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Sialyl-Tn antigen expression and *O*-linked GalNAc-Thr synthesis by *Trypanosoma cruzi*

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Abstract

Most *Trypanosoma cruzi O*-glycans are linked to Thr/Ser residues via *N*-acetylglucosamine. We report that the mucin-type carcinoma-associated sialyl-Tn antigen (NeuAc-GalNAc-*O*-Ser/Thr) is expressed by *T. cruzi*. A specific MAb allowed us to localize the antigen on the surface of epimastigotes and to identify reactive components in parasite lysates (32, 60, and 94 kDa). In addition, ppGalNAc-T activity was characterized in epimastigotes, and direct evidence was obtained for the in vitro incorporation of GalNAc to a synthetic peptide derived from a *T. cruzi* mucin. These results add an as yet unknown complexity to the pathways of *O*-glycan biosynthesis in this protozoan parasite.

Keywords: *O*-glycosylation; Sialyl-Tn antigen; UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase; *Trypanosoma cruzi*

The glycobiology of *Trypanosoma cruzi*, the causative agent of Chagas' disease, is characterized by several interesting features: (i) like other trypanosomatids, this organism is unable to synthesize dolichol-phosphate-glucose; thus, the biosynthesis of *N*-linked oligosaccharides involves the transfer of a truncated, non-glucosylated $Man_9GlcNAc_2$ saccharide to a nascent peptide chain [1]. (ii) Mucin-like (TcMUC) molecules, encoded by a very large family of genes (about 500 per haploid genome), are expressed on the surface of the parasite and attached via glycosylphosphatidylinositol anchors [2]. These highly glycosylated proteins are strongly implicated in invasion of the mammalian host cells [3]. (iii) Although unable to synthesize sialic acid, *T. cruzi* expresses a surface *trans*-sialidase that catalyzes the transfer of sialic acid from host sialoglycoconjugates to parasite glycoconjugates, mainly to terminal β-galactosyl residues of TcMUC [4,5]. Sialylation of the parasite surface occurs in all developmental stages except in amastigotes, which lack *trans*-sialidase activity [6]. The acquisition of sialic acid appears to be important for parasite infectivity and survival in the vertebrate host [7]; for example, for evasion from the early complement-mediated response [8], and for adhesion and invasion of host cells [9]. (iv) TcMUC are unusual because their glycans are linked to Thr/Ser residues in the protein core via *N*-acetylglucosamine (GlcNAc) rather than *N*-acetylgalactosamine (GalNAc), as is usually found in vertebrate mucins [10]. Very little is known about the biosynthesis of the TcMUC *O*-glycans, the only report being the characterization of UDP-GlcNAc: polypeptide-α-*N*-acetylglucosaminyltransferase (ppGlcNAc-T), an enzyme located in the microsomal membrane that transfers GlcNAc to threonine residues [11].

O-linked carbohydrate chains are attached to mammalian proteins by means of a GalNAc linked to serine or threonine residues [12]. In many human cancer cells, the elongation of the *O*-linked saccharide chain does not occur, thus leading to the generation of antigenic determinants such as Tn (GalNAc-*O*-Ser/Thr), sialyl-Tn (NeuAc-GalNAc-*O*-Ser/Thr), and T (Gal-GalNAc-*O*-Ser/Thr) [13]. Evidence has recently accumulated indicating that the carcinoma-associated Tn and sialyl-Tn antigens are expressed by helminth parasites. Tn

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was initially observed both in the schistosomula and in the adult worm of the trematode *Schistosoma mansoni* [14]. In addition, we have recently shown that *Echinococcus granulosus*, the cestode responsible for cystic echinococcosis and *Fasciola hepatica*, the digenean trematode that causes fasciolosis, express Tn and sialyl-Tn antigens [15,16]. Furthermore, the presence of Tn would be widespread among helminth parasites; in fact, we also detected Tn in *Taenia hydatigena*, *Mesocestoides corti*, *Nippostrongylus brasiliensis*, and *Toxocara canis* [17]. Thus, this antigen appears to be expressed in representatives from all major taxonomic helminth groups, suggesting that truncated *O*-glycosylation does not constitute an aberrant phenomenon in these organisms.

The present study was undertaken in order to investigate whether the synthesis of simple mucin-type carcinoma-associated antigens also occurs in protozoan parasites, such as *T. cruzi.* We identified and characterized sialyl-Tn-bearing glycoproteins in cultured epimastigotes; in addition, a direct evidence was obtained for the in vitro incorporation of GalNAc to a synthetic peptide derived from a *T. cruzi* mucin.

Materials and methods

Parasite culture and preparation of parasite lysates. The CL Brener strain of *T. cruzi* was used throughout this work. Epimastigotes were grown in liver infusion tryptose (LIT) medium supplemented with 10% heat-inactivated fetal calf serum (FCS) at 28 °C. Extracts were prepared by washing the parasites five times with PBS and resuspending them in 20 mM Tris–HCl, pH 8, containing 2% NP-40, and the following protease inhibitors (all from Sigma Aldrich, St. Louis, MO): 5 μg/ml pepstatin A, 40 μg/ml bestatin, 15 μg/ml leupeptin, 1 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 1 mM NaF, 1 mM TLCK, 1 mM benzamidine, and 1 mM PMSF (lysis buffer).

Antibodies. The MAb 83D4 (IgM), which binds specifically to the Tn antigen [18], was raised against a human breast cancer [19]. It was precipitated from ascites fluids by dialysis against demineralized water at 4 °C, dissolved in a small volume of 0.5 M NaCl in PBS, and purified by gel-filtration chromatography on Sephacryl S-200. The MAb B72.3 (IgG_1), which recognizes the sialyl-Tn determinant [20], was established by immunizing a mouse with human colon cancer cells [21]. It was purified by chromatography over protein A–Sepharose.

Polyclonal antibodies recognizing *E. granulosus* ppGalNAc-T1 (Accession No. AY353720, Freire et al., submitted) were produced against the catalytic domain of the enzyme (amino acids 357–461). The corresponding cDNA fragment was PCR-amplified using sense and antisense primers EgDC-F, 5′-cgggatccTGGGGTGGTGAGAACTT GGAG-3′ and EgDC-R, 5′-cgaagcttctaAGGAGCAATGTTGTCAA GAAACC-3′ (the sequences in lowercase characters indicate restriction sites for *Bam*HI and *Hin*dIII, respectively). The amplified product was digested with *Bam*HI and *Hin*dIII, and cloned in the pQE30 expression vector (Qiagen, USA). The recombinant protein was produced in *Escherichia coli* strain M15 by induction with 0.5 mM IPTG, purified over a Ni^{2+}–nitriloacetic acid column under denaturing conditions according to the manufacturer's instructions (Qiagen, USA), and used to raise polyclonal antibodies. Rabbits were immunized with 200 μg of the protein in Freund's Complete Adjuvant (Sigma), boosted three times with the same dose in Freund's Incomplete Adjuvant (Sigma), and bled 10 days after the last booster. After checking the serum reactivity by ELISA, the IgG fraction was isolated by protein A chromatography.

Immunofluorescence analysis. This was carried out as previously described [22]. Briefly, parasites were washed three times with cold PBS, resuspended at 2×10^6 cells/ml, fixed in 2.5% paraformaldehyde, and incubated for 8 min in acetone at −20 °C. The slides were treated for 1 h at 37 °C with MAbs 83D4 (IgM) or B72.3 (IgG) in PBS/0.1% bovine serum albumin (IFI buffer). Then, they were washed three times in IFI buffer and incubated for 1 h at 37 °C with fluoresceine-conjugated anti-mouse IgM or IgG (Sigma) diluted 1:200 in IFI buffer, containing 0.01% Evans blue. After washing again three times, the slides were examined under a fluorescent microscope. Controls using PBS instead of MAbs were carried out in parallel.

ELISA of T. cruzi extracts. Microtiter wells were coated with 100 μl of different dilutions of parasite extracts in 0.1 M carbonate buffer, pH 9.6, by overnight incubation at room temperature. The wells were washed, blocked with gelatine, washed again three times, and incubated for 2 h at 37 °C with 100 μl MAb 83D4 or B72.3 (10 μg/ml). The plates were washed again and then treated with a biotinylated anti-mouse antibody (Sigma, St. Louis, MO), for 1 h at 37 °C. Unbound material was subsequently washed off and 100 μl of 1/2000 avidin/peroxidase complex (Sigma) was added to each well and incubated for 1 h at 37 °C. Peroxidase activity was developed by incubation with ABTS [2,2-azin-bis(3-ethylbenz-thiazoline-6-sulfonic acid)] and hydrogen peroxide in phosphate-citrate buffer, pH 5.0. The concentration of Tn and sialyl-Tn in the samples was estimated using a standard curve prepared with asialo-ovine submaxillary mucin or ovine submaxillary mucin, respectively. The reactivity determined in 10 ng of mucin was defined as one unit. All samples were assayed in duplicate.

SDS–PAGE and Western blotting. T. cruzi protein extracts were resolved by SDS–PAGE (3–15% gradient) under reducing and non-reducing conditions [23], and visualized by silver staining of the gels according to standard protocols; or electro-transferred onto nitrocellulose sheets (Amersham) for Western blots [24]. These filters were then blocked overnight at 4 °C with 3% bovine serum albumin in PBS. After washing with Buffer A (0.1% Tween 20, 1% BSA in PBS), blots were incubated with appropriate dilutions of MAbs 83D4 and B72.3 in buffer A, for 3 h at 37 °C; washed again three times with buffer A, incubated for 1 h at room temperature with peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma), and developed with enhanced chemoluminescence (ECL, Amersham). Polyclonal antibodies against *E. granulosus* ppGalNAc-T1 and an alkaline phosphatase-conjugated anti-rabbit IgG antibody (Dako) were used for the detection of ppGalNAc-T. In this case, membranes were washed twice (10 min) with developing buffer (100 mM NaCl, 100 mM Tris–HCl, pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween 20), and developed with nitroblue tetrazolium and 5-bromo-1-chloro-3-indolyl phosphate (Roche).

Purification of ppGalNAc-T. ppGalNAc-T was affinity-purified from *T. cruzi* epimastigote extracts using anti-ppGalNAc-T1 from *E. granulosus* polyclonal IgG antibodies immobilized onto CNBr-activated Sepharose. Cell lysates were re-circulated through the column for 8 h at 4 °C. Then, the column was washed with PBS containing 0.7 M NaCl and eluted with 0.1 M triethylamine buffer, pH 10.5. Purified fractions were immediately neutralized with phosphate buffer and the proteins, visualized by silver staining after SDS–PAGE (10%).

Evaluation of ppGalNAc-T activity. This was done as described before [16]. Briefly, the standard reaction mixture contained 50 mM imidazole–HCl, pH 7.2, 10 mM $MnCl_2$, 0.5% Triton X-100, 150 μM UDP-[^{3}H]GalNAc (approximately 80,000 dpm), 2 mM of a synthetic peptide acceptor, and epimastigote protein extract (about 100 μg), in a final volume of 50 μl. The synthetic peptides used—derived from the tandem repeat sequences of highly glycosylated proteins from *T. cruzi*, *T. brucei*, and *S. mansoni*, and from different human mucins (MUC1, MUC2, MUC5B, and MUC6)—are

Table 1
Sequence of the synthetic peptides used to evaluate ppGalNAc-T activity

Peptide	Amino acid sequence	Reference
Human MUC1 (a)	PDTRPAPGSTA	[45]
Human MUC1 (b)	HGVTSAPDTRP	[45]
Human MUC2 (a)	PTTTPITTTTTV	[46]
Human MUC2 (b)	VTPTPTPTGTQT	[46]
Human MUC5B	VLTTTATTPTA	[47]
Human MUC6	GTTPPPTTLK	[48]
T. brucei mucin	SSLLSSFASSAVG	[49]
T. cruzi mucin	KPPTTTTTTTTKPP	[10]
S. mansoni mucin	ISTSPSPSNITTTT	[50]

indicated in Table 1. The rate of GalNAc transfer to peptides was measured by incubating the mixture at 28 °C for 2 h. The reaction was terminated by addition of 50 μl of 250 mM EDTA. The glycosylated peptides were separated from unreacted UDP-[^{3}H]GalNAc on a 1 ml AG1X-8 (Cl^- form, Sigma) column with 2.6 ml of water as eluent. The radioactivity was measured with an LS Analyzer Beckman scintillation counter. The enzyme activity was expressed as pmoles of UDP-[^{3}H]GalNAc transferred per mg of protein and per hour. All experiments were done in triplicate. Reactions lacking the acceptor substrate yielded background values that were averaged for each set of assays and subtracted from every triplicate value.

Analysis of an in vitro glycosylated synthetic peptide. A synthetic peptide derived from a *T. cruzi* MUC gene 1 (KPTTTTTTTTPK) was in vitro glycosylated as described above, and the reaction products were purified by HPLC using a C18 reverse phase column, eluted with an acetonitrile–0.1% trifluoracetic acid (TFA) gradient. The purified glycopeptides (0.2 mg) were hydrolyzed with 2 M TFA (0.3 ml) at 100 °C for 3 h. After freeze-drying, the aqueous layer and part of the material was reduced with $NaBH_4$ (10 mg) in 1 M NH_4OH (1 ml) for 30 min at room temperature. $NaBH_4$ excess was quenched with AcOH and the boric acid was removed by codistillation with MeOH (3×1 ml). The resulting alditols were acetylated with a mixture of Ac_2O-pyridine (1:1, 1 ml) at 120 °C for 30 min and analyzed by gas-chromatography followed by mass spectroscopy (GLC–MS) using authentic samples as references [25]. The alditol acetates were separated on a 17A Shimadzu gas-chromatograph, equipped with a fused silica capillary column SE-52 (25 mm × 0.25 mm i.d. × 0.25 μm, Mega, Italy), using a temperature program from 140 °C (held for 3 min), increased at 5 °C/min to 260 °C (held for 2 min), connected to a QP5050 Shimadzu quadrupole mass spectrometer. Detection was performed using either the total ion chromatogram (TIC) or the selective ion monitoring (SIM) mode, monitoring the ions at *m/z* 102, 114, 144, 156, and 258.

Results

Evaluation of Tn and sialyl-Tn antigens in Trypanosoma cruzi

In order to assess whether the *O*-glycosylated Tn and sialyl-Tn antigens are expressed by *T. cruzi*, cultured epimastigotes were analyzed by immunofluorescence using the anti-Tn MAb 83D4 and the anti-sialyl-Tn MAb B72.3. The periphery of the parasites was strongly stained with the latter MAb (Fig. 1B), whereas no sign of the Tn determinant was observed (Fig. 1A). An ELISA, where the solid phase was coated with the parasite extract, was also used to detect Tn and sialyl-Tn in epimastigotes. In agreement with the immunofluorescence results, only the anti-sialyl-Tn MAb was found to be reactive (Fig. 2), and this reactivity was abolished after chemical desialylation of the extract (data not shown). Analysis of the pattern of sialyl-Tn-bearing glycoproteins by SDS–PAGE followed by Western blotting allowed the identification of three components migrating as bands of 32, 60, and 94 kDa relative molecular mobility (Fig. 3, lane 1). The band of 94 kDa was not observed under reducing conditions, and a new band of 51 kDa was visualized instead, indicating that some sialyl-Tn-bearing components contain disulfide bonds (Fig. 3, lane 2).

Identification and characterization of ppGalNAc-T activity

Considering that no *O*-linked GalNAc has been identified to date in *T. cruzi*, and that the detection of sialyl-Tn antigen indicates that sialic acid linked to Thr/Ser-α-GalNAc is present in epimastigotes; we proceeded to evaluate the ppGalNAc-T activity of parasite lysates using a synthetic peptide (KPTTTTTTTTPK) derived from a *T. cruzi* mucin as acceptor substrate, and UDP-GalNAc as donor substrate. Transferase activity was indeed observed in the epimastigote extract and appeared to be maximal at 28 °C and 5.5–6.6 pH units (Fig. 4A). It was also found to be ion dependent, with Co^{2+}, Ni^{2+}, and Mn^{2+} behaving as the best activators

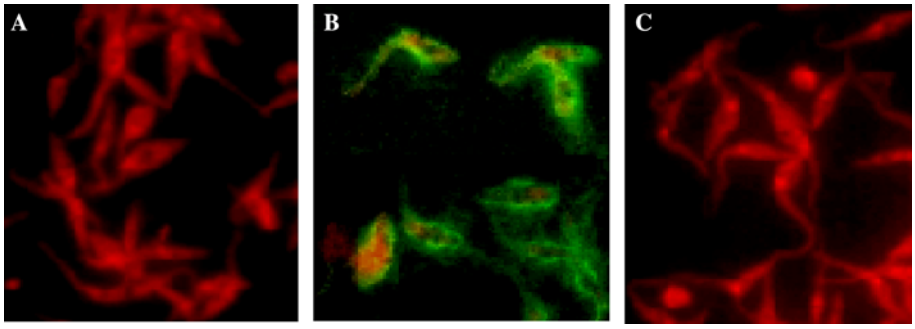

Fig. 1. Detection of Tn and sialyl-Tn antigens in *T. cruzi* epimastigotes. Immunofluorescence analysis of parasites incubated with the anti-Tn MAb 83D4 (A), the anti-sialyl-Tn MAb B72.3 (B), and without primary antibody (negative control), which showed no reactivity (C).

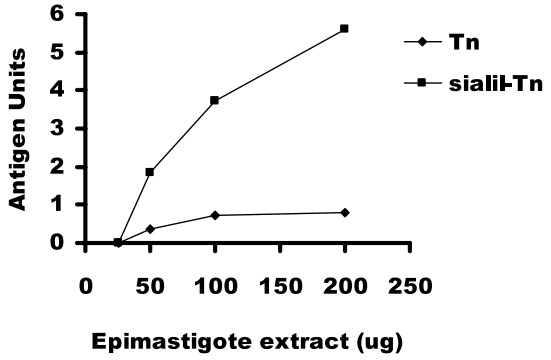

Fig. 2. Assessment of Tn and sialyl-Tn antigen levels in *T. cruzi* extracts. Tn and sialyl-Tn antigen levels were analyzed by ELISA in different dilutions of *T. cruzi* epimastigote extracts using, respectively, MAb 83D4 and MAb B72.3. The antigen content is expressed in units, defined on the basis of the reactivity of a standard mucin. All samples were processed in duplicate. The antigen levels were measured in different extract preparations. The figure shows the results obtained in one representative experiment.

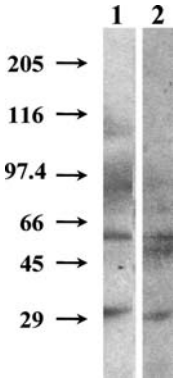

Fig. 3. Identification of sialyl-Tn-bearing glycoproteins in *T. cruzi* epimastigotes. Western immunoblot of a parasite lysate probed with the anti-sialyl-Tn MAb B72.3. SDS–PAGE of the sample was carried out using non-reducing (lane 1) or reducing (lane 2) conditions. No reactivity was observed in the negative controls that were incubated with PBS instead of primary antibody (data not shown).

among the tested divalent cations (Table 2). Then, we analyzed the effect of different acceptor substrates on enzyme activity, using a panel of nine synthetic peptides derived from *O*-glycosylated proteins of *S. mansoni*, *T. cruzi*, and *T. brucei*, and from human mucins (MUC1, MUC2, MUC5B, and MUC6) (Table 1). *T. cruzi* extracts preferentially glycosylated the peptide sequences derived from parasite proteins, whereas the ones designed from human mucins were poor substrates of epimastigote ppGalNAc-T (Fig. 4B).

An attempt was carried out to identify the protein(s) responsible for this activity by Western blotting analysis of a *T. cruzi* epimastigote extract using polyclonal antibodies raised against the conserved catalytic domain of *E. granulosus* ppGalNAc-T1. Two closely migrating

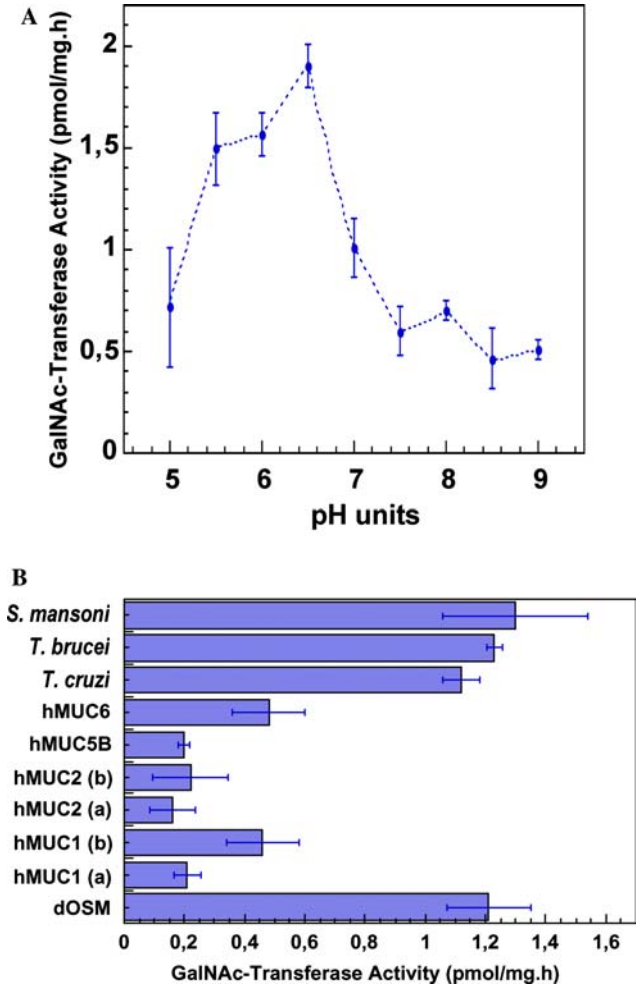

Fig. 4. Evaluation of ppGalNAc-T activity. (A) Influence of pH. ppGalNAc activity using the *T. cruzi* peptide (KPTTTTTTTTPK) and UDP-[^{3}H]GalNAc was measured at various pH values. Results are expressed in pmol of transferred [^{3}H]GalNAc per mg of protein and per hour. Bars correspond to the standard deviation of three determinations. (B) Activity towards synthetic peptide acceptors. ppGalNA-T activity was determined using nine synthetic peptides (2 mM). Results are expressed in pmol of transferred [^{3}H]GalNAc per mg of protein and per hour. Bars correspond to the standard deviation of three determinations. The activity with deglycosylated ovine submaxillary mucin (dOSM, 5 μg) was measured in parallel, for comparison.

Table 2
Evaluation of cation dependence of ppGalNAc-T activity

Cation (10 mM)	Transferase activity (pmol/mg h)	Relative activity (%)
Mn^{2+}	1.17 ± 0.28	100
Co^{2+}	2.55 ± 0.31	218
Ca^{2+}	1.16 ± 0.03	99
Cu^{2+}	1.00 ± 0.02	85
Ni^{2+}	1.62 ± 0.22	138
Zn^{2+}	0.65 ± 0.22	56
Mg^{2+}	0.95 ± 0.18	81
—	0.69 ± 0.02	59

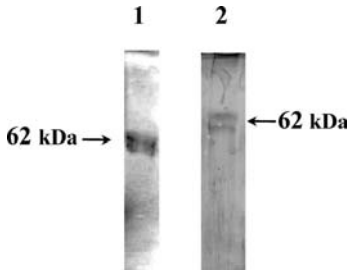

Fig. 5. Identification of ppGalNAc-T from *T. cruzi* epimastigotes. Lane 1: SDS–PAGE of a parasite lysate followed by Western blot using polyclonal antibodies raised against *E. granulosus* ppGalNAc-T1. Lane 2: SDS–PAGE followed by silver staining of the affinity-purified fraction from an epimastigote lysate, eluted from a column containing anti-Eg-ppGalNAc-T1 immobilized IgG.

bands of about 62 kDa were thus identified (Fig. 5, lane 1). We, therefore, tried to affinity purify the active fraction by loading the parasite extract onto a Sepharose column of immobilized anti-Eg-ppGalNAc-T1 IgG. In agreement with the result previously obtained by Western blot, SDS–PAGE followed by silver staining of the eluted proteins showed two close components of 62 kDa (Fig. 5, lane 2), which were found to have ppGalNAc-T activity (not shown).

Identification of GalNAc in an in vitro glycosylated peptide

The product from the ppGalNAc-T activity assay was analyzed so as to obtain direct evidence for the in vitro incorporation of GalNAc to the synthetic peptide (KPTTTTTTTTPK) derived from *T. cruzi* mucin. A peak with the same retention time as the acetate of GalNAc alditol was observed in the TIC of the alditol acetates derived from the *O*-glycosylated peptides. To confirm the presence of this derivative in the sample, GLC–MS analyses were performed using the SIM and selecting the diagnostic ions at *m/z* 102, 114, 144, 156, and 258. These ions typically arise from the fragmentation of the molecular ion [M^+] of the alditol acetates of 2-desoxy-2-acetamido-hexoses [26]. A peak with the same elution time as the one obtained for the corresponding GalNAc derivative (19.59 min) was detected with all the selected diagnostic ions (Fig. 6); this peak is different from the one corresponding to the GlcNAc derivative. Confirmation of the identity of the incorporated saccharide was obtained through co-injection of the sample with the alditol acetate of GalNAc.

Discussion

In this work, we report the presence of the sialyl-Tn antigen in epimastigotes of the protozoan parasite *T. cruzi*, as judged by immunofluorescence analysis, ELISA, and Western blot using the anti-sialyl-Tn MAb B72.3. Sialyl-Tn was detected mainly on the parasite surface, including the flagellum. MAb B72.3, which was established after immunization with a human metastatic breast cancer [21], shows highly specific reactivity with various human cancers and restricted reactivity with the respective normal tissues [27,28]. Initially, two independent works reported the molecular structure of the epitope as NeuAcα2-6GalNAc-*O*-Ser/Thr [20,29]; more recently, clusters of two sialyl-Tn residues were found to be essential for MAb recognition [30]. In *T. cruzi*, we identified the sialyl-Tn-bearing glycoproteins of an epimastigote lysate as components of 23–60 kDa under reducing conditions; thus, they could correspond to the mucin-like proteins already described in this parasite stage [10]. The presence of sialylated

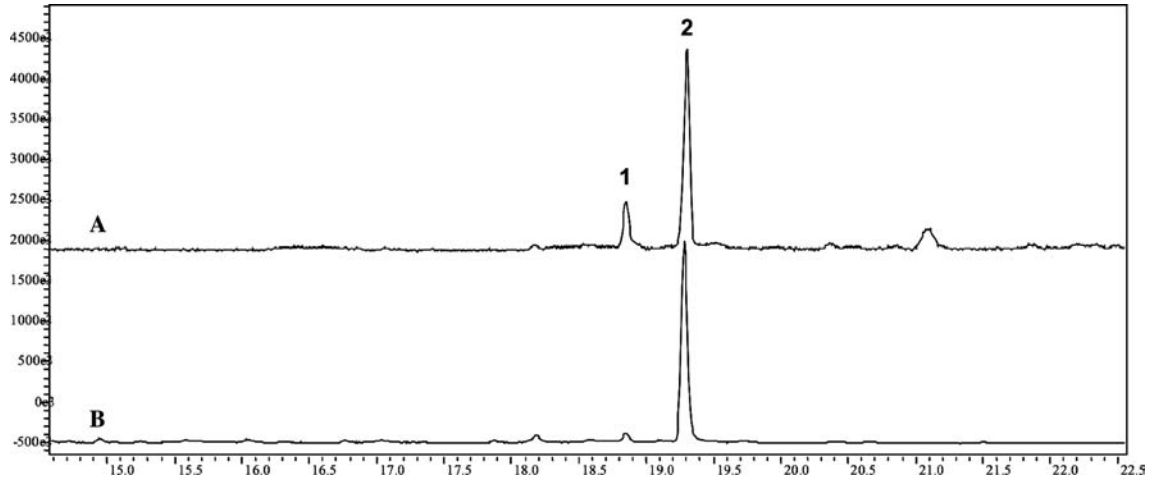

Fig. 6. Identification of GalNAc in an in vitro glycosylated peptide. The peptide KPTTTTTTTTPK was in vitro *O*-glycosylated with an epimastigote extract and the incorporated GalNAc was analyzed by GLC–MS. The TICs correspond to: (A) a standard mixture of *N*-acetyl-glucosamine alditol pentaacetate (**1**) and *N*-acetyl-galactosamine alditol pentaacetate (**2**), and (B) the acetate of the alditols from the *O*-glycosylated peptide.

galactose residues on glycosylated chains of TcMUC has previously been reported [10]. Our results suggest that another oligosaccharide structure, i.e., sialic acid linked to a GalNAc residue, is also present in *T. cruzi* epimastigotes; since the MAb B72.3 does not bind to Gal, GlcNAc or to lactose residues [20,29]. In turn, the putative presence of sialyl-Tn on the surface of epimastigotes raises the question of whether there is a *trans*-sialidase capable of transferring sialic acid to GalNAc residues. As already mentioned, *trans*-sialidases catalyze the transfer of sialic acid to *O*-glycans on the parasite surface; they constitute a very large family that includes several hundreds of related genes [31]. It has been clearly shown that *trans*-sialidase activity involves the interaction of the enzyme with sialic acid residues α2-3 ligated to terminal βGal, both in donor and acceptor glycans [32]. However, *T. cruzi trans*-sialidase also binds to NeuAc α2-6 [33]; moreover, a point mutation (Trp-312-Ala) renders the enzyme capable of hydrolyzing both α2-3 and α2-6 linked sialyl-molecules [34]. In this context, the existence of an enzyme capable of transferring sialic acid to GalNAc residues appears worthy of further investigation.

The sialyl-Tn antigen has been found to be capable of inducing an effective immune response against cancer cells [35,36]. In addition, studies of protective immunity to infection have frequently focused on molecules present on the parasite surface that may serve as targets for aggressive immune attack. The fact that sialyl-Tn was detected on the surface of *T. cruzi* suggests that sialyl-Tn-bearing glycoproteins could be immunogenic during *T. cruzi* infection. Moreover, the expression of a cancer-associated structure such as the sialyl-Tn antigen in *T. cruzi* raises the interesting possibility that cross-reactive immune responses may alter host susceptibility to cancer or to parasite infection. In fact, a low level of colon cancer induced by 1,2-dimethylhydrazine (DMH) was recently reported in rats chronically infected with *T. cruzi* [37]. Since DMH-induced rat colon cancer cells also express the sialyl-Tn antigen [38], we are tempted to hypothesize that the *T. cruzi* sialyl-Tn antigen could be involved in the induction of a cross-reactive immune response, which would be effective against colon cancer cells.

The glycan chains that are *O*-linked to the *T. cruzi* surface mucins have been extensively analyzed. As already mentioned, threonine residues in the peptide backbone have been found to be predominantly ligated through α-linked GlcNAc, rather than through GalNAc, as commonly observed in mammalian mucins [39,40]. However, this finding does not exclude the possibility of a small amount of GalNAc-linked glycans being also present in *T. cruzi* mucins. In fact, analysis of the oligosaccharide alditols obtained from the GPI-anchored TcMUC indicated that other *N*-acetylhexosaminitols apart from GlcNAc-ol are present, in proportions ranging from 10 to 30% depending on the sample [41]. Studies involving the use of lectins have also detected GalNAc-Thr in different *T. cruzi* stages [42]. Here we provide the first evidence that *T. cruzi* extracts are capable of adding GalNAc residues to synthetic acceptor peptides in vitro. This reaction is catalyzed by ppGalNAc-T (EC 2.4.1.41), an enzyme responsible for the transfer of *N*-acetylgalactosamine from UDP-GalNAc to serine or threonine residues on an acceptor polypeptide [43]. To discard a potential interference from a 4-GlcNAc-epimerase activity (not demonstrated in *T. cruzi*, as far as we are aware), unambiguous evidence for GalNAc incorporation to the synthetic peptide acceptor substrate was obtained through the identification of GalNAc-ol by GLC–MS. In addition, the affinity-purified enzyme(s) from *T. cruzi* extracts displayed ppGalNAc-T activity. The results obtained using a panel of nine synthetic peptides, derived from parasite and human mucin-type glycoprotein motifs, suggest that the primary sequence of the acceptor site is a determining factor for the rate of *O*-glycosylation. The best acceptor substrates were peptide sequences derived from parasite glycoproteins, even those containing only threonine (*T. cruzi*) or serine (*S. mansoni*) residues that were *O*-glycosylated equally well.

Although the structure of *T. cruzi O*-linked glycans has been extensively studied, very little is known about their biosynthesis. As noted before, the only report so far available describes the biochemical characterization of a ppGlcNAc-T [11], an enzyme incorporating *O*-linked GlcNAc to peptide backbones. We characterized *T. cruzi* ppGalNAc-T activity using a peptide sequence derived from a *T. cruzi* mucin (KPTTTTTTTTPK), precisely the one that was previously used by Previato et al. [11] for the evaluation of ppGlcNAc-T activity. We found the ppGalNAc-T activity to be maximal at 28 °C and 5.5–6.6 pH units, whereas 37 °C and 7.5 pH units had been determined as the optimum conditions for ppGlcNAc-T. Similar to other ppGalNAc-Ts, the activity from *T. cruzi* epimastigotes requires divalent metal cations for activity, with Co^{2+} being the most effective. The difference between our data and those previously determined for ppGlcNAc-T indicates that they correspond to two different activities. In any case, since *T. cruzi* ppGlcNAc-T has neither been purified nor cloned yet, we cannot exclude the possibility that the same protein could display both activities.

Our results offer clear perspectives for future work and, even, may open new horizons for research on *T. cruzi* glycobiology. The expression sialyl-Tn, so far assessed by means of immunochemical techniques, should be confirmed through chemical analysis. It will also be of interest to study the immunogenicity of *T. cruzi* sialyl-Tn-bearing glycoproteins, their role in the host–parasite relationship. It has been suggested that

sialylation could influence parasite differentiation and/or migration in the insect, for example, by providing a signal for its detachment from the mucous membrane in *Triatominae* vectors [44]. In addition, it remains to be demonstrated whether the ppGalNAc-T activity observed in vitro plays any role in vivo. Since we purified two protein bands with ppGalNAc-T activity, attempts should be made to clone and characterize the gene(s) codifying for these proteins. Considering that each member of the ppGalNAc-T family has different substrate specificities and acts in a coordinate and sequential form [43], the molecular cloning and functional characterization of ppGalNAc-T as well as ppGlcNAc-T from *T. cruzi* could provide a basis to understand the regulation of *O*-glycan biosynthesis in this organism and its relevance along its life cycle.

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