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# Structural features of the lipopeptidophosphoglycan from *Trypanosoma cruzi* common with the glycophosphatidylinositol anchors

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The lipopeptidophosphoglycan (LPPG) from *Trypanosoma cruzi*, a major constituent of the plasma membrane of epimastigote forms, has been now extracted with butanol/water from delipidated cells and purified by hydrophobic chromatography. We have found that the LPPG undergoes two reactions, characteristic of the glycosylphosphatidylinositol anchors: (a) cleavage of the ceramide by phosphatidylinositol-specific phospholipase C (PtdIns-specific phospholipase C) from *Bacillus thuringiensis*, (b) nitrous acid deamination of the non-N-acylated glucosamine. Palmitoylsphinganine, palmitoylsphingosine, lignoceroylsphinganine and, as minor components, the stearoylceramides were identified by gas liquid chromatography/mass spectrometry.

The presence of cross reacting determinant (CRD) epitopes in the glycophosphoinositol released by PtdIns-specific phospholipase C was investigated by direct and inhibition ELISA. A sample of glycophosphoinositol containing 5 μg carbohydrate caused 60% inhibition of the binding of anti-CRD antibodies raised against the soluble form of variant surface glycoprotein.

The lipopeptidophosphoglycan (LPPG) from *Trypanosoma cruzi* is a major constituent of the plasma membrane of epimastigote forms [1, 2]. A partial structure of glycophosphoinositol-ceramide was reported for the LPPG [3]. Further studies revealed that LPPG has a very complex structure. Amino acids (3–5%), and in particular aminophosphonic acids, were detected in acid hydrolysates [4, 5]. Structural studies on the oligosaccharide moiety showed the presence of β-D-galactofuranose [6] as terminal units attached to position 3 of (1→2)-linked mannoses [7,8].

In recent years, LPG containing inositol have received a great deal of attention. They have been shown to act as anchors of membrane proteins of eukaryotic cells [9] and of a phosphosaccharide in *Leishmania species* [10, 11]. The structure of the glycosylphosphatidylinositol which anchors the variant cell surface glycoprotein (VSG) of *Trypanosoma brucei* was elucidated [12]). A lyso-1-O-alkylphosphatidylinositol was identified in the LPG of Leishmania [10, 13]. Both glycerolipids could be released by bacterial PtdIns-specific phospholipase C [12, 13].

Although glycosyl-PtdIns-anchored proteins have been detected in metacyclic trypomastigotes of *T.cruzi* by treatment with PtdIns-specific phospholipase C [14], the structure of the anchor was not elucidated. A recent publication described a ceramide glycan anchor for a glycoprotein of *Dictyostelium discoideum* [15], although the lipid was not cleaved by PtdIns-specific phospholipase C.

In this paper we report three characteristic features of the LPPG of *T.cruzi* common to the glycosyl-PtdIns anchors. First, the ceramide could be released by bacterial PtdIns-specific phospholipase C. Second, we found cross-reacting determinant (CRD) activity in the phosphoinositolglycan liberated by PtdIns-specific phospholipase C. Finally, we demonstrated the presence of non-*N*-acylated glucosamine.

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*Abbreviations.* LPPG, lipopeptidophosphoglycan; VSG, variant surface glycoprotein of *Typanosoma brucei*; sVSG, soluble form of VSG; LPG, lipophosphoglycans; CRD, cross-reacting determinant; BSA, bonne serum albumin.

## MATERIALS AND METHODS

Sugars, inositol, lipids, Tes and phosphatase substrate tablets of *p*-nitrophenyl phosphate, disodium salt (Sigma 104), were purchased from Sigma Chemical Company. Octyl-Sepharose CL-4B was obtained from Pharmacia. Bio-Gel P-2 was purchased from Bio-Rad Laboratories. All the solvents were distilled. Affinity-purified swine anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase was from Dakopatts a/s (Denmark).

Phosphatidylinositol-specific phospholipase C from *B. thuringiensis* was a kind gift from Dr. Martin G. Low (Columbia University). Soluble form of VSG (sVSG variant MITat 1.4) and anti-CRD antiserum were generous gifts from Dr. Michael A.J. Ferguson (Dundee University).

### *Parasites*

Epimastigote forms of *T. cruzi* (Y strain) cultivated in LIT medium [16] were provided by the laboratory of Dr. Walter Colli (São Paulo University, Brazil).

### *Analytical methods*

Slab gel electrophoresis was performed on 10–15% polyacrylamide gradient slab gels in the presence of 0.1% sodium dodecylsulphate. Gels were stained with periodic acid/Schiff [17]. Paper chromatography was conducted by the descending method on Whatman lo.1 paper using 1-butanol/pyridine/water (6:4:3). Radioactivity was determined on paper chromatograms by cutting longitudinal strips into 1-cm horizontal sections which were measured for radioactivities in 3 ml toluene scintillation solution, in a Beckman spectrometer.

TLC was performed on 0.25-mm silica gel 60 coated plates (Merck) with the following solvent systems: (a) chloroform/methanol (38:3) and (b) chloroform/methanol/2.5 M $NH_4OH$ (40:10:1). Detection was effected by immersion in 5% sulfuric acid and subsequent heating, or with ninhydrin spray [18].

Capillary GLC was performed with a Hewlett-Packard 5890 gas chromatograph with nitrogen as the carrier gas. The following conditions were used: (a) SP 2330 fused silica column (0.25 mm x 15 m), flow rate 4 ml/min; $t_i$, 220°C; $t_d$, 220°C; $t_c$, 160°C; (b) SP 2340 fused silica column (0.25 mm × 15 m), flow rate, 4 ml/min; $t_i$, 220°C; $t_d$, 220°C; $t_c$, 180°C; (c) SP 2250 (0.20 mm × 12.5 m), flow rate 3.8 ml/min; $t_i$, 220°C; $t_d$, 220°C; $t_c$, 190°C. Sugars were analysed as alditol acetates [19].

The mass spectra were recorded with a Varian Mat CH-7A mass spectrometer at 70 eV. A glass column (2 mm × 1.2 m) packed with 1% SE-30 on Chromosorb WAW (80–100 μm mesh) was used, column temperature 100–320°C with a gradient of 20°C/min, helium 38 ml/min. A Varian 1400 gas chromatograph was used.

High-performance liquid chromatography was performed on a Micromeritics liquid chromatograph equipped with a 771 refractive index detector and a Rheodyne injection valve. A column (200 mm, Hewlett-Packard) of silica 100 (particle size, 10 μm) and hexane/2-propanol (93:7) were used for analysis of the lipid. Carbohydrate was measured by the phenol/sulfuric acid method [20] with a mixture of mannose and galactose (3:2) as standard. Amino acids were analysed after hydrolysis with 6M HCl at 105°C for 24 h, on a Beckman 119 CL amino acid analyser. Phosphorus was analysed by Bartlett's method [21].

*Isolation of LPPG*

Lyophilized cells ($2 \times 10^{12}$) were sequentially extracted with 17 vol. chloroform/methanol (2:1, by vol.) and twice with the same volume of chloroform/methanol (1:1, by vol.). The delipidated residue (14.8 g) was extracted with water saturated with 1-butanol (50 mg/ml) overnight at 4°C with stirring and the extract was separated after centrifugation at 10000 × *g* for 20 min. Butanol was removed by evaporation under diminished pressure at room temperature, and the aqueous solution was lyophilized to a volume of 30 ml. After standing overnight at 5°C a precipitate was removed by centrifugation. The water solution was lyophilized to half of the initial volume and chloroform/methanol (1:1, by vol.) was added to obtain a final proportion of chloroform/methanol/water (10:10:3, by vol.). After shaking overnight at room temperature, the supernatant was separated from insoluble material and the LPPG (fraction A) was precipitated by the addition of an equal volume of methanol and storing at −20°C for 18 h. The precipitate was collected by centrifugation and the LPPG was further purified by chromatography on octyl-Sepharose CL-4B. The column (1.2 cm × 34 cm) was equilibrated with 0.1M buffer Tes, pH 7.0, containing 5% (by vol) 1-propanol. The sample (70 mg) was dissolved in the same buffer and eluted with a linear gradient of 1-propanol (5–70%). Fractions were analysed for carbohydrate and protein ($A_{280}$); they were appropriately pooled, propanol removed under diminished pressure and lyophilized. The LPPG (fractions 35–46) was dissolved in 1 ml water and precipitated with 4 vol. of ethanol. After standing for 1 h at 5°C the material was centrifuged and washed twice with cold ethanol/water (4:1).

*Acid hydrolysis*

Component sugars were determined after acid hydrolysis of the sample with 2M trifluoroacetic acid (1–2 mg/ml) at 105°C for 3 h. 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 50 W. The acid eluate (1M HCl) was further hydrolysed with 4M HCl at 105°C for 15 h and hexosamines were analysed in this fraction.

*Treatment of LPPG with PtdIns-specific phospholipase C*

A sample of LPPG (7 mg) was dissolved in 1 ml 50 mM buffer Tris/HCl, pH 7.2, containing 0.1% deoxycholate and incubated with 4 units phospholipase C from *B. thuringiensis* for 3 h at 37°C. The lipid was extracted with ether (3 × 2 ml) and analysed by HPLC and TLC.

*Alkaline hydrolysis*

LPPG (5 mg) was treated with 0.25 M KOH in methanol (2 ml) for 16 h at 28°C. The methanol was evaporated, the residue was suspended in water, neutralized with 1 M HCl and extracted with ether. Strong alkaline hydrolysis for release of the long-chain bases was performed by treatment of the ceramides, separated by HPLC, with 1M NaOH (1 ml) for 36 h at 105°C in a Teflon flask. The mixture was extracted with ether and analysed.

*Nitrous acid cleavage of the carbohydrate of LPPG*

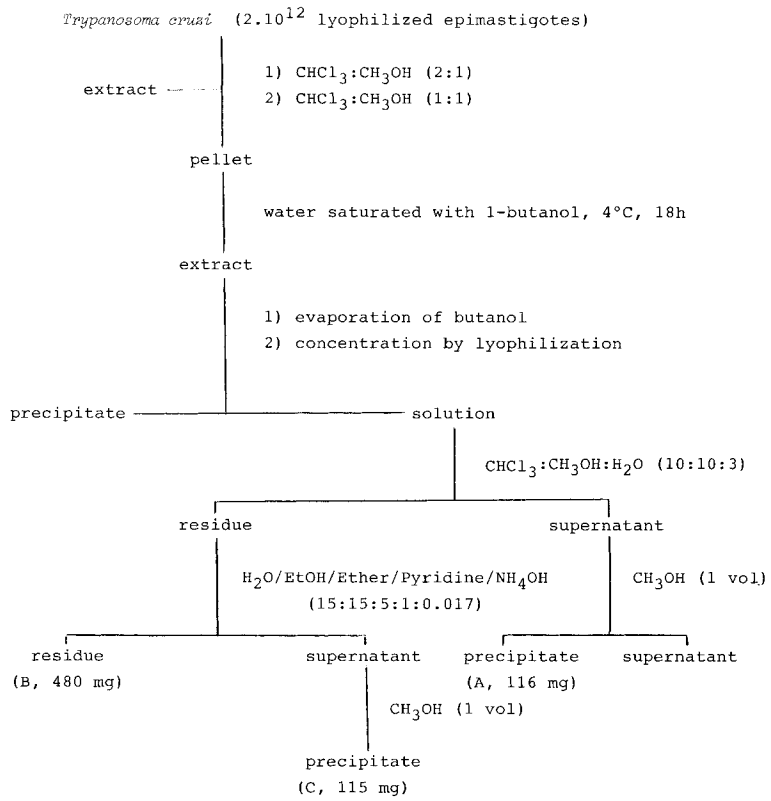
The aqueous phase from the PtdIns-specific phospholipase C treatment (80 μg sugar) in 50 mM sodium acetate, pH 3.5 (total volume 0.4 ml) was deaminated by the addition of $NaNO_2$ (1 mg) for 3 h at 0°C. The reaction mixture was neutralized with 1 M sodium carbonate and reduced with $NaB^3H_4$ (250 μCi, 347 mCi/mmol) for 2 h. Sodium borohydride (1 mg) was then added and after 1 h the sample was hydrolysed with 2M trifluoroacetic acid. The acid was evaporated, the sample was desalted by passage through a small column of Amberlite MB-3 and analysed by paper chromatography.

*Mild periodate oxidation*

Periodate oxidation was performed on the carbohydrate cleaved by alkaline methanolysis of LPPG and purified on Bio-Gel P-2. A sample (1 mg sugar) was treated with 0.02M sodium metaperiodate (1 ml) for 30 min in the dark at room temperature. The reaction was stopped with ethylene glycol (0.1 ml) and after evaporation the residue was dissolved in water and reduced with NaBH4 (1 mg) for 1 h. Excess reagent was decomposed with acetic acid, neutralized with barium hydroxide and the precipitate was separated by centrifugation. The sample was hydrolysed with 1M trifluoroacetic acid for 1 h at 105°C and the monosaccharides analysed as alditol acetates using conditions (a).

*Enzyme-linked immunosorbant assay (ELISA) for CRD detection*

*Direct assay.* The CRD was detected by the binding of anti-sVSG antibody MITat 1.2 to microtitre plate wells, coated with glycophosphoinositol obtained by treatment of




Scheme 1. *Extraction of glycophospholipids from* T. cruzi *epimastigotes*

LPPG with PtdIns-specific phospholipase C from *B. thuringiensis*. Each well was coated with 2 μg (in sugar) glycophosphoinositol dissolved in 100 μl 0.05M $Na_2CO_3$ buffer, pH 9.6, and incubated for 3 h at 37°C. Bovin serum albumin (BSA) controls were used. All wells were washed four times with phosphate-buffered saline containing Tween 20 [NaCl/$P_i$/Tween 20, 15 mM sodium phosphate, 0.15 M sodium chloride, pH 7.2, 0.05% (by vol) Tween 20] and were incubated with 100 μl NaCl/$P_i$/Tween 20 containing 1% BSA, for 30 min at 37°C to block remaining adsorption sites. The plates were washed as before and allowed to incubate with a series of doubling dilutions of anti-(sVSG MITat 1.2) antibody in NaCl/$P_i$/Tween 20 for 1 h at 37°C. Bound antibodies were detected by incubation with anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase (1:500 dilution in NaCl/$P_i$/Tween 20) for 1 h at 37°C. The wells were washed again and the assay developed with sodium *p*-nitrophenyl phosphate (1 mg/ml in 1% diethanolamine, 0.01% $MgCl_2 \cdot 6H_2O$ pH 9.8) for 45 min at room temperature. The reaction was terminated by the addition of 3M NaOH and absorbance measured at 410 nm with a Microelisa Minireader 590 (Dynatech).

*Inhibition assay.* The CRD was also detected by measuring the ability of glycophosphoinositol (*T. cruzi*) to inhibit the binding of anti-MITat 1.2 antiserum to microtitre plate wells coated with sVSG MITat 1.4. Each well was coated with 2 μg sVSG in 100 μl 0.05M $Na_2CO_3$ buffer pH 9.6 and was incubated for 3 h at 37°C. Wells were washed with NaCl/$P_i$/Tween 20 and allowed to incubate with NaCl/$P_i$/Tween 20 containing 1% BSA to block remaining adsorption sites. The anti-(sVSG MITat 1.2) antibodies (1:10 dilution in NaCl/$P_i$/Tween 20) was incubated with 5, 20 and 50 μg glycophosphoinositol (*T. cruzi*) for 90 min at room temperature. The incubated samples were transferred to coated microtitre plate wells and incubations continued in the wells for a further 1 h at 37°C. Bounded antibodies were detected as in the direct assay.

## RESULTS

### *Isolation of LPPG*

The *T. cruzi* LPPG was isolated from delipidated epimastigote cells with water saturated with 1-butanol and the extract was fractionated as shown in Scheme 1. Three main fractions (A, B and C) were obtained. Analysis by SDS/PAGE (Fig.1) showed that all three fractions contained a glycoconjugate with a mobility similar to the LPPG previously isolated by phenol/water extraction and purified by its solubilization in chloroform/methanol/water (10:10:3) [22, 23]. Moreover, the pattern shown by fraction B was similar to the glycoconjugate complex isolated with phenol/water. All the fractions contained about 50 % of carbohydrate (phenol/sulfuric acid assay). When analysed, after acid hydrolysis, by GLC using conditions (a) they showed mannose as main sugar, and also galactose and a trace of inositol (Table 1). The acid eluates from the separation of neutral and basic components gave, by further acid hydrolysis, inositol and glucosamine (GLC, conditions b), which suggested that this two constituents were originally linked as in the known anchor structures [9].

Fraction A was further purified by hydrophobic interaction chromatography on octyl-Sepharose (Fig. 2A). The first peak (peak I, Fig. 2A) is a minor glycoprotein contaminant which gave by acid hydrolysis glucose and mannose in the molar ratio 3:2 and a trace of galactose (GLC conditions a). It is noteworthy that the large proportion of glucose is not a

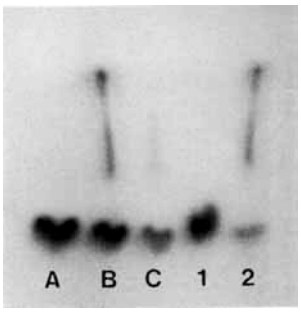



Fig. 1. *Polyacrylamide gel electrophoresis of glycolipid fractions obtained from a water saturated with 1-butanol extract of delipidated* T. cruzi *epimastigote cells.* Fractions obtained according to Scheme 1 were analysed by electrophoresis in polyacrylamide (8–15%), with 0.1% SDS. Gels were stained with periodic acid/Schiff. (1) LPPG, isolated with the phenol/water procedure and purified by chloroform/methanol/water (10:10:3) extraction; (2) glycoconjugates extracted with phenol/water

Table 1. *Composition of glycophospholipid fractions obtained from* T. cruzi *epimastigotes as in Scheme 1*

| Sample | Neutral sugars | Peak area of | | | GlcN | Inositol | Phosphate |
|---|---|---|---|---|---|---|---|
| | | Man | Gal | Glc | | | |
| | % | % | | | | | % |
| A | 59 | 76.2 | 23.1 | 0.7 | + | + | 4 |
| B | 57 | 54.9 | 45.1 | – | + | + | + |
| C | 63 | 66.4 | 32.1 | 1.5 | + | + | + |

Neutral sugars were estimated by phenol-sulfuric acid [20].
GlcN was determined by GLC analysis as alditol acetates.
Phosphate was measured by Bartlett's method [21].
Where + and – are used, the compounds were detected qualitatively but not determined.

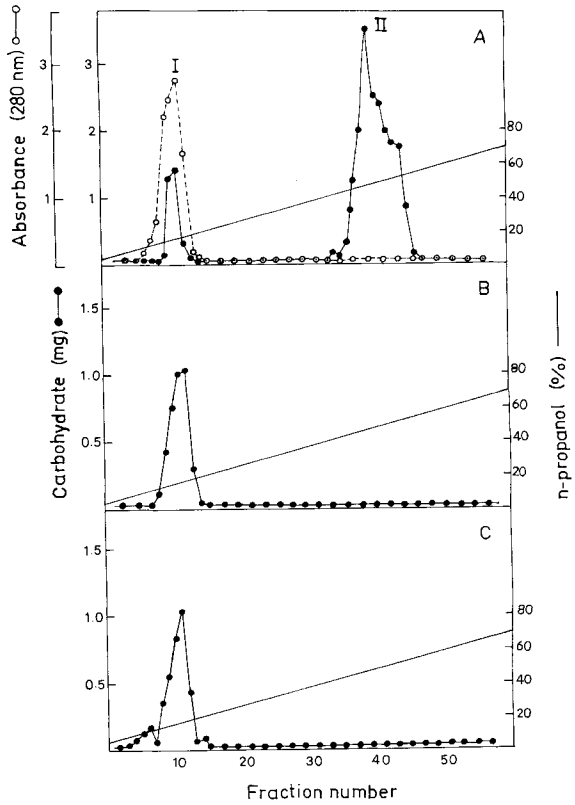



Fig. 2. *Purification by octyl-Sepharose CL-4B chromatography of LPPG and carbohydrate fractions obtained from LPPG.* (A) Purification of LPPG (fraction A, Scheme 1). The column was equilibrated with 0.1 M buffer Tes, pH 7.0 containing 5% (by vol.) 1-propanol. The sample (70 mg) was dissolved in the same buffer and eluted with a linear gradient of 1-propanol (5–70%). 3 ml volumes were collected and aliquots were analysed for carbohydrate. (B) Reaction of peak II from (A) with PtdIns-specific phospholipase C from *B. thuringiensis.* A sample (7 mg) was incubated with the enzyme in Tris/HCl, pH 7.2, containing 0.1% deoxycholate, for 3 h at 37 °C. The lipid was extracted with ether and the aqueous phase chromatographed as in (A). (C) Alkaline hydrolysis of peak II, (A). A sample (5 mg) was treated with 0.25M KOH in $CH_3OH$ (2 ml) for 16 h at 28 °C and extracted with ether. The aqueous phase was chromatographed as above

common constituent of glycopeptides. This glycoconjugate was not further investigated.

The major fraction (peak II, Fig. 2A) was eluted at 42–54% 1-propanol and it migrated in SDS/PAGE as the LPPG previously described. Peak I could not be detected by the periodic acid/Schiff reagent when a similar amount of carbohydrate was analysed. This explains why the original material (Fig.1) appeared homogeneous on SDS/PAGE. The LPPG now isolated contained 45–50% neutral sugars and 2.5–3% phosphorus which corresponds to 7–8 mol neutral sugar/2 phosphates

*Release of the lipid from LPPG by PtdIns-specific phospholipase C or mild alkaline treatment*

Incubation of LPPG with PtdIns-specific phospholipase C from *B. thuringiensis* cleaved the lipid and the sample did not further interact with the octyl-Sepharose (Fig. 2B). The lipid was also released by treatment of LPPG with alkali in methanol (Fig. 2C). In both cases the reaction mixture was extracted with ether and analysed by TLC with solvent a (Fig. 3). Both extracts showed spots with a mobility similar to a commercial sample of *N*-lignoceroylsphinganine. A standard of 1-*O*-hexadecylglycerol was also included because we have identified this alkylglycerol by GLC/MS (unpublished results) in a hydrolysate of fraction C (Scheme 1). Release of the ceramide under mild alkaline conditions is facilitated by the formation of a cyclic phosphate, which implies that it should be a free *cis*-oriented hydroxyl group with respect to the phosphate (Fig. 4). This would be the case if the phosphoceramide is linked to C1 of inositol, as was demonstrated for the *T. brucei* anchor [12]. Inositol phosphoceramide from *Neurospora crassa* showed similar alkaline lability [24].

HPLC of the ether extract from the PtdIns-specific phospholipase C incubation gave one main peak which cochromatographed with *N*-lignoceroylsphinganine and a small peak which was eluted as a shoulder (Fig. 5). The two peaks were collected from the HPLC and separately hydrolysed with 1M NaOH. The hydrolysed lipids remained at the starting point when analysed by TLC in solvent a. An alkylglycerol would be stable to the hydrolysis conditions. With the more polar solvent b both samples gave a major spot with the same $R_f$ as sphinganine and a faint spot with the $R_f$ of sphingosine; both were detected with ninhydrin (not shown).

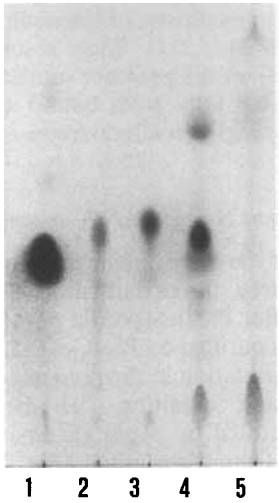



Fig. 3. *TLC of products, following different treatments of LPPG.* (3) Ether extract after treatment of LPPG with 0.25M KOH in $CH_3OH$; (4) ether exract after cleavage of LPPG with PtdIns-specific phospholipase C; (5) ether extract of a control of LPPG in the same buffer used for treatment with the enzyme; (1, 2) authentic 1-*O*-hexadecylglycerol and *N*-lignoceroylsphinganine standards. Solvent, chloroform/methanol (38:3). Detected by immersion in 5% sulfuric acid and subsequent heating

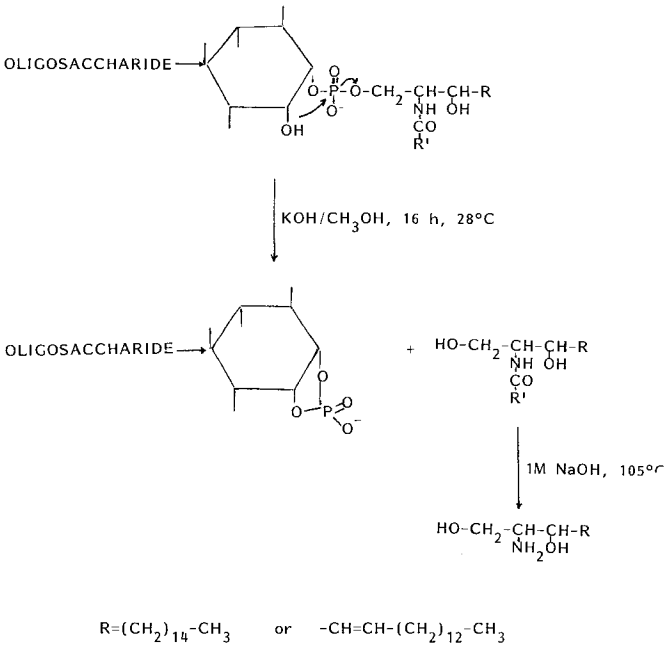



Fig. 4. *Alkaline hydrolysis of LPPG*

GLC of the long chain bases as trimethylsilyl derivatives using conditions c showed the presence of sphinganine and sphingosine. Analysis of varios isolates of LPPG gave a different ratio of the long chain bases, being always sphinganine the main constituent.

On the other hand, the lipid extract from the PtdIns-specific phospholipase C treatment was purified by preparative TLC (solvent a) and the ceramides were analysed by GC/MS as trimethylsilyl derivatives (Fig. 6). Mass fragments (Table 3) were compared with reported values [25]. The main

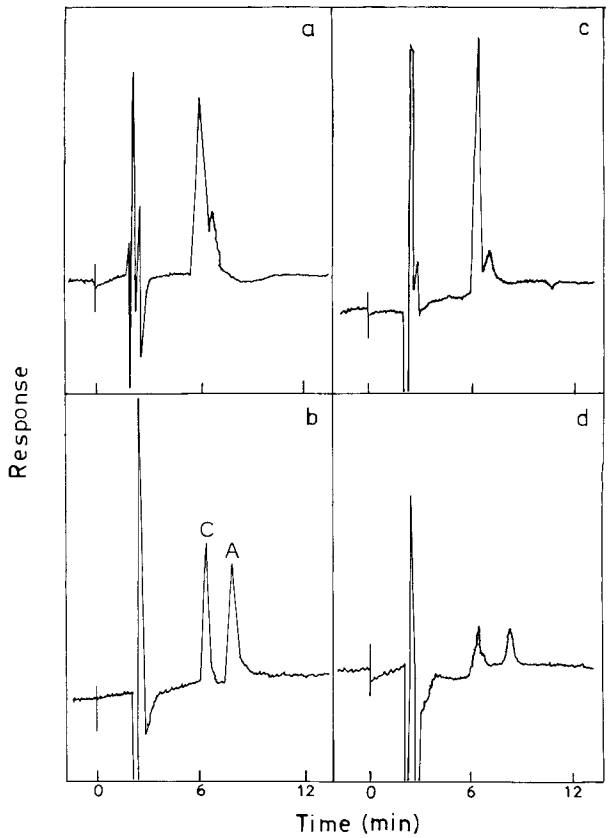


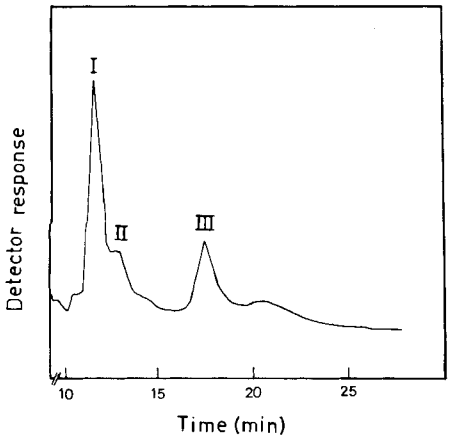

Fig. 5. *HPLC of the lipid released from LPPG by PtdIns-specific phospholipase C.* LPPG was incubated with PtdIns-specific phospholipase C from *B. thuringiensis* in Tris/HCl, pH 7.2, with 0.1% deoxycholate, for 3 h at 37 °C. The lipid was extracted with ether and analysed on a Micromeritics liquid chromatograph equipped with a 771 refractive index detector and a Linear 1200 recorder (Linear Instruments Corporation). A column of Silica 100 (particle size 10 μm) with hexane/2-propanol (93:7) as solvent was used. (a) PtdIns-specific phospholipase C treated LPPG; (b) a mixture of 100 μg each of 1-*O*-hexadecylglycerol (A) and *N*-lignoceroylsphinganine (C); (c) cochromatography of (a) and (C); (d) cochromatography of (a) and (A)

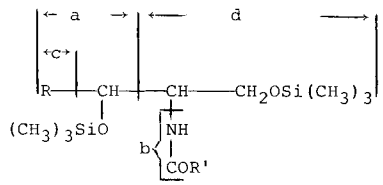
component (peak I, Fig. 6) corresponded to a mixture of *N*-palmitoylsphinganine and *N*-palmitoylsphingosine which could not be resolved. Thus, fragments which are characteristic of the $C_{18}$ saturated and unsaturated long chain bases, resulting from cleavage between C2 and C3, appeared at *m/z* 313 (40%), and 311 (50%) respectively. Some contribution to the 311 peak is due to the sphinganine ceramide. Also two fragments of highest mass appeared at *m/z* 668 and 666 (*M* − 15). The intense fatty acid fragments (*M* − *a*) at *m/z* 370 (51%), (*M* − *a* + 73) at m/z 443 (30%) and (*M* − *a* − 89) at *m/z* 281 (30%) are indicative of the palmitoyl residue.

The second important peak (peak III, Fig. 6), corresponds to lignoceroylsphinganine. The M-90 fragment appeared at *m/z* 705 (5.7%). The sphinganine diagnostic ion at *m/z* 313 was the base peak of the spectrum as in a commercial sample of lignoceroylsphinganine. The fatty acid fragments appeared at *m/z* 482 (M-313), *m/z* 555 (*M* − 313 + 73) and m/z 393 (M-313-89). The ion at *m/z* 217 which is characteristic of sphinganine ceramide derivatives was present in the spectrum of both main ceramides.

In the shoulder (peak II, Fig. 6), emerging after the *N*-palmitoylceramides, ions characteristic of the trimethylsilyl

derivative of *N*-stearoylsphingosine were detected. Thus, the M-15 and M-103 fragments appeared at *m/z* 694 and 606 and the diagnostic ion for sphingosine at *m/z* 311 was the base peak. The *m/z* 398 ion (*M* − a) is indicative of the $C_{18}$ fatty acid constituent. It could also be observed the presence in this spectrum of ions characteristic of the analogous sphinganine ceramide (*m/z* 608, 313, 217). These results agree with our previous report [23] on the presence of about equal amounts of the 16:0 and 24:0 fatty acids bound as amide in LPPG with the 18:0 fatty acid as a minor component.




| | | | | |
|---|---|---|---|---|
| I | R | $CH_3-(CH_2)_{14}-$ | or | $CH_3-(CH_2)_{12}-CH=CH-$ |
| | R' | $CH_3-(CH_2)_{14}-$ | | |
| II | R | $CH_3-(CH_2)_{14}-$ | or | $CH_3-(CH_2)_{12}-CH=CH-$ |
| | R' | $CH_3-(CH_2)_{16}-$ | | |
| III | R | $CH_3-(CH_2)_{14}-$ | | |
| | R' | $CH_3-(CH_2)_{22}-$ | | |

Fig. 6. *GLC/MS of the $Me_3Si$ derivatives of the ceramides released by PtdIns-specific phospholipase C treatment of LPPG.* Conditions as described under Materials and Methods. Main fragments are listed in Table 3

*Determination of non* N*-acylated glucosamine*

To determine if the glucosamine present in LPPG [22] has the amino group free, the carbohydrate recovered from the octyl-Sepharose after PtdIns-specific phospholipase C treatment (Fig. 2B) was purified on Bio-Gel P-2 and was subjected to nitrous acid degradation in the presence of $NaB^3H_4$. After acid hydrolysis and desalting, $^3$H-labeled 2,5-anhydromannitol was identified by paper chromatography, using a standard prepared from glucosamine (Fig. 7). This result indicates that LPPG has non-*N*-acylated glucosamine, as reported for anchor structures [9].

*Identification of galactofuranose*

We have previously shown that most of the galactose in LPPG is present in the furanoic configuration [6]. We confirmed that galactofuranose is a constituent of the LPPG now purified by hydrophobic interaction chromatography. After mild periodate oxidation of the carbohydrate, followed by acid hydrolysis, arabinose was detected by GLC. One third of the total galactose was not oxidized (Table 2). Methylation studies had shown a trace of terminal galactopyranose [8]. Also Gal*f* with a minor amount of Gal*p* was found in an oligosaccharide obtained by hot alkaline treatment of LPPG [7]. A variable ratio of Gal*f*:Gal*p* in different isolates of LPPG could explain our failure to $^3$H label the glycoconjugate by the galactose oxidase/$NaB^3H_4$ method [26] whereas Ferguson et al. [27] succeeded in $^3$H labeling their LPPG preparation.

The starting carbohydrate for the periodate oxidation was obtained from LPPG by mild alkaline hydrolysis. As is shown in Table 2, the relative proportion of glucose in the sample is higher than in the LPPG directly hydrolysed with 2M trifluoroacetic acid. We have consistently found a higher proportion of glucose in samples of carbohydrate released from LPPG by alkali. The presence of a substituent in glucose, more labile under alkaline than acid conditions, could explain this result, but additional experiments are necessary.

Table 2. *Composition (molar ratio) of LPPG and LPPG fractions*
Man, Gal, Glc and Ara were measured by GLC as alditol acetates after hydrolysis with 2M trifluoroacetic acid at 105 °C for 2 h and separation of the neutral fraction on Dowex 50 W. n.d. not determined. Carbohydrate was released from LPPG by 0,25M KOH, as described in Materials and Methods, and purified on Bio-Gel P-2. The carbohydrate obtained as above was oxidized with 20 mM sodium metaperiodate and analyzed as described in Materials and Methods

| Sample | Man | Gal | Glc | Ara | GlcN | Inositol | Phosphate |
|---|---|---|---|---|---|---|---|
| | mol/mol | | | | | | |
| LPPG | 6.2 | 2.6 | trace | 0 | 1.2[a] | 1[a] | 2.9[b] |
| Fraction released by alkali | 5.0 | 2.8 | 1.0 | 0 | + | + | n.d. |
| Fraction oxidized with metaperiodate | 4.8 | 1.0 | 1.1 | 2.2 | + | + | n.d. |

[a] Analysed by GLC as alditol acetates in the acid eluate from the previous hydrolisis (a), further hydrolysed with 4M HCl as described under Materials and Methods. Xylitol was used as internal standard and the response of the inositol and glucosaminitol acetates were respectively compared with that of authentic samples.
[b] Measured by Bartlett's method [21].

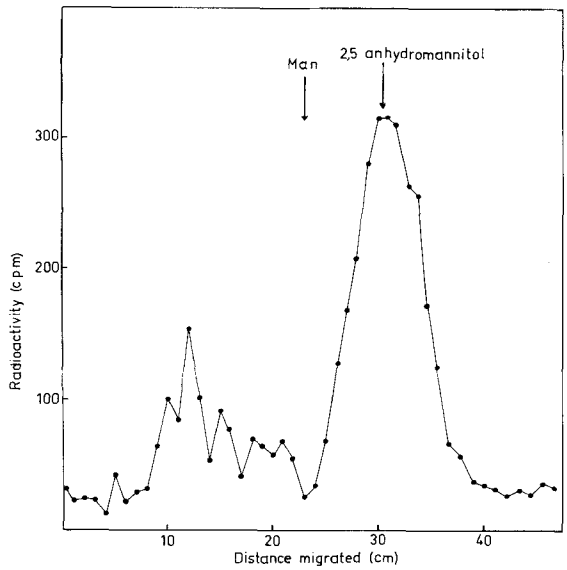



Fig. 7. *Paper chromatographic identification of 2,5-anhydromannitol as the degradation product of glucosamine of LPPG.* The carbohydrate recovered from the octyl-Sepharose after PtdIns-specific phospholipase C digestion (B, Fig. 2) was purified on Bio-Gel P-2 and subjected to nitrous acid degradation in the presence of $NaB^3H_4$. Following hydrolysis with 2 M trifluoroacetic acid, 3 h at 105°C the sample was chromatographed using butanol/pyridine/water (6:4:3). The paper was cut into 1-cm strips and radioactivity was determined in a scintillation spectrometer

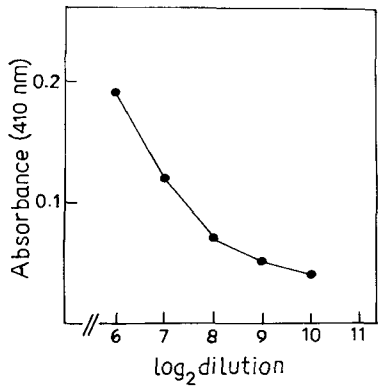



Fig. 8. *CRD activity determination by ELISA.* CRD was detected by the binding of anti-(sVSG MITat 1.2) antibodies to glycosyl PtdIns obtained by treatment of LPPG with PtdIns-specific phospholipase C from *B. thuringiensis*. The assay was carried out as described in Materials and Methods

Table 3. *Diagnostic fragments for structural determination of the ceramides*
The terms used for the fragments are defined in Fig. 6. With *m/z* of peak I the base peak corresponded to *m/z* 73

| Fragment | Peak | | | | | |
|---|---|---|---|---|---|---|
| | I | | II | | III | |
| | *m/z* | % | *m/z* | % | *m/z* | % |
| *M*−15 | 668 | 4.8 | | | | |
| | 666 | 1.6 | 694 | 3.0 | | |
| *M*−90 | 593 | 1.3 | 621 | 2.6 | 705 | 5.7 |
| | 591 | 1.8 | | | | |
| *M*−103 | 580 | 5.2 | 608 | 2.4 | | |
| | | | 606 | 2.5 | | |
| *M*−*a*+73 | 443 | 30.6 | 471 | 22.6 | 555 | 68.7 |
| *M*−*b*+1 | 428 | 3.2 | 428 | 6.7 | 428 | 8.3 |
| | 426 | 14.4 | 426 | 34.9 | | |
| *M*−*a* | 370 | 51.6 | 398 | 22.5 | 482 | 57.6 |
| a | 313 | 39.8 | 313 | 100.0 | 313 | 100.0 |
| | 311 | 50.4 | 311 | 95.0 | 311 | 28.3 |
| *M*−a−$Me_3SiO$ | 281 | 30.1 | 309 | 17.9 | 393 | 82.0 |
| *M*−(*b*+*l*+*c*) | 217 | 12.9 | 217 | 18.6 | 217 | 32.9 |
| 157 | 157 | 43.3 | 157 | 62.5 | 157 | 22.6 |
| $H_2NCHCH_2$ OMe$_3$Si | 132 | 53.0 | 132 | 73.5 | 132 | 52.2 |
| $CH_2OMe_3Si$ | 103 | 19.2 | 103 | 23.0 | 103 | 11.4 |

### *Analysis of amino acids*

The same amino acids previously reported [5, 27], although in a different proportion, have been found in a sample of LPPG purified by octyl-Sepharose chromatography. As before, glutamic acid, glycine, aspartic acid and serine were the major constituents; aminoethylphosphonic acid was also detected. From the analysis it was calculated that 3.5% of the amino acids in LPPG and a molar ratio of aminoethylphosphonic acid to neutral sugars and inositol of 1:8:0.9.

### *CRD activity*

CRD activity in the glycophosphoinositol moiety released by PtdIns-specific phospholipase C was investigated by direct and inhibition ELISA assays. Fig. 8 shows that anti-(sVSG MITat 1.2) antibody binding to glycophosphoinositol, obtained by treatment of LPPG with PtdIns-specific phospholipase C, decreases with the dilution of the antibody, in the direct assay.

The CRD activity was also measured by the ability of the glycophosphoinositol from *T. cruzi* to inhibit the binding of anti-(sVSG MITat 1.2) antiserum to sVSG MITat 1.4. A sample of glycophosphoinositol containing 5 μg carbohydrate caused 60% inhibition, which increased to 77% with a 20 μg sugar sample. Further increase in the glycophosphoinositol concentration gave no higher inhibition.

## DISCUSSION

The present work conclusively proves that specific bacterial PtdIns-specific phospholipase C also cleaves the ceramide phosphate inositol moiety of LPPG. This is a significant finding because the enzymatic reaction is usually taken as indicative of the presence of an acyl or alkylglycerol lipid anchor in glycoconjugates. It was suggested that cleavage of the lipid could also serve to generate second messengers [9]. In this respect, sphingolipid breakdown products play an important role in cellular regulation [28]. It has been recently reported that the cell adhesion glycoprotein of *Dictyostelium discoideum* is anchored to the membrane by a ceramide phosphoglycan. However the lipid was not cleaved by PtdIns-specific phospholipase C from *Staphylococcus aureus* [15]. On the other hand, inositol sphingophospholipids from *Leishmania donovani* have been hydrolysed with PtdIns-specific phospholipase C from *Bacillus cereus* [29]. A substituent in the inositol ring could be responsible for the resistance of the glycoconjugate of *D. discoideum* to cleavage by the PtdIns-specific phospholipase C, as reported for the anchor

of human erythrocyte acetylcholinesterase [30]. Systematic work with PtdIns-specific phospholipases C from different origins could provide information on their general ability to hydrolyse ceramide phosphate linkages.

We had previously reported on the presence of sphinganine and 17-methylsphinganine in the ceramide of LPPG [3]. The branched long chain base could not be detected in the LPPG now further purified by octyl-Sepharose chromatography. Some of the samples previously analysed would contain variable amounts of the lipophosphoglycan with 1-*O*-hexadecylglycerol as a constituent (unpublished results). Moreover, we found that the trimethylsilyl derivative of the latter has a retention time very close to the sphinganine and sphingosine derivatives in several chromatographic columns used for GLC analyses. Also, several of the mass fragments obtained by MS analysis are common for the trimethylsilyl derivatives of the alkylglycerol and the long chain bases. Thus, it is most likely that the peak assigned to the branched $C_{19}$ sphinganine corresponded to a mixture of alkylglycerol and one of the long-chain bases.

A characteristic feature of the LPPG from *T. cruzi* is the presence of β-galactofuranose as terminal units. Gal*f* was also reported as a constituent of the core in the LPG from *L. donovani*, but in that case it was present in the α configuration in an internal position of the oligosaccharide [11].

It was shown that mannose was linked (1→2) and (1→6) in a ceramide containing fraction obtained by partial acid hydrolysis of LPPG [8]. Both linkages were also found in the core of the VSG anchor of *T. brucei*. A small amount of Gal*p* and 3,6 di-*O*-substituted mannose were also detected.

It was recently reported that polyclonal antisera to glycoproteins of *T. cruzi* trypomastigotes recognize β-D-galactofuranosyl epitopes [31]. However, galactofuranose could not be identified in the oligosaccharides released by endoglycosidase H treatment of glycoproteins of *T. cruzi* [32]. It is possible that the sugar recognized by the antisera is part of the anchor of the glycoproteins. The hypothesis that this function is accomplished by LPPG needs further investigation. The fact that a small peptide moiety is present in LPPG [5, 27] could reflect the action of proteolytic enzymes during the purification steps. A cysteine proteinase from epimastigotes of *T. cruzi* capable of degrading soluble proteins from the parasite was described [33]. Although the presence of anchored proteins in metacyclic trypomastigotes was reported [14], the structure of the anchor was not elucidated.

The detection of non *N*-acylated glucosamine in LPPG is another characteristic feature of anchor oligosaccharides. In fact, inositol 1,2-(cyclic)phosphate and glucosamine are important CRD epitopes [34]. The chemical results explain the detection of CRD structures in the glycophosphoinositol released from LPPG by PtdIns-specific phospholipase C. The fact that the maximum inhibition attained in ELISA was 77% could be due to the presence of terminal galactose mainly in the furanoic configuration whereas α-Gal*p* units contribute to some of the CRD epitopes of the sVSG [34].

We have not detected ethanolamine phosphate, which was found to link the protein to the known glycosyl-PtdIns anchors [9], but the carbon-phosphorous analogous, 2-aminoethylphosphonic acid [4, 5] and 2-amino-3-phosphonopropionic acid [5] were identified in LPPG hydrolysates. If aminophosphonic acids could play the same role as ethanolamine phosphate remains to be established.

We have also isolated a glycophospholipid (Scheme 1C) containing an alkylacylglycerol linked to inositol phosphate (unpublished results).

It is intriguing if both, ceramide and the glycerol lipid can indistinctively act as anchors of *T. cruzi* glycoconjugates.

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