Structural studies on a lipoarabinogalactan of *Crithidia fasciculata*

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The monosaccharide D-arabinopyranose has only been found in glycoconjugates of the trypanosomatid parasites *Leishmania major*, *Endotrypanum schaudinni* and *Crithidia fasciculata*. The donor molecule for the relevant arabinosyltransferases is known to be GDP-α-D-Ara*p* in *L. major* and *C. fasciculata*, and the latter organism is being used to study the biosynthesis of GDP-α-D-Ara*p*. In this study, we describe the structure of the terminal product of arabinose metabolism in *C. fasciculata*, namely lipoarabinogalactan. This molecule was purified by hydrophobic-interaction chromatography and studied by a variety of techniques, including gas chromatography–mass spectrometry, electrospray mass spectrometry and chemical and enzymic digestions. These data show that lipoarabinogalactan contains a previously described D-arabino-D-galactan polysaccharide component covalently attached to a glycosylphosphatidylinositol type of membrane anchor that is similar to, but not identical with, that found in the lipophosphoglycans of the *Leishmania*.

INTRODUCTION

The protozoan flagellates of the genus *Crithidia* are monogenetic trypanosomatid parasites that colonize the digestive tract of infected flies. These parasites produce two types of major polysaccharide: (1) a β1-2-linked D-mannan and (2) an arabinogalactan made of β1-3-linked D-galactan partially substituted in the 2 positions by single D-arabinopyranose residues [1,2]. In addition to *Crithidia* arabinogalactan, the uncommon D-Ara*p* residue has been described in a glycolipid of the trypanosomatid *Endotrypanum schaudinni* [3] and in the lipophosphoglycan (LPG) of *Leishmania major* [4,5]. *L. major* is a digenetic trypanosomatid parasite which is the causative agent of cutaneous leishmaniasis, a disease affecting a large number of people in various tropical areas. The D-Ara*p* residues of *L. major* are significant because they are most abundant in the LPG of the mammalian-infective metacyclic stage of the parasite [6]. The capping of LPG terminal βGal residues by D-Ara*p* abolishes the binding of the parasite to the midgut epithelium of the insect vector, allowing infective parasites to detach and migrate forward to the mouth parts of the sandfly [7]. In *L. major*, the activated biosynthetic precursor of D-Ara*p* is GDP-α-D-Ara*p* [8]. The same sugar nucleotide has been found in *Crithidia fasciculata*, and this organism was chosen to investigate the biosynthesis of GDP-α-D-Ara*p* [9] because it contains more GDP-Ara than *L. major* and because it is easier to grow. During these studies, it became apparent that the arabinose was transferred to a hydrophobic glycoconjugate instead of the expected hydrophilic arabinogalactan [1]. In the present report, we show that the arabinogalactan of *C. fasciculata* is linked at its reducing end to a glycosylphosphatidylinositol (GPI) anchor that is similar to, but not identical with, the GPI anchor of *Leishmania* LPGs.

MATERIALS AND METHODS

Materials

NaB^3H_4 (9–11 Ci/mmol) and D-[6-^{3}H]GlcN (36 Ci/mmol) were purchased from DuPont/NEN (Little Chalfont, Bucks., U.K.). D[5-^{3}H]Ara (36 Ci/mmol) was prepared by allowing D-[6-^{3}H]GlcN to react with ninhydrin, as described previously [8]. Coffee-bean α-galactosidase, jack-bean α-mannosidase and bovine testes β-galactosidase were obtained from Boehringer (Mannheim, Germany). Schneider's *Drosophila* medium (minus Glc) was from Life Technologies Inc. Silica-gel 60 high-performance TLC (HPTLC) and cellulose TLC were from Merck (Darmstadt, Germany). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was a gift from Dr. M. G. Low (Columbia University, New York, U.S.A.).

Cell culture and biosynthetic labelling

C. fasciculata strain HS6 was maintained at 27 °C in Schneider's *Drosophila* medium supplemented with 5% fetal calf serum and subcultured twice a week. For larger cultures, cells were grown at 27 °C in modified Schaefer's medium as described previously for *L. major* [10,11], except that BSA and fetal calf serum were omitted. Biosynthetic labelling was performed for 30 min at 27 °C in Schneider's *Drosophila* medium lacking Glc, yeast extract and fetal calf serum, but supplemented with 2.5% BSA and D-[5-^{3}H]Ara (1 μCi/ml), essentially as described previously [8].

Extraction of lipoarabinogalactan (LAG) and glycoinositolphospholipids (GIPLs)

Cell pellets were extracted twice with chloroform/methanol/water (1:2:0.8, by vol.); the cell pellet was assumed to be water

Abbreviations used: AHM, 2,5-anhydromannitol; ES-MS, electrospray mass spectrometry; GPI, glycosylphosphatidylinositol; GIPL, glycoinositolphospholipid; HPAEC, high-pH anion-exchange chromatography; HPTLC, high-performance thin-layer chromatography; IPC, inositolphosphoceramide; LAG, lipoarabinogalactan; LPG, lipophosphoglycan; PI-PLC, phosphatidylinositol-specific phospholipase C; GU, glucose unit; CID, collision-induced dissociation.

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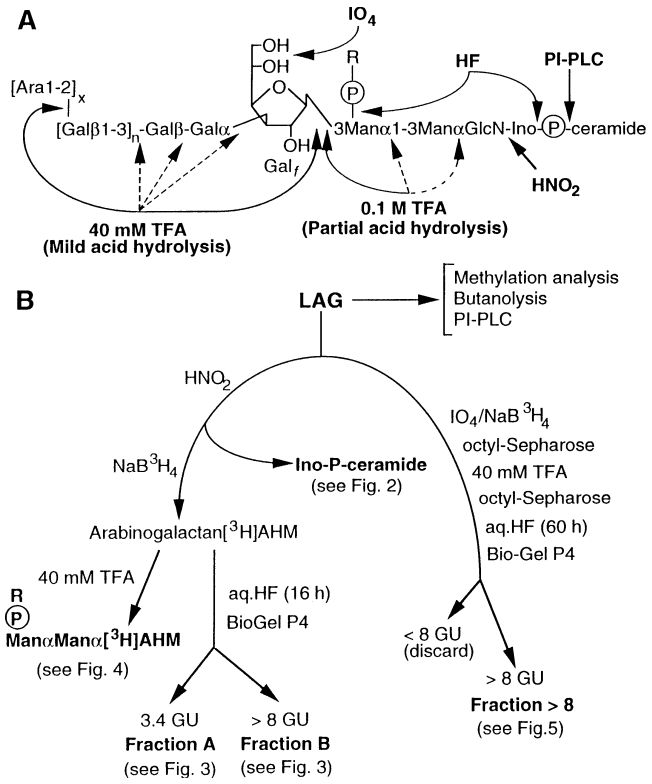

Figure 1 Summary of the experiments performed on LAG

(**A**) The sites of action of the various chemical and enzymic treatments are shown on the schematic structure of LAG. The solid arrows indicate the sites of quantitative cleavage and the dotted arrows indicate sites of partial cleavage. (**B**) The protocols used in the analysis of LAG are summarized. The figures showing further analysis of the various fragments and derivatives generated from the LAG molecule are indicated. TFA, Trifluoroacetic acid.

for adjusting the solvent volumes in the first extraction. After centrifugation, the pellet was saved for the extraction of LAG (see below), and water (0.6 vol.) was added to the combined supernatants to form a biphasic mixture. The upper phase was dried under a stream of N_2 and a polar GIPL fraction was purified by octyl-Sepharose chromatography, as described below. The pellet was briefly dried under a stream of N_2 and re-extracted twice with 4 vol. of 9% butan-1-ol in water with sonication in a sonicating water-bath. The pooled 9% butan-1-ol supernatants were taken to dryness, redissolved in 0.1 M ammonium acetate containing 5% propan-1-ol and applied at 6 ml/h to a column (1 cm × 10 cm) of octyl-Sepharose in the same buffer. The column was washed with 10 ml of the same buffer and eluted at 15 ml/h with a 60 ml gradient of 5–60% propan-1-ol. Fractions of 1.5 ml were collected and carbohydrate-containing fractions were detected by spotting 1 μl of each fraction on silica HPTLC and staining by spraying with orcinol reagent (20 mg/ml orcinol monohydrate in ethanol/conc. H_2SO_4/water, 75:10:5, by vol.) and heating for 5 min at 110 °C. LAG was eluted at about 35% propan-1-ol. Fractions of interest were freeze-dried twice and stored in 40% propan-1-ol.

Methylation analysis

Methylation analysis was performed as described previously [12].

Butanolysis

Enantiomerically pure 2-(−)-butanolic HCl (1 M with respect to HCl) was prepared by adding acetyl chloride to 2-(−)-butanol. Samples, and D- and L-sugar standards, were taken to dryness and subjected to butanolysis in 50 μl of butanolic HCl. The resulting 2-(−)-butyl glycoside diastereoisomers were derivatized with a trimethylsilylating reagent and the products were analysed by gas chromatography–mass spectrometry (GC–MS) [13].

HNO_2 deamination of biosynthetically labelled LAG

Biosynthetically labelled LAG (10000 c.p.m.) was deaminated at 60 °C in 66 mM sodium acetate (pH 4.0)/0.3 M $NaNO_2$ (three additions of 22 μl, 1 h apart). In a negative control, NaCl was used instead of $NaNO_2$. The reaction mixture was adjusted to 5% with respect to propan-1-ol and applied to a 200 μl octyl-Sepharose column. The column was washed with 5 × 1 ml of 5% propan-1-ol in 0.1 M ammonium acetate and eluted with 1 ml of 40% propan-1-ol and 1 ml of 50% propan-1-ol in water. Radioactivity in the flow-through and in the eluate was quantified by liquid-scintillation counting.

HNO_2 deamination and NaB^3H_4 reduction of LAG

Unlabelled LAG (45 μg of carbohydrate) was deaminated as described above. The reaction mixture was extracted twice with 2 vol. of water-saturated butan-1-ol. The aqueous phase of the deamination reaction was taken to dryness and reduced in 0.4 M borate/NaOH buffer (15 μl, pH 9.5) with 30 mM NaB^3H_4 in 0.1 M NaOH (5 μl; 1 h; room temperature). Excess reagent was destroyed by acidification with acetic acid, cations were removed by passage through a column of AG 50X12 (H^+ form) (200 μl) and the eluate was dried. Boric acid was removed by co-evaporation (four times) with 250 μl of methanol. The sample was subjected to descending paper chromatography on Whatman 3MM paper for 48 h in butan-1-ol/ethanol/water (4:1:0.6, by vol.) and the origin was eluted with water. This fraction contained arabinogalactan labelled at the reducing terminus with a 2,5-[1-^{3}H]anhydromannitol (AHM) residue.

Electrospray mass spectrometric (ES-MS) analysis of the inositolphospholipid fraction released by deamination

ES-MS data were obtained using a VG Quattro triple-quadrupole mass spectrometer (Fisons Instruments). The pooled butan-1-ol extracts, obtained after HNO_2 deamination of LAG and containing the released inositolphospholipids, were back-extracted with water, dried and dissolved in chloroform/methanol (2:3, v/v) for ES-MS analysis. Samples were introduced into the electrospray ion source in the same solvent at 10 μl/min. Negative-ion spectra were recorded after optimization of the source conditions using a 20 pmol/μl sample of soya-bean phosphatidylinositol. Collision-induced daughter ion spectra were recorded after acceleration of the $[M-H]^-$ pseudomolecular ions through a potential difference of 65 V into a collision cell containing argon 0.23 Pa. Mass-spectral data were processed using MassLynx software.

Periodate oxidation and NaB^3H_4 reduction of LAG

Unlabelled LAG (45 μg of carbohydrate) was oxidized in 40 μl of 0.1 M ammonium acetate containing a 4 mM $NaIO_4$ (10 min on ice and in the dark). The reaction was quenched by the addition of 2 μl of ethylene glycol, dialysed against water for 5 h in a microdialyser with a 2000 Da cut-off membrane and freeze-dried. Reduction was performed in 0.1 M NH_4OH/20% propan-1-ol

Table 1 Methylation linkage analyses of LAG before and after mild acid hydrolysis

Peak areas were taken from the GC–MS total-ion chromatograms. These values are not corrected for molar response. + indicates that a trace of these components was positively identified from characteristic retention times and mass spectra. n.d., Not determined.

Partially methylated alditol acetates	Origin	Peak area	
		Native LAG	Mild acid-treated LAG
1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methyl-arabinitol	Terminal-Ara*p*	0.3	0.2
1,4-Di-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinitol	Terminal-Ara*f*	0.0	0.1*
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galacitol	3-*O*-Substituted Gal*p*	0.3	0.8
1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methyl-galacitol	2,3-Di-*O*-substituted Gal*p*	0.7	0.2
1,3,4-Tri-*O*-acetyl-2,5,6-tri-*O*-methyl-galacitol	3-*O*-Substituted Gal*f*	+	n.d.
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-mannitol	3-*O*-Substituted Man*p*	+	n.d.
6-*O*-Acetyl-1,2,3,4,5-penta-*O*-methyl-*myo*-inositol	6-*O*-Substituted *myo*-inositol	+	n.d.

* This Ara*f* derivative is formed as the result of the acid-catalysed release of free Ara, which gives rise to a mutorotation mixture of the pyranose and furanose forms.

Table 2 Recovery of neutral or hydrophilic LAG products after various chemical treatments

Neutral counts is radioactivity that did not bind to AG 50X12 (H^+ form) over AG 3X4 (OH^- form). Hydrophilic counts is radioactivity that did not bind to octyl-Sepharose. TFA, Trifluoroacetic acid.

Sample	Treatment	Percentage of neutral counts	Percentage of hydrophilic counts
Biosynthetically labelled LAG*	None	–	4
	HNO_2	–	75
HNO_2/NaB^3H_4-labelled LAG†	None	2	–
	40 mM TFA (1 h)	2	–
	HF (60 h)	79	–
IO_4/NaB^3H_4-labelled LAG‡	None	1	4
	40 mM TFA (1 h)	61	86

* Biosynthetically labelled with D-[^{3}H]Ara and purified by octyl-Sepharose chromatography.

† Labelled in the AHM residue (formerly GlcN) at the reducing end of the molecule. Purified by paper chromatography. This material still contains a proportion of charged radioactive contaminants from the commercial NaB^3H_4.

‡ Labelled in the acid-labile internal L-Ara*f* residue (formerly Gal*f*). Purified by octyl-Sepharose chromatography. This material still contains a proportion of hydrophobic (and possibly charged) radioactive contaminants from the commercial NaB^3H_4.

(20 μl) by the addition of 30 mM NaB^3H_4 in 0.1 M NaOH (5 μl; 1 h; room temperature). Excess reductant was destroyed with 5 μl of 1 M acetic acid, and 500 μl of 0.1 M ammonium acetate containing 5 % propan-1-ol was added. The mixture was loaded on to an octyl-Sepharose column (1 ml) which was washed with 8 ml of 0.1 M ammonium acetate containing 5 % propan-1-ol and batch-eluted with 40 % propan-1-ol. The eluate, containing IO_4^-/NaB^3H_4-labelled LAG, was freeze-dried and redissolved in 40 % propan-1-ol.

Liquid chromatography

Neutral monosaccharides were resolved by Dionex high-pH anion-exchange chromatography (HPAEC) using a Carbopack PA-1 column (4 mm × 250 mm) eluted isocratically at 0.6 ml/min in 15 mM NaOH. The elution positions of internal monosaccharide standards (50 nmol each of D-Ara, D-Glc, D-Gal and D-Man) were monitored by pulsed amperometric detection, and radioactivity was detected by liquid-scintillation counting of the fractions. Labelled oligosaccharides were separated according to their hydrodynamic volume on Bio-Gel P4, using an Oxford Glycosystem GlycoMap, and detected by liquid-scintillation counting of the fractions. The sizes of the oligosaccharides were expressed as glucose units (GU), relative to the elution positions of co-injected unlabelled α1-6-linked D-Glc dextran oligomers which were detected by refractive-index monitoring.

Chemical and enzymic hydrolyses

LAG was subjected to digestion by *B. thuringiensis* PI-PLC (30 m-units in 5 μl of 20 mM Tris/acetate, pH 7.5/0.1 % Triton X-100) for 2 h at 37 °C. Labelled oligosaccharides were digested for 16 h at 37 °C with jack-bean α-mannosidase (25 units/ml; 0.1 M sodium acetate, pH 5.0; 20 μl), coffee-bean α-galactosidase (25 units/ml; 0.1 M sodium citrate/phosphate buffer, pH 6.0; 20 μl) or bovine testes β-galactosidase (1.8 units/ml; 0.1 M sodium citrate/phosphate buffer, pH 4.5; 20 μl). Enzymes were inactivated by heating for 5 min at 100 °C, and the radiolabelled neutral products were desalted by passage through AG 50X12 (H^+ form) over AG 3X4 (OH^- form) (200 μl each).

Mild acid hydrolysis was performed in 40 mM trifluoroacetic acid (200 μl; 100 °C; 1 h). These conditions are known to preferentially hydrolyse hexofuranosidic linkages as well as the D-Ara*p* (β1-2)Gal glycosidic bond [4]. Partial acid hydrolysis was achieved in 0.1 M trifluoroacetic acid (200 μl; 100 °C; 4 h). These conditions are known to partially hydrolyse αMan glycosidic linkages and result in a mixture of oligosaccharides of different sizes [14]. Constituent monosaccharides were quantitatively released by treatment with 2 M trifluoroacetic acid (200 μl; 100 °C; 2 h). Acid was removed by evaporation, and labelled products were desalted as described above.

Oligosaccharides were dephosphorylated with aq. 48 % HF (50 μl; 0 °C; 16 h or 48 h). Samples were neutralized with

saturated LiOH, centrifuged, and the supernatant was desalted by passage through AG 50X12 (H^+ form) over AG 3X4 (OH^- form) (400 μl each).

TLC

Monosaccharides were resolved on cellulose TLC in ethyl acetate/pyridine/water (4:2:1, by vol.) (solvent A) [15]. Oligosaccharides were separated on silica-gel 60 HPTLC plates developed twice in propan-1-ol/acetone/water (9:6:4, by vol.) (solvent B) [15], and glycolipids were analysed on silica-gel 60 HPTLC plates developed in chloroform/methanol/1 M NH_4OH (10:10:3, by vol.) (solvent C). Radioactivity was detected by fluorography after spraying the TLC or HPTLC plates with En^{3}Hance (DuPont/NEN).

Phenol/H_2SO_4 assay for carbohydrates

The phenol/H_2SO_4 assay for the estimation of carbohydrate content was adapted from the method of Rao and Pattabiraman [16] and performed as follows: samples in water (100 μl) were mixed with 5% phenol (100 μl), and conc. H_2SO_4 (500 μl) was added. After 30 min, 200 μl of the reaction mixture was transferred to a 96-well plate and the A_{490} determined in an ELISA plate reader. Carbohydrate content was estimated via a standard curve using D-Glc.

RESULTS AND DISCUSSION

A summary of the experiments performed on *C. fasciculata* LAG and of the effects of various chemical and enzymic treatments of this molecule are shown in Figure 1.

Identification of a LAG in *C. fasciculata*

The protocol which is routinely used for the extraction of *Leishmania* LPG [4] was applied to *C. fasciculata*. The latter parasite was found to contain an amphiphilic molecule which could be extracted from delipidated cell pellets with 9% butan-1-ol and bound to octyl-Sepharose. This fraction contained about 0.45 mg of carbohydrate/10^{10} cells, as determined by the phenol/H_2SO_4 assay. This is comparable with the amount of arabinogalactan extracted by a different procedure by Gorin *et al.* [1]. Most of the carbohydrate material in this fraction was large and stayed at the origin of a silica-gel 60 HPTLC plate developed in solvent C, but some contaminating polar glycolipids were also present in some preparations. These glycolipids migrated in the same region as the ethanolamine phosphate-containing GIPL standards ($EPiM_3$, $EPiM_4$) from *Leishmania mexicana* [17] and were not studied further. When *C. fasciculata* cells were labelled with D-[^{3}H]Ara, the label was readily incorporated into LAG. The only radioactive monosaccharide that could be released from LAG by strong acid hydrolysis was co-eluted with authentic D-Ara on Dionex HPAEC (results not shown), indicating that D-Ara was not metabolized to other monosaccharides.

Monosaccharide and inositol analysis showed the presence of Ara, Gal, Man and *myo*-inositol (in a molar ratio of 8:12:1:1), and analysis of the monosaccharides as diastereoisomers [13] [after butanolysis with enantiomerically pure 2-(−)-butanolic HCl] showed that Ara, Gal and Man were exclusively in the D-absolute configuration (results not shown). Methylation analysis (Table 1) showed the presence of terminal D-Ara*p*, 2,3-di-O-substituted Gal residues and some 3-O-substituted Gal residues. On mild acid hydrolysis, using conditions that cleave the relatively acid-labile β-D-Ara*p*(1-2)β-D-Gal*p* glycosidic bond found in *L. major* LPG [4], most of the Ara residues were released as free monosaccharides and the majority of the 2,3-di-O-substituted Gal residues were replaced by 3-O-substituted Gal residues (Table 1). These data suggest that the D-Ara*p* of LAG was originally located at the 2-position of about 75% of the Gal residues and that the majority of the Gal residues form 1-3-linked linear galactan chains.

Taken together, these data indicate that *C. fasciculata* produces a lipo-D-arabino-D-galactan (LAG).

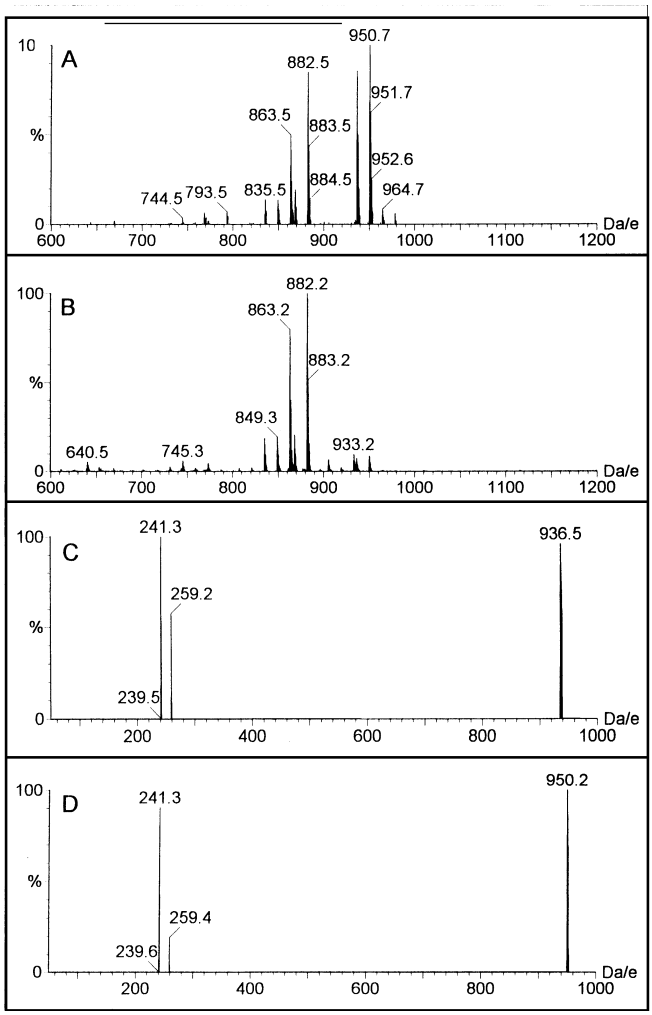

Figure 2 Analysis of the inositolphospholipid fraction of LAG by ES-MS

(**A**) Negative-ion ES-MS spectrum (*m/z* 600–1200) of the inositolphospholipid fraction released from LAG by HNO_2 deamination; (**B**) negative-ion ES-MS spectrum (*m/z* 600–1200) of the inositolphospholipid fraction released from the *C. fasciculata* polar GIPL fraction by HNO_2 deamination; (**C**) negative-ion ES-MS-(CID)-MS daughter-ion spectrum of the LAG-specific parent ion at *m/z* 936; (**D**) negative-ion ES-MS-(CID)-MS daughter-ion spectrum of the LAG-specific parent ion at *m/z* 950.

LAG contains a glucosaminylinositolphosphoceramide

The interaction between LAG and octyl-Sepharose was completely abolished by treatment of LAG with PI-PLC. Similarly, about 75% of the D-[^{3}H]Ara radioactivity biosynthetically incorporated into LAG was rendered soluble by deamination with HNO_2 (Table 2). These data support the presence of non-N-acetylated glucosamine (GlcN) residue linked to an inositol-

Table 3 ES-MS and ES-MS-MS assignments of the inositolphospholipids released from LAG and from the polar GIPL fraction

IPC, Inositolphosphoceramide; PI, phosphatidylinositol.

Parent ion $[M-H]^-$	Daughter ions (m/z)		Type of inositol-phospholipid	Possible lipid components
	Inositolphosphate	Fatty acids		
951*	259, 241		IPC	C_{18} phytosphingosine + $C_{27:0}$ fatty acid or C_{18} sphinganine + $C_{27:0}$-OH fatty acid
937*	259, 241		IPC	C_{18} phytosphinogosine + $C_{26:0}$ fatty acid or C_{18} sphinganine + $C_{26:0}$-OH fatty acid
883	259, 241		IPC	C_{18} phytosphingosine + $C_{21:0}$-OH fatty acid
869	259, 241		IPC	C_{18} phytosphingosine + $C_{20:0}$-OH fatty acid
864	241	283, 281	PI	$C_{18:0}$ fatty acid + $C_{18:1}$ fatty acid
850	241	281, 269	PI	$C_{18:1}$ fatty acid + $C_{17:0}$ fatty acid or $C_{18:1}$ fatty acid + $C_{16:1}$-OH fatty acid
836	241	281, 255	PI	$C_{18:1}$ fatty acid + $C_{16:0}$ fatty acid

* Species unique to the LAG sample.

phospholipid. Inositolphospholipids released by the latter treatment were extracted with butan-1-ol and analysed by negative-ion ES-MS (Figure 2A) Each of the $[M-H]^-$ pseudomolecular ions between m/z 800 and m/z 1000 were subjected to collision-induced dissociation (CID) and the daughter ions were analysed using ES-MS-(CID)-MS. The assignments made from the MS data are shown in Table 3. The presence of the [inositol 1,2-cyclic phosphate]$^-$ fragment ion (m/z 241) in all of the daughter-ion spectra indicated that all of the parent ions were due to inositolphospholipids [18]. In some daughter-ion spectra, identifiable carboxylate ions were detected, indicating the presence of diacylglycerol-containing phosphatidylinositols (PIs), whereas in other spectra the lack of carboxylate ions and the presence of an intense [inositol monophosphate]$^-$ fragment ion (m/z 259) implied the presence of inositolphosphoceramides (IPCs). The LAG preparation used for this analysis was found to be contaminated with some GIPL molecules, as judged by HPTLC analysis using solvent C and orcinol staining. Thus a sample of inositolphospholipids released by HNO_2 from the polar GIPL fraction of a chloroform/methanol/water extract was also analysed by ES-MS (Figure 2B) and ES-MS-MS. In this case, all of the inositolphospholipid species were present in similar ratios except for those corresponding to the two IPCs at m/z 936 and 950 which are the most abundant species in the LAG sample (Figure 2A). We cannot fully define the nature of the ceramide moieties in these two LAG-specific species, since the daughter-ion spectra of IPCs do not contain fragment ions other than inositol 1,2-cyclic phosphate and inositol monophosphate (Figures 2C and 2D). However, the masses of these species suggest that they are fully saturated and contain either a hydroxylated fatty acid or a hydroxylated sphinganine long-chain base, i.e. phytosphingosine (see Table 3 for possible assignments).

Taken together, these data show that LAG contains a glucosaminylinositolphosphoceramide (GlcN-IPC).

LAG is linked to IPC via an R_1-3Gal*f*-(R_2-PO_4)Man(α1-3)Man(α1-4)GlcN structure

The distinctive derivative of a 3-O-substituted Gal*f* residue [19] was observed in the methylation analysis of LAG (Table 1). The presence of an acid-labile galactofuranosidic linkage was exploited as described below.

Soluble LAG released by HNO_2 deamination was reduced with NaB^3H_4, thus introducing a label into the AHM residue at the reducing end of the carbohydrate chain. The label was incorporated into a structure that was judged to contain phosphoryl/phosphonyl group(s) because 98% of the counts were lost on

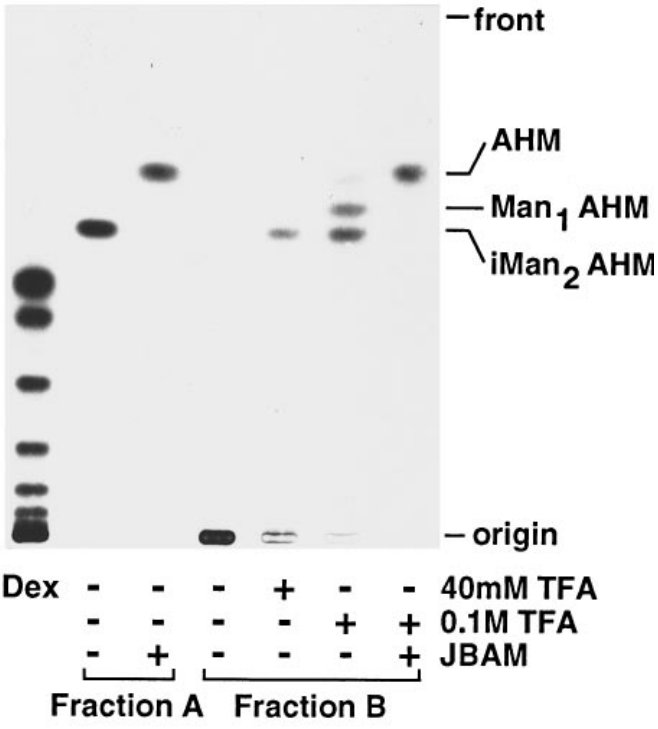

Figure 3 Analysis of AHM-labelled LAG

LAG labelled in the AHM residue by the HNO_2 deamination/NaB^3H_4 reduction procedure was dephosphorylated with aq. HF and fractionated into the neutral fractions A and B by size-exclusion chromatography on Bio-Gel P4. The fractions were submitted to the indicated treatments, desalted and analysed by silica-gel 60 HPTLC (solvent B). Radioactivity was detected by fluorography. The migration positions of authentic AHM, Man(α1-4)AHM (Man_1AHM) and Man(α1-3)Man(α1-4)AHM ($iMan_2$AHM) are indicated on the right-hand side of the chromatogram. Dex, NaB^3H_4-reduced dextran oligomers; 40 mM TFA, 40 mM trifluoroacetic acid for 1 h at 100 °C; 0.1 M TFA, 0.1 M trifluoroacetic acid for 4 h at 100 °C; JBAM, jack-bean α-mannosidase.

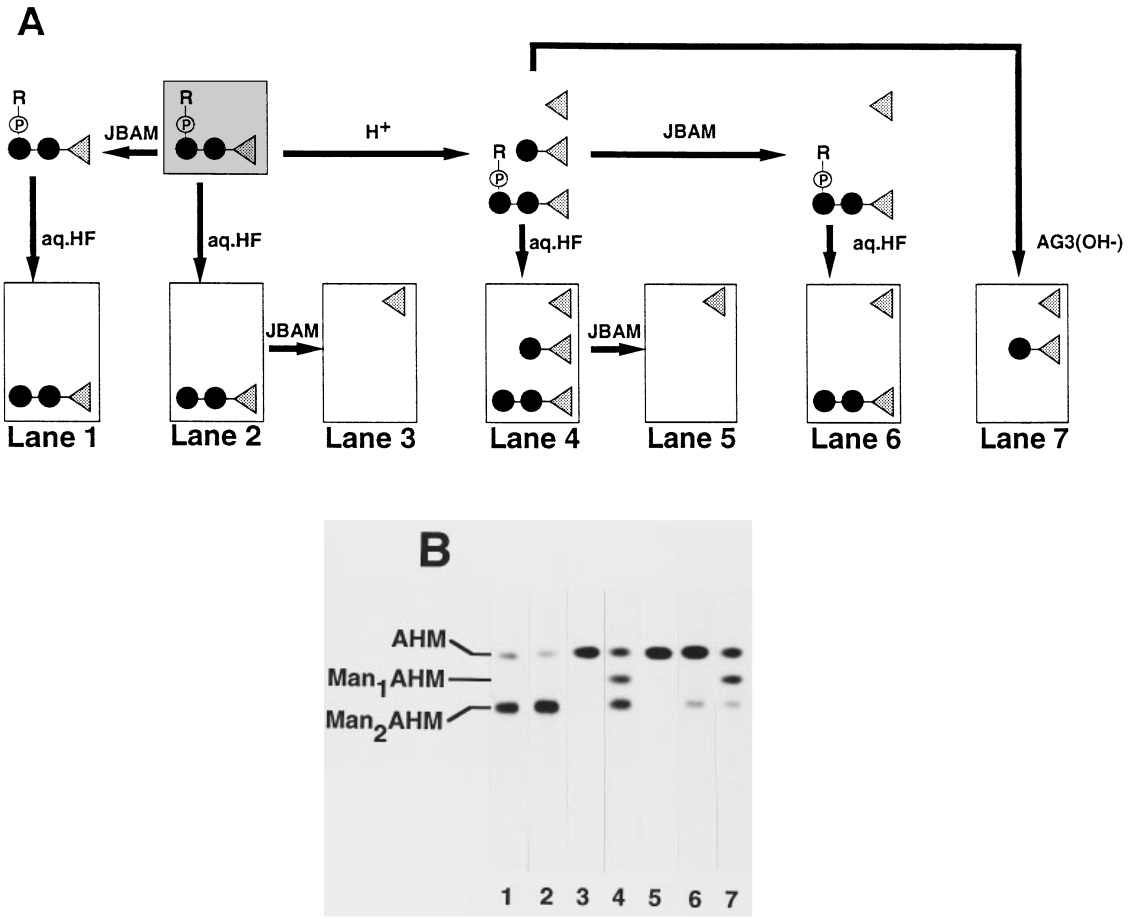

Figure 4 Location of phosphoryl substituents

Deaminated and NaB^3H_4-reduced LAG was treated with acid to cleave the galactofuranosidic linkage, and the resulting negatively charged radiolabelled product (shown in the shaded box) was treated in a variety of ways, as indicated (**A**), before HPTLC and fluorography (**B**). The structure in the shaded box corresponds to R-PO_4-Man(α1-3)Man(α1-4)AHM. Only the major products are indicated in (**A**). The migration positions of authentic AHM, Man(α1-4)AHM (Man_1AHM) and Man(α1-3)Man(α1-4)AHM ($iMan_2$AHM) are indicated on the left-hand side of the chromatogram. JBAM, jack-bean α-mannosidase.

desalting, but were rendered neutral after aq. HF treatment for 60 h (Table 2). When aq. HF treatment was performed for 16 h {conditions that caused only 40 % cleavage of the hexofuranosidic bond of a Gal(α1-6)Gal(α1-3)Gal_f(β1-3)Man(α1-3)Man(α1-4)[^{3}H]AHM standard}, two major neutral fractions, called A and B, were recovered after size-exclusion chromatography on Bio-Gel P4. Fraction A co-migrated with authentic Man(α1-3)Man(α1-4)AHM on Bio-Gel P4 (with a size of 3.4 GU) and on HPTLC, and yielded AHM after digestion with α-mannosidase (Figure 3, fraction A). The remainder of the radioactivity was heterogeneous and was eluted throughout the Bio-Gel P4 profile (results not shown). Only the fractions corresponding to material of a size greater than 8 GU were pooled in fraction B. This was done to omit small oligosaccharides that could have been derived from the polar GIPL contaminants present in the original LAG fraction. This cut-off was effective because the radioactivity in fraction B did not move from the origin of the HPTLC plate (Figure 3, fraction B), whereas neutral glycolipid headgroups comprising up to six monosaccharides are known to migrate in this system [15]. Fraction B was converted into the α-mannosidase-sensitive Man(α1-3)Man(α1-4)AHM structure by mild acid hydrolysis and yielded a mixture of Man(α1-3)Man(α1-4)AHM, Man(α1-4)AHM and AHM on partial acid hydrolysis (Figure 3, fraction B). These data suggest that all of the LAG structures contain a common core glycan of R-3Gal_f-Man(α1-3)Man(α1-4)GlcN, which is the same as that found in all *Leishmania* LPG molecules [5].

When the aq. HF step was omitted, mild acid hydrolysis was unable to generate a good yield of neutral Man(α1-3)Man(α1-4)AHM, indicating that this structure is substituted with one or more phosphoryl/phosphonyl substituents. The location(s) of this (these) substituent(s) was determined using various combinations of partial acid hydrolysis, α-mannosidase digestion, aq. HF dephosphorylation and desalting [14] (Figure 4A). The production of neutral Man(α1-3)Man(α1-4)AHM (together with a small amount of AHM) after aq. HF dephosphorylation of the mild acid hydrolysate is shown in Figure 4(B), lane 2. Digestion of the mild acid hydrolysate with α-mannosidase before aq. HF dephosphorylation generated slightly more AHM, but the majority of the Man(α1-3)Man(α1-4)AHM band was unchanged (Figure 4B, lane 1). Since all of the Man(α1-3)Man(α1-4)AHM band is converted into AHM if aq. HF dephosphorylation precedes α-mannosidase digestion (Figure 4B, lane 3), we can conclude that most of the non-reducing terminal αMan of Man(α1-3)Man(α1-4)AHM is originally substituted by a phosphoryl/phosphonyl component. Given the precedent that *Leish-*

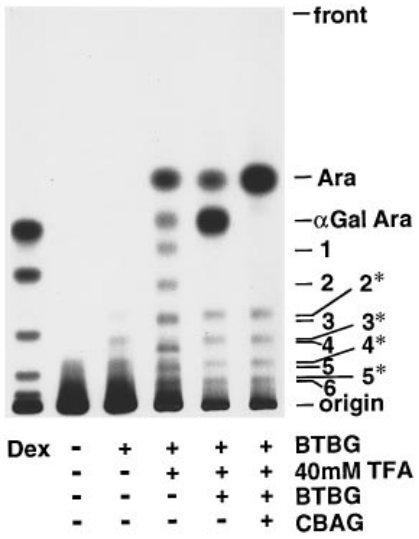

Figure 5 Microsequencing of IO_4^-/NaB^3H_4-labelled LAG

LAG labelled in its former Gal*f* residue by the IO_4^-/NaB^3H_4 procedure was submitted to mild acid hydrolysis, HF dephosphorylation and size-exclusion chromatography on Bio-Gel P4. Oligosaccharides bigger than 8 GU were pooled and submitted to the indicated treatments (in the indicated order, from top to bottom). Samples were desalted and analysed by silica-gel 60 HPTLC (solvent B). Radioactivity was detected by fluorography. The migration positions of authentic Ara and Gal(α1-3)Ara (αGalAra) standards are indicated on the right-hand side. Numbers 1–6 indicate the migration positions of putative Gal(α1-3)Ara structures substituted with 1 to 6 βGal residues. Numbers 2*–5* indicate the migration positions of putative Gal(α1-3)Ara structures substituted with 2–5 βGal residues plus a single terminal Ara residue. Dex, NaB^3H_4-reduced dextran oligomers; BTBG, bovine testes β-galactosidase; 40 mM TFA, 40 mM trifluoroacetic acid for 1 h at 100 °C; CBAG, coffee-bean α-galactosidase.

mania LPGs contain Glcα1-PO_4 attached to the 6-position of the comparable αMan residue [5], we tentatively suggest that *C. fasciculata* LAG may also be similarly substituted with Glcα1-PO_4.

When the mild acid hydrolysate was subjected to partial acid hydrolysis and then aq. HF treatment, a ladder of three structures [Man(α1-3)Man(α1-4)AHM, Man(α1-4)AHM and AHM] was seen (Figure 4B, lane 4). If the same partial acid hydrolysate was treated with α-mannosidase and then aq. HF, the middle band [Man(α1-4)AHM] disappeared whereas the Man(α1-3)Man(α1-4)AHM band was substantially resistant (Figure 4B, lane 6). Since the Man(α1-3)Man(α1-4)AHM band was completely sensitive to α-mannosidase after aq. HF dephosphorylation (Figure 4, lane 5), these data again show that the terminal αMan residue was substituted with a phosphoryl/phosphonyl substituent and that the inner αMan residue was not substituted by such substituents. To investigate whether the AHM residue (derived from the original GlcN residue) might also contain a phosphoryl/phosphonyl substituent (as has been reported in a number of kinetoplastid GPI-related structures, reviewed in [5]), the partial acid hydrolysate was passed through a Dowex AG 3X4 (OH^- form) anion-exchange column before HPTLC analysis (Figures 4, lane 7). In this case, some Man(α1-3)Man(α1-4)AHM was found in the flow-through (i.e. the small fraction that does not contain the phosphoryl/phosphonyl substituent on the non-reducing terminal αMan residue) together with all of the Man(α1-4)AHM and AHM material, suggesting that the AHM residue does not carry a negative charge.

Taken together, the data described so far indicate that LAG contains the sequence R_1-3Gal*f*-(R_2-PO_4)Man(α1-3)ManαGlcN-IPC, where R_1 represents additional carbohydrate and R_2 may be αGlc.

Structure of the neutral glycan of LAG

To gain information on the R_1 substituent of the Gal*f* residue, the exocyclic glycol structure of the Gal*f* residue was selectively labelled by mild periodate oxidation followed by reduction with NaB^3H_4 [20]. As a result of these treatments, the D-Gal*f* residue of LAG was converted into L-[5-^{3}H]Ara*f*. Labelled LAG was repurified by octyl-Sepharose chromatography, treated with mild acid to cleave the arabinofuranosidic linkage, and the labelled glycan fraction was recovered in the flow-through of a second octyl-Sepharose chromatography step. After aq. HF dephosphorylation, desalting and gel filtration on Bio-Gel P4, labelled structures larger than 8 GU (fraction > 8) were pooled. Complete acid hydrolysis of fraction > 8 generated a single labelled monosaccharide that co-migrated with Ara on both Dionex HPAEC and cellulose TLC (results not shown), confirming that the Gal*f* residue had been selectively oxidized.

Fraction > 8 was successively submitted to β-galactosidase digestion, mild acid hydrolysis, a second β-galactosidase treatment and α-galactosidase digestion. A sample of fraction > 8 was analysed by HPTLC after each of these steps (Figure 5). This experiment showed that the fraction was substantially resistant to β-galactosidase but that subsequent mild acid hydrolysis (which preferentially hydrolyses the Ara*p*1-2Gal glycosidic linkage [4]) and re-digestion with β-galactosidase resulted in the accumulation of an α-galactosidase-sensitive disaccharide that co-migrated with an authentic Gal(α1-3)[^{3}H]Ara standard derived from *L. major* GIPL-2 (Figure 5). This indicates that position 3 of the Gal*f* residue of LAG is substituted with an αGal residue that in turn carries the arabinogalactan chain. Interestingly, the mild acid hydrolysate, shown in Figure 5, yielded a number of oligosaccharides that migrated as a series of doublets. The lower (major) bands of each doublet were susceptible to β-galactosidase digestion, indicating that their structures were (Galβ1-3)$_n$Gal(α1-3)[^{3}H]Ara, whereas the upper (minor) bands of the doublets were resistant to β-galactosidase, suggesting that their structures were Ara1-2(Galβ1-3)$_{n-1}$Gal(α1-3)[^{3}H]Ara. However, no upper band was apparent for $n = 1$ and $n = 2$, suggesting that the αGal and the first βGal residues of LAG are not substituted with an Ara residue (see Figure 6).

When IO_4^-/NaB^3H_4-labelled LAG was submitted to mild acid hydrolysis, more than 80 % of the radioactivity was converted from an amphiphilic into an hydrophilic form (Table 2), consistent with the cleavage of the furanosidic linkage resulting in the loss of the lipid. The same treatment converted about 60 % of the radioactivity from a charged into a neutral state (Table 2), indicating that the arabinogalactan moiety of LAG does not carry a significant amount of mild acid-resistant charged substituents, such as the phosphoryl group present in the glycan core described above.

Taken together, these results support the structure of LAG shown in Figure 6. This molecule most probably corresponds to the soluble neutral arabinogalactan described previously in the same parasite by Gorin *et al.* [1]. The purification method used by these authors included exposure to mild acid and desalting with both anion- and cation-exchange resins. Under these conditions, most of the LAG was probably hydrolysed at the Gal*f* residue, yielding a neutral arabinogalactan headgroup. Any remaining unhydrolysed glycolipid-containing LAG would have been removed by interaction with the anion-exchange resin and therefore not detected. Hydrophilic arabinogalactan is unlikely to occur naturally in significant amounts since the majority of

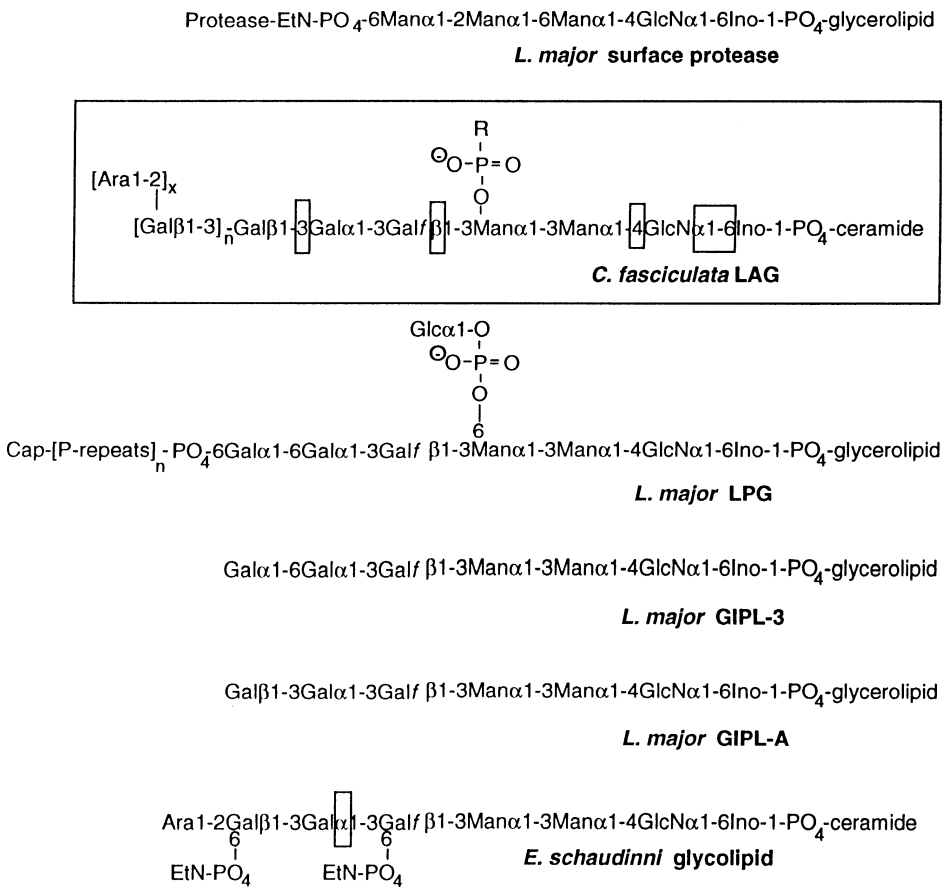

Figure 6 Structure of LAG and comparison with other parasite glycoconjugates

The structure deduced for *C. fasciculata* LAG (large box), where the average values for *x* and *n* are 8 and 12 respectively, is compared with the GPI anchor of *L. major* promastigote surface protease [21], *L. major* LPG [4], *L. major* GIPL-3 and GIPL-A [22,23] and an *E. schaudinni* glycolipid [3]. Cap, neutral oligosaccharide at the non-reducing end of LPG; P-repeats, phosphorylated disaccharide repeats [-6Gal(β1-4)Manα1-PO_4^-] that can carry various oligosaccharide side chains which for *L. major* LPG contain mainly D-Ara*p* and βGal residues [4,6]. The features in the small boxes are not supported by direct experimental data but are inferred by analogy with related structures.

D-[^{3}H]Ara was biosynthetically incorporated into the amphiphilic LAG [9].

Protozoan parasites make wide use of GPI structures as either free glycolipids or anchors to attach proteins and other macromolecules to the plasma membrane [5,24]. Among this latter class, it is now possible to recognize three major glycolipid structures: (1) those based on the sequence ethanolamine phosphate-6Man(α1-2)Man(α1-6)Man(α1-4)GlcN(α1-6)*myo*-inositol-1-PO_4-lipid (where the lipid can be glycerolipid or a ceramide), which are used to anchor proteins in both higher and lower eukaryotes (Figure 6); (2) those based on Gal(α1-6)Gal(α1-3)Gal*f*(β1-3)Man(α1-3)Man(α1-4)GlcN(α1-6)*myo*-inositol-1-PO_4-lipid (where the lipid is a *lyso*-alkyl glycerolipid), used to anchor phosphoglycan in *Leishmania*; (3) the structure described in this study, based on Gal(β1-3)Gal(α1-3)Gal*f*(β1-3)Man(α1-3)Man(α1-4)GlcN(α1-6)inositol-1-PO_4-lipid (where the lipid is a ceramide), which anchors the arabinogalactan in *C. fasciculata* (Figure 6). This last structure is also found in a free glycolipid of *E. schaudinni* [3], and GIPL-A of *L. major* has the same glycan structure, attached to an alkylacylglycerol lipid moiety [5,22] (Figure 6). The GIPL-A structure in *L. major* does not appear to be further galactosylated with Galβ1-3 residues, as is the case in LAG. This could indicate that *C. fasciculata* possesses two distinct Galβ1-3-transferases, one that would be responsible for the addition of the first βGal residue (to an αGal residue), and the second that would generate the Galβ1-3 backbone of the arabinogalactan. In *L. major*, the short repeats of Galβ1-3 residues found in the side chains of *L. major* LPG [4,5] could be added by the action of further distinct Galβ1-3-transferase(s). Although LPG is more negatively charged than LAG, because of the presence of the phosphorylated repeats, these two molecules have several similarities. Both are large molecules containing a glycolipid anchor and both contain several copies of the sequence Ara1-2Gal(β1-3)Gal in their structure. This raises the possibility that *C. fasciculata* LAG, like *L. major* LPG [7], could be a surface molecule involved in the interaction of the parasite with its insect host.

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