Structure of the glycosylphosphatidylinositol-anchor of the 
trans-sialidase from Trypanosoma cruzi metacyclic 
trypomastigote forms

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Abstract

Both, culture-derived and metacyclic trypomastigotes of Trypanosoma cruzi shed a glycoprotein, the shed acute phase antigen, that is responsible for the trans-sialidase activity. In the present work the structure of the glycosylphosphatidylinositol membrane anchor of the trans-sialidase isolated from metacyclic forms was determined. Parasites were metabolically labelled with [9, 10(n)3H]-palmitic acid and the glycoprotein was purified by immunoprecipitation with a monoclonal antibody directed against the repetitive aminoacid sequence. Treatment of the glycoprotein with phosphatidylinositol phospholipase C (PI-PLC) from Bacillus thuringiensis rendered a lipid that comigrated in TLC with a standard of ceramide. No alkylglycerol was detected in contrast with the results previously obtained for the trans-sialidase isolated from culture-derived trypomastigotes where both lipids were found. Chemical and chromatographic analysis showed that the lipid moiety is palmitoyldihydrosphingosine with a minor amount of stearoyldihydrosphingosine. The glycan constituent of the glycosylphosphatidylinositol-anchor was analysed by nitrous acid deamination of the aqueous phase of the PI-PLC treatment, followed by reduction with NaBH₄ and hydrolysis of the phosphodiester with aqueous hydrofluoric acid. A major oligosaccharide was obtained and enzymatic treatment with exoglycosidases and further chromatography in a high pH anion exchange system showed that the trimannosyl core backbone is substituted by an α-galactose. A comparison between the lipid constituent of

Abbreviations: AHM, 2, 5-anhydro-α-mannitol; DU, Dionex units; GPI, glycosylphosphatidylinositol; HPAE-PAD, high pH anion exchange chromatography with pulse amperometric detection; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol phospholipase C; SAPA, shed acute phase antigen; Tc-85, 85 kDa glycoprotein of Trypanosoma cruzi; VSG, variant surface glycoprotein.

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the glycosylphosphatidylinositol anchor of several proteins and their spontaneous shedding by the action of an endogenous PI-PLC was made. © 1998 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Trypanosoma cruzi expresses a superfamily of surface antigens some of which are shed into the medium [1]. One member of the super-family, originally identified as a major parasite antigen named shed acute-phase antigen (SAPA), contains most of the trans-sialidase and neuraminidase activities of the parasite [2]. The T. cruzi enzyme is exceptional in that it is more efficient in transferring than in hydrolysing sialic acid [2,3]. The trans-sialidase catalyses the transfer of (α2-3) linked sialic acid from host macromolecules to parasite surface molecules. It has been observed that the attachment to and invasion of mammalian cells by trypanomastigotes is regulated by addition of sialic acid to a family of glycoproteins collectively designated stage-specific antigens (Ssp3) [4]. Trans-sialidase is a family of several proteins with molecular masses ranging from 120 to 200 kDa. The size differences would be due mainly to different number of repeats rather than to differential glycosylation [5].

In T. cruzi, most membrane proteins are linked via glycosylphosphatidylinositol (GPI) to the carboxy-terminus of the protein. The lipid moieties can be quite variable, being a ceramide in the Ssp4 antigen of amastigote forms [6] and the mucins of metacyclic trypomastigotes [7]. 1-O-hexadecyl-2-O-acetylglcerol in the 1G7 from metacyclics [8] and the mucins of epimastigotes [7,9] and trypanomastigotes [10] and 1-O-hexadecylglycerol in the 85 kDa glycoprotein specific of the trypomastigote forms (Tc-85) [11]. However, there are no firm data as to whether the lipid moieties found in a protein are determined by the species, the stage of the parasite or the protein itself.

We have already characterized the lipid moiety of the GPI-anchor of the trans-sialidase from cell culture trypanomastigote forms. Ceramide and 1-O-hexadecylglycerol in a 3:1 ratio were found [12]. In this article we have determined the structure of the lipid and glycan moieties of the trans-sialidase isolated from axenic-culture-derived metacyclic trypanomastigotes.

2. Materials and methods

2.1. General methods

Radioactivity was determined in a 1214 Rack-beta Wallack liquid scintillation counter using a scintillation cocktail (Optiphase’hisafe 3, LKB) for aqueous and non aqueous samples. For fluorography, TLC plates were sprayed with EN3HANCE (New England Nuclear) and were exposed to Kodak X-OMAT AR films at −70°C.

2.2. Metabolic labelling of parasites

Metacyclic trypomastigotes of Trypanosoma cruzi (Tulahuen strain) were obtained by spontaneous differentiation from epimastigotes in M16 medium [13]. Parasites (6.4 × 10⁹, 70% of metacyclic forms) were metabolically labelled with [9, 10 (n)³H]-palmitic acid (Amersham, 500 mCi mmol⁻¹, 5 mCi ml⁻¹) coupled with delipidized BSA (1 mg ml⁻¹). After incorporation of the precursor for 3 h, microscopic observation showed that the parasites did not suffer morphological modifications. The cells were harvested by centrifugation, washed twice with DME medium and lysed in 50 mM Tris–HCl buffer pH 7.6, 150 mM NaCl containing 1 mM phenylmethylsulfonyl fluoride, 10 mM p-chloro-mercury-phenyl sulfonic acid, 0.5 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 0.2% SDS and 1% Triton X-100. Before the immunoprecipitation the lysate was diluted with an equal volume of Tris–HCl buffer and the immunoprecipitation was performed as previously described. Contami-
nation with the trans-sialidase from epimastigote forms that lacks the SAPA aminoacid repeats was avoided by employing a monoclonal antibody against this region obtained by immunization of mice with the recombinant antigen [2,12].

2.3. Thin layer chromatography

TLC was performed on silica gel 60 Merck precoated plates using the following solvents: A, isopropylether/acetic acid (96:4, vol.); B, chloroform/methanol (1:1, vol.); C, chloroform/methanol/2,5 M NH$_4$OH (40:10:1, vol.). Reverse phase TLC (RP-TLC) was performed on RP-18 f 254 (Merck) plates using acetonitrile/acetic acid (1:1, vol.) as solvent (solvent D).

2.4. Acid hydrolysis

The ceramide was hydrolyzed by heating for 18 h at 70–80°C with concentrated HCl/methanol/water (3:29:4, vol.). The acid was eliminated by successive evaporations with methanol. Total hydrolysis of the oligosaccharide was performed with 3 N HCl for 3 h at 100°C. The acid was eliminated as above.

2.5. Nitrous acid deamination

The aqueous phase obtained from the specific phospholipase C treatment, was freeze–dried and redissolved in 50 mM sodium acetate pH 3.5 (1 ml). Sodium nitrite (3 mg) was added and left for 4 h at room temperature. After addition of NaNO$_2$ the reaction was kept at room temperature for 8 h. The mixture was neutralized with 1M NaOH and reduced with NaBH$_4$ (400 mCi, 347 mCi/mmol) for 8 h at room temperature. Sodium borohydride (1 mg) was then added and after another 12 h the pH was lowered with acetic acid to pH 5. The sample was further desalted by Bio-Gel P-2 using water as eluent. The excluded fractions containing the deaminated sample were joined and freeze–dried.

2.6. Dephosphorylation

The deaminated sample was treated with 50% HF for 48 h at 5°C. The sample was neutralized with a saturated solution of LiOH at 0°C, salts were separated by centrifugation and labelled products were analysed by high pH anion exchange chromatography with pulse amperometric detection (HPAE-PAD).

2.7. Enzymatic digestions

Digestion with phosphatidylinositol-specific phospholipase C (PI-PLC) was as follows: The labelled trans-sialidase was suspended in 0.5 ml of 50 mM Tris–HCl buffer pH 7.4 and extracted with ether to remove adsorbed lipids until no appreciable radioactivity could be extracted. PI-PLC digestion was then performed for 90 min at 37°C with 0.1 U of PI-PLC of Bacillus thuringiensis in the presence of 0.1% deoxycholate. The lipid was extracted with ether (3 × 1 ml) and analyzed by TLC.

For digestion with α-mannosidase, labelled oligosaccharides were dissolved in 100 ml of 0.1 M sodium acetate and 0.1 U of α-mannosidase from jack bean (Sigma) was added. Digestion was performed during 18 h at 37°C. The mixture was boiled for 1 min for enzyme inactivation and was passed through AG50X12(H$^+$), dried, and analyzed by HPAE-PAD. For digestion with α-galactosidase, the oligosaccharide was dissolved in 20 ml of 50 mM sodium acetate, pH 5 and incubated at 37°C for 18 h with 0.1 U of α-galactosidase from coffee bean (Sigma) The sample was analysed as above. For digestion with β-N-acetylhexosaminidase, the oligosaccharide was dissolved in 100 μl of 150 mM citrate–phosphate buffer pH 4.4 and incubated at 37°C for 18 h with 0.1 U of β-N-acetylhexosaminidase from Jack bean (Sigma) The products were desalted by passage through 0.1 ml AG50X12(H$^+$) over 0.2 ml AG3X4(OH) and elution with 1.5 ml water. The sample was analysed as above.

2.8. High pH anion exchange chromatography with pulse amperometric detection

A Dionex BioLC system fitted with a Carbo-Pack PA1 anion exchange column and pulsed amperometric detector (PAD) was used with the
Fig. 1. (A) Thin layer chromatography of the lipid obtained from metabolically labelled trans-sialidase after treatment with PI-PLC. Solvent: chloroform/methanol (19:1.5, vol.) C, palmitoyldihydrosphingosine; A, 1-O-hexadecylglycerol; DAG, 1,2-di-O-palmitoyl glycerol; AAG, 1-O-hexadecyl-2-O-palmitoyl glycerol. (B) TLC of the lipid released by PI-PLC treatment after methanolysis. Solvent: chloroform/methanol/2.5M NH₄OH (40:10:1, vol.). PS, phytosphingosine; DHS, dihydrosphingosine; SP, sphingosine; C, palmitoyldihydrosphingosine; FAME, palmitic acid methyl ester. (C) RP-TLC of the methyl esters obtained from (B) Solvent: acetonitrile/acetic acid (1:1, vol.). C₂₂:₀, behenic acid methyl ester; C₂₀:₀, arachidic acid methyl ester; C₁₈:₀, stearic acid methyl ester; C₁₆:₀, palmitic acid methyl ester; C₁₄:₀, myristic acid methyl ester; C₁₂:₀, lauric acid methyl ester.

following programme: gradient elution starting at 95% buffer A (0.15 M NaOH) changing to 80% buffer A, 20% buffer B (0.25 M sodium acetate in 0.15 M NaOH) at 50 min, followed by a wash cycle to 100% buffer B at 55 min held for 15 min, flow rate 0.6 ml min⁻¹. For analytical determinations, non radioactive internal standards of glucose, maltose, maltotriose and maltotetraose were added. The elution position of radioactive glycanes were expressed in Dionex units (DU) as suggested by Guther et al. [8].

3. Results

Metacyclic forms of T. cruzi were metabolically labelled with [9, 10(n)³H]-palmitic acid and the trans-sialidase glycoprotein was purified as previously described [12]. The immunoprecipitate was dissolved in the PI-PLC buffer and was exhaustively extracted with ether until no counts in the organic phase were detected. This extract contained only remaining precursor when analyzed by TLC in solvent A (not shown). The glycoprotein was further incubated with PI-PLC of Bacillus thuringiensis. Extraction with ether showed 93% (28 000 cpmp) of the radioactivity in the organic phase. In contrast with the results obtained for the trans-sialidase glycoprotein purified from the cell-culture derived trypomastigote stage, only one spot coincident with an authentic standard of ceramide was observed when the extract was chromatographed in solvent sys-
tem B (Fig. 1A). The lipid was recovered from the plate, subjected to methanalysis with HCl/methanol/water (3:29:4) and the products were analysed in solvent C. Fig. 1B shows that the ceramide was completely cleaved obtaining a long-chain base migrating as an authentic sample of dihydrospingosine and the concomitant formation of fatty acid methyl ester. The latter was eluted from the plate and rechromatographed on RP-TLC in solvent D (Fig. 1C). Spots coincident with C16:0 and C18:0 fatty acid derivatives were detected.

The aqueous phase obtained from PI-PLC digestion was deaminated with NaNO₂, reduced with NaB₃H₄ and desalted on a Bio-gel P-2 column. The excluded radioactive peak was freeze-dried, dephosphorylated with aq. 50% HF, neutralized and the supernatant separated by centrifugation. When the crude extract was subjected to preparative HPAE-PAD, a main radioactive peak was obtained (Fig. 2). Fractions corresponding to peaks I and II were recovered separately and used for further analysis. When peak I was rechromatographed, it eluted at 3.4 DU (Fig. 3A). However, this oligosaccharide could not be completely hydrolysed to 2,5-anhydro-D-mannitol (AHM) with α-mannosidase. After digestion with the enzyme, the main product eluted at 2.7 DU between the standards of Man₃ AHM (2.5 DU) and Man₄ AHM (3 DU) (Fig. 3A). The neutral oligosaccharides obtained from the epimastigote lipopeptidophosphoglycan were used for comparison [14]. After β-N-acetylhexosaminidase digestion, the oligosaccharide was not affected (not shown). Total acid hydrolysis rendered a peak eluting as a standard of AHM (Fig. 3B). The nature of the sugar substituent was determined by α-galactosidase treatment of the original peak I. A compound eluting as the standard of Man₃ AHM was obtained, showing the presence of an α-galactose unit substituting the trimannosyl core (Fig. 3B). Susceptibility to α-mannosidase before α-galactosidase treatment indicated that the last mannose residue was not substituted and suggested branching at the mannose residue linked to the GlcNH₂ as occurs in the α-galactose-containing anchors of the variant surface glycoprotein (VSG) from T. brucei [15]. This is in accordance with the elution position (3.4 DU) for the original oligosaccharide (I). In fact, the elution position given for Manₙ(α1-2)Manₙ(α1-6)[Galₘ(α1-3)]Manₙ(α1-4)AHM is 3.6 DU [16].

Peak II (Fig. 4), when rechromatographed, eluted at 3 DU (Fig. 3C), coincident with the standard of Man(α1-2)Man(α1-2)Man(α1-6)Man(α1-4)AHM. When this oligosaccharide was incubated with
x-mannosidase, it was completely hydrolysed and eluted as an authentic sample of AHM (Fig. 3C).

4. Discussion

In this paper we describe the structure of the GPI anchor of the trans-sialidase obtained from metacyclic forms of *T. cruzi*.

Sialyltransferase activity has been reported in three of the four stages of *T. cruzi*. It is not present in intracellular and dividing amastigotes, but starts to be synthesized when *T. cruzi* transforms into trypomastigotes [3]. The enzyme present in epimastigote forms has similar kinetic properties and the same substrate specificity as the trypomastigote enzyme but lacks the 12 aminoacids SAPA repeat region and is not anchored to the membrane by a glycosylphosphatidylinositol [17]. Upon differentiation of epimastigotes into metacyclic trypomastigotes in vitro, a *trans*-sialidase similar to that of bloodstream and tissue-cultured derived trypomastigotes is expressed [18]. We have previously proved that *trans*-sialidase isolated from trypomastigote forms is anchored via a GPI to the membrane [12]. In this paper we show that the same happens with the enzyme obtained from metacyclic forms. However, some differences have been detected in their structures:

1. While the anchor of metacyclic forms is completely susceptible to PI-PLC digestion, showing no acylation of the inositol ring, in tissue-culture derived trypomastigotes 25% of the GPI remains resistant to the enzyme [12].

2. The structural determination described in this paper revealed differences in the lipidic moiety. Ceramide is the only constituent obtained from the anchor of metacyclic forms. Its structure as stearoyl and palmitoyl dihydrosphingosine was shown by methanolysis. It must be considered that elongation of fatty acids may not be an efficient process, as previously shown for trypomastigotes [19]. On the other hand, ceramide and an alkylglycerol in a 3:1 ratio were detected in the trypomastigote *trans*-sialidase [12].

Changes in the anchor structure during parasite differentiation have been described. Thus, mucins

![Fig. 3. HPAE-PAD analysis and exoglycosidase treatment of fractions I and II. Panel A: —O—, peak I; —■—, peak I digested with Jack bean x-mannosidase; Panel B: —O—, peak I; —Δ—, total hydrolysis of peak I; —○—, peak I digested with x-galactosidase; Panel C: —O—, peak II; —○—, peak II digested with Jack bean x-mannosidase. Numbers indicate the elution position of glucose (1) and maltooligosaccharides (2–4).](image-url)
from epimastigote forms contain alkylacylglycerol and they acquire 70% of inositolphosphoceramides when they differentiate into metacyclic forms [7]. The relationship between the ceramide-PI type GPI structures and the glycerolipid-PI type GPI structures is not clear. 

Proteins containing glycerolipid-PI type GPI structures such as 1G7 from T.cruzi [8], VSG [20] and procyclic acidic repetitive protein (procyclin) from T.brucei [21–23] and promastigote surface protease (PSP) from Leishmania major [24] are retained on the surface of the live parasite. On the contrary, Ssp4 [6] and mucins from T.cruzi [7] and contact site A glycoprotein (csA) from Dytiscostelium discoideum [25,26] present a ceramide-PI type GPI structure and are actively shed by the action of endogenous PI-PLCs. It is important to point out that even though Tc-85 from the trypomastigote stage of T.cruzi carries an alkylglycerol-PI structure [11] it can also be recovered from the culture medium. However, only PI-PLC resistant Tc-85 is released by an alternative mechanism of vesiculation [27,28]. In Table 1, the lipid structure of different glycoproteins in relation to their spontaneous shedding is presented. 

All the GPI anchors that have been characterized to date contain a Man(α1-2)Man(α1-6)Man(α1-4)GlcN(α1-6)-myo-inositol backbone. Trans-sialidase from metacyclic forms contains a major oligosaccharide. Treatment with α-mannosidase before and after α-galactosidase digestion showed substitution by an α-galactose residue. 

Galactose was first described as a component of the VSG anchor of T.brucei. Several variants of VSG containing different amounts of galactose in linear or branched chains have been identified.
The level of galactosylation correlates with the subclass, defined by the aminoacid to which the GPI-anchor is attached [20,29]. On the other hand, trans-sialidase obtained from metacyclic forms of T. cruzi presents an α-galactose-containing structure. Even though in the trypomastigote stage of T. cruzi, a galactose α(1-3) epitope has been found in the N-linked oligosaccharide of Tc-85 [30] and in the O-glycosidic chains of the mucins [31], this is the first report of the presence of α-galactose in an anchor of T. cruzi.

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References


