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Structural variation in the glycoinositolphospholipids of different strains of *Trypanosoma cruzi*

JOÃO C. CARREIRA[2], CHRISTOPHER JONES[3], ROBIN WAIT[4], JOSÉ O. PREVIATO[1] and LUCIA MENDONÇA-PREVIATO[1]*

[1]*Instituto de Microbiologia, Centro de Ciências da Saúde, Bloco I, Universidade Federal do Rio de Janeiro, 21 944 970, Cidade Universitária, Rio de Janeiro, RJ and* [2]*Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil*

[3]*Laboratory for Molecular Structure, NIBSC, Herts EN6 3QG and* [4]*CAMR, Porton Down, Salisbury, SP4 0JG, UK*

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The structures of the glycoinositolphospholipids (GIPLs) from five strains of the protozoan parasite *Trypanosoma cruzi* have been determined. Two series of structures were identified, all but one containing the same Man_4(AEP)GlcN-Ins-PO_4 core. Series 1 oligosaccharides are substituted at the third mannose distal to inositol (Man 3) by ethanolamine-phosphate or 2-aminoethylphosphonic acid, as are some glycosyl-phosphatidylinositol-protein anchors of *T. cruzi*. The core can be further substituted by terminal (1-3)-linked β-galactofuranose units. In contrast, Series 2 oligosaccharides do not have additional phosphorus-containing groups attached to Man 3, the latter being substituted instead by a single side chain unit of β-galactofuranose. Series 1 oligosaccharides are present in all strains (G, G-645, Tulahuen CL, and Y) whereas Series 2 structures are present mainly in CL and Y strains. The lipid moiety in the GIPLs from the G, G-645 and Tulahuen strains is predominantly ceramide, as reported for the Y strain, whilst that from the CL strain is a mixture of ceramide and alkylacylglycerol species. The lipid moiety of the GIPLs, and probably also the phosphoinositol-oligosaccharide structures may play an important immunomodulatory role in infection by *T. cruzi*.

Keywords: *Trypanosoma cruzi*, glycoinositolphospholipids, nuclear magnetic resonance, FAB-mass spectrometry

Abbreviations: GIPL, glycoinositolphospholipid; LPPG, lipopeptidophosphoglycan; GPI, glycosylphosphatidylinositol; AEP, 2-aminoethylphosphonic acid; PI, phosphoinositol; GC, gas-liquid chromatography; MS, mass spectrometry; FAB, fast atom bombardment; NMR, nuclear magnetic resonance; DQF-COSY, double quantum-filtered correlation spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; EtNP, ethanolaminephosphate; HMQC, heteronuclear multiple quantum coherence; Man, mannose; Gal*f*, galactofuranose; GlcN, glucosamine; Ins, inositol; InsP, inositolphosphate; Man 3, third mannose distal to inositol; NOE, nuclear Overhauser effect; $[M+H]^+$, protonated molecule; $[M-H]^-$, deprotonated molecule; RMM, relative molecular mass (monoisotopic).

Introduction

The protozoan parasite *Trypanosoma cruzi* is the causative organism of Chagas' disease in humans, which is endemic on the American continent. Cell surface glycoconjugates from different developmental stages of the parasite [1] have been implicated in processes such as host cell invasion [2], morphological transition [3], protection against complement mediated lysis [4], induction of protective lytic antibodies [5] and down-regulation of T-lymphocyte activation *in vitro* and *in vivo* [6]. Structural characterization of the surface glycoconjugates of *T. cruzi* is therefore crucial to an understanding of the molecular mechanisms of its virulence.

The structures of two major surface glycoconjugates from epimastigote forms of the Y-strain of *T. cruzi* have been extensively investigated. One is the low molecular mass glycoinositolphospholipid (GIPL) [7, 8], formerly known as lipopeptidophosphoglycan (LPPG) [9]. The other is a major cell surface glycoprotein which in strain

*To whom correspondence should be addressed.

Y is membrane-attached via a glycosylphosphatidylinositol (GPI) anchor in which the nonacetylated glucosamine residue is substituted at *O*-6 by 2-aminoethylphosphonic acid (AEP) [10], a feature not previously reported in GPI-protein anchors [11]. This protein, and the corresponding glycoprotein from strain G [12] are heavily *O*-glycosylated with oligosaccharide chains linked mainly via *N*-acetylglucosamine residues, which act as acceptors for sialic acid units transferred by a *trans*-sialidase reaction [10, 13]. Even though epimastigotes are unable to invade mammalian cells, several studies have shown that the sialic acid acceptor glycoproteins of infective metacyclic-trypomastigotes [14, 15] are structurally similar to those of epimastigotes [12]. In trypomastigotes derived from cell culture the sialic acid residues are transferred to the oligosaccharide chains of the glycoproteins collectively known as F2/F3, whose structures have not yet been determined. These glycoproteins are highly immunogenic, are recognised by protective lytic antibodies and are implicated in the invasion of host cells [5].

The GIPL from epimastigote forms of *T. cruzi* was the first glycolipid to be isolated from a trypanosomatid species [9] that is closely related to the structure of the GPI-protein anchors. The primary sequence of the GIPL of *T. cruzi* Y strain was characterized [7, 8] and shown to have the major structure: Gal$f\beta$1-3Manα1-2(Gal$f$ β1-3)Manα1-2-Manα1-6Manα1-4(2-AEP-6)GlcNα1-6Ins-PO_4 linked to *N*-lignoceroyldihydrosphingosine.

Although GIPLs are the most abundant glycoconjugates of epimastigote forms of *T. cruzi*, their precise role in host-parasite interactions is unclear. However, it is noteworthy that GIPLs from malaria parasites activate signal transduction pathways in host cells, resulting in secretion of the pro-inflammatory cytokine TNF-α [16]. Recently it has been demonstrated that *T. cruzi* GIPLs are able to block murine T-lymphocyte activation *in vitro* and *in vivo*, and that this suppressive activity depends on the ceramide domain [6]. Thus the subversion of host cellular defence mechanisms by *T. cruzi* GIPLs may play an important role in the establishment and maintenance of chronic infection. The varying patterns of infectivity and pathogenicity in mice exhibited by strains of *T. cruzi* [17, 18], may therefore be attributable, at least in part, to differences in the structures of potentially immunomodulatory molecules such as GIPLs. This possibility prompted us to investigate the occurrence of structural heterogeneity among GIPLs isolated from a range of *T. cruzi* strains.

Materials and methods

Parasite strains

Trypanosoma cruzi G and CL strains were kindly supplied by Dr N. Yoshida (Universidade Federal de São Paulo, São Paulo) and Dr Z. Brener (Fundação Oswaldo Cruz, Belo Horizonte, Minas Gerais), respectively. The G-645 strain was isolated from *Didelphis marsupialis* captured in Teresópolis, Rio de Janeiro state and the Tulahuen and Y strains were from our laboratory collection.

Isolation of glycoinositolphospholipids (GIPLs)

Trypanosoma cruzi epimastigotes were grown in brain heart infusion medium (without fetal calf serum) supplemented with 10 mg l^{-1} hemin, as previously described [13]. Frozen cells were thawed, extracted with cold water, centrifuged and the resulting pellet was extracted with 45% (v/v) aqueous phenol at 75 °C. The aqueous layer was dialysed, freeze dried, dissolved in water and applied to a column (2 × 100 cm) of Bio-Gel P-60. The excluded material was lyophilized and the GIPLs were recovered by extraction (twice) with chloroform/methanol/water (10:10:3). The extracts were combined, evaporated to dryness, dissolved in water and lyophilized. The yield of GIPLs was approximately 90 mg per 5.10^{11} cells.

Isolation of phosphoinositol (PI) oligosaccharides

Phosphoinositol-oligosaccharides were isolated from intact GIPLs by alkaline degradation (1 M KOH, 48 h at 37 °C) [19]. After neutralization with acetic acid, nonpolar material was removed by chloroform extraction, and the aqueous layer was passed through a column (2 × 40 cm) of Dowex 50-X8 (25–50 mesh; H^+ form). The PI-oligosaccharides were eluted with water, and fractionated on a Bio-Gel P-4 (extra fine) column (1 × 130 cm). Fractions of 1.0 ml were collected and assayed by spotting 5 μl portions onto a thin layer chromatography plate which was stained with orcinol-H_2SO_4 [20], ninhydrin [21], and molybdate [22] reagents to detect carbohydrate, nitrogen and phosphorus respectively.

Carbohydrate analysis

For analysis of neutral sugars, GIPLs were methanolysed with 0.5 M HCl in methanol containing mannitol as internal standard (18 h at 80 °C) followed by heptane extraction and trimethylsilyl derivatization with bis-(trimethylsilyl) trifluoroacetamide/pyridine (1:1 v/v for 2 h at room temperature). The products were analysed by gas-chromatography (GC) on a DB-1 fused silica column (30 m × 0.25 mm internal diameter) using hydrogen (0.7×10^5 Pa) as the carrier gas. The column temperature was programmed from 120 to 240 °C at 2 °C min^{-1}. For analysis of inositol and glucosamine, GIPLs were treated with 3 M methanolic HCl (containing xylitol as internal standard) for 18 h at 80 °C. The dried methanolysates were then hydrolysed with 6 M HCl for 18 h at 100 °C, reduced with sodium borohydride, acetylated (acetic anhydride/pyridine 9:1), and analysed by GC as described above, except that the column temperature was programmed from 120 to 240 °C at 3 °C min^{-1}.

Lipid analysis

For analysis of fatty acids and alkylglycerols the GIPLs were methanolysed with 0.5 M HCl in methanol for 18 h at 80 °C. The methanolysate was extracted with heptane and the resulting mixture of fatty acid methyl esters and alkylglycerols was analysed by GC after trimethylsilylation using a DB-1 fused silica column, with temperature programming from 180 to 310 °C at 3 °C min^{-1}. Peaks were identified by their retention times and by GC-mass spectrometry (GC-MS). For analysis of long chain sphingosine bases, GIPLs were treated with 1 M HCl in methanol (made 10 M with respect to water) for 18 h at 80 °C [23]. The pH was adjusted to 11 with aqueous NaOH, and the methanolysate was extracted three times with 2 volumes of diethyl ether. The combined extracts were dried with sodium sulfate, evaporated to dryness under nitrogen, dissolved in methanol and *N*-acetylated with acetic anhydride. The *N*-acetyl derivatives were *O*-trimethylsilylated by treatment with bis-(trimethylsilyl) trifluoroacetamide/pyridine (1:1 v/v) for 2 h at room temperature and were analysed by GC-MS as described for fatty acid methyl esters and alkylglycerols.

Deamination with HNO_2

The method for nitrous acid deamination of intact GIPLs was modified from Schneider and Ferguson [24]. Samples of GIPL (50–100 μg) were dissolved in 50 μl sodium acetate buffer (pH 4), and 25 μl of a freshly prepared 1 M solution of sodium nitrite was added. The solution was incubated at 37 °C for 1 h, after which a further 25 μl of nitrite solution was added, and the incubation was continued for one more hour. The liberated inositolphospholipids were recovered by extraction (twice) with 200 μl of water-saturated butanol, and the pooled butanol phases were dried in a vacuum centrifuge. The residue was dissolved in 10 μl chloroform/methanol (1:1) prior to analysis by negative ion fast atom bombardment-mass spectrometry (FAB-MS).

Fast atom bombardment-mass spectrometry

Fast atom bombardment-mass spectra were recorded using a Kratos MS80RFA spectrometer equipped with an Ion Tech FAB gun using xenon atoms as the bombarding particles. Underivatized samples were dissolved in 30% acetic acid to a concentration of about 10 $\mu g\,\mu l^{-1}$ and 1 μl was mixed with an equal volume of a 1:1 mixture of glycerol and dithiothreitol (5:1) on the stainless steel target. Triethanolamine was used as matrix for negative ion analysis of the inositolphospholipids obtained by HNO_2 deamination of the intact GIPLs. The instrument was operated at 4 kV accelerating voltage and spectra were obtained at scan rates of 10 or 30 s per decade of mass at a resolution of 1000. Spectra were processed using either DS90 or Mach3 software running on Data General DG30 or Sun Microsystems Sparcstation IPX computers respectively. Collision-induced dissociation was performed using helium as the collision gas, at a pressure sufficient to attenuate the precursor ion beam to 30% of its initial intensity and daughter ions were analysed by means of linked scans at constant B/E ratio.

Nuclear magnetic resonance (NMR) spectroscopic methods

Sample preparation and NMR spectroscopy were carried out as previously described [10, 12] except that samples were clarified by passage through a 0.22 μ reconstituted cellulose filter (Millipore, Watford, UK) in a microcentrifuge prior to deuterium exchange. Spectra were recorded at an indicated probe temperature of 30 °C. The mixing times for TOCSY and ROESY spectra were of 80 ms and 150 ms respectively. Proton spectra were referenced to internal acetone at 2.225 ppm or internal acetate anion at 1.908 ppm, ^{31}P spectra to internal 85% H_3PO_4 (in sealed capillary) at 0 ppm, and ^{13}C spectra indirectly to acetone at 31.5 ppm via the ethanolaminephosphate (EtNP) C-2 resonance. HMQC spectra were recorded without heteronuclear decoupling.

Other analytical methods

Total neutral sugars were analysed by the phenol/sulfuric acid procedure [25]. Total phosphorus was determined by the method of Ames [26] and acid hydrolyzable phosphorus by the method of Bartlett [27]. The procedure of Lauter and Trams [28] was used for the quantitative analysis of the long chain bases in methanolysates of GIPLs.

Results

Chemical composition of GIPLs isolated from T. cruzi G, G-645, Tulahuen, CL and Y strains

Epimastigotes of five strains of *T. cruzi* were subjected to phenol-water extraction and the GIPLs were recovered by treatment of the freeze-dried aqueous layer with chloroform/methanol/water. Compositional analysis showed that GIPLs of Tulahuen strain contained mannose (Man), glucosamine (GlcN), inositol (Ins) and phosphorus, and those from the other strains contained additionally galactose (Table 1). GC and GC-MS of acid methanolysates of the GIPLs revealed fatty acid methyl esters, long chain bases and (mainly in the CL strain) alkylglycerol (Table 1). FAB-MS of inositolphospholipids obtained by nitrous acid deamination confirmed these results and showed that whereas the long chain base (sphinganine) could be *N*-acylated with lignoceric, stearic and palmitic acids, hexadecylglycerol is esterified exclusively with palmitic acid.

Table 1. Chemical composition of GIPLs from *T. cruzi* strains

	T. cruzi strain			
	G	*G-645*	*CL*	*Tulahuen*
Mannose*[a]	2.6	2.5	4.0	3.0
Galactose*[a]	1.2	1.2	2.0	0.1
Glucosamine*[b]	1.0	0.9	0.9	1
Inositol*[b]	1.0	1.0	1.0	1
Phosphorus*[c]	3.0	3.0	2.2	3.0
Long chain base‡[d]				
Sphinganine	80	80	60	100
Sphingosine	20	20	tr	–
Alkyl of alkylglycerol‡[d]				
16:00	tr	tr	40	tr
Fatty acid‡[d]				
16:00	30	30	70	17
18:00	5	5	tr	13
24:00	65	65	30	70

[a]determined by GC as trimethylsilyl derivatives of methylglycosides; [b]determined by GC as alditol acetate derivatives; [c]determined by colorimetric methods; [d]determined by GC-MS; *the values are expressed in mol/mol; ‡the values are expressed in mol%; tr, traces.

Isolation and structural analysis of PI-oligosaccharides from GIPLs of T. cruzi

Chromatography on Bio Gel P-4 of the base hydrolysed GIPLs from strains G, G-645 and Tulahuen produced one major fraction which had the same elution volume as Manα1-2(EtNP-6)Manα1-2Manα1-6Manα1-4(2-AEP-6) GlcNα1-6Ins-PO_4 [10]. The CL strain produced, in addition, a slightly later eluting minor component. Four fractions were obtained from the Y strain, the fastest moving of which (Fraction A) eluted in the same volume as the major fraction from the other strains.

The structures of the PI-oligosaccharides were determined by chemical methods, high field NMR spectroscopy and FAB-MS. The protonated and deprotonated molecules observed in the positive and negative mode FAB-mass spectra, and their associated compositions are summarized in Table 2. Figure 1 shows the anomeric regions of the one-dimensional proton NMR spectra of the PI-oligosaccharides from GIPLs of strains G, G-645, CL and Y (Fraction A). The spectra of the G, G-645 and Tulahuen (not shown) PI-oligosaccharides were similar, apart from the absence of galactofuranose in the Tulahuen strain compound. Those of the CL and Y strains however were more complex and will be discussed separately.

G, G-645 and Tulahuen strains PI-oligosaccharides

The proton and carbon-13 spectral signals of PI-oligosaccharides from G, G-645, and Tulahuen strains were assigned by double quantum filtered COSY, TOCSY and HMQC experiments and sequence and linkage were

Table 2. Summary of the FAB-MS data on the PI-oligosaccharides of *T. cruzi* strains

T. cruzi strain	$[M+H]^+$	$[M-H]^-$	*Composition*	*RMM*[1]
	1462.4	1460.3	$(Hex)_5$-HexN-InsP-EtNP-AEP	1461.38
G	1446.3	1444.3	$(Hex)_5$-HexN-InsP-$(AEP)_2$	1445.389
	1300.3	1298.3	$(Hex)_4$-HexN-InsP-EtNP-AEP	1299.332
	1284.2	1282.3	$(Hex)_4$-HexN-InsP-$(AEP)_2$	1283.33
	1501.2	1499.1	$(Hex)_6$-HexN-InsP-AEP	1500.429
	1462.2	1460.2	$(Hex)_5$-HexN-InsP-EtNP-AEP	1461.38
G-645	1446.2	1444.2	$(Hex)_5$-HexN-InsP-$(AEP)_2$	1445.389
	1339.2	1337.2	$(Hex)_5$-HexN-InsP-AEP	1338.376
	1300.2	1298.2	$(Hex)_4$-HexN-InsP-EtNP-AEP	1299.332
Tulahuen	1300.5	1298.3	$(Hex)_4$-HexN-InsP-EtNP-AEP	1299.332
	1663.2	1661.6	$(Hex)_7$-HexN-InsP-AEP	1662.48
	1501.4	1499.5	$(Hex)_6$-HexN-InsP-AEP	1500.429
CL	1462.4	1460.4	$(Hex)_5$-HexN-InsP-EtNP-AEP	1461.38
	1446.4	1444.6	$(Hex)_5$-HexN-InsP-$(AEP)_2$	1445.389
	1339.6	1337.4	$(Hex)_5$-HexN-InsP-AEP	1338.376
	1663.3	1661.9	$(Hex)_7$-HexN-InsP-AEP	1662.48
	1501.8	1499.7	$(Hex)_6$-HexN-InsP-AEP	1500.429
	1462.2	1460.3	$(Hex)_5$-HexN-InsP-EtNP-AEP	1461.38
Y (Fraction A)	1446.2	1444.4	$(Hex)_5$-HexN-InsP-$(AEP)_2$	1445.389
	1339.2	1337.3	$(Hex)_5$-HexN-InsP-AEP	1338.376
	1300.2	1298.3	$(Hex)_4$-HexN-InsP-EtNP-AEP	1299.332
	1284.2	1282.3	$(Hex)_4$-HexN-InsP-$(AEP)_2$	1283.33

[1]Relative Molecular Mass (monoisotopic).

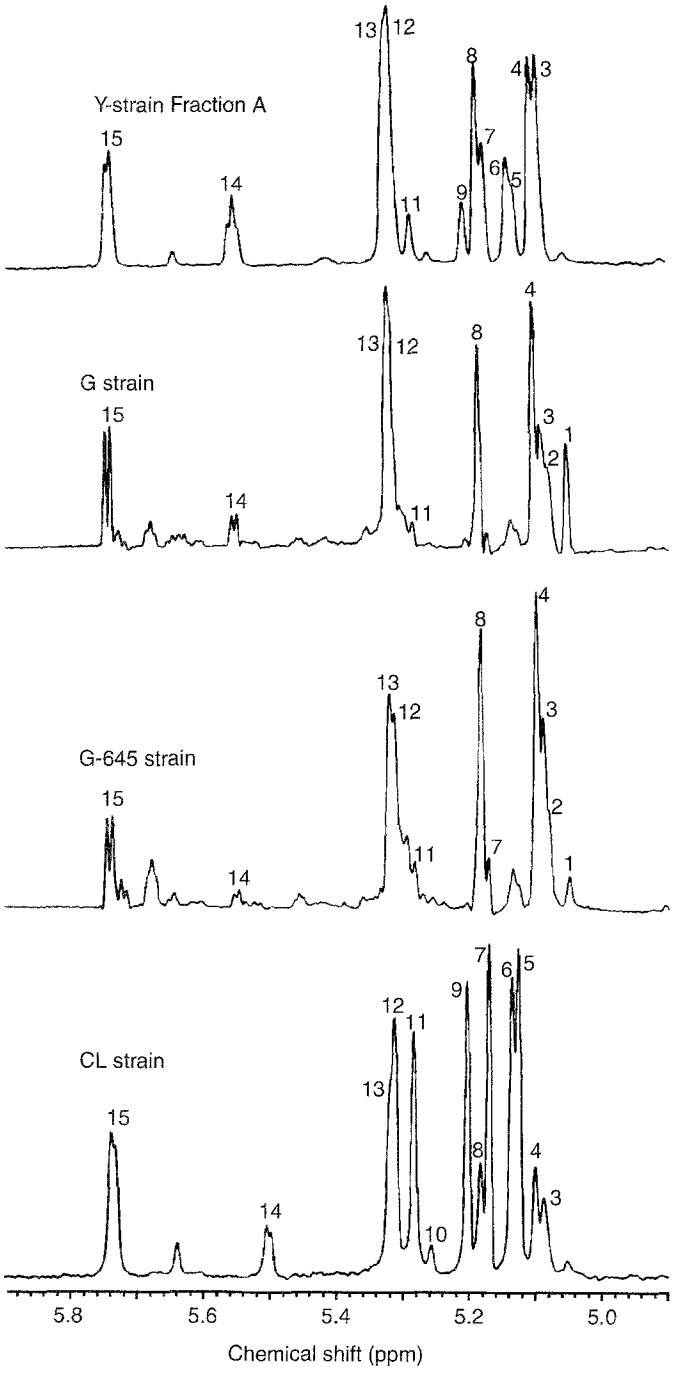

Figure 1. Region of the proton NMR spectra of the PI-oligosaccharides from *T. cruzi* Y strain (Fraction A), G, G-645 and CL strains arising from the anomeric protons. Peak labelling, from highfield, is as follows (see Table 5 for structures): (1) Terminal α-Man H-1 in **II** and, perhaps, **IV**; (2) $\rightarrow$2)-α-Man-(1$\rightarrow$6 H-1 in **II** and **IIa**; (3) $\rightarrow$2)-α-Man-(1$\rightarrow$6 H-1 in **I** and **Ia**; (4) $\rightarrow$3)-α-Man-(1$\rightarrow$2 H-1 in **I** and **Ia**; (5) $\rightarrow$2)-α-Man-(1$\rightarrow$6 H-1 in **III**; (6) $\rightarrow$3)-α-Man-(1$\rightarrow$2 H-1 in **III**; (7) Terminal β-Gal*f* H-1 in **III**; (8) Terminal β-Gal*f* H-1 in **I**; (9) Sidechain β-Gal*f* H-1 in **III**, and probably **IV**; (10) $\rightarrow$2,3)-α-Man-(1$\rightarrow$2 H-1 in **IV**; (11) $\rightarrow$2,3)-α-Man-(1$\rightarrow$2 H-1 in **III**; (12) $\rightarrow$6)-α-Man-(1$\rightarrow$4 H-1 in **I**, **II**, **III**, and **IV**; (13) $\rightarrow$2)-α-Man-[6-substituted]-(1$\rightarrow$2 in **I**; (14) $\rightarrow$4)-α-GlcN-(1$\rightarrow$6)-Ins-2-phosphate H-1; (15) $\rightarrow$4)-α-GlcN-(1$\rightarrow$6)-Ins-1-phosphate H-1.

established from ROESY and carbon chemical shift data (Tables 3 and 4). Resonances at 2.0 and 3.2 ppm in the one-dimensional proton spectrum were attributable to AEP residues, which was consistent with the observation of complex resonances at ~22 ppm in the one-dimensional ^{31}P spectrum; the latter experiment also indicated the presence of EtNP residues. Two-dimensional proton NMR confirmed these observations, and also indicated (in strain G) the presence of two separate AEP spin systems.

The one-dimensional spectra indicate that the GIPL from Tulahuen strain is similar to the GPI anchor of the sialic acceptor glycoprotein of epimastigote forms of *T. cruzi* Y strain [10], in their carbohydrate structures, and differed from the G-645 strain (Fig. 1) by the presence, in the PI-oligosaccharide of G-645, of a single terminal β-Gal*f* residue. These structures, I, containing the Gal*f* residue, and II are shown in Table 5. The PI-oligosaccharide from G strain was a 7:3 ratio of structures I and II, as judged by the relative intensities of the β-Gal*f* and terminal α-Man H-1 resonances at 5.184 and 5.052 ppm (Fig. 1). The FAB-MS data was in reasonable agreement with this (Table 2).

Some heterogeneity was apparent in the NMR spectra due to base-induced migration of the phosphate group from Ins *O*-1 to *O*-2 [29], and to the pattern of AEP or EtNP substitution (Fig. 1). The relative intensities of the resonance assigned to these latter groups (in G and G-645 strains) imply substitution of the $\rightarrow$2)-α-Man-(1$\rightarrow$2) residue (Man 3) by either EtNP or AEP. These are located on *O*-6 by the observation of a lowfield C-6 resonance at 66.28 ppm correlated in an HMQC experiment to protons at 4.138 ppm (for the EtNP substituent). FAB-MS confirmed the presence of AEP or EtNP on Man 3, because of the *m/z* increments between the Y_4 glycosidic cleavage ions at *m/z* 853 and the Y_5 fragments at *m/z* 1138 (in the case of EtNP substitution) or *m/z* 1122 (for AEP). The relative intensities of these ions suggested that EtNP predominated at this site. The glucosamine appeared to be exclusively substituted by AEP, because Y_2 glycosidic cleavage fragments were observed only at *m/z* 529, and not at *m/z* 545. The positive FAB spectrum of the major PI-oligosaccharide from the Tulahuen strain is shown in Fig. 2.

The relative ratio of AEP and EtNP substitution in the PI-oligosaccharides from various *T. cruzi* strains was estimated by integration of the AEP and EtNP $-CH_2-NH_2$ resonances at 3.21 and 3.29 ppm respectively (Fig. 3). In the Tulahuen oligosaccharide, the phosphorus containing substituent were present in a 1:1 ratio, whereas AEP predominated in G-645 (1.3:1.0) and in G strains (3.3:1.0). The integrations imply approximately 15% of the G-645 PI-oligosaccharide has an AEP substituent on Man 3 (Structure Ia), whilst G strain has an approximately equal substitution of AEP and EtNP on Man 3 (Structures I and Ia, and II and IIa) (Table 5).

Table 3. ^{1}H and ^{13}C assignments (ppm) of the major PI-oligosaccharide of *T. cruzi* G and G-645 strains

			EtNP | 6			*2-AEP* | 6			
	β-Galf(1→3)	*α-Manp(1→2)*	*α-Manp(1→2)*	*α-Manp(1→6)*	*α-Manp(1→4)*	*α-GlcN(1→6)*		*-Ins-*	*OPO_3*
						major	*minor*	*major*	*minor*
H-1	5.184	5.101	5.318	5.091	5.312	5.737	5.543	4.062	
H-2	4.158	4.268	4.138	4.023	4.064	3.364	3.390	4.192	4.505
H-3	4.072	3.933	3.994	3.992	3.840	4.097	4.113	3.564	3.466
H-4	4.084[a]		3.864	[3.693]	[3.693]	3.791	3.775	3.694	3.720
H-5	3.840				[3.780]	4.258	4.301	3.393	3.249
H-6	[3.615]	[3.728]	[4.138]			4.141		3.866	3.864
H-6′	[3.738]	[3.84]	[4.138]		[3.853]	4.141			
$POCH_2$			4.144						
PCH_2						2.047			
NCH_2			3.307			3.234			
C-1	105.87	103.16	101.76	99.56	102.32	96.82			
C-2	82.55	68.16	78.21	79.78	71.90	54.44			[73.33]
C-3	79.60	[76.81]						[72.10]	
C-4	84.20	67.95[b]	67.48[b]	68.50[b]	68.50[b]	79.18			
C-5						[71.39]		[74.59]	
C-6	64.40	62.18	66.28	62.56	[67.56]	64.62		[78.32]	
$POCH_2$			63.44						
PCH_2						25.56			
NCH_2			41.54			36.59			

[ ] assignment tentative; [a]proton assignment from HMQC; [b]assignment may be interchanged.

Table 4. ^{1}H and ^{13}C assignments (ppm) of the major PI-oligosaccharides of *T. cruzi* Tulahuen strain

			EtNP | 6		*2-AEP* | 6			
	α-Manp(1→2)	*α-Manp(1→2)*	*α-Manp(1→6)*	*α-Manp(1→4)*	*α-GlcN(1→6)*		*-Ins-OPO_3*	
					Major	*Minor*	*Major*	*Minor*
H-1	5.050	5.311	5.080	5.316	5.643	5.503	4.151	
H-2	4.070	4.118	4.021	4.062	3.400	3.420	4.198	
H-3	3.846	3.996	[3.985]	3.836	4.107	4.127	3.564	
H-4	[3.698]	3.846	[3.669]		3.796	3.791	3.693	
H-5	[3.79]		[3.79]		4.284	4.295	3.415	3.407
H-6	3.886	4.135	3.886	4.025	4.138		3.910	3.819
H-6′	3.762	4.135	3.762	3.775	4.138			
$POCH_2$		4.139						
PCH_2					2.035			
NCH_2		3.298			3.231			
C-1	103.39	101.83	99.61	102.24	96.39		77.10	
C-2	71.36[a]	79.70	79.82	71.60[a]	55.15		72.91	
C-3					[71.44]		71.77	
C-4	68.21		[68.21]		76.40		74.20	
C-5					71.08		74.06	
C-6	62.24	65.74	62.34	67.34	64.26		78.67	
$POCH_2$		63.14						
PCH_2					25.73			
NCH_2		41.43			36.64			

[ ] assignment tentative; [a]assignment may be interchanged.

Table 5. Proposed structures of PI-oligosaccharides present in *T. cruzi* GIPLs of G, G-645, Tulahuen, CL and Y strains

GIPL	*PI-oligosaccharides*	*Structures*	*T. cruzi strain*
Series 1	EtNP, 2-AEP Gal*f*β(1-3)Manα(1-2)Manα(1-2)Manα(1-6)Manα(1-4)GlcNα(1-6)InsPO$_4$	I	G, G-645, CL, Y
	2-AEP, 2-AEP Gal*f*β(1-3)Manα(1-2)Manα(1-2)Manα(1-6)Manα(1-4)GlcNα(1-6)InsPO$_4$	Ia	G-645, CL[1], Y[1]
	EtNP, 2-AEP Manα(1-2)Manα(1-2)Manα(1-6)Manα(1-4)GlcNα(1-6)InsPO$_4$	II	G, G-645[1], Tulahuen, Y[1]
	2-AEP, 2-AEP Manα(1-2)Manα(1-2)Manα(1-6)Manα(1-4)GlcNα(1-6)InsPO$_4$	IIa	G, Y[1]
Series 2	β-Gal*f*, 2AEP Gal*f*β(1-3)Manα(1-2)Manα(1-2)Manα(1-6)Manα(1-4)GlcNα(1-6)InsPO$_4$	III	CL, Y, G[1], G-645[1]
	β-Gal*f*, 2AEP Manα(1-2)Manα(1-2)Manα(1-6)Manα(1-4)GlcNα(1-6)InsPO$_4$	IV	CL, Y
	β-Gal*f*, 2AEP Manα(1-2)Manα(1-6)Manα(1-4)GlcNα(1-6)InsPO$_4$	V	Y[2]

[1]less than 5% of total GIPL structures; [2] [8].

CL strain PI-oligosaccharide

Two-dimensional NMR spectra of the main fraction from the CL-strain indicated that the two predominant PI-oligosaccharide structures were present in an approximate ratio of 4:1. This was in agreement with the FAB-MS results, which also suggested that several additional minor molecular species were present (Table 2). The TOCSY spectrum (Fig. 4) and the proton and carbon resonance assignments (Table 6) showed that the major component contains two β-Gal*f* residues (Structure III, Table 5) and is identical to the major GIPL (previously called LPPG) of *T. cruzi* Y strain [7, 8].

The component present at the 20% level had an EtNP substituent in place of the sidechain Gal*f*, and is identical to structure I (Table 5). This is supported by the H-1 resonance of the →2)-α-Man-(1→6 residue which is sensitive to substitution of the adjacent residue by β-Gal*f*, and appears at 5.135 ppm in Structure III (Table 6), compared to 5.091 ppm in Structure I (Table 3).

Lederkremer *et al.* [8] assigned the resonance at 5.17 ppm as the H-1 of both β-Gal*f* residues, and that at 5.20 ppm as the H-1 of Man in a minor, undefined variant. We have assigned the resonance at 5.171 ppm (Table 6) as the H-1 resonance of the terminal β-Gal*f* and the resonance at 5.202 ppm as the H-1 of the sidechain β-Gal*f* residue. The corresponding ^{13}C signals in HMQC were 105.88 and 106.2 respectively (Table 6). The n.O.e. observed [8] between the resonance at 5.20 ppm and H-2 of Man 3 is thus explained.

In the TOCSY spectrum (Fig. 4) of this material a minor crosspeak between Man H-1 at 5.261 ppm and H-2 at 4.338 ppm was consistent with a →2,3) substituted Man, probably attributable to a minor PI-oligosaccharide which lacks the terminal β-Gal*f* (structure IV, Table 5). Another minor α-Man spin system with H-1 at 5.132 ppm, H-2 at 3.998 ppm and H-3 at 3.904 ppm was tentatively assigned as a terminal Man in the same Man α1-2(Gal*f*β1-3) Man (α1→ substructure. The ratio of the intensity of the H-1 resonance at 5.261 ppm to that of the →2,3)-α-Man H-1 in Structure III suggested that

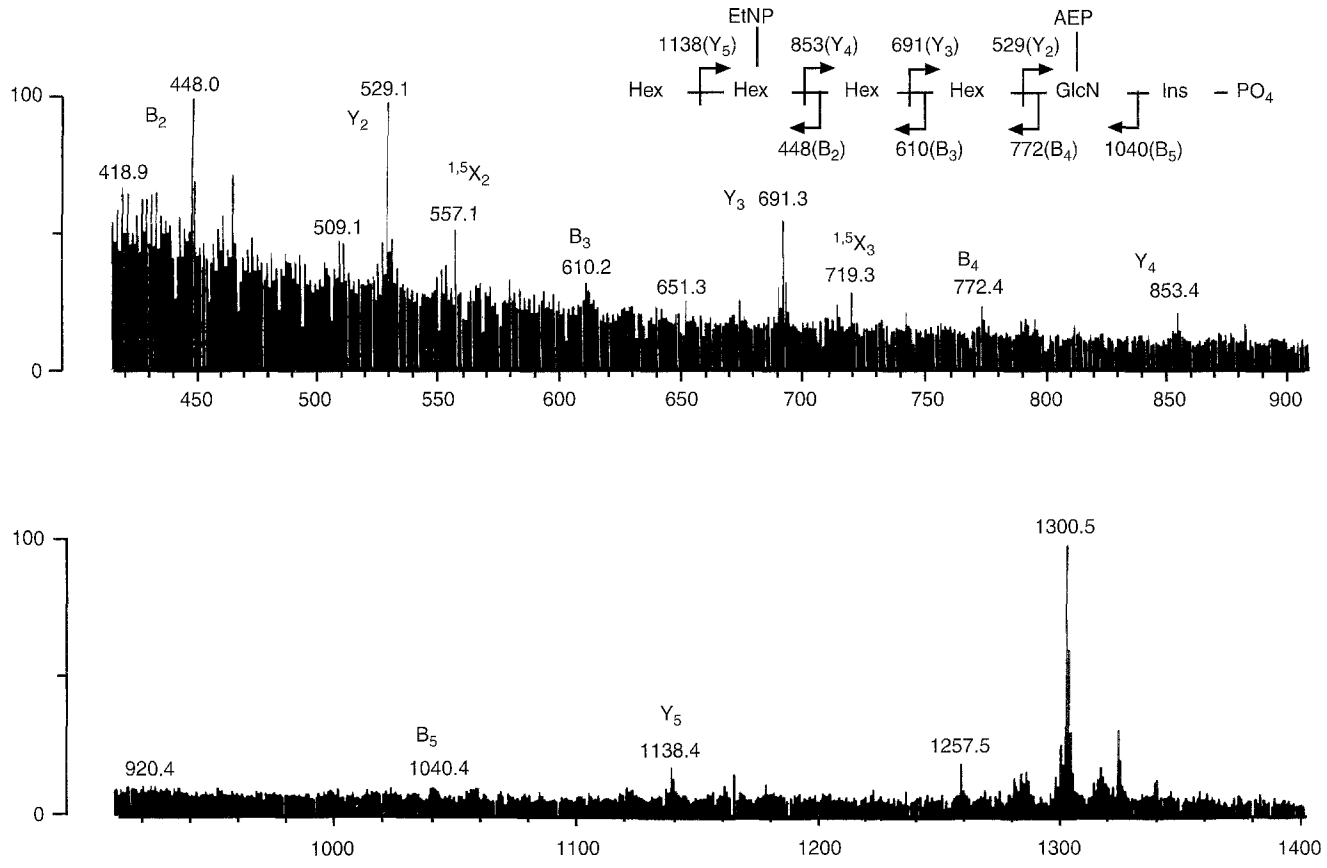

Figure 2. Positive ion fast atom bombardment mass spectrum of the major PI-oligosaccharide from the Tulahuen strain of *T. cruzi*.

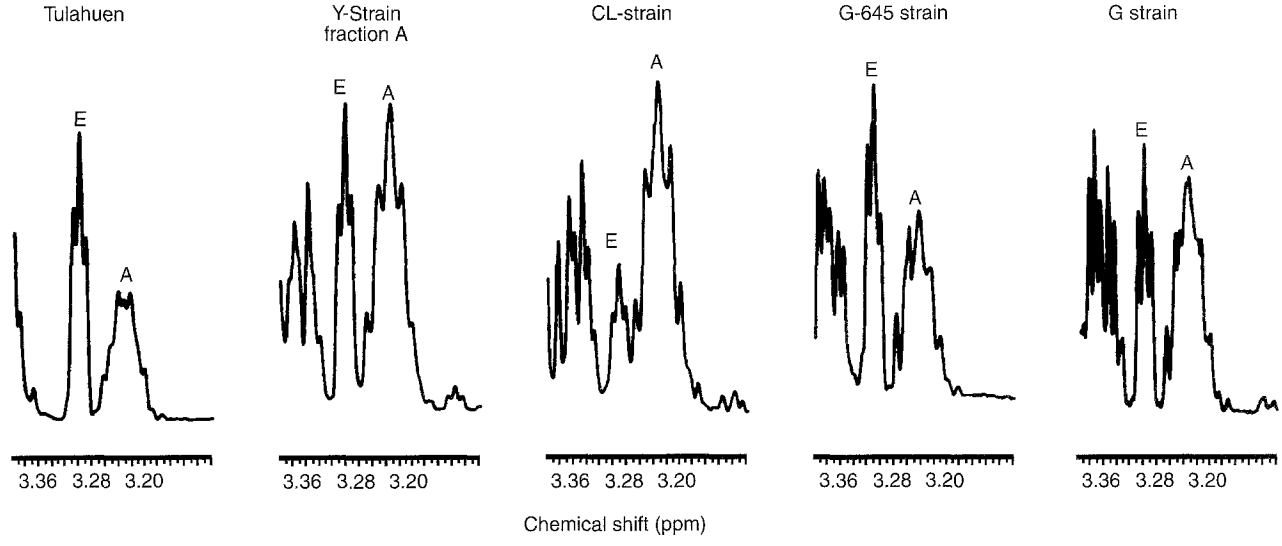

Figure 3. Partial 500 MHz ^{1}H NMR spectra of the *T. cruzi* strains PI-oligosaccharides showing the AEP $-CH_2NH_2$ resonance (labelled A) and the EtNP $-CH_2NH_2$ resonance (labelled E). In the Y and CL strains, the AEP resonance is largely due to Series 1 GIPLs, but for the Tulahuen, G and G-645 GIPLs the ratio of AEP to EtNP substitution can be derived by integration of these resonances.

structure IV (Table 5) comprised about 10% of the fraction.

Y strain PI-oligosaccharides

In our original characterization of the GIPLs from strain Y, ^{31}P NMR and chemical analysis indicated the presence of phosphorylated substituents in addition to those on GlcN and Inositol [7], though these were not characterized further at that time. The detection of EtNP or AEP substitution on Man 3 in the CL, Tulahuen and G strains prompted us to reanalyse the PI-oligosaccharide structures of the Y strain GIPLs. These results (Fig. 1; Tables 2 and

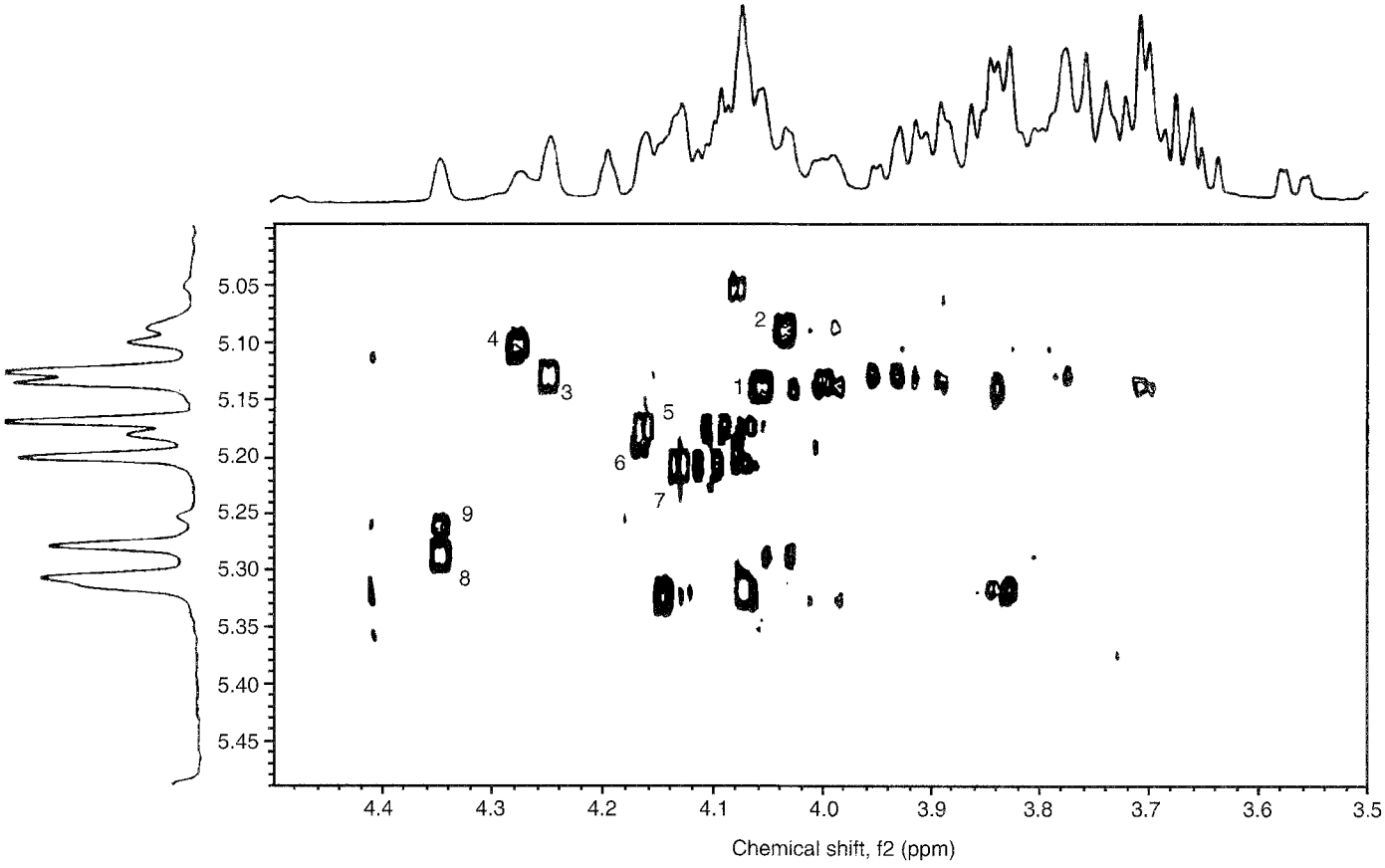

Figure 4. Partial 500 MHz TOCSY spectrum, obtained with an 80 msec mixing time, of the PI oligosaccharides from the CL strain. The spectrum shows correlations between the α-Man and β-Gal*f* H-1 resonances and the ring protons. Important resonances discussed in the text are (see Table 5 for structures): (1) α-Man (1→6) H-1/H-2 in **III**; (2) α-Man (1→6) H-1/H-2 in **I** and **II**; (3) →3)-Man-(α1→2) H-1/H-2 in **III**; (4) →3)-Man-(α1→2) H-1/H-2 in **I**; (5) Terminal β-Gal*f* H-1/H-2 in **III**; (6) Terminal β-Gal*f* in **I**; (7) Sidechain βGal*f* H-1/H-2 in **III**; (8) →2,3)-Man(α1→2) H-1/H-2 in **III**; (9) →2,3)-Man(α1→2) H-1/H-2 in **IV**.

5) show that in addition to the previously described structures [7, 8], additional PI-oligosaccharide in which Man 3 is substituted with EtNP or AEP are present. The most abundant of these were structures I and Ia, but low levels of II and IIa were detected by FAB MS (Table 2). In addition a further component containing only one (the sidechain) β-Gal*f* was present, which seems to be identical to structure IV (Table 5). These PI oligosaccharides have not been characterized before [7, 8].

Discussion

We have compared the carbohydrate and lipid structures of GIPLs isolated from five strains of *T. cruzi*. The carbohydrate structures are depicted in Table 5.

Although these GIPLs share the same Man_4-GlcN-Ins-PO_4 core sequence, they can be classified into two series on the basis of the substituent on the third mannose residue distal to inositol (Man 3). Series 1 has a phosphorus containing group (EtNP or AEP), and Series 2 has a β-D-Gal*f* unit. It is noteworthy that Series 1 GIPLs contains EtNP or AEP linked to the *O*-6 position of the Man 3 together with AEP linked to *O*-6 of GlcN, a feature that has been reported for the GPI anchor of the sialic acid acceptor glycoprotein of epimastigote forms of *T. cruzi* Y strain [10]. Indeed, the major GIPL from the Tulahuen strain is identical to this GPI anchor, and the other Series 1 structures differ only by the presence of terminal β-D-Gal*f* residues. Substitution of the third mannose distal to inositol with EtNP is typical of protein linked GPI molecules, but is not usual in trypanosomatid GIPLs, in which phosphorus-containing substituents (when present) are on the second mannose in the glycan core or on GlcN [30], though EtNP substitution of Man 3 has recently been reported in GIPLs from a *Phytomonas* sp. [31]. However, trypanosomatids of the genus *Phytomonas* are a group of specialized parasites of plants, which constitute a relatively recent diverging lineage within the Trypanosomatidae family [32, 33].

Although the GPI anchor of the sialic acid acceptor glycoprotein and the GIPLs of *T. cruzi* differ in their lipid composition, the former containing mostly ceramide

Table 6. ^{1}H and ^{13}C assignments (ppm) of the di-Gal*f*-containing PI-oligosaccharide of *T. cruzi* CL strain

	β-Galf | 3						*2-AEP* | 6			
	β-Galf(1→3)	*α-Manp(1→2)*	*α-Manp(1→2)*		*α-Manp(1→6)*	*α-Manp(1→4)*	*α-GlcNα(1→6)*		*-Ins-OPO$_3$*	
			β-Galf	*α-Manp*			*Major*	*Minor*	*Major*	*Minor*
H-1	5.171	5.128	5.202	5.284	5.135	5.315	5.734	5.524	4.072	
H-2	4.158	4.244	4.127	4.346	4.052	4.068	3.356	3.315	4.189	4.490
H-3	4.092	3.939	4.095	4.037	4.010	3.830	4.084	4.072	3.564	3.492
H-4	4.076	3.787	4.076	3.779		[3.8]	3.799	3.760	3.685	3.740
H-5	3.842		3.856	[3.812]			4.256	4.292	3.403	3.353
H-6	[3.69]	[3.902]	[3.73]	[3.915]	[3.92]		4.124	4.124	3.865	3.854
H-6′	[3.65]	[3.732]	[3.684]	3.750	[3.76]		4.124	4.124		
PCH_2							2.039			
NCH_2							3.239			
C-1	105.88	102.79	106.20	101.92	99.61	102.41	96.30			
C-2	82.50		82.50	75.58	76.40					
C-3	78.00	76.49	78.00	[79.78]						
C-4	84.00		84.00				76.20			
C-5										
C-6	63.86	62.00	63.86	62.00	62.00		64.06			
PCH_2										
NCH_2							36.17			

[ ] assignment tentative.

and the latter alkylacylglycerol, it seems likely that the same transferases are responsible for the synthesis of their common glycan structure and substituents:

EtNP 2-AEP
| |
6 6
Manα1-2Manα1-2Manα1-6Manα1-4GlcNα1-6Ins

The Series 1 GIPLs could therefore originate from excess GPI anchor precursors by a lipid modification in which the alkylglycerol is replaced by ceramide. Further substitution with terminal βGal*f* would give rise to structures I and Ia (Table 5). It remains to be determined, however, whether the kind of remodelling described in *Saccharomyces cerevisiae* [34] also occurs in *T. cruzi*. An alternative possibility is the formation of both inositolphosphoglycerolipid and inositolphosphosphingolipid intermediates via separate biosynthetic pathways.

The Series 2 GIPLs are substituted with βGal*f* on Man 3 rather than with EtNP or AEP. Their biosynthesis implies the participation of a specific β-galactofuranosyl transferase and may take place in a different cellular compartment. Further extension of the glycan chain may need a (1→2) mannosyl transferase which differs in specificity from the one involved in Series 1 biosynthesis. Accordingly, the biosynthesis of the Series 2 GIPLs could involve structures V→IV→III (Table 5).

It has been suggested that in the GPI anchors and GIPLs of *T. cruzi*, the shift from glycerolipid to ceramide is developmentally regulated [15, 35]. Both alkylacylglycerol based (GIPL A) and sphingolipid based GIPLs (GIPL B) have been found in the early phase of culture growth, whereas in stationary phase only a glycosylated inositolphosphoceramide (LPPG) was expressed [35].

In the present study we have isolated GIPLs from epimastigotes of different strains of *T. cruzi*, in the late stationary phase of growth, which contain varying amounts of phosphoglycerol and phosphosphingolipids. The G, G-645, Y and Tulahuen strains synthesize mainly ceramide-containing GIPLs whereas CL strain has in addition, high levels of 1-*O*-hexadecyl-2-acylglycerol (Table 1), suggesting that these differences are strain dependent. The significance of these strain related differences in the GIPL profiles of epimastigotes is unclear. In the case of infective forms of *T. cruzi* the expression of inositolphosphoglycerolipids vs inositolphosphosphingolipids may have a role in the host immunological response.

Recently, it has been demonstrated that purified GIPLs from both strains Y and G, are immunosuppressive for cell-mediated responses *in vitro*, and block murine T-cell activation *in vivo* [6]. This down-regulation effect was mapped to the GIPL ceramide domain, and it was suggested that GIPLs could be involved in subverting the local immune response against *T. cruzi*. Both Y and G strain GIPLs have similar T-cell suppressive capacity, and

their lipid moieties consist mainly of *N*-lignoceroyldihydrosphingosine, which is also suppressive in the purified form. Commercially available *N*-palmitoyldihydrosphingosine, is inactive [6], suggesting that the specific fatty acid rather than the long chain base of the ceramide is pivotal to this process. In a variety of host cell types, including lymphoid cells, ceramides are recognized as important mediators of growth arrest and apoptosis signalled by cytokine and other surface antigen receptors [36, 37]. The evolutionary relationship between the ability of ceramides to cause cell cycle arrest/apoptosis in cells of the immune system, and the occurrence of inositolphosphingolipids in GIPLs of trypanosomes might be more than accidental. In this regard, it is interesting to note that *T. cruzi* is the only trypanosomatid pathogen of mammals in which ceramide containing GIPLs have been found. The strain dependent differences in GIPL structure, (such as the high proportion of 1-*O*-hexadecyl-2-acylglycerol in the CL strain) can be correlated to biological activity. Trypomastigotes of the CL strain are 20–30-fold less infective than those of the Y strain and have a different tropism for the host cells [18, 38]. It is known also that a myotropic CL strain is not immunosuppressive for human T cells *in vitro* [39] and is an inducer of high level of host macrophage activation *in vivo* [40]. The high levels of 1-*O*-hexadecyl-2-acylglycerol expression in CL strain GIPL, may therefore impart less immunomodulatory potency to this compound. In fact, the lipid domain of *Phytomonas serpens* GIPL is an alkylacylglycerol [41], and the purified GIPL lacks immunosuppressive activity [16].

Further studies are presently been conducted to determine the role of the GIPL oligosaccharide as an immunomodulatory determinant which could influence the infection by *T. cruzi*.

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