJ, Epand RM. Transbilayer inhibition of protein kinase C by the lipophosphoglycan from *Leishmania donovani*. Proc Natl Acad Sci USA 1996;93:11634–9.

[12] Frankenburg S, Leibovici V, Mansbach N, Turco SJ, Rosen G. Effect of glycolipids of *Leishmania* parasites on human monocyte activity: Inhibition by lipophosphoglycan. J Immunol 1990;145:4284–9.

[13] Descoteaux A, Turco SJ, Sacks DL, Matlashewski G. *Leishmania donovani* lipophosphoglycan selectively inhibits signal transduction in macrophages. J Immunol 1991;146:2747–53.

[14] Carver MA, Turco SJ. Cell-free biosynthesis of lipophosphoglycan from *Leishmania donovani*. Characterization of microsomal galactosyltransferase and mannosyltransferase activities. J Biol Chem 1991;266:10974–81.

[15] Carver MA, Turco SJ. Biosynthesis of lipophosphoglycan from *Leishmania donovani*: Characterization of mannosylphosphate transfer in vitro. Arch Biochem Biophys 1992;295:309–17.

[16] Greis KD, Turco SJ, Thomas JR, McConville MJ, Homans SW, Ferguson MAJ. Purification and characterization of an extracellular phosphoglycan from *Leishmania donovani*. J Biol Chem 1992;267:5876–81.

[17] King DL, Turco SJ. A ricin agglutinin-resistant clone of *Leishmania donovani*. Mol Biochem Parasitol 1988;28:285–94.

[18] Elhay M, Kelleeher M, Bacic A, McConville MJ, Tolson DL, Pearson TW, Handman E. Lipophosphoglycan expression and virulence in ricin-resistant variants of *Leishmania major*. Mol Biochem Parasitol 1990;40:255–68.

[19] Ryan KA, Garraway LA, Descoteaux A, Turco SJ, Beverley SM. Isolation of virulence genes directing surface glycosyl-phosphatidylinositol synthesis by functional complementation of *Leishmania*. Proc Natl Acad Sci USA 1993;90:8609–13.

[20] Ma D, Russell DG, Beverley SM, Turco SJ. Golgi GDP-mannose uptake requires *Leishmania LPG2*-A member of a eukaryotic family of putative nucleotide-sugar transporters. J Biol Chem 1997;272:3799–805.

[21] Descoteaux A, Garraway LA, Ryan KA, Garrity LK, Turco SJ, Beveley SM. Identification of genes by functional complementation in the protozoan parasite *Leishmania*. In: Adolph KW, editor. Methods in Molecular Genetics. San Diego: Academic Press, 1994:22–48.

[22] Tolson DL, Turco SJ, Beecroft RP, Pearson TW. Immunochemical and cell surface arrangement of the *Leishmania donovani* lipophosphoglycan determined using monoclonal antibodies. Mol Biochem Parasitol 1989;35:109–18.

[23] Orlandi PA Jr., Turco SJ. Structure of the lipid moiety of the *Leishmania donovani* lipophosphoglycan. J Biol Chem 1987;262:10384–91.

[24] McConville MJ, Bacic A. A family of glycoinositol phospholipids from *Leishmania major:* Isolation, characterization, and antigenicity. J Biol Chem 1989;264:757–66.

[25] Volwerk JJ, Wetherwax PB, Evans LM, Kuppe A, Griffith OH. Phosphatidylinositol-specific Phospholipase C from *Bacillus cereus*: Improved purification, amino acid composition, and amino-terminal sequence. J Cell Biochem 1989;39:315–25.

[26] Mengeling BJ, Zilberstein D, Turco SJ. Biosynthesis of *Leishmania* lipophosphoglycan: solubilization and partial characterization of the initiating mannosylphosphoryltransferase. Glycobiology 1997;7:847–53.

[27] Ilg T, Harbecke D, Wiese M, Overath P. Monoclonal antibodies directed against *Leishmania* secreted acid phosphatase and lipophosphoglycan--Partial characterization of private and public epitopes. Eur J Biochem 1993;217:603–15.

[28] McConville MJ, Blackwell JM. Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*. Characterization of the promastigote and amastigote glycolipids. J Biol Chem 1991;266:15170–9.

[29] Huang C, Turco SJ. Defective galactofuranose addition in lipophosphoglycan biosynthesis in a mutant of *Leishmania donovani*. J Biol Chem 1993;268:24060–6.

[30] Gahmberg CG, Hakomori S. External labeling of cell surface galactose and galactosamine in glycolipid and glycoprotein of human erythrocytes. J Biol Chem 1973;248:4311–7.

[31] Butcher BA, Turco SJ, Hilty BA, Pimenta PF, Panunzio M, Sacks DL. Deficiency in β1,3-galactosyltransferase of a *Leishmania major* lipophosphoglycan mutant adversely influences the *Leishmania* sand fly interaction. J Biol Chem 1996;271:20573–9.

[32] McConville MJ, Homans SW. Identification of the defect in lipophosphoglycan biosynthesis in a non-pathogenic strain of *Leishmania major*. J Biol Chem 1992;267:5855–61.

[33] Elhay MJ, McConville MJ, Curtis JM, Bacic A, Handman E. Identification of truncated forms of lipophosphoglycan in mutant cloned lines of *Leishmania major* that are deficient in mature lipophosphoglycan. Parasitol Res 1993;79:435–88.

[34] Opat A, Ng K, Currie G, Handman E, Bacic A. Characterization of lipophosphoglycan from a Ricin-resistant mutant of *Leishmania major*. Glycobiology 1996;6:387–97.

[35] Brown GM, Millar AR, Masterson C, Brimacombe JS, Nikolaev AV, Ferguson MAJ. Synthetic phosphooligosaccharide fragments of lipophosphoglycan as acceptors for *Leishmania major* α-D-mannosylphosphate transferase. Eur J Biochem 1996;242:410–6.

[36] Pimenta PFP, Turco SJ, McConville MJ, Lawyer PG, Perkins PV, Sacks DL. Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. Science 1992;256:1812–5.

[37] Sacks DL, Saraiva EM, Rowton E, Turco SJ, Pimenta PF. The role of the lipophosphoglycan of *Leishmania* in vector competence. Parasitology 1994;108:55–62.

[38] Pimenta PFP, Saraiva EMB, Rowton E, et al. Evidence that the vectorial competence of phlebotomine sand flies for different species of *Leishmania* is controlled by structural polymorphisms in the surface lipophosphoglycan. Proc Natl Acad Sci USA 1994;91:9155–9.

[39] Sacks DL, Pimenta PFP, McConville MJ, Schneider P, Turco SJ. Stage-specific binding of *Leishmania donovani* to the sand fly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. J Exp Med 1995;181:685–97.

[40] Sacks DL. Metacyclogenesis in *Leishmania* promastigotes. Exp Parasitol 1989;69:100–3.

[41] Puentes SM, Sacks DL, Da Silva RP, Joiner KA. Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. J Exp Med 1988;167:887–902.

[42] Puentes SM, Da Silva RP, Sacks DL, Hammer CH, Joiner KA. Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. J Immunol 1990;145:4311–6.

[43] Desjardins M, Descoteaux A. Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. J Exp Med 1997;185:2061–8.

[44] Miao L, Stafford A, Nil S, Turco SJ, Flanagan TD, Epand RM. Potent inhibition of viral fusion by the lipophosphoglycan of *Leishmania donovani*. Biochemistry 1995;34:4676–83.