

Hexadecylpalmitoylglycerol or ceramide is linked to similar glycosphosphoinositol anchor-like structures in *Trypanosoma cruzi*

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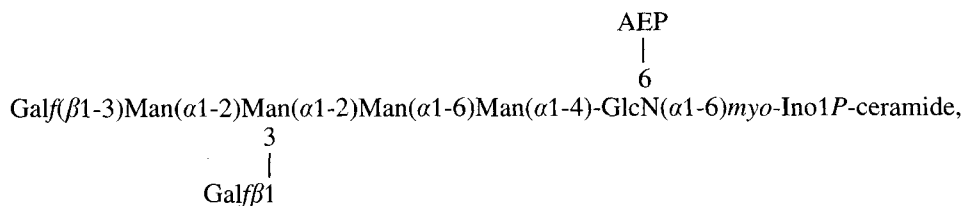
(Received June 14/August 7, 1993) – EJB 93 0892/4

The lipopeptidophosphoglycan from *Trypanosoma cruzi* is a glycosylated inositol-phosphoceramide isolated from epimastigotes at the stationary phase of growth (4–5 days). We have now purified two similar glycosylphospholipids (glycosylphospholipid A and glycosylphospholipid B) from epimastigotes after the second day of culture growth. [³H]Palmitic acid was incorporated into 1-*O*-hexadecyl-2-*O*-palmitoylglycerol in glycosylphospholipid A and into ceramide in glycosylphospholipid B. The lipids were released by incubation with glycosylphosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* or by chemical methods. After alkaline hydrolysis, the lipids were analysed by GLC/MS. In glycosylphospholipid A the resulting lipids corresponded to 1-*O*-hexadecylglycerol and palmitic acid. The ceramide components in glycosylphospholipid B are sphinganine, palmitic acid and lignoceric acid. The oligosaccharides could be degraded by nitrous acid and further enzymic treatment showed that the two glycosylphospholipids isolated from *T. cruzi* share the common core structure of the glycosylphosphatidylinositol membrane anchors. The microheterogeneity was determined, as well as the substitution by galactose, and was mainly in the furanose configuration as was previously described for lipopeptidophosphoglycan. However, methylation analysis indicated that 20% of the galactose is in the pyranose form. Both glycosylphospholipids mainly differ in the lipid moiety.

Trypanosoma cruzi, the agent of Chagas' disease (South American trypanosomiasis) has a complex life cycle between the mammalian host and the insect vector [1]. The infective trypomastigote circulates in the peripheral blood of vertebrate hosts and is capable of penetrating cells. Cell penetration is followed by differentiation to the dividing amastigote stage. The intracellular amastigotes differentiate back to trypomastigotes which lyse the host cells and invade other neighboring cells or return into the circulation. When parasites are ingested by the insect vector (reduviid) during a blood meal, the trypomastigotes differentiate into dividing epimastigotes in the insect midgut. In the hindgut, epimasti-

In recent years, glycosylphospholipids have received a great deal of attention due to their function as the anchors of membrane proteins [3, 4]. In addition, glycosylphospholipids with anchor-like structures have been described in protozoa [5, 6].

The elucidation of the complete structure of the glycan from the lipopeptidophosphoglycan of *T. cruzi* epimastigotes [7] revealed a core structure common to the known glycosylphosphatidylinositol (glycosylPtdIns) membrane anchors. A microheterogeneity with three major oligosaccharide structures has been detected in lipopeptidophosphoglycan. The major species (65%) has the following structure:



gotes are transformed to metacyclic trypomastigotes which are deposited near the wound with faeces and urine and are competent to invade the mammalian host [2].

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Abbreviations. PtdIns-PLC, glycosylphosphatidylinositol-specific phospholipase C; AEP, aminoethylphosphonic acid.

with an additional Man α -2 linked to the last mannose residue of the glycosylPtdIns-conserved core and antigenic galactofuranose terminal units (AEP represents aminoethylphosphonic acid) [8, 9]. A characteristic feature of the lipopeptidophosphoglycan is the presence of AEP linked to the 6 position of the glucosamine residue. Interestingly, AEP is the C-*P* analog of ethanolamine phosphate which is the link to the protein in the known glycosylPtdIns anchors.

The lipid component in lipopeptidophosphoglycan is a ceramide which could be released by glycosylphosphatidyl-

inositol-specific phospholipase C (PtdIns-PLC). Some of the analysed samples contained 1-*O*-hexadecylglycerol as a constituent of lipopeptidophosphoglycan [10], which was routinely isolated from parasites reaching the stationary phase (4–5 days).

To examine the possible influence of the transition from the log growth phase to the stationary phase on the structure of the lipid, the incorporation of [³H]palmitic acid into *T. cruzi* glycoinositolphospholipids isolated from epimastigotes of the second day of growth (logarithmic growth) has been studied. The lipid moieties and glycans have been analysed using chemical and enzyme degradation methods.

MATERIALS AND METHODS

Parasites

Epimastigote forms of *T. cruzi*, Y strain, were cultivated in Liver infusion tryptose (LIT medium) as described [11]. Cells were harvested at the logarithmic phase of growth (day 2) by centrifugation at 800×g for 10 min and were washed four times with 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2 (NaCl/P_i).

Metabolic labelling

Cells were cultured for 2 days in LIT medium, transferred to TAU-3AG medium [12] containing 0.2% fetal calf serum at 1×10⁸–2×10⁸ parasites/ml and were incubated for 6 h at 28°C. Labelling was performed with [9,10³H]palmitic acid (54Ci/mmol; 28 µCi/ml for 17 h at 28°C. After incorporation, microscopic observation showed that all of the parasites remained viable.

Analytical methods

Gel electrophoresis was performed using 10% polyacrylamide in the presence of 0.1% SDS. Gels were prepared for fluorography as in [13].

TLC was performed using silica-gel 60 (Merck) with the solvent systems hexane/isopropanol (23:2, by vol.; solvent A) and propanol/ethanol/water (7:1:2, by vol.; solvent B). For fluorography, TLC plates were sprayed with EN³HANCE (New England Nuclear) and were exposed to Kodak X-Omat A-R5 films at –70°C.

Capillary GLC was performed with a Hewlett-Packard 5890 gas chromatograph with nitrogen as the carrier gas. SP-2330 (0.25 mm×30 m; Supelco) and HP-5 (0.32 mm×50 m; Hewlett-Packard) columns were used; flow rate, 1.4 ml/min; column temperature (*t_c*), 165°C; injector temperature (*t_i*), 220°C; detector temperature (*t_d*), 220°C.

GLC/MS of the lipids was performed with a Varian Mat CH-7A mass spectrometer at 70 eV using a glass column (2 mm×1.2 m) of 3% OV-17 on Chromosorb WAW. Trimethylsilylation of the lipids was performed with 20–50 µl Sylon HTP (Supelco) by heating at 70°C for 10 min. Samples were centrifuged at 1000 g before injection. Carbohydrate was measured by the phenol/sulfuric acid method [14].

Purification of the glycoinositolphospholipids

Lyophilized cells were delipidated with chloroform/methanol and the glycoinositolphospholipids were extracted with

water saturated with 1-butanol and purified on octyl-Sepharose as described previously [10].

Sugar analysis

Neutral sugars were determined after hydrolysis with 2 M trifluoroacetic acid (200 µg sugar/ml) at 105°C for 3 h. The hydrolysates were extracted with ether and the aqueous phase was evaporated under diminished pressure with repeated additions of water. Neutral sugars were separated from basic components on Dowex 50W-X8 (H⁺) (1.5 cm×1 cm). Monosaccharides in the neutral eluate were analysed as alditol acetates [15] by capillary GLC using the SP-2330 column, flow rate 0.8 ml/min; *t_c*, 220°C; *t_i*, 250°C; *t_d*, 250°C. The acid eluate (1 M HCl) from the Dowex column was further hydrolysed with 4 M HCl at 100°C for 15 h and analysed as before, but using *t_c*, 240°C; *t_i* and *t_d*, 270°C. A sample of the glycan obtained by dephosphorylation with HF was also analysed for sugar composition.

Enzymic digestions

Phosphatidylinositol phospholipase C

Digestions with PtdIns-PLC from *Bacillus thuringiensis* (a gift from Dr M. Low, Columbia University, New York) were performed as described previously [10]. For *Bacillus cereus* PtdIns-PLC (Sigma) digestions, radioactive samples were treated with 0.1 U enzyme in 0.1 ml 10 mM Tris/HCl, pH 7.4, 144 mM NaCl, 0.01% Triton X-100, for 24 h at 25°C. The lipid was extracted with ether (3×0.5 ml) and analysed by TLC.

Exo-glycosidases

Radiolabelled reduced oligosaccharides were incubated in 20 µl 0.1 M sodium acetate, pH 5, at 37°C, for 18 h with 0.8 U jack bean α -mannosidase (Sigma) or 30 µU α -mannosidase from *Aspergillus phoenicis* (Oxford Glycosystems). Digestions were terminated by heating for 1 min at 80°C. The samples were desalted by passage through a tandem column of 0.4 ml AG50 X 12 (H⁺), 0.8 ml of AG3X4 (OH⁻), 0.4 ml QAE-Sephadex A-25 and were concentrated.

Lipid analysis

The labelled lipids released from the glycoinositolphospholipids by PtdIns-PLC were extracted with ether and analysed by TLC using solvent A followed by fluorography. Strong alkaline hydrolysis of samples of the extracted lipids (100000 cpm) was performed by heating with 1 M NaOH (0.5 ml) for 24 h at 100°C in Teflon tubes. The mixture was neutralized, the lipids extracted with ether and samples were analysed by TLC as described above. For GLC/MS the glycoinositolphospholipids (1 mg) were treated with 0.25 M KOH in methanol (1 ml) for 16 h at 28°C. The methanol was evaporated, the residue was suspended in water, acidified with 1 M HCl and extracted with ether. The lipids in glycoinositolphospholipid A were analysed as trimethylsilyl derivatives by GLC/MS. The lipid released from glycoinositolphospholipid B was further hydrolysed by heating with 1 M NaOH at 100°C before GLC/MS analysis.

Aqueous HF treatment of the glycoinositolphospholipids

The glycoinositolphospholipids (700 µg in sugar) were treated with 100 µl 48% aqueous HF at 0°C for 60 h. The

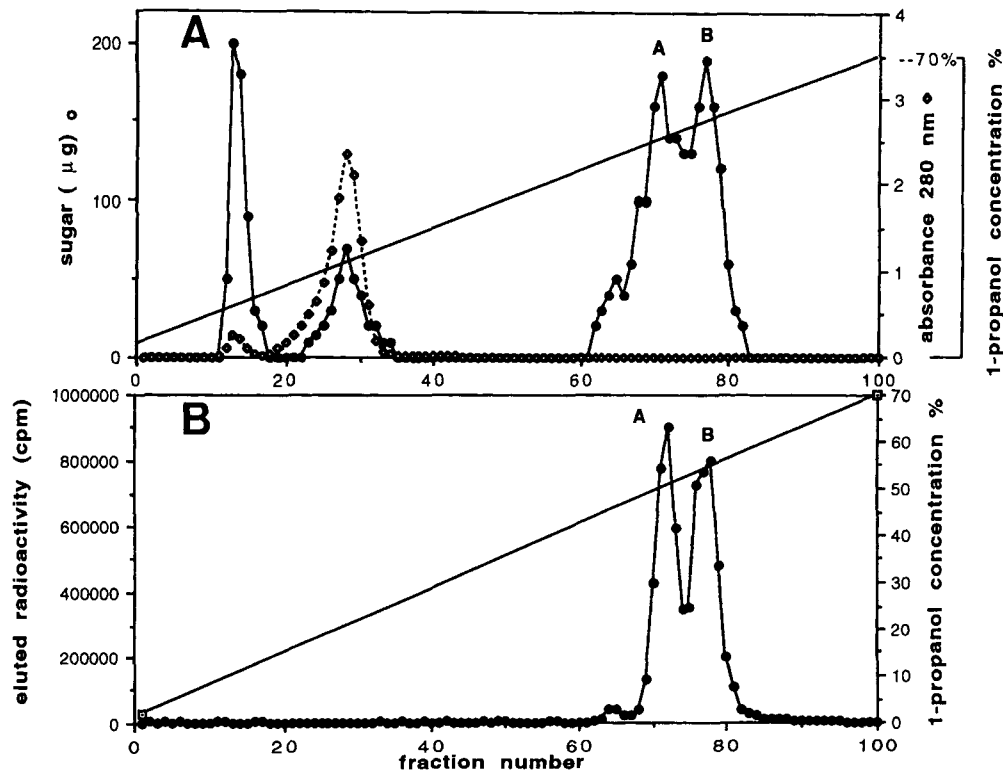


Fig. 1. Purification by octyl-Sepharose chromatography of glycoinositolphospholipids A and B. The column (1.2 cm×30 cm) was equilibrated with 0.1 M Tes, pH 4.5, containing 2% 1-propanol. (A) A 20-mg sample of the glycoconjugates solubilized with chloroform/methanol/water and precipitated by the addition of methanol [10] was dissolved in the starting buffer and eluted with a linear gradient of 1-propanol (2–70%). Fractions of 1 ml were collected and aliquots were analysed for carbohydrate. (B) [³H]palmitic-acid-labelled glycoinositolphospholipids. Aliquots were analysed for radioactivity. The presence of glycoinositolphospholipids A and B is indicated.

samples were neutralized with cold saturated LiOH and LiF was removed by centrifugation at 3000 *g* and washed twice with 200 μl water at 0°C. The pooled supernatants were extracted with diethyl ether to remove the lipid, passed through a column of Dowex 50W-X8 (2 ml) and eluted with 10 ml water, followed by 20 ml 1 M HCl. The aqueous fraction was analysed by GLC chromatography. The acid fraction was dried under vacuum and the residue was evaporated several times with water to remove the acid. The sample, dissolved in water, was passed through a column of AG3-X4 (OH⁻) (0.8 ml), eluted with 10 ml water and with 15 ml 1 M NH₄OH. In the ammoniacal eluate, AEP was identified by paper electrophoresis on Whatman 3MM paper in 1 M acetic acid/1 M formic acid, pH 2.2, at 45 V/cm for 90 min. Detection was performed with ninhydrin [16] and with the molybdate spray of Hanes and Isherwood as reported, for phosphate [17]. The fraction which eluted with water from the AG3-X4 column was used for methylation analysis.

Preparation of radioactive neutral oligosaccharides

Samples containing approximately 20 μg of sugar were dephosphorylated with 48% HF as described above. After neutralization with LiOH and centrifugation of the LiF, the supernatants were dried and deaminated in 0.5 ml 50 mM sodium acetate, pH 3.5, with 5 mg NaNO₂ at 0°C for 3 h with stirring. After deamination, the sample was adjusted to pH 8 with 5% Na₂CO₃ and reduced with 2 ml 25 mM NaB³H₄ (10Ci/mmol) in 0.1 M NaOH for 1 h at 20°C. An excess of NaBH₄ was added and the reduction was continued overnight

at 5°C. The excess of reagent was destroyed with 5% acetic acid followed by several evaporations with methanol. The samples were dissolved in water and desalted as described above.

Methylation analysis

Samples of glycoinositolphospholipid A and glycoinositolphospholipid B, containing 600 μg of sugar, were digested with PtdIns-PLC and the lipid was extracted with ether. The aqueous solutions were treated with HNO₂/NaBH₄ as described above. Boric acid was eliminated by repeated evaporation with methanol, the residue was dissolved in water and cations were removed by passing the sample through 1 ml of Dowex AG50X12 (H⁺) and eluting with water. The solutions were dried in a Speed-vac and were methylated by a modification of the Ciucanu and Kerek procedure [18] as previously reported [7]. The permethylated samples were hydrolysed with 100 μl 0.25 M sulfuric acid in 93% acetic acid for 2.5 h at 80°C. After neutralization with 1 M NaOH the samples were evaporated under vacuum and the residues were dissolved in 50 μl 1 M NH₄OH and reduced with 200 μl 0.25 M NaBD₄ for 3 h at 20°C. The reduced mixtures were processed as usual and were acetylated with 0.25 ml acetic anhydride/pyridine (1:1, by vol.) at 100°C for 2 h. The reagent was removed under vacuum and the partially methylated alditol acetates were analysed by capillary GLC using a HP-5 column as described above. The dephosphorylated glycans were also methylated and analysed. The identity of the peaks was confirmed by GLC/MS.

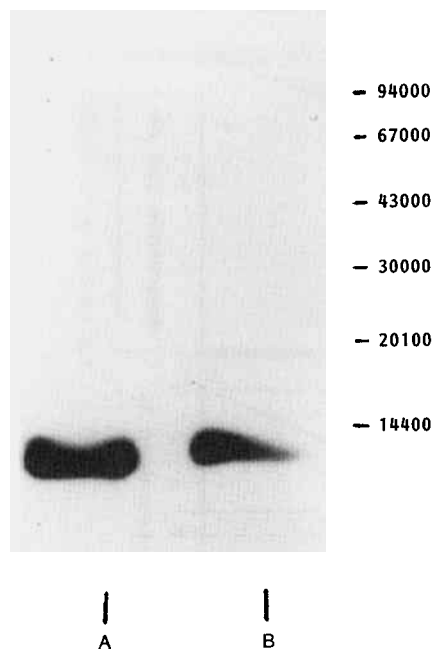


Fig. 2. SDS/PAGE of glycoinositolphospholipids A and B obtained after purification by octyl-Sepharose chromatography. The samples, metabolically labelled with [^3H]palmitic acid were analysed in 10% polyacrylamide with 0.1% SDS gels followed by fluorography. The M_r values of standards are indicated. A, glycoinositolphospholipid A; B, glycoinositolphospholipid B.

Table 1. PtdIns-PLC release of radioactive lipids from [^3H]palmitic-acid-labelled glycoinositolphospholipids. A sample (1×10^5 – 2×10^5 cpm) obtained from [^3H]palmitic-acid-labelled epimastigotes was digested with 1 mU PtdIns-PLC from *B. thuringiensis* for 3 h at 37°C in 50 mM Tris/HCl, pH 7.2, containing 0.1% deoxycholate. The lipid was extracted with ether and the total radioactivity in the ether extract and aqueous phase was determined. No radioactivity was found in the ether extract of a mock-treated sample. A second sample was treated as above with PtdIns-PLC from *B. cereus* for 24 h at 25°C in 10 mM Tris/HCl, pH 7.4, 144 mM NaCl, 0.01% Triton X-100. The radioactivity in the ether extract and aqueous phase was determined. GIPL, glycoinositolphospholipid.

PtdIns-PLC from	GIPL A radioactivity in		GIPL B radioactivity in	
	ether phase	aqueous phase	ether phase	aqueous phase
	cpm			
<i>B. thuringiensis</i>	142 000	27 000	125 000	34 000
<i>B. cereus</i>	109 000	12 000	81 100	31 500

RESULTS

Isolation and purification of glycoinositolphospholipids

The *T. cruzi* glycoinositolphospholipids were isolated from delipidated epimastigote cells after two days of culture growth. In typical experiments, 1.5×10^{12} epimastigotes or 5×10^9 [^3H]palmitic-acid-labelled parasites were extracted with chloroform/methanol followed by water saturated with 1-butanol. The extract was fractionated with solvents as pre-

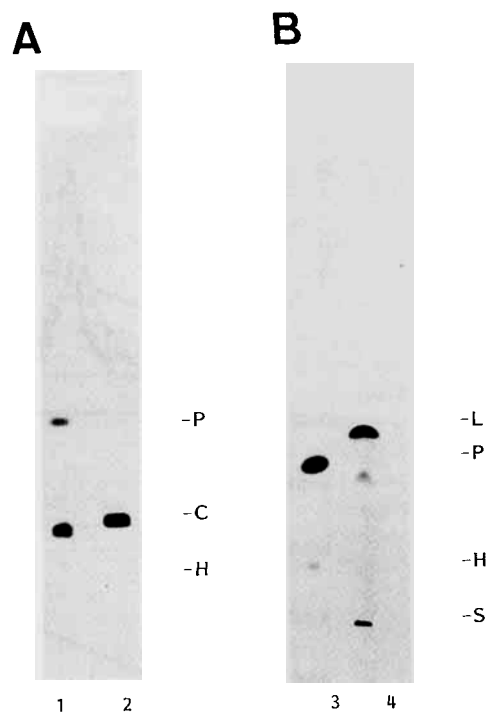


Fig. 3. TLC followed by fluorography of the [^3H]palmitic-acid-labelled lipids released from glycoinositolphospholipids A and B. (A) Ether extract after treatment with PtdIns-PLC from *B. thuringiensis* of glycoinositolphospholipid A (lane 1) and glycoinositolphospholipid B (lane 2). (B) Ether extract after hydrolysis with 1 M NaOH for 24 h at 100°C followed by acidification with 1 M HCl of glycoinositolphospholipid A (lane 3) and glycoinositolphospholipid B (lane 4). The position of standards are indicated as follows: L, lignoceric acid; P, palmitic acid; C, ceramide; H, 1-*O*-hexadecylglycerol; S, sphinganine. The solvent was hexane/2-propanol (23:2, by vol.).

viously described for the lipopeptidophosphoglycan [10] and the glycolipids were separated on octyl-Sepharose (Fig. 1). The two glycoinositolphospholipids eluted very closely with 48–53% (glycoinositolphospholipid A) and 53–60% (glycoinositolphospholipid B) 1-propanol. Both radioactive glycoinositolphospholipids were also obtained in similar amounts (Fig. 1B). For further studies each peak was chromatographed again or, alternatively, analysis was performed only for the major individual fractions collected from the octyl-Sepharose column. The radioactive glycoinositolphospholipids A and B behaved similarly when analysed by SDS/PAGE giving fast-migrating broad bands (Fig. 2).

Lipid analysis

The incubation of radioactive (1×10^5 – 2×10^5 cpm) glycoinositolphospholipids with PtdIns-PLC from *B. thuringiensis* or *B. cereus* released 70–90% of the radioactivity (Table 1). Analysis by TLC showed a spot with the mobility of a commercial sample of *N*-lignocerylsphinganine in glycoinositolphospholipid B and a slightly slower moving lipid in glycoinositolphospholipid A (Fig. 3A). The latter corresponded to 1-*O*-alkyl-2-acylglycerol since, after alkaline hydrolysis, the lipid from glycoinositolphospholipid A gave an intense spot having the mobility of palmitic acid and a less intense spot with the R_f value of hexadecylglycerol. The lipid from glycoinositolphospholipid B was hydrolysed to compo-

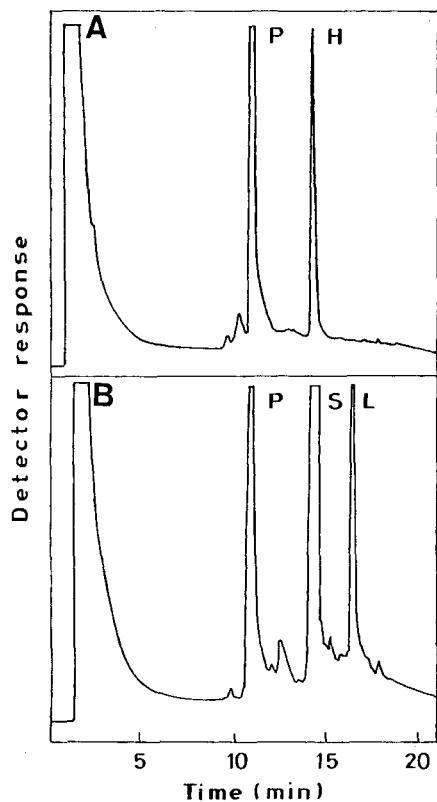


Fig. 4. GLC-MS of the Me_3Si derivatives of the lipid components of the glycoinositolphospholipids. (A) Glycoinositolphospholipid A was hydrolysed with 0.25 M KOH in methanol for 18 h at 25°C. After acidification, the lipids were extracted with ether and analysed as trimethylsilyl derivatives using a Varian Mat CH-7A mass spectrometer at 70 eV. A column of 3% OV-17 was used. (B) Glycoinositolphospholipid B was hydrolysed with 1 M NaOH for 24 h at 100°C and analysed as described for glycoinositolphospholipid A. The compounds are assigned as described in the legend of Fig. 3.

nents with the mobilities of lignoceric acid, palmitic acid and sphinganine (Fig. 3B).

The lipids could also be released by treatment of the non labelled glycoinositolphospholipids with 0.25 M KOH in methanol for 16 h at 28°C, as previously reported for lipopeptidophosphoglycan [10] and were analysed as trimethylsilyl derivatives by GLC/MS (Fig. 4). The derivatives of palmitic acid and 1-*O*-hexadecylglycerol were identified by a comparison of retention times and characteristic mass spectra with authentic standards (Table 2). The base peak at m/z 205 is characteristic of 1-monoalkylglycerols [19] and is clearly different from the base peaks for 2-alkylglycerols (m/z 218) and sphinganines (m/z 132). The diagnostic ions M-15, M-90 and M-103 are consistent with a $\text{C}_{16:0}$ alkyl chain.

The first major peak in the GLC/MS analysis corresponds to the trimethylsilyl ester of palmitic acid and, accordingly, the molecular ion appeared at m/z 328; an intense M-15 ion (64%) was also observed.

Attempts to analyse the lipid released from glycoinositolphospholipid A by PtdIns-PLC without previous deacylation were not successful, confirming the absence of lysoalkylglycerol which yields the volatile 1-*O*-alkyl-2,3-di-*O*-trimethylsilyl derivative on silylation. Similarly the 1-*O*-alkyl-2-*O*-acyl-3-*O*-trimethylsilyl glycerol derived from the anchor of the *Leishmania major* surface protease, could not be detected by GLC [20].

The lipids in glycoinositolphospholipid B were analysed after strong alkaline hydrolysis. Two main fatty acids with m/z 328 (M^+) and m/z 440 (M^+), corresponding to palmitic acid and lignoceric acid, respectively, were detected (Fig. 4B). In the same analysis, the presence of sphinganine was evident by the base peak at m/z 132 and the diagnostic fragments at M-15, M+1- $\text{CH}_2\text{OSiMe}_3$ and M-132. The lipids from glycoinositolphospholipid B correspond to the major ceramides found in lipopeptidophosphoglycan previously isolated from a 4-day culture of *T. cruzi* [10].

Glycan structures

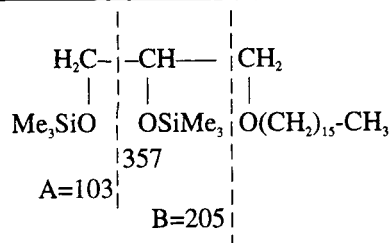
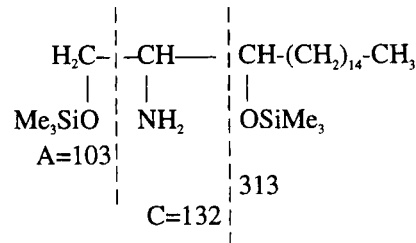
The analysis of neutral sugars as alditol acetates by GLC established that the mannose/galactose ratio was 2.7:1 for both glycoinositolphospholipids. After hydrolysis of the glycan with 2 M trifluoroacetic acid, followed by passage of the sample through Dowex 50 W (H^+), the acid eluate was further hydrolysed with 4 M HCl for 15 h at 100°C to cleave the stronger GlcN-inositol linkage. Inositol, but not glucosamine, could be detected by GLC. Glucosamine could only be analysed when the glycoinositolphospholipids were previously dephosphorylated with HF suggesting that AEP was linked to GlcN, as in lipopeptidophosphoglycan [7].

To establish whether the galactose in the glycoinositolphospholipids was present in the furanose configuration as in lipopeptidophosphoglycan, the acid lability of the galactosidic linkages was investigated. Galactose was the only monosaccharide released by 20 mM trifluoroacetic acid as shown by GLC analysis of the alditol acetate. The oligosaccharides from both glycoinositolphospholipids were prepared and analysed according to Fig. 5. Treatment with aqueous HF for 60 h at 0°C cleaved all the phosphate bonds, leaving the sugar in the aqueous phase after partition with ether. Additionally, the galactofuranose was selectively cleaved as previously reported [7]. Under similar conditions Mayor et al. found pyranosidic galactose and mannose linkages to be stable [21]. Further treatment with nitrous acid, followed by NaB^3H_4 reduction yielded anhydromannitol-labeled oligosaccharides. TLC and fluorography (Fig. 6), using conditions previously reported [22] showed a main component (I) with the mobility of Man_4 anhydromannitol* (Man_4 [1- ^3H] 2,5-anhydromannitol), $R_f = 0.20$, and minor components (II and III) with $R_f = 0.30$ and 0.39 migrating as Man_3 anhydromannitol*, and a standard of galactitol, respectively. Accordingly, the fastest moving compound was not affected when eluted from the plate and treated with jack bean α -mannosidase. The oligosaccharides used for comparison were obtained from lipopeptidophosphoglycan, and were separated from free galactose as reported [22]. Digestion with *A. phoenicis* α -mannosidase was performed for identifying $\text{Man}(\alpha 1-2)$ - Man linkages. When this treatment was applied to lipopeptidophosphoglycan a single spot corresponding to $\text{Man}(\alpha 1-6)$ - $\text{Man}(\alpha 1-4)$ anhydromannitol* was obtained [22]. The glycans from glycoinositolphospholipids A and B were only partly hydrolysed by the *A. phoenicis* α -mannosidase as is shown by the decrease in intensity of components I and II with concomitant intensification of the spot of higher mobility. In this case the Man_2 anhydromannitol* was not well resolved from galactitol. Treatment with jack bean α -mannosidase hydrolysed most of the oligosaccharides to anhydromannitol, although a minor amount was resistant to the enzyme (Fig. 6).

Methylation analysis was performed on the oligosaccharides obtained from the glycoinositolphospholipids by cleav-

Table 2. Diagnostic fragments for structural determination of 1-*O*-hexadecylglycerol in glycoinositolphospholipid A (peak H, Fig. 4A) and sphinganine in glycoinositolphospholipid B (peak S, Fig. 4B).

Peak H			Peak S		
fragment	<i>m/z</i>	relative intensity	fragment	<i>m/z</i>	relative intensity
M-Me	445	3.8	M+1-Me	431	0.6
M-Me ₃ SiOH	370	3.2	M-Me	430	0.4
M-A	357	2.4	M-A	342	6.0
M-147	313	5.7	M-C	313	2.4
B	205	100.0	B	205	14.9
Me ₃ SiOSiMe ₂	147	31.5	Me ₃ SiOSiMe ₂	147	7.4
Me ₃ SiOCH ₂ -CHOH	133	35.2	C	132	100.0
Me ₃ SiOCH-CHO	131	17.5	HNHC-CH ₂ OSiMe ₃	131	8.1
Me ₂ SiOCH ₂ -CHO	117	31.8	HNHC-CH ₂ OSiMe ₂	116	14.0
CH ₂ OSiMe ₃	103	17.5	—	—	—

1-*O*-Hexadecylglycerol (Me₃Si derivative)Sphinganine (Me₃Si derivative)**Table 3. Methylation analysis of the oligosaccharide fractions obtained from glycoinositolphospholipid A and glycoinositolphospholipid B.** Fraction I was obtained by treatment of the glycoinositolphospholipids with PtdIns-PLC followed by deamination with HNO₂/NaBH₄. Fraction II was prepared by dephosphorylation of the glycoinositolphospholipids with HF. The glycan was recovered in the acid eluate of a Dowex 50 column and from the aqueous eluate of an AG3-X4 column. GIPL, glycoinositolphospholipid.

<i>O</i> -Methyl sugar	Structural feature	GIPL A		GIPL B	
		I	II	I	II
2,3,4,6-Man	terminal Man	0.65	0.80	0.53	0.70
2,3,5,6-Gal	terminal Gal <i>f</i>	1.75	—	1.36	—
2,3,4,6-Gal	terminal Gal	0.32	0.19	0.23	0.18
3,4,6-Man	2- <i>O</i> -substituted Man	2.02	1.52	1.70	1.44
2,4,6-Man	3- <i>O</i> -substituted Man	0.90	0.17	0.74	0.23
2,3,4-Man	6- <i>O</i> -substituted Man	1.00	1.00	1.00	1.00
4,6-Man	2,3-di- <i>O</i> -substituted Man	0.62	—	0.60	—
1,2,3,4,5-Ino	6- <i>O</i> -substituted Ino	—	0.47	—	0.54
2,3,6-GlcNAc	4- <i>O</i> -substituted GlcN	—	0.31	—	0.45

age of the lipid with PtdIns-PLC, followed by nitrous acid deamination and reduction with NaBH₄. The data in Table 3 indicate the presence of a branched oligosaccharide with terminal non-reducing Gal_f residues and, in a lesser proportion, terminal Man and terminal Gal_p residues. The low amount of the latter indicates a small proportion of molecules being substituted by Gal_p. The presence of 4,6-di-*O*-methylmannose is due to 3-*O* substitution of the 2-*O* Man units by Gal_f as is observed in lipopeptidophosphoglycan [7]. The fact that the methylation analysis of samples of the glycoinositolphospholipids after HF treatment gave no dimethylmannose indicates that only Gal_f is branching from the glycan core. The small peak of 2,4,6-Man would agree with a Gal_p(1-3)Man linkage.

The large ratio of 2-*O*-substituted mannose/6-*O*-substituted mannose, could mean that the latter is in part cryptic

to the methylation linkage analysis, perhaps by phosphate substitution, mainly in glycoinositolphospholipid A. As expected, when the HF treated samples were analysed, the ratio of 2-*O*-Man/6-*O*-Man was substantially reduced. Methylation analysis by MS allowed the identification of 4-*O*-substituted GlcN and mono-substituted inositol in the HF treated samples. The low proportions obtained for these components was also observed in the methylation analysis of other glycosylPtdIns glycans [20].

DISCUSSION

The results presented in this study demonstrate that two different kinds of lipids are linked through phosphate bridges

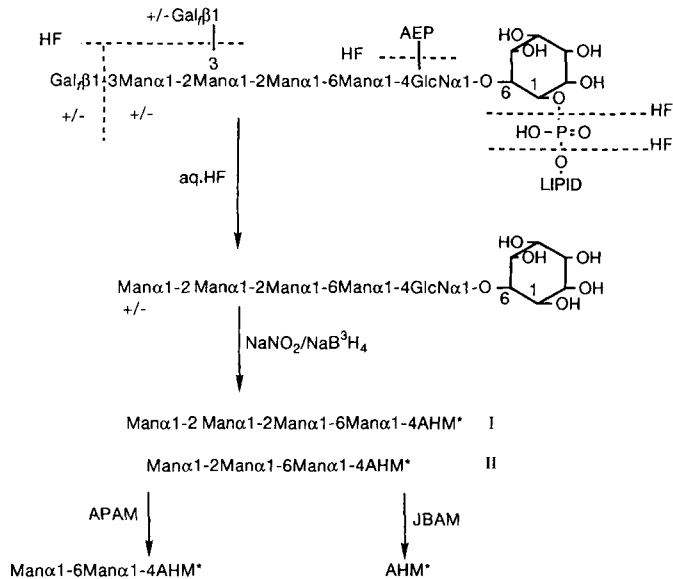


Fig. 5. Reactions used for the analysis of the oligosaccharides from both glycoinositolphospholipids. The structure corresponds to the main oligosaccharides in glycoinositolphospholipid A and glycoinositolphospholipid B. Compounds I and II were obtained from lipopeptidophosphoglycan and used as standards. APAM, *A. phoenicis* α -mannosidase; JBAM, jack bean α -mannosidase.

to similar inositolglycans in glycoinositolphospholipids isolated from *T. cruzi*. A glycosylPtdIns anchor-like structure was confirmed for both lipids and their similarity with the previously characterized lipopeptidophosphoglycan [7] has been shown. Both glycoinositolphospholipids were efficiently labeled with [3 H]palmitic acid and practically all the label was released with the specific PtdIns-PLC from *B. thuringiensis*.

Glycoinositolphospholipid A belongs to the class of glycerol lipids found in glycoprotein anchors [20, 23] and in the lipophosphoglycan of *Leishmania sp.* [24]. However, the alkyl chain usually has a higher number of carbon atoms compared to glycoinositolphospholipid A. The hexadecylglycerol, 2-*O*-palmitoyl structure was determined by GLC/MS. The same alkylglycerol, probably acylated, has been found in the glycosylPtdIns anchor of the 1G7 antigen from *T. cruzi* metacyclic forms, although esterification could not be demonstrated since the antigen studied had been previously deacylated [25]. An alkylglycerol was identified in the anchor of the TC-85 glycoprotein from *T. cruzi* trypomastigotes [26]. Both anchors and glycoinositolphospholipid A may have a common glycosylPtdIns precursor.

A sphinganine-containing ceramide was released by PtdIns-PLC from glycoinositolphospholipid B. In *Saccharomyces cerevisiae*, glycoproteins are anchored either by a glycerolipid or a C₁₈-phytosphingosine-containing ceramide [27]. A suggestion has been made that the base sensitive lipid moiety is exchanged by ceramide during glycoprotein maturation. Lipid remodeling of glycosylPtdIns anchors has been described in *Trypanosoma brucei* [28]. In our case, we have concomitantly found alkylacylglycerol or ceramide linked to the same glycan structure in the glycoinositolphospholipids extracted from epimastigotes collected at the logarithmic phase of growth (day 2). However, epimastigotes do not grow synchronously and, thus, part of the culture could

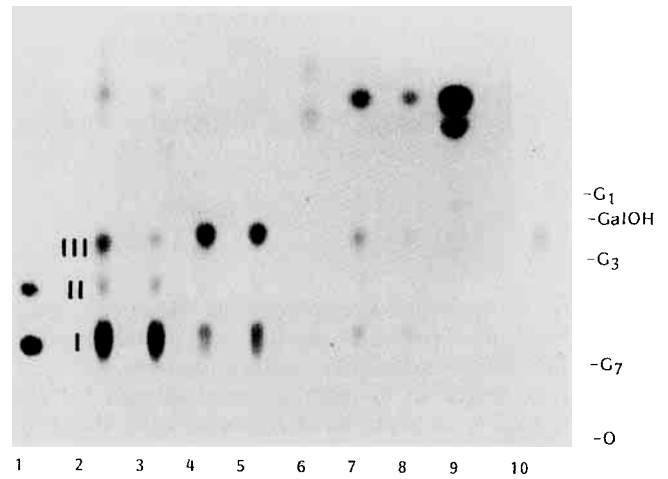


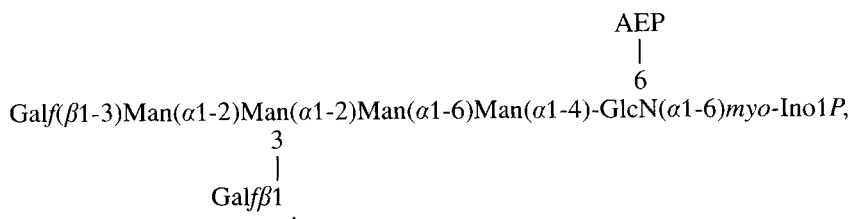
Fig. 6. TLC on silica-gel 60 of the oligosaccharides obtained from glycoinositolphospholipid A and glycoinositolphospholipid B of *T. cruzi* by the chemical modifications summarized in Fig. 5 followed by digestions with α -mannosidases. Lane 1, oligosaccharides obtained from lipopeptidophosphoglycan as shown in Fig. 5 and further purified on Bio-Gel P-2; lane 2, oligosaccharides obtained from glycoinositolphospholipid A; lane 3, oligosaccharides obtained from glycoinositolphospholipid B; lane 4, sample 2, after digestion with *A. phoenicis* α -mannosidase (APAM); lane 5, sample 3, after digestion with APAM; lane 6, sample 1, after digestion with jack bean α -mannosidase (JBAM); lane 7, sample 2, after treatment with JBAM; lane 8, sample 3, after treatment with JBAM; lane 9, [3 H]-labelled 2,5-anhydromannitol obtained from glucosamine; lane 10, compound III from lane 2 eluted from the plate and treated with JBAM. The solvent was 1-propanol/ethanol/water (7:1:2, by vol.) and detection was by fluorography. Standards: GalOH, galactitol; G₁, glucose; G₃, maltotriose; G₇, maltoheptaose.

have been already committed to differentiation to the metacyclic stage. Only ceramide could be detected in lipopeptidophosphoglycan [10], essentially isolated by the same purification method from *T. cruzi* epimastigotes at the stationary phase of growth (day 4).

To our knowledge a ceramide has not yet been reported to be a constituent of glycoprotein anchors in *T. cruzi*, particularly in epimastigote or axenic culture-derived metacyclic forms. Our results suggest that either lipid remodeling can take place in *T. cruzi* glycoinositolphospholipids or, alternatively, glycosyltransferases could indiscriminately transfer monosaccharides irrespective of the lipid moiety nature.

The fact that an alkylglycerol-containing glycoinositolphospholipid A, with an otherwise analogous lipopeptidophosphoglycan structure, has not been found in lipopeptidophosphoglycan preparations may be explained by assuming that during development from the logarithmic phase to the stationary phase of growth glycoinositolphospholipid A is modified to give a macromolecule with a larger glycan structure which is no longer soluble in chloroform/methanol/water (10:10:3, by vol.). In fact, from the insoluble fraction we have previously extracted with water/ethanol/ether/pyridine/ammonia a glycoinositolphospholipid with a higher content of neutral sugars [10]. The alkaline solvent has been previously used for the extraction of lipophosphoglycan from *Leishmania sp.* [30].

The glycan moiety of the glycoinositolphospholipids described in this study shows microheterogeneity. The main structure would correspond to:



as is found in lipopeptidophosphoglycan. However, minor structures with the Man_3GlcN -inositol core are also part of both glycoinositolphospholipids. Galactofuranose and AEP, previously identified in lipopeptidophosphoglycan [7, 29], have been found in the glycoinositolphospholipids. However, in contrast to lipopeptidophosphoglycan, 20% of the galactose is in the pyranose configuration. Developmental changes in the glycan structures of *Leishmania donovani* glycoinositolphospholipids have been reported [6]. The detection of Galp in *T. cruzi* glycoinositolphospholipids could account for an early finding of terminal galactopyranosyl residues in a lipopeptidophosphoglycan fraction [31].

The exchange of terminal Galp for Galf residues will render a glycoconjugate insensitive to the action of exoglycosidases which require a substrate with the pyranoside configuration.

This investigation received financial support from the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, Consejo Nacional de Investigaciones Científicas y Técnicas and Universidad de Buenos Aires to R. M. de Lederkremer; Fundación Antorchas to R. M. de Lederkremer and W. Colli, Fundação de Amparo a Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Científico e Tecnológico to W. Colli. We are grateful to Dr Martin G. Low for the gift of PtdIns-PLC from *B. thuringiensis*.

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