Glycoinositol phospholipids from *Endotrypanum* species express epitopes in common with saccharide side chains of the lipophosphoglycan from *Leishmania major*

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We have characterized glycoinositol phospholipids (GIPLs) from three strains of the trypanosomatid parasites *Endotrypanum schaudinni* and *Endotrypanum monterogei*. Methanolysis of the intact GIPLs liberated methyl esters of tetracosanoic acid, docosanoic acid, octadecanoic acid and hexadecanoic acid and C₂₀ and C₂₁ phytosphingosines. Phosphoinositol oligosaccharides were released from the GIPLs by mild base treatment, and their structures were determined by compositional analysis, fast-atom-bombardment MS and NMR spectroscopy. Similar compounds were detected in all three strains, although their

relative proportions varied. The predominant components in *E. schaudinni* strain LV59 and *E. monterogei* LV88 were Galpβ1-3Galpβ1-3Manα1-3Manα1-4GlcNα1-6Ins-1-*P* and Arapβ1-2Galpβ1-3Galpβ1-3Manα1-3Manα1-4GlcNα1-6Ins-1-*P*, and the major phosphoinositol oligosaccharide in *E. schaudinni* LV58 was the hybrid-type GIPL Manα1-2(EtNP-6)Manα1-6(Galpβ1-3Manα1-3)Manα1-4GlcNα1-6Ins-1-*P* (where EtNP is ethanolamine phosphate). Several minor oligosaccharides containing additional galactose and/or arabinose residues were also detected.

INTRODUCTION

Endotrypanum is a genus of heteroxenous trypanosomatid parasites of two-toed (*Choloepus* spp.) and three-toed sloths (*Bradypus* spp.) indigenous to Central and South America [1,2]. Uniquely among trypanosomatids, the bloodstream forms of *Endotrypanum* inhabit the erythrocytes of its mammalian host. Two-toed sloths are important animal reservoirs for the *Leishmania braziliensis* complex, which is an important cause of cutaneous leishmaniasis in the New World [3]. Sloths have also been implicated as reservoir hosts for at least five other human-pathogenic *Leishmania* species [4]. It is thought that *Endotrypanum* is transmitted, like New World *Leishmania*, via sandflies of the genus *Lutzomyia* [3,5–7]. Natural and experimental infections of sandflies are indistinguishable, and are characterized by high concentrations of promastigotes in the pyloric region of the hindgut, thus resembling the insect development stage of *Leishmania* of the subgenus *Viannia* [8], although unlike *Endotrypanum*, the latter do not develop in the Malpighian tubules of *Lutzomyia* [9].

Two species of *Endotrypanum* are currently recognized, *E. schaudinni* and *E. monterogei*, which were originally differentiated morphologically (*E. monterogei* produces intraerythrocytic trypomastigotes rather than epimastigotes) [1]. Subsequent studies, however, have questioned the division into two species. Isoenzyme analysis and kinetoplast DNA buoyant-density measurements failed to distinguish the two [10], and neither did transmission electron microscopy [11] or a panel of 10 *Endotrypanum*-specific monoclonal antibodies [12]. Analysis of nuclear DNA restriction patterns of 17 *Endotrypanum* strains [13] indicated the existence of three groups (A, B and C). *E. monterogei* clustered in group B, together with several *E. schaudinni* strains, which suggests that its retention as a species distinct from *E. schaudinni* should be reconsidered. The divergence of nuclear

DNA between the three groups was comparable with that within some *Leishmania* species complexes [14], and suggests that *Endotrypanum* may also constitute a species complex. Recent molecular phylogenetic studies [15,16] have indicated that the organisms known as *Leishmania herreri* and *Leishmania hertigi* may be more appropriately classified as *Endotrypanum* species. Methods for differentiating *Leishmania* from *Endotrypanum* included isoenzyme profiles [7], monoclonal antibodies [12] and restriction length polymorphism [13]. DNA hybridization has also been used, but insufficient discrimination between the two genera was observed with a total *Leishmania* DNA probe [17], and an *Endotrypanum* kinetoplast DNA probe showed cross-hybridization with *L. braziliensis* [18]. However, a hybridization probe derived from the mini-exon intergenic region of *E. schaudinni* strain LV86 satisfactorily distinguishes *Endotrypanum* from common New World *Leishmania* species [19].

Although genetic and biological evidence implies a close relationship between *Endotrypanum* and New World *Leishmania* of the subgenus *Viannia*, there are some intriguing differences. Whereas *Leishmania* are able to colonize a range of mammalian hosts, including humans and sloths, *Endotrypanum* infections are unique to two-toed, and more rarely three-toed, sloths. Attempts to experimentally infect other mammals with either bloodstream or culture-derived *Endotrypanum* have invariably been unsuccessful [1]. In contrast with *Leishmania*, there is no evidence of *in vivo* invasion or multiplication in phagocytic cells, although transient *in vitro* infections of mouse macrophages by *Endotrypanum* promastigotes have been reported [20]. It thus seems likely that *Endotrypanum* does not possess the ability to evade destruction by macrophages to the same extent as *Leishmania*, which may explain its inability to colonize hosts other than sloths. In *Leishmania*, survival in the macrophage is attributable, in part, to the presence of surface lipophosphoglycan (LPG) [21]. It is therefore possible that differences in cell-surface glyco-

Abbreviations used: EtNP, ethanolamine phosphate; FAB, fast atom bombardment; GIPLs, glycoinositol phospholipids; HMQC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; LPG, lipophosphoglycan; nOe, nuclear Overhauser effect; [M+H]⁺, protonated molecule; [M–H][–], deprotonated molecule; PAS, periodate–Schiff's reagent; PI, phosphoinositol.

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conjugates explain the restricted host range of *Endotrypanum* compared with that of *Leishmania*, either because it lacks a protective component such as LPG or possibly because its surface is highly immunogenic and triggers a powerful immune response in species other than sloths. Although the surface glycoconjugates of *Leishmania* species are well characterized [22], little is known about those of *Endotrypanum*. We have therefore characterized glycoinositol phospholipids (GIPLs) from three *Endotrypanum* strains, using chemical analysis, fast-atom-bombardment (FAB)-MS and NMR spectroscopy. These compounds comprise a novel family of linear and hybrid-type galactose-containing GIPLs, expressing terminal sequences which are also present in the saccharide side chains of the LPG from *Leishmania major* [22] and *Leishmania tropica* [23].

MATERIALS AND METHODS

Trypanosomatid strains and growth conditions

MBRA/PA/67/LV58 and MCHO/PA/68/LV59 strains of *E. schaudinni* were supplied by Dr. Jeffrey J. Shaw (Wellcome Parasitology Unit, Instituto Evandro Chagas, Fundação Nacional de Saúde, Belém, Pará, Brasil). *E. monterogeii* strain MCHO/CR/62/LV88,A-9 was donated by Dr. M. Chance (Liverpool School of Tropical Medicine, Liverpool, U.K.) and *E. schaudinni* (strain MCHO/BR/80/M6159) was provided by Dr. A. H. C. Lopes (Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Brasil). *Crithidia fasciculata* was from our laboratory culture collection. Promastigotes of all strains were grown in a brain/heart infusion medium containing 5% fetal calf serum at 26 °C for 3–4 days. The cells were harvested by centrifugation, washed three times with 0.9% NaCl and frozen at –20 °C until use.

Extraction of GIPLs

Harvested promastigotes (5×10^{10}) were extracted at 80 °C with aq. 45% phenol for 10 min. After dialysis and freeze-drying, the aqueous layer was applied to a column (2 cm $\times$ 100 cm) of Bio-Gel P-60. The excluded material was freeze-dried and the GIPLs recovered by extraction (twice) with chloroform/methanol/water (10:10:3, by vol.). The extracts were combined, evaporated to dryness, dissolved in water, and freeze-dried as previously described [24].

SDS/PAGE

GIPLs were analysed by SDS/PAGE under reducing conditions using a discontinuous buffer system on 6–7 cm slab gels containing 15% polyacrylamide [25]. Carbohydrate and lipids were detected by periodate–Schiff's Reagent (PAS) [26] and Sudan Black staining [27] respectively.

Isolation of phosphoinositol (PI) oligosaccharides

PI-oligosaccharides were liberated from intact GIPLs by hydrolysis with 1 M KOH, for 72 h at 37 °C [28]. After neutralization with acetic acid, non-polar material was removed by chloroform extraction. The aqueous layer was passed through a column (2 cm $\times$ 20 cm) of Dowex 50W-X8 (H⁺) resin, and the desalted PI-oligosaccharides were fractionated on a column of Bio-Gel P-4 using water as eluent.

Analytical procedures

Neutral sugars were analysed by GLC of their trimethylsilylated methylglycosides, as previously described [29]. Inositol and glucosamine were quantified by GLC after methanolysis (3 M

methanolic HCl for 18 h at 80 °C), hydrolysis with 6 M HCl (18 h; 100 °C), NaBH₄ reduction and acetylation with acetic anhydride/pyridine. The absolute configurations of arabinose and galactose were determined by GLC of their trimethylsilylated (–)-2-butylglycosides [30]. For fatty acid analysis, GIPLs were methanolysed in 0.5 M HCl in methanol for 18 h at 80 °C, the solution was extracted with heptane and the resulting mixture of fatty acid methyl esters analysed by GLC and identified by comparison of retention times with those of authentic standards. Long-chain bases were identified by GLC and GLC/MS after hydrolysis of intact GIPLs with 1 M HCl in methanol (made 10 M with respect to water) for 18 h at 80 °C. The pH was adjusted to 11 with aq. NaOH, and the long-chain bases were extracted three times with 2 vol. of diethyl ether. The combined extracts were dried with Na₂SO₄, evaporated to dryness under nitrogen, dissolved in methanol, *N*-acetylated and trimethylsilylated [31]. The procedures of Ames [32] and Bartlett [33] were used to measure total and acid-hydrolysable phosphate respectively.

GLC

GLC was performed with a DB-1 fused silica column (0.25 mm internal diameter $\times$ 30 m) using hydrogen (0.7×10^5 Pa) as carrier gas. For analysis of trimethylsilylated methylglycosides and acetylated inositol and glucosaminitol, the column oven was temperature programmed from 120 to 240 °C at 2 °C/min and held at the upper temperature. For GLC of fatty acid methyl esters, a temperature program of 110 to 240 °C (4 °C/min, then hold) was used. A program of 180 to 310 °C (4 °C/min, then hold) was employed for GLC of *O*-trimethylsilyl ethers of *N*-acetyl-sphingosine bases.

GLC/MS

Capillary GLC/MS of *O*-trimethylsilyl ethers of *N*-acetyl-sphingosine bases was carried out using a silicone OV-101 capillary column (0.2 mm internal diameter $\times$ 25 m) and helium at 5 kPa as carrier gas, with temperature programming from 180 to 310 °C at 4 °C/min.

NMR spectroscopy

NMR spectra of PI-oligosaccharides were obtained using a Varian Unity 500 spectrometer equipped with a 5 min triple-resonance probe at an indicated probe temperature of 30 °C, as previously described [34], except that some TOCSYCHO spectra employed a 100 ms spinlock period. Proton chemical shifts were referenced to internal acetate anion at 1.908 p.p.m. Some heteronuclear multiple quantum coherence (HMQC) spectra were obtained without heteronuclear decoupling to enable measurement of $^1J_{CH}$ values. The field-gradient-enhanced heteronuclear single quantum coherence (HSQC) spectra were obtained on a Varian Unity 600 spectrometer, using a pulse sequence derived from that of Wider and Wüthrich [35] and optimized for $^1J_{CH}$ of 150 Hz or $^nJ_{CH}$ of 10 Hz. Susceptibility-matched low-volume NMR tubes were used (Shigemi, Tokyo, Japan).

FAB-MS

FAB mass spectra were recorded using a Kratos MS80RFA spectrometer equipped with an Ion Tech FAB gun using xenon atoms as the bombarding particles. PI-oligosaccharides were dissolved in 30% acetic acid to a concentration of about 10 $\mu\text{g}/\mu\text{l}$ and 1 μl was mixed with an equal volume of matrix [dithiothreitol/dithioerythritol (5:1, w/w) mixed 1:1 (v/v) with glycerol]. Spectra were recorded in both positive- and negative-ion

modes at a resolution of 1000. Collisional activation was performed with helium (50 % precursor beam attenuation) in the field-free region between the ion source and the electrostatic analyser, and daughter ion spectra were recorded by means of computer-generated B/E scans.

Antisera

Anti-*E. schaudinni* LV58) serum was obtained as described by Mendonça-Previato et al. [36]. Rabbits were immunized by intramuscular injection of 10^8 promastigotes emulsified in complete Freund's adjuvant (two injections, with an interval of 7 days). One week after the second injection, the rabbits were boosted three times (every other day) by the intravenous route. The animals were bled 5–7 days after the final booster injection.

Indirect immunofluorescence studies

Endotrypanum strains (M6159, LV58, LV59 and LV88) and *Crithidia fasciculata* (2.5×10^7 parasites) were fixed in methanol for 20 min at 4 °C. After resuspension in PBS, the parasites were incubated with anti-LV58 serum at dilutions of 1:500; 1:1000; 1:2000; 1:3000 and 1:4000 for 1 h at room temperature. The parasites were then washed three times with PBS and incubated for 30 min at room temperature with anti-rabbit IgG conjugated with FITC. To investigate the presence of LPG-like material, the *Endotrypanum* strains were analysed by immunofluorescence, using the mouse IgM monoclonal antibody CA7AE (provided by Professor S. Turco, Department of Biochemistry, University of Kentucky Medical Center, Lexington, CT, U.S.A.), which is specific for the phosphorylated disaccharide repeats of *Leishmania donovani* LPG [37]. Fixed cells were incubated with monoclonal antibody CA7AE diluted to 1:200, for 1 h at room temperature. Binding was detected by incubation with goat anti-IgM-FITC at 1:50. Fluorescence was observed with a Zeiss confocal laser-scan microscope.

RESULTS

Chemical composition of total GIPLs isolated from *E. schaudinni* and *E. monterogeii*

Promastigotes of strains LV58 and LV59 of *E. schaudinni* and strain LV88 of *E. monterogeii* were extracted with phenol/water, and the GIPLs were removed from the aqueous layer with chloroform/methanol/water. The GIPLs were revealed by SDS/

Figure 1 PAGE of GIPLs from *Endotrypanum*

Lane A, LV58 strain; lane B, LV59 strain; lane C, LV88 strain. The gel was stained with PAS.

Figure 2 Analysis of long-chain base (by GLC/MS) of *Endotrypanum* GIPL (strain LV88)

Inset, total-ion chromatogram of *N*-acetyl-*O*-trimethylsilyl bases after electron-impact ionization: peak A, trimethylsilyl ether of *N*-acetyl- C_{20} -phytosphingosine; peak B, trimethylsilyl ether of *N*-acetyl- C_{21} -phytosphingosine. (A) and (B) Mass spectra of peaks A and B (inset) respectively.

PAGE as broad bands in the 12–15 kDa range which were stained with PAS (Figure 1) and Sudan Black reagents. Compositional analysis demonstrated the presence of galactose, mannose, arabinose, glucosamine, inositol, tetracosanoic acid, docosanoic acid, octadecanoic acid and hexadecanoic acid. Acid methanolysis of the unfractionated GIPLs released long-chain bases, which were analysed by GLC (Figure 2 inset) and GLC/MS (Figure 2) after *N*-acetylation and *O*-trimethylsilylation. Two major GLC peaks were present in all strains in approximately similar proportions. The electron-ionization mass spectra of these contained a prominent fragment at m/z 276 attributable to cleavage between C-3 and C-4 of phytosphingosine. No molecular ions were present, but the spectrum of the peak eluted first had an $M-15$ fragment at m/z 588 whereas in the other component the $M-15$ fragment was observed at m/z 602, which suggested that the two bases were C_{20} -phytosphingosine and C_{21} -phytosphingosine respectively.

Structural analysis of PI-oligosaccharides from GIPL of *Endotrypanum*

$^1\text{H-NMR}$ spectroscopy of the fractions from gel-filtration chromatography indicated that the same components were present in all three strains, although their relative proportions varied and there was evidence of cross-contamination between fractions.

Table 1 Structures of PI-oligosaccharides from GIPLs of *Endotrypanum*

PI-oligosaccharide	Structure
A0	$\alpha\text{-Man-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 4)\text{-GlcN-(1} \rightarrow 6)\text{-Ins-}P$
A1	$\beta\text{-Galp-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 4)\text{-GlcN-(1} \rightarrow 6)\text{-Ins-}P$
A2	$\beta\text{-Galp-(1} \rightarrow 3)\text{-}\beta\text{-Galp-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 4)\text{-GlcN-(1} \rightarrow 6)\text{-Ins-}P$
A3	$\beta\text{-D-Arap-(1} \rightarrow 2)\text{-}\beta\text{-Galp-(1} \rightarrow 3)\text{-}\beta\text{-Galp-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 4)\text{-GlcN-(1} \rightarrow 6)\text{-Ins-}P$
A4	$\beta\text{-Galp-(1} \rightarrow 3)\text{-}\beta\text{-Galp-(1} \rightarrow 3)\text{-}\beta\text{-Galp-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 3)\text{-}\alpha\text{-Man-(1} \rightarrow 4)\text{-GlcN-(1} \rightarrow 6)\text{-Ins-}P$
B1	$\begin{array}{c} \text{EtNP} \\ \downarrow \\ \alpha\text{-Man(1} \rightarrow 2)\text{-}\alpha\text{-Man(1} \rightarrow 6) \\ \begin{array}{cc} (4) & (3) \end{array} \end{array}$ $\begin{array}{c} \diagup \quad \diagdown \\ \beta\text{-Galp(1} \rightarrow 3)\text{-}\alpha\text{-Man(1} \rightarrow 3) \quad \alpha\text{-Man-(1} \rightarrow 4)\text{-GlcN-(1} \rightarrow 6)\text{-Ins-}P \\ (1) \quad (2) \end{array}$
B2	$\begin{array}{c} \text{EtNP} \\ \downarrow \\ \alpha\text{-Man(1} \rightarrow 2)\text{-}\alpha\text{-Man(1} \rightarrow 6) \\ \begin{array}{cc} (4) & (3) \end{array} \end{array}$ $\begin{array}{c} \diagup \quad \diagdown \\ \beta\text{-Galp(1} \rightarrow 3)\text{-}\beta\text{-Galp(1} \rightarrow 3)\text{-}\alpha\text{-Man(1} \rightarrow 3) \quad \alpha\text{-Man-(1} \rightarrow 4)\text{-GlcN-(1} \rightarrow 6)\text{-Ins-}P \\ (2) \quad (1) \end{array}$
B3	$\begin{array}{c} \text{EtNP} \\ \downarrow \\ \alpha\text{-Man(1} \rightarrow 2)\text{-}\alpha\text{-Man(1} \rightarrow 6) \\ \begin{array}{cc} (4) & (3) \end{array} \end{array}$ $\begin{array}{c} \diagup \quad \diagdown \\ \beta\text{-D-Arap-(1} \rightarrow 2)\text{-}\beta\text{-Galp(1} \rightarrow 3)\text{-}\beta\text{-Galp(1} \rightarrow 3)\text{-}\alpha\text{-Man(1} \rightarrow 3) \quad \alpha\text{-Man-(1} \rightarrow 4)\text{-GlcN-(1} \rightarrow 6)\text{-Ins-}P \\ (1) \quad (2) \end{array}$

The fractions were further characterized by chemical methods, FAB-MS and two-dimensional homo- and hetero-nuclear NMR. The structures of major and minor PI-oligosaccharides are shown in Table 1, and the NMR assignments are listed in Table 2.

Characterization of PI-oligosaccharide A0

The smallest component, Man α 1-3Man α 1-4GlcN α 1-6Ins1/2-*P*, was observed in significant amount in *E. schaudinni* strain LV58, but was also detected by FAB-MS in *E. monterogii*. Protonated molecules were observed at m/z 746.2 in the positive-ion FAB spectrum, and an $[M-H]^-$ signal at m/z 744.2 by negative-ion FAB-MS. The daughter-ion spectrum obtained by collisional activation of the $[M+H]^+$ ion at m/z 746 contained Y_3 and Y_2 fragments at m/z 584 and 422 respectively, and an abundant B_3 ion at m/z 486, which was consistent with the structure Hex-Hex-HexN-Ins-*P*. A TOCSY spectrum with 100 ms mixing time showed four spin systems consistent with glucosamine, inositol and two mannose residues. Phosphate migration from Ins-*O*-1 to -*O*-2 during base hydrolysis accounts for the heterogeneity observed in the spectrum [24]. The assigned proton chemical shifts (H-1 to H-4) of one of the α -Man residues were consistent with a terminal location, whereas those of the other were identical with those of the -3Man α 1-4 in the higher homologues (Table 2). This compound is structurally related to a minimal-core oligosaccharide of type-2 GIPLs of *Leishmania* [22].

Characterization of PI-oligosaccharide A1

PI-oligosaccharide A1 was observed in all strains, and represents one of the more abundant components from *E. schaudinni* strain LV59, and *E. monterogii* strain LV88. FAB-MS analysis indicated the presence of an extra hexose compared with the previous compound ($[M+H]^+$ at m/z 908.3; $[M-H]^-$ at 906.3). Glycosidic cleavage fragments at m/z 746 (Y_4), 584 (Y_3) and 422 (Y_2) suggested a linear sequence of hexose residues. The NMR spectrum of oligosaccharide A1 differed from compound A0 by the presence of an additional β -Galp spin system (Figure 3A). The 1H and ^{13}C chemical shifts of this residue were consistent with a terminal location, and the value of the chemical shift of the C-3 of the distal mannose residue (79.28 assigned from an HMQC experiment) indicated that this is the glycosylation site, showing that this GIPL has the non-reducing terminal sequence Galp β 1-3Man α 1-3Man.

Characterization of PI-oligosaccharide A2

PI-oligosaccharide A2 is one of most abundant components in all three strains. The molecular mass determined by FAB-MS was 1069.4 Da ($[M+H]^+ = 1070.4$; $[M-H]^- = 1068.4$), suggesting the presence of an additional hexose compared with PI-oligosaccharide A1. Fragment ions at m/z 906.2 (Y_5), 744 (Y_4), 582 (Y_3) and 422 (Y_2) were consistent with a linear sequence of

Table 2 ^1H - and ^{13}C -NMR chemical shifts (p.p.m.) of PI-oligosaccharides from *Endotrypanum* GIPLs

Values in square brackets mean that the assignment is tentative.

PI-oligosaccharide	H-1	H-2	H-3	H-4	H-5	H-6	H-6'	C-1	C-2	C-3	C-4	C-5	C-6
A0													
Man (2)	5.146	4.085	3.895	3.657									
Man (1)	5.278	4.225	3.954										
GlcN	Major	5.640	3.372	4.087	3.790	4.196	3.865	3.865					
	Minor	5.469	3.469	4.110									
Ins-1- <i>P</i>	4.154	4.210	3.580	3.707	3.429	3.909							
A1													
Galp (1)	4.561	3.615	3.688	3.945				102.20	71.68		69.81		
Man (2)	5.203	4.282	4.141					103.07	68.91	79.28			
Man (1)	5.279	4.229	3.955					102.58	71.06	78.91			
GlcN	Major	5.617	3.394	4.096	3.796	4.210	3.865	3.865	96.47	55.11	71.56	76.79	71.93
	Minor	5.469	3.392										61.20
Ins-1- <i>P</i>		4.214	3.582	3.714	3.437	3.936			72.68	71.68	73.43	73.92	78.53
A2													
Galp (2)	4.635	3.629	3.682	3.937		3.790	3.760	105.40	72.20	73.68	69.76		62.16
Galp (1)	4.619	3.779	3.860	4.220		3.790	3.760	101.77	71.12	83.28	69.61		62.16
Man (2)	5.201	4.282	4.155	3.816		[3.944]	[3.785]	102.98	68.88	79.28	67.06		[62.31]
Man (1)	5.279	4.226	3.957	3.796		[3.900]	[3.785]	102.46	71.01	78.82	66.42		[61.84]
GlcN	Major	5.686	3.346	4.076	3.782	4.174	3.863	96.42	55.03	71.18	76.80	71.82	61.16
	Minor	5.476	3.385	4.110	3.807	4.170							
Ins-1- <i>P</i>	4.104	4.203	3.579	3.701	3.422	3.881			72.92	71.72		74.02	78.76
Ins-2- <i>P</i>			3.531	3.721	3.279	3.869							
A3													
Arap (1)	5.373	3.866	3.905	4.005	4.399	3.633		100.81				64.48	
Galp (2)	4.739	3.712	3.882	3.921				104.11	77.96				62.16
Galp (1)	4.575	3.734	3.860	4.208				102.1	71.16	82.44	70.02		62.16
Man (2)	5.200	4.276	4.144	3.805	3.8	3.936		102.94	69.01	79.27	67.92		[62.32]
Man (1)	5.278	4.226	3.958	3.778	[3.813]	3.889	3.957	102.46	71.05	78.84	66.40		[61.82]
GlcN	Major	5.686	3.348	4.077	3.781	4.173	3.862	96.43	55.01	71.79	76.80	71.82	61.01
	Minor	5.480	3.383	4.110									
Ins-1- <i>P</i>	4.102	4.204	3.580	3.700	3.421	3.875			72.96	71.70			78.82
A4													
Galp (3)	4.640	3.642	3.684	3.942									
Galp (2)	4.702	3.804	3.865	4.213									
Galp (1)	4.622	3.787	3.873	4.222									
Man (2)	5.199	4.287	4.167										
Man (1)	5.279	4.231											
GlcN	Major	5.708	3.352	4.074	3.767	4.191							
	Minor	5.489	3.398										
Ins-1- <i>P</i>	4.093	4.206	3.583	3.707	3.427	3.879							
Ins-2- <i>P</i>		4.515											
B1													
Galp (1)	4.564	3.615	3.695	3.946	3.732	3.79	3.79	102.21	71.96	73.98	69.81	75.92	62.09
Man (2)	5.193	4.283	4.145	3.811	[3.852]	3.944	3.798	103.07	68.99	79.41	66.51	74.25	62.30
Man (4)	5.061	4.103	3.887	3.775	3.902	3.900	3.782	103.57	71.06	71.42	67.48	73.19	62.11
Man (3)	5.135	4.041	3.987	3.724		4.126	4.152	99.46	80.03	71.42	68.10	73.97	65.97
Man (1)	5.248	4.237	3.959	3.905		4.018	3.790	102.83	71.06	79.24	66.90	73.58	67.07
GlcN	Major	5.708	3.352	4.074	3.767	4.191	3.87	96.47	54.93	71.80	77.29	71.92	61.22
	Minor	5.489	3.398	4.089	3.784	4.165	3.87	98.32	55.88				
Ins-1- <i>P</i>	4.093	4.206	3.583	3.707	3.427	3.879		76.31	73.05	71.73	73.61	73.92	78.91
Ins-2- <i>P</i>		4.515	3.503	3.755	3.370	3.849				72.51		74.16	
EtNP	3.311	4.160						41.24	63.14				

hexose residues. The NMR spectrum was similar to that of PI-oligosaccharide A1, except for the presence of an additional β -Galp spin system (Figure 3B). This residue was shown to be 1-3-linked to Galp β 1-3Man α 1- from the low-field value of the chemical shift of C-3 of the other Galp (83.28 p.p.m.). The linkage pattern was established from the ^{13}C chemical shifts (determined from HMQC spectra of both the LV58 and LV59 PI-oligosaccharides) and by comparison of the β -Galp ^1H chemical shifts with model systems [38]. A related component,

with a protonated molecule at m/z 1232.5 and containing an additional Galp β 1-3 spin system in its NMR spectrum, was detected in strain LV58 (Structure A4, Table 1).

Characterization of PI-oligosaccharide A3

PI-oligosaccharide A3 expressed by all strains is the major component from *E. schaudinni* LV59 and *E. monterogeii* LV88.

Figure 3 Partial 500 MHz NMR spectra of PI-oligosaccharides from *Endotrypanum* GIPL (strain LV58)

(A) PI-oligosaccharide A1 (containing about 25% of A0); (B) PI-oligosaccharide A2; (C) PI-oligosaccharide A3; (D) PI-oligosaccharide B1. The labels indicate the resonances of the anomeric protons.

FAB-MS data ($[M+H]^+ = 1202.5$; $[M-H]^- = 1200.5$) were consistent with the addition of a pentose residue to compound A2. A facile loss of 132 from the protonated and deprotonated molecules suggested that this pentose was terminally located. Fragment ions in the negative-ion spectrum at m/z 1068 (Y_6), 906 (Y_5), 744 (Y_4), 582 (Y_3) and 420 (Y_2) suggested that the terminal pentose was linked to a linear chain of hexose residues (Figure 4). In the positive-ion collision-induced daughter-ion spectrum, in addition to the Y_n series of fragments, an abundant B_6 ion was observed at m/z 942, consistent with loss of inositol phosphate from the reducing terminal. NMR spectroscopy showed that the pentose was arabinose, and GLC of its trimethylsilylated (–)-2-butylglycoside indicated that it had the unusual D configuration. Inter-residue nuclear Overhauser effects (nOes) (from a rotating-frame nuclear Overhauser enhancement spectroscopy spectrum) and ^{13}C chemical-shift evidence (from

HMQC spectra) showed that the terminal D-Arap residue was β 1-2-linked to β -Galp. Other nOes confirmed the remaining linkage positions in the Galp β 1-3Man α 1-3Man α 1-4GlcN α 1-6Ins-1/2-P core (Table 1).

Characterization of PI-oligosaccharide B1

FAB-MS of PI-oligosaccharide B1 suggested a molecular mass of 1354.5 Da ($[M+H]^+ = 1355.2$; $[M-H]^- = 1353.5$), which is consistent with a composition of five hexose units, hexosamine, inositol phosphate and one equivalent of ethanolamine phosphate (EtNP). Fragment ions in the positive-ion collision-induced dissociation spectrum were observed at m/z 1193, 1031, 908 and 422, but not at m/z 746 or 584, suggesting the presence of a dihexosyl branch on the innermost hexose. The absence of an abundant fragment at m/z 545 suggests that the EtNP substituent

Figure 4 Analysis by FAB-MS of the PI-oligosaccharide A3 from *Endotrypanum* GIPL (strain LV59)

Partial negative-ion FAB mass spectrum.

Figure 5 Analysis by NMR of the PI-oligosaccharide B1 from *Endotrypanum* GIPL (strain LV58)

Partial 600 MHz gradient-enhanced HSQC spectrum of a fraction containing predominantly, PI-oligosaccharide B1 from *E. schaudinni* strain LV58. This region of the spectrum shows long-range correlations between the α -Man H-1 and ^{13}C resonances in the ring or across the glycosidic linkage.

is not located on hexosamine, and it cannot be attached to either of the terminal hexoses because there is no Y_4 fragment at m/z 1070 in the daughter-ion spectrum of m/z 1355. The presence of a B_2 fragment at m/z 448, however, strongly suggests that one of the subterminal hexoses bears the EtNP substituent; a similar fragment is found in the spectrum of the EtNP-substituted hybrid GIPL, EPiM4, found in *Leishmania mexicana* [39] and *Leishmania adleri* [40]. It is not possible to say from the FAB-MS data which of the two subterminal residues is substituted. Four α -Man spin systems, and one equivalent of EtNP were observed in the NMR spectra of PI-oligosaccharide B1 (Figure 3D). The

^1H -NMR spectra were assigned by conventional two-dimensional methods, and the ^{13}C spectra by means of gradient-enhanced HSQC experiments at 600 MHz (Figure 5). The residue sequence and linkages were determined from nOe measurements and long-range ^1H - ^{13}C correlations observed in an HSQC experiment optimized for $^nJ_{\text{CH}}$ of 10 Hz, and were confirmed by ^{13}C chemical-shift evidence. The EtNP residue was located to an O-6 by the presence of a low-field resonance (at 65.97 p.p.m.) coupled to low-field H-6 and H-6' resonances (at 4.15 and 4.13 p.p.m. respectively), which were in turn correlated to the phosphoester resonances by mean of a ^1H - ^{31}P correlation experiment. It

proved difficult to identify unambiguously the spin system to which the C-6 and H-6 resonances belong, and the assignment to the Man α 1-6 residue is by elimination, all other sugar spin systems having been fully assigned.

FAB-MS also provided evidence for the presence of higher EtNP-containing homologues with an additional hexose ($[M+H]^+$ at m/z 1517) or a hexose and a pentose ($[M+H]^+$ at m/z 1649) which are tentatively assigned as structures B2 and B3 (Table 1). NMR evidence also suggested the presence of hybrid structures lacking the EtNP substituent, as found in GIPLs from *L. adleri* [40].

Immunofluorescence assay

The antigenic properties of the *Endotrypanum* surface were investigated by indirect immunofluorescence. Antiserum raised against LV58 cells showed a diffuse continuous fluorescent pattern over the body and flagella of promastigotes of homologous cells. Cross-reaction was observed with heterologous strains, including *E. schaudinni* strain LV59 and *E. monterogeii* strain LV88. The serum was titrated in 0.1 M PBS and the most suitable working dilution was found to be between 1:3000 and 1:4000. Cross-reaction was observed with *E. schaudinni* M6159 and *C. fasciculata* only when the anti-LV58 serum was diluted less than 1:50. To verify the presence of LPG-like structures in *Endotrypanum*, promastigotes were investigated by immunofluorescence, using a mouse IgM monoclonal antibody CA7AE that recognizes the phosphorylated disaccharide repeats of LPG from *L. donovani* [37]. Whereas intense fluorescence was observed with the surface of *L. donovani* promastigotes, no fluorescence was observed with strains LV58, LV88 and M6159 or with cells of *C. fasciculata*. Most cells of *Endotrypanum* strain LV59 gave a negative reaction, but poor fluorescence was observed with a small proportion.

DISCUSSION

In this study, we have characterized the first members of a novel family of glycolipids from the trypanosomatid genus *Endotrypanum*. The three strains investigated, representing the species *E. schaudinni* and *E. monterogeii*, were shown to synthesize several structurally related GIPLs of which those designated A1, A2, A3 and B1 (Table 1) predominated, although their relative proportions varied between strains. Although similar in glycan-core structures to the glycerol-linked type-2 and hybrid GIPLs synthesized by the leishmanial parasites of mammals [22] and reptiles [40], the compounds from *Endotrypanum* are sphingolipids. Several of these compounds contain terminal β -D-Ara residues, a feature that has not been observed in the GIPLs from *Leishmania*. In the galactose-containing type-2 GIPLs of *Leishmania*, the conserved Man α 1-3Man α 1-4GlcN α 1-6Ins glycosylphosphatidylinositol core sequence is connected to the galactopyranose extension via a β -Galp residue, whereas in the linear (A-series) GIPLs from *Endotrypanum*, the link residue is another galactopyranose unit. The GIPLs of the three *Endotrypanum* strains also differ in that all the galactose residues have the β configuration, whereas *Leishmania* GIPLs contain predominantly α -linked galactopyranose residues. In contrast with their counterparts in *Leishmania*, the hybrid (B-series) GIPLs found in *Endotrypanum* LV58 have their α (1-3)mannosyl arms extended by Galp β 1-3 (in B1) and by Galp β 1-3Galp or Arap β 1-2Galp β 1-3Galp in PI-oligosaccharides B2 and B3 (Table 1).

In a previous paper, we showed that the major GIPL from *E. schaudinni* strain MCHO/BR/80/M6159 was the phytosphingosine-linked PI-oligosaccharide Arap β 1-2Galp β 1-3Galp α 1-

3Galp β 1-3[EtNP6-O]Manp α 1-3Manp α 1-4[EtNP6-O]GlcN1-6InsP [41]. This structure resembles PI-oligosaccharides from strains LV58, LV59 and LV88 to the extent that it is capped by a terminal β -D-Arap1-2Galp β 1-3 disaccharide, but is also similar to the galactose-containing type-2 GIPLs from *Leishmania* in that the terminal three residues (one of which is α -Galp) are connected to the α 1-3-linked mannose via galactofuranose. The structure is also closely related to the ceramide-linked galactofuranose-containing glycosylphosphatidylinositol anchor of the lipoarabinogalactan from *C. fasciculata* [42], a monoxenous trypanosomatid which constitutes a sister lineage to the dioxenous genera *Endotrypanum* and *Leishmania*. Unlike the crithidial lipoarabinogalactan anchor, or any previously described leishmanial GIPL, the GIPL from *E. schaudinni* strain M6159 contains two EtNP substituents, one of them on O-6 of glucosamine. Interestingly, it has recently been shown that strain M6159 displays some serological differences from other *Endotrypanum* strains investigated [43].

The distinctive GIPL compositions of *Endotrypanum* compared with lizard and mammalian *Leishmania* could be interpreted as being consistent with recent molecular phylogenetic data [15,16] which show *Endotrypanum* branching off the leishmanial lineage before the *Sauroleishmania*, or members of the subgenera *Leishmania* and *Viannia*. However, since the GIPLs of strain M6159 differ from those described here, and since intra-specific polymorphisms in GIPL profile appear to be quite common, having also been described in *L. major* [44] and *Trypanosoma cruzi* [45], the inference of phylogenetic relationships from such data is probably unwise. It is similarly unclear that our data enable resolution of the question whether *E. schaudinni* and *E. monterogeii* constitute separate species, although the GIPLs of strains LV58, LV59 and LV88 exhibit greater homogeneity in both lipid and PI-oligosaccharide composition than is found between *Leishmania* species.

The terminal saccharides linked to the α (1-3)mannose (Galp β 1-3 in A1 and B1, Galp β 1-3Galp in A2 and B2, Arap β 1-2Galp β 1-3Galp in A3 and Galp β 1-3Galp β 1-3Gal in A4) are closely related to the saccharide side chains that replace the C-3 position of galactose in the phosphosaccharide backbone of the LPG of *L. major* and *L. tropica* [22,23]. Comparable oligosaccharides are absent from the LPG of *L. mexicana* and *L. donovani* [22]. Monoclonal antibodies have indicated the presence of several epitopes which are common to the surfaces of *Endotrypanum* and *Leishmania* species. Of particular interest is the observation that monoclonal antibody WIC79.3 recognizes *L. major*, *L. tropica* and *Endotrypanum* [2]. This antibody is highly specific for the galactose-containing side chains of the LPG from *L. major* [46]. Thus the antigenic relationship between *Endotrypanum* and *Leishmania* could be attributable to the presence of common carbohydrate epitopes, which are carried on LPG in *L. major* and on GIPLs in *Endotrypanum* strains. It is unlikely that the reaction of the WIC79.3 antibody with *Endotrypanum* is due to the presence of LPG-like molecules, because indirect immunofluorescence, using the monoclonal antibody CA7AE which recognizes the phosphorylated disaccharide repeats of LPG [37], did not detect LPG at all in strains LV58 and LV88 and gave an extremely poor reaction with strain LV59.

LPG has been shown to play a crucial role in the interaction of *L. major* with its natural vector *Phlebotomus papatasi* [47]. Attachment of procyclic promastigotes to the insect midgut wall involves binding to a lectin-like receptor which has high specificity for the β -Galp side chains of LPG. Metacyclogenesis is associated with structural alteration to *L. major* LPG, including an increase in the number of phosphorylated repeat units, a reduction in the number of β -Galp and Galp(β 1-3)Galp(β 1- side chains and an

increase in the proportion of side chains capped with terminal β -D-Arap residues. These D-arabinopyranose substituents mask the β -galp-recognition site and enable the parasites to detach from the insect midgut and migrate to the foregut before infection of a mammalian host [48]. Other *Leishmania* species lack the β -linked galactose residues characteristic of *L. major* LPG, and are therefore unable to bind to the midgut of *P. papatasi* and do not undergo their complete developmental cycle when experimentally inoculated into it. *Phlebotomus argentipes*, by contrast, is readily infected by most *Leishmania* species tested, including *L. major* [47], suggesting that different ligands and receptors are involved in colonization. It is not known if the terminally exposed galactose residues on the GIPLs of *Endotrypanum* are required for attachment to and colonization of the midgut of its sandfly vectors, but it does seem to be the case that many *Lutzomyia* species are refractory to infection with *Endotrypanum*, which would be consistent with a highly specific ligand-receptor interaction. Our results predict that *Endotrypanum* should be able, like *L. major*, to bind to the midgut β -Galp receptors of the Old World sandfly *P. papatasi* and possibly establish infections. Certainly there is no *a priori* reason why New World *Leishmania* cannot infect Old World sandflies, since experimental infections of *P. argentipes* with *L. mexicana* (strain Josepha) are readily established [47]. A recent report suggested that strain ISHA/BR/80/IM1111 of *E. shadinni* can infect *P. papatasi* under laboratory conditions [49], although infection rates were low (27%) in comparison with those of *Lutzomyia shannoni* (100%). However, nothing is known about the GIPL composition of the *Endotrypanum* strain used in these experiments.

We thank Professor S. J. Turco from the Department of Biochemistry, University of Kentucky Medical Center, Lexington, CT, U.S.A., for the monoclonal antibody CA7AE. We thank the MRC Biomedical NMR Centre, Mill Hill, for access to the 600 MHz NMR spectrometer, Dr. T. A. Fenkiel and Dr. M. J. Gradwell (MRC) for the gradient-enhanced HSQC experiment and Mr. X. Lemercinier for the ^1H - ^{31}P correlation spectrum, Mrs Lucy Jacinto do Nascimento and Mr. Orlando Augusto Agrellos Filho and Joab Pinto da Silva for technical assistance. This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/RHAE and PADCT), Financiadora de Estudos e Projeto (FINEP), Conselho de Ensino para Graduados (CEPG/UFRJ), British Council/CNPq Science Link Programme, and Programa Núcleo de Excelência (PRONEX). L.M.-P. is a Howard Hughes International Research Scholar.

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