Heterogeneity in the Biosynthesis of Mucin *O*-Glycans from *Trypanosoma cruzi* Tulahuen Strain with the Expression of Novel Galactofuranosyl-Containing Oligosaccharides†

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ABSTRACT: Sialoglycoprotein from *Trypanosoma cruzi* strains participates in important biological functions in which the O-linked glycans play a pivotal role, and their structural diversity may be related to the parasite's virulence pattern. To provide supporting evidence for this idea, we have determined the structure of novel linear and branched α-*O*-GlcNAc-linked oligosaccharides present on the mucins of the *T. cruzi* Tulahuen strain. The *O*-glycans were isolated as oligosaccharide alditols by reductive *β*-elimination, purified, and characterized by nuclear magnetic resonance spectroscopy and methylation analysis. Two core families were synthesized by the parasite: the Gal*f*β1→4GlcNAc and Gal*p*β1→4GlcNAc. The Gal*f*β1→4GlcNAc core yields three series of O-chain structures. In the first, the Gal*f* residue is nonsubstituted, while in the other series it is elongated by the activity of galactopyranosyl or galactofuranosyl transferases giving rise to Gal*p*-*β*-(1→2)-Gal*f*-*β*-(1→4) or Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→4) substructures not previously observed. The three series can arise by further galactopyranosylation of the GlcNAc O-6 arm. Sialylation was the only observed elaboration of the Gal*p*β1→4GlcNAc core family. Thus the determination of the structures of the *O*-glycans from *T. cruzi* Tulahuen mucins confirms the strain specificity of the glycosylation and predicts a relationship between it and parasite pathogenicity and the epidemiology of Chagas' disease.

Trypanosoma cruzi, the parasite responsible for Chagas' disease, infects 18−20 million people in South and Central America (*1*). *T. cruzi* is a heterogeneous group of strains that establish infection in a wide range of mammalian hosts, exhibiting tropism for different tissue types (*2*), varying in the pathology and clinical manifestation of infection, and leading to death or serious damage to the heart or digestive tract during its chronic phase (*3*). The causes of this wide variability are not known. However, recently a correlation between the clinical variations and the genetic diversities of *T. cruzi* was proposed (*4, 5*). Several grouping schemes for *T. cruzi* strains have been developed in order to understand the role of parasite diversity in the pathogenesis of the disease (*4*). On the basis of biochemical and molecular studies, it has been observed that *T. cruzi* strains can be divided into two major groups (*6*−*8*), which have been recently standardized as *T. cruzi* I and *T. cruzi* II (*9*). Current biological and epidemiological studies provide evidence for an association of *T. cruzi* II with the domestic cycle, mainly involved in human infection, whereas *T. cruzi* I is associated with the sylvatic cycle, affecting marsupials and edentates (*10*), and rarely and asymptomatically infects humans (*11*).

Presumably variability observed during infection by different *T. cruzi* strains is a result of diversity in parasite/host interactions resulting from variability of the macromolecules expressed on both the parasite and host cell surface. *T. cruzi* is an intracellular parasite and must invade cells of the vertebrate host in order to replicate and liberate infective forms (trypomastigotes) to complete its life cycle. Specific *T. cruzi* surface sialoglycoproteins, known as mucin-like molecules, are implicated in the interaction of the parasite with host cells and modulation of the host immune system (*12*, *13*). The protein expressed by *T. cruzi* mucin genes contains a short hypervariable N-terminal region, a threonine-(Thr-)[1] rich central domain where O-glycosylation occurs, and a C-terminus containing the GPI-anchor sequence. Recent data suggest that the *T. cruzi* mucins are stage-

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[1] Abbreviations: Thr, threonine; GPI, glycosylphosphatidylinositol; GalNAc, *N*-acetylgalactosamine; Ser, serine; GlcNAc, *N*-acetylglucosamine; Gal*f*, galactofuranose; Gal*p*, galactopyranose; Neu5Ac, *N*-acetylneuraminic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; PGC, porous graphitic carbon; GC, gas−liquid chromatography; NMR, nuclear magnetic resonance spectroscopy; Man, mannose; Glc, glucose; Ins, inositol; NOE, nuclear Overhauser enhancement; GlcNAc-ol, *N*-acetylglucosaminitol; ManNAc-ol, *N*-acetylmannosaminitol; HexNAc-ol, *N*-acetylhexosaminitol; ROESY, rotating frame NOE spectroscopy; TOCSY, total correlation spectroscopy.

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specific, being divided into two families (*14*). The first family contains in its central domain tandem-repeated regions. The extensive O-glycosylation of this molecule may lead to the production of the higher molecular mass (60−200 kDa) mucins found in the trypomastigotes. The second family lacks the repeats in the central domain and may form lower molecular mass mucins (35−50 kDa) expressed in all stages of the parasite.

Structural characterization of the *O*-glycans or *O*-sialylglycans from *T. cruzi* epimastigote mucins has been reported (*15*−*18*). Unlike mammalian mucins that contain an α-GalNAc residue as the sugar linked to Ser and/or Thr, in *T. cruzi* sialoglycoproteins the O-linked glycans are attached to the peptide by α-GlcNAc-*O*-Thr linkages (*19*). The α-GlcNAc-*O*-Thr of the *T. cruzi* O-linked oligosaccharides are elongated by *β*-Gal*f*, *β*-Gal*p*, and α-2,3-linked Neu5Ac in a strain-specific pattern of substitution (*15*−*18*). Among all of the strain-specific variations described, probably the most important is the presence of a *β*-Gal*f* residue attached to GlcNAc O-4 in G and Dm28c strains (*T. cruzi* I), while in the Y and CL-Brener strains (*T. cruzi* II) a *β*-Gal*p* residue is present. Indeed, there is a perfect match between the structure of O-linked glycans already studied and the two major parasite phylogenetic groups *T. cruzi* I and *T. cruzi* II.

In this work we describe the high structural diversity of the biosynthesis of *O*-glycans isolated from the mucins of *T. cruzi* Tulahuen strain, which expresses two core families of oligosaccharides with substitution of GlcNAc O-4 by either *β*-Gal*f* or *β*-Gal*p*. A series of not previously described structures was characterized. A biosynthetic route for *O*-glycans from *T. cruzi* Tulahuen mucin is discussed.

EXPERIMENTAL PROCEDURES

Parasite. The *T. cruzi* Tulahuen strain was obtained from our laboratory collection.

Isolation of Mucins from T. cruzi. *T. cruzi* Tulahuen epimastigotes were grown in brain heart infusion medium containing 5% fetal calf serum, supplemented with 10 mg/L hemin and 20 mg/L folic acid at 28 °C with shaking (100 rpm). After 7 days cells were harvested by centrifugation, washed three times with 0.9% NaCl, and frozen at −20 °C. Frozen cells were thawed and extracted three times with cold water. The pellet recovered by centrifugation was extracted with 45% (v/v) aqueous phenol at 75 °C. The aqueous phase of the phenol extract was dialyzed, lyophilized, redissolved in water, and applied to a column of Bio-Gel P-100 (2 × 60 cm). The excluded volume, containing carbohydrate, was lyophilized and suspended in chloroform/methanol/water (10:10:3 v/v). The insoluble glycoproteins in this solvent mixture, containing the mucin-like molecules, were analyzed by 15% SDS−PAGE.

Isolation and Purification of Oligosaccharide Alditols. The mucins were treated with 0.01 M NaOH in the presence of 0.3 M $NaBH_4$ at 37 °C for 48 h. The solution was neutralized and passed through Dowex 50W-X8 H^+ form and lyophilized. The residue was dissolved in deionized water, and the oligosaccharide alditols were fractionated on a Bio-Gel P-4 column (1 × 100 cm). Fractions of 1.25 mL were collected, and elution was monitored by spotting 5 μL portions onto a TLC plate and staining with orcinol−H_2SO_4 reagent and H_2SO_4 spray. The carbohydrate-positive fractions were further fractionated by HPLC on porous graphitized carbon (PGC; 7 m, 4.6 × 100 mm) (Lifescience International, Basingstoke, U.K.). An acetonitrile gradient to a final concentration of 20% (*20*) was used for the fractionation of small oligosaccharide alditols, while a final acetonitrile concentration of 30% was used for analytical runs and some preparative runs for late-eluting components. Analytical separations were monitored at 206 nm with typically 50 μg of carbohydrate loaded. Preparative separations were monitored at 215 nm with typically 1−1.5 mg of carbohydrate loaded.

Carbohydrate Analysis. The sugar composition was determined by methanolysis (*21*), in 0.5 M methanolic HCl, for 18 h at 80 °C. After hexane extraction the methyl glycosides were O-trimethylsilylated with bis(trimethylsilyl)-trifluoroacetamide/pyridine (1:1 v/v) for 1 h at room temperature. The products were analyzed by gas−liquid chromatography (GC) on a fused silica column of DB-1 (30 m × 0.25 mm i.d.) using hydrogen as carrier gas. The column temperature was programed from 120 to 240 °C at 2 °C/min.

Methylation Analysis. Oligosaccharide alditols were permethylated as described by Ciucanu and Kerek (*22*) and modified by Previato et al. (*23*). Permethylated samples were methanolyzed (0.5 M HCl in methanol, 18 h, 80 °C), and the products were dried under a stream of nitrogen, acetylated with acetic anhydride/pyridine (9:1) for 24 h at 25 °C, and analyzed by GC as described above. The O-acetylated, partially O-methylated methyl glycosides were identified by their retention time and by GC−mass spectrometry (24) and quantified by peak area.

NMR Methods. NMR spectra were obtained on a Varian Unity 500 spectrometer equipped with a 5 mm triple resonance probe at an indicated probe temperature of 30 °C, as previously described (*15*). Proton chemical shifts are referenced to internal TSP-d_4 at 0 ppm. The preparation and full NMR assignment of reference compounds have been described (*25*).

RESULTS

Isolation and Chemical Composition of Mucins from the T. cruzi Tulahuen Strain. Sialoglycoproteins from the *T. cruzi* Tulahuen strain were extracted with hot aqueous phenol and partially purified as described by Previato et al. (*15*), giving on SDS−PAGE analysis two broad bands with apparent molecular masses of 35 and 50 kDa, which were strongly stained by periodate Schiff reagent. The carbohydrate content of these glycoproteins was found to be 72%, containing as monosaccharides Gal, GlcNAc, Man, Glc, Neu5Ac, and Ins. Amino acid analysis of purified (*26*) sialoglycoprotein indicated that approximately 50% of the residues are Thr and only 0.9% Ser.

Release and Purification of O-Linked Glycans of Mucins. Treatment of *T. cruzi* sialoglycoproteins with sodium hydroxide under reducing conditions released the *O*-glycans, which were isolated as oligosaccharide alditols by chromatography on PGC, yielding 14 neutral carbohydrate-positive fractions (Figure 1). Because the column profile was too complex for preparative purification and structural studies, the *β*-elimination products were first subjected to gel filtration chromatography on a Bio-Gel P-4 column, giving five fractions, four containing neutral *O*-glycans and one contain-

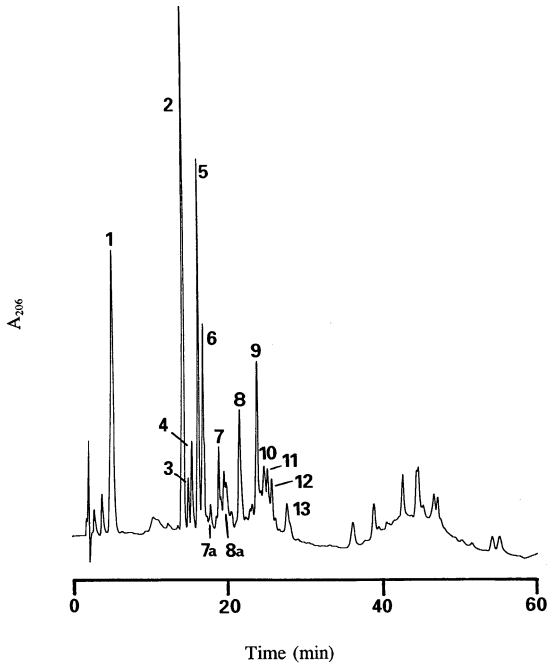

FIGURE 1: HPLC on porous graphitic carbon (PGC) of the total material released from the *T. cruzi* Tulahuen strain mucins using a gradient to 30% acetonitrile. Fractions isolated and characterized are numbered. Fraction 9 was found not to be carbohydrate.

ing sialylated *O*-glycans. One- and two-dimensional NMR analysis of these fractions allowed a large number of assignments to be made and some glycosidic linkages assigned from the patterns of interresidue NOEs, but it was apparent that each contained a number of distinct molecular species.

Structural Characterization. HPLC fractions were characterized by one- and two-dimensional homonuclear NMR, and by methylation analysis. The earliest eluting carbohydrate-containing fraction (Figure 1, peak 1) had an NMR spectrum corresponding to that of GlcNAc-ol (by comparison with the spectrum of authentic material) (*25*). Other fractions contained oligosaccharides in which the GlcNAc-ol was substituted at the O-4 position by either a β-Gal*f* or a β-Gal*p* unit, giving rise to two oligosaccharide alditol core families. As shown previously (*25*, *27*), unsubstituted or substituted GlcNAc undergoes facile base-catalyzed epimerization to the ManNAc analogue without apparent degradation and is trapped by borohydride reduction. We have therefore discussed the two homologues together, when appropriate, although they are often chromatographically resolved.

*Characterization of the Gal*p*-β-(1→4)-GlcNAc-ol Core Family.* NMR analysis of peak 2 (Figure 1) demonstrated the presence of two components as judged by the relative intensities of the anomeric proton resonance. The major component was identified as Gal*p*-β-(1→4)-GlcNAc-ol by comparison of the chemical shifts with those of an authentic standard (*16*), and this was confirmed by methylation analysis (Table 1). The minor component was identified as Gal*p*-β-(1→4)-ManNAc-ol, again by comparison with the spectrum of an authentic sample (*25*).

The only sialylated *O*-glycans, isolated from the Tulahuen mucin, were generated by sialylation of the Gal*p*-β-(1→4)-GlcNAc core. NMR assignments of the sialylated *O*-glycans obtained from chromatography on a Bio-Gel P-4 column showed that the presence of Neu5Ac-α-(2→3)-Gal*p*-β-(1→4)-GlcNAc-ol corresponded to that of an authentic sample prepared by sodium borohydride reduction of commercial 3′-sialyl *N*-acetyllactosamine. Traces of Neu5Ac-α-(2→3)-Galp-β-(1→4)-ManNAc-ol (Table 2) were also characterized.

*Characterization of the Gal*f*-β-(1→4)-GlcNAc-ol Core Family.* The NMR spectrum of peak 5 (Figure 1) showed a single intense anomeric signal with the small coupling constant ($^3J_{H1,H2}$ = 2.3 Hz) and low-field position (5.154 ppm) typical of a β-Gal*f* residue, and the HexNAc-ol was identified as GlcNAc-ol from the low-field H-2 resonance at 4.154 ppm, compared to 3.921 ppm for the HexNAc-ol H-2 in Gal*f*-β-(1→4)-ManNAc-ol (Table 2). The Gal*f*-β-(1→4)-GlcNAc-ol structure was confirmed by the presence of a terminal Gal*f* and a 4-O-substituted GlcNAc in the methylation analysis (Table 1).

Two disaccharide alditols, and their epimerization products, were identified in which the β-Gal*f* residue has been substituted, by the addition of either β-Gal*p* (Figure 1, peak 7) or β-Gal*f* (Figure 1, peak 8) units. The 1D ^{1}H NMR spectrum of peak 7 (Figure 1) showed the presence of a single β-Gal*p* anomeric resonance and a low-field unresolved anomeric resonance typical of the Gal*p*-β-(1→2)-Gal*f*-(β1→ system (*15*). The chemical shifts closely matched those of the Gal*p*-β-(1→2)-Gal*f*-β-(1→4)-GlcNAc-ol substructure found in the largest oligosaccharide from the *T. cruzi* G strain (*15*). An interresidue NOE was observed between the Gal*p* H-1 and the corresponding Gal*f* H-2 in the ROESY spectrum of the mixture obtained by gel filtration. The HexNAc-ol residue was identified as →4)-GlcNAc-ol from the relative positions of the H-2 and H-3 resonances (δ_H = 4.130 and 3.922 ppm, respectively). The Gal*p*-β-(1→2)-Gal*f*-β-(1→4)-GlcNAc-ol structure was confirmed by detection of a terminal Gal*p*, a 2-O-substituted Gal*f*, and a 4-O-substituted GlcNAc in the methylation analysis (Table 1). The assignments of this novel O-linked glycan are reported in Table 2. The 1D ^{1}H NMR spectrum of the epimer of this compound (Figure 1, peak 7a) contained a single high-field anomeric doublet (δ_H = 4.586 ppm, $^3J_{H1,H2}$ = 8 Hz) and a low-field unresolved anomeric proton (δ_H = 5.353 ppm) correlating to a low-field H-2 resonance (δ_H = 4.368 ppm) in the TOCSY spectrum. The HexNAc-ol residue was identified as →4)-ManNAc-ol from the positions of the H-2 and H-3 resonances (relatively high-field H-2 at δ_H = 4.034 and relatively low-field H-3 at δ_H = 3.979 ppm), compared, for example, with the data for the analogous compound containing GlcNAc-ol. The 1D ^{1}H NMR of peak 8 (Figure 2) showed two low-field unresolved anomeric protons (δ_H = 5.295 and 5.209 ppm) coupled to H-2 resonances at δ_H = 4.229 and 4.111 ppm, respectively. An interresidue NOE was observed in the ROESY spectrum between the anomeric resonance at 5.209 ppm and the H-2 of the other Gal*f* residue, consistent with the presence of a Gal*f*-β-(1→2)-Gal*f*-β-(1→ substructure. The HexNAc-ol was identified as →4)-GlcNAc-ol from the close agreement of its chemical shifts with those of this residue in the oligosaccharide alditol Gal*f*-β-(1→4)-GlcNAc-ol. The structure Gal*f*-β-(1→2)-Gal*f*-β-(1→4)-GlcNAc-ol was confirmed by the methylation analysis (Table 1). This is also a novel compound, and the assignments are reported in Table 2. The ^{1}H NMR spectrum of the epimer Gal*f*-β-(1→2)-Gal*f*-β-(1→4)-ManNAc-ol, eluted at peak 8a (Figure 1), showed the presence of two low-field unresolved anomeric reso-

Table 1: Methylation Analysis of PGC/HPLC-Purified Oligosaccharide Alditols[a]

partially methylated and acetylated methyl glycoside	O-linked oligosaccharides present in PGC chromatographic peaks (Figure 1)													linkage
	2	3	4	5	6	7	8a	8	10	11	12	Neu5Ac-containing *O*-glycans		
2,3,5,6-Gal*f*[b]			0.85	0.9	0.15		1.05	1.0	0.5	0.9	0.9			Gal*f*(1→
2,3,4,6-Gal*p*[b]	1.05					1.0	1.2		2.3	1.8	1.9			Gal*p*(1→
2,3,4,6-Glc*p*[b]		0.9			0.7									Glc*p*(1→
3,5,6-Gal[c]						1.1		1.1	0.4	1.0				→2)Gal*f*-(1→
2,4,6-Gal*p*[c]												1.3	1.0	→3)Gal*p*-(1→
4,6-Gal*p*[c]									1.0	1.0	1.0			→2,3)Gal*p*-(1→
1,2,3,5,6-GlcNAc-ol[d]	1.0			1.0	1.0	1.0		1.0				0.4	1.0	→4)GlcNAc-ol
1,2,3,5,6-ManNAc-ol[d,e]	0.2	1.0	1.0			0.1	0.4					1.0		→4)ManNAc-ol
1,2,3,5-GlcNAc-ol[d]							1.0		1.0	1.0	1.0			→4,6)GlcNAc-ol
1,2,3,5-ManNAc-ol[d,e]									0.2					→4,6)ManNAc-ol
4,6,7,8-Neu5Ac[f]												1.3	0.9	Neu5Ac(2→

[a] Partially methylated and acetylated methyl glycosides were analyzed by GC-MS, and results are expressed as molar proportion related to 2,3,6-HexNAc-ol set equal to 1.0. [b] 2,3,5,6-Gal*f* represents methyl 2,3,5,6-tetra-*O*-methylgalactofuranoside etc. [c] 3,5,6-Gal*f* represents methyl 3,5,6-tri-*O*-methyl-2-*O*-acetylgalactofuranoside etc. [d] 1,2,3,5,6-GlcNAc-ol represents 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methyl-*N*-acetylglucosaminitol etc. [e] ManNAc-ol derivatives are from GlcNAc epimerization during the reductive β-elimination reaction (*20*). [f] 4,6,7,8-Neu5Ac represents methyl-4,7,8,9-tetra-*O*-methyl-*N*-acetylneuraminic acid.

nances (δ_H = 5.277 and 5.197 ppm) consistent with the presence of a Gal*f*-β-(1→2)-Gal*f*-β-(1→ substructure. The HexNAc-ol was identified as →4)-ManNAc-ol by the chemical shift of the *N*-acetyl methyl resonance (δ_H = 2.032 ppm).

*Formation of Branched Oligosaccharide Alditols by Galactopyranosylation of the GlcNAc O-6 from the Gal*f*-*β*-(1→4)-GlcNAc-ol Core.* A series of oligosaccharides formed by β-galactopyranosylation of the GlcNAc O-6 of a Gal$f\beta$1−4GlcNAc core was observed. The alditols Gal*f*-β-(1→4)-[Gal*p*-β-(1→6)]GlcNAc-ol, Gal*f*-β-(1→4)[Gal*p*-β-(1→3)-Gal*p*-β-(1→6)]GlcNAc-ol, Gal*f*-β-(1→4)[Gal*p*-β-(1→3){Gal*p*-β-(1→2)}Gal*p*-β-(1→6)}GlcNAc-ol, and Gal*p*-β-(1→2)-Gal*f*-β-(1→4)[Gal*p*-β-(1→3){Gal*p*-β-(1→2)}Gal*p*-β-(1→6)}GlcNAc-ol were present in peaks 8a, 12, and 10 (Figure 1), respectively. These *O*-glycans were fully characterized in a previous study of the oligosaccharide alditols released from the *T. cruzi* G-strain mucins (*15*). The 1D ^{1}H NMR spectrum of the principal component of peak 13 (Figure 1) showed the presence of a Gal*f*-β-(1→2)-Gal*f*-β-(1→4)-GlcNAc-ol substructure and a Gal*p* H-1 resonance at a chemical shift consistent with assignment as the Gal*p*-β-(1→3)-Gal*p*-β-(1→6) system. The resonance from the anomeric proton of the terminal Gal*p*-β-(1→3) is visible at the expected chemical shift, but its intensity is reduced by its proximity to the HOD resonance, which has been subjected to presaturation. The structure of this novel *O*-glycan is Gal*f*-β-(1→2)Gal*f*-β-(1→4)[Gal*p*-β-(1→3)-Gal*p*-β-(1→6)]GlcNAc-ol, and the NMR assignments are reported in Table 2.

The 1D ^{1}H NMR (Figure 3) and TOCSY spectra (Figure 4) of peak 11 (Figure 1) showed the presence of two β-Gal*f* spin systems with chemical shifts typical of a Gal*f*-β-(1→2)-Gal*f*-β-(1→4) substructure, and three β-Gal*p* spin systems in a pattern typical of Gal*p*-β-(1→2)[Gal*p*-β-(1→3)]Gal*p*-β-(1→6). The HexNAc-ol residue was identified as →4,6)-GlcNAc-ol by its chemical shifts (Table 2) and is consistent with the presence of a novel compound: Gal*f*-β-(1→2)-Gal*f*-β-(1→4)[Gal*p*-β-(1→3){Gal*p*-β-(1→2)}Gal*p*β-(1→6)}-GlcNAc-ol. Also, minor components were detected in Figure 3. The presence of two Gal*f* spin systems was typical of a Gal*f*-β-(1→2)-Gal*f*-β-(1→4) substructure, while the chemical shift of the *N*-acetyl resonance (δ_H = 2.040 ppm) indicated the presence of Gal*f*-β-(1→4)-ManNAc-ol. Other resonances were assigned as arising from the branched Gal*p*-β-(1→3)-[Gal*p*-β-(1→2)]Gal*p*-β-(1→6) substructure (Table 2). The existence of these structures was consistent with the methylation analysis (Table 1) showing the presence of 2-O-substituted Gal*f* and derivatives corresponding to nonreducing terminal Gal*p* and Gal*f* in a molar ratio of 1.8:0.9.

*Characterization of the Glc-*β*-(1→4)-GlcNAc-ol Core: A Minor Component*. The NMR spectrum of the monosaccharide alditol fraction from gel filtration chromatography contained a number of resonances between 3.3 and 3.6 ppm characteristic of β-Glc residues. Two Glc-containing fractions were isolated by HPLC and characterized as Glc-β-(1→4)-ManNAc-ol (Figure 1, peak 3) and Glc-β-(1→4)GlcNAc-ol (Figure 1, peak 6) by one- and two-dimensional NMR (data not shown) and by methylation analysis (Table 1). The assignments are given in Table 2. No further elaboration of this core was detected.

DISCUSSION

Protozoan trypanosomatids of medical interest present on their surface elaborate glycoconjugates, such as the lipophosphoglycan (LPG) of *Leishmania* spp. and the variant surface glycoproteins (VSG) of *Trypanosoma brucei*, which are known to be involved in parasite virulence (*28*). In *T. cruzi*, the major external glycoconjugates are sialylated GPI-anchored mucin-like molecules (*16*). The sialic acid present on these molecules is transferred from host sialyl glycoconjugates (*23*) by a cell surface modified sialidase, *trans*-sialidase (*26*, *29*). *trans*-Sialidase activity is able to extensively remodel the *T. cruzi* cell surface. Alternatively, the enzyme may sialylate host cell glycomolecules to generate receptors used by the trypanosome for adherence to, and penetration of, target cells, playing an important role in parasite−host interactions. *T. cruzi* infects a broad range of hosts. In humans it causes Chagas' disease, which presents a wide variety of pathological manifestations such as cardiomyopathy, peripheral nervous system damage, or dysfunction of the digestive tract, often leading to megaesophagus and/or megacolon (*1*). These clinical variations may be

Table 2: ^{1}H NMR Data on the HPLC-Purified Oligosaccharide Alditols

	H-1	H-1′	H-2	H-3	H-4	H-5	H-6	H-6′	NAc
Neu5Ac-α-(2→3)-Gal*p*-*β*-(1→4)-ManNAc-ol									
Neu5Ac-α-(2→3)				1.794/2.761	3.704	3.842	3.648		2.031
→3)-Gal*p*-*β*-(1→3)	4.554		3.588	4.094	3.955	3.686	3.754	3.754	
→4)-ManNAc-ol			4.106	4.057	3.818				2.017
Gal*f*-*β*-(1→4)-GlcNAc-ol									
β-Gal*f*	5.154		4.147	4.094	4.092	3.828	3.724	3.648	
GlcNAc-ol	3.752	3.682	4.154	3.930	3.768	3.917	3.819	3.689	2.056
Gal*f*-*β*-(1→4)-ManNAc-ol									
β-Gal*f*	5.138		4.132	4.079	4.066	3.802	3.711	3.638	
ManNAc-ol	3.773	3.685	3.921	3.798	3.778	4.058	3.793	3.984	2.033
Gal*p*-*β*-(1→2)-Gal*f*-*β*-(1→4)-GlcNAc-ol									
t-Gal*p*-*β*-(1→2)	4.595		3.536	3.670	3.948				
→2)-Gal*f*-*β*-(1→4)-GlcNAc-ol	5.364		4.378	4.265	4.134	3.865	3.739	3.647	
GlcNAc-ol	3.746	3.669	4.130	3.922		3.930	3.782	3.707	2.057
Gal*p*-*β*-(1→2)-Gal*f*-*β*-(1→4)-ManNAc-ol									
t-Gal*p*-*β*-(1→2)	4.586		3.530	3.667	3.945				
→2)-Gal*f*-*β*-(1→4)-ManNAc-ol	5.353		4.368	4.240	4.079				
→4)-ManNAc-ol			4.034	3.979		3.950	3.802		2.031
Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→4)-GlcNAc-ol									
t-Gal*f*-*β*-(1→)	5.209		4.111	4.079	4.017	3.836	3.732	3.639	
→2)-Gal*f*-*β*-(1→4)	5.295		4.229	4.119	[4.119]	3.848	3.732	3.664	
GlcNAc-ol	3.748	3.672	4.140	3.924	[3.770]	3.922	3.818	3.696	2.057
Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→4)-ManNAc-ol									
t-Gal*f*-*β*-(1→2)	5.197		4.101	3.999					
→2)-Gal*f*-*β*-(1→4)-ManNAc-ol	5.277		4.218	4.069					
→4)-ManNAc-ol									2.032
Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→4)[Gal*p*-*β*-(1→3)-Gal*p*-*β*-(1→6)]GlcNAc-ol									
Gal*f*-*β*-(1→**2**)-Gal*f*-*β*-(**1**→4)	5.360		4.246	[4.24]	4.121				
Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→4)	5.213		4.111	4.091	4.012				
Gal*p*-*β*-(1→3)	4.619		3.610	3.662	3.922				
→3)-Gal*p*-*β*-(1→	4.479		3.721	3.824	4.189				
GlcNAc-ol									2.056
Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→4)[Gal*p*-*β*-(1→3)-{Gal*p*-*β*-(1→2)}Gal*p*-*β*-(1→6)]GlcNAc-ol									
→2)-Gal*f*-*β*-(1→4)	5.424		4.256	4.230	4.124	3.854			
t-Gal*f*-*β*-(1→2)	5.214		4.133	4.111	4.022				
t-Gal*p*-*β*-(1→2)	4.844		3.561	3.641	3.928				
t-Gal*p*-*β*-(1→3)	4.657		3.609	3.659	3.924				
→2,3)-Gal*p*-*β*-(1→6)	4.573		3.954	4.000	4.199				
→4,6)-GlcNAc-ol	3.672	3.752	4.141	3.932	[3.850	4.068]	3.786	4.184	2.057
Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→4)[Gal*p*-*β*-(1→3)-{Gal*p*-*β*-(1→2)}Gal*p*-*β*-(1→6)]ManNAc-ol									
t-Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→	5.203		4.144	4.118	4.084				
Gal*f*-*β*-(1→2)-Gal*f*-*β*-(1→	5.372		4.232	4.062					
Gal*p*-*β*-(1→2)-Gal*p*	4.805		3.531	3.644	3.927				
Gal*p*-*β*-(1→3)	4.662		3.618	3.662	3.926				
→2,3)-Gal*p*-*β*-(1→	4.600		3.938	4.002	4.202				
ManNAc-ol									
Glc*p*-*β*-(1→4)-GlcNAc-ol									
β-Glc*p*	4.506		3.335	3.454		3.295	3.978	3.646	
GlcNAc-ol	3.730	3.648	4.151	3.988	3.830				2.056

related, in part, to genetic aspects and the immune competence of the host, but it has been suggested that they are caused largely by the genetic diversity of *T. cruzi*. In addition, it is evident that the complex communication between parasite and host molecules determines the outcome of *T. cruzi* infection. The sialoglycoproteins expressed on the cell surface of *T. cruzi* are thought to be involved in parasite−host interaction and escape from the host immune system (*30*). Structural characterization of *O*-glycans from the *T. cruzi* epimastigote mucins has demonstrated strain-specific variations (*15*, *18*) that may be phylogenetic markers, in agreement with the clustering of *T. cruzi* strains (*T. cruzi* I and II) based on molecular and zymodeme analysis (*6*). Strains expressing Gal*f*-containing mucins (G, Dm28c) group together in *T cruzi* I, and strains whose mucins lack Gal*f* are in *T cruzi* II. In this paper we report the structures of the O-linked oligosaccharides released from the GPI-anchored mucin from the *T. cruzi* Tulahuen strain, classified as *T. cruzi* I (*9*). Several oligosaccharides are described, many of which are novel (Figure 5). The oligosaccharide alditols were purified by a combination of chromatographic techniques including gel filtration and HPLC on PGC (*20*). The primary structures of the oligosaccharide alditols were characterized by NMR spectroscopy and methylation analysis.

By comparison of the structures of the O-linked oligosaccharide chains described in this work with those previously investigated from other *T. cruzi* strains, two distinct patterns were observed. While in the Y- and CL-Brener strains the substituent on the GlcNAc-ol O-4 is *β*-Gal*p*, in the G strain and the Dm28c clone it is *β*-Gal*f*. While all of these strains then add a *β*-Gal*p* residue to the GlcNAc O-6, in the Y- and CL-Brener strains further extension of the 6-arm occurs by

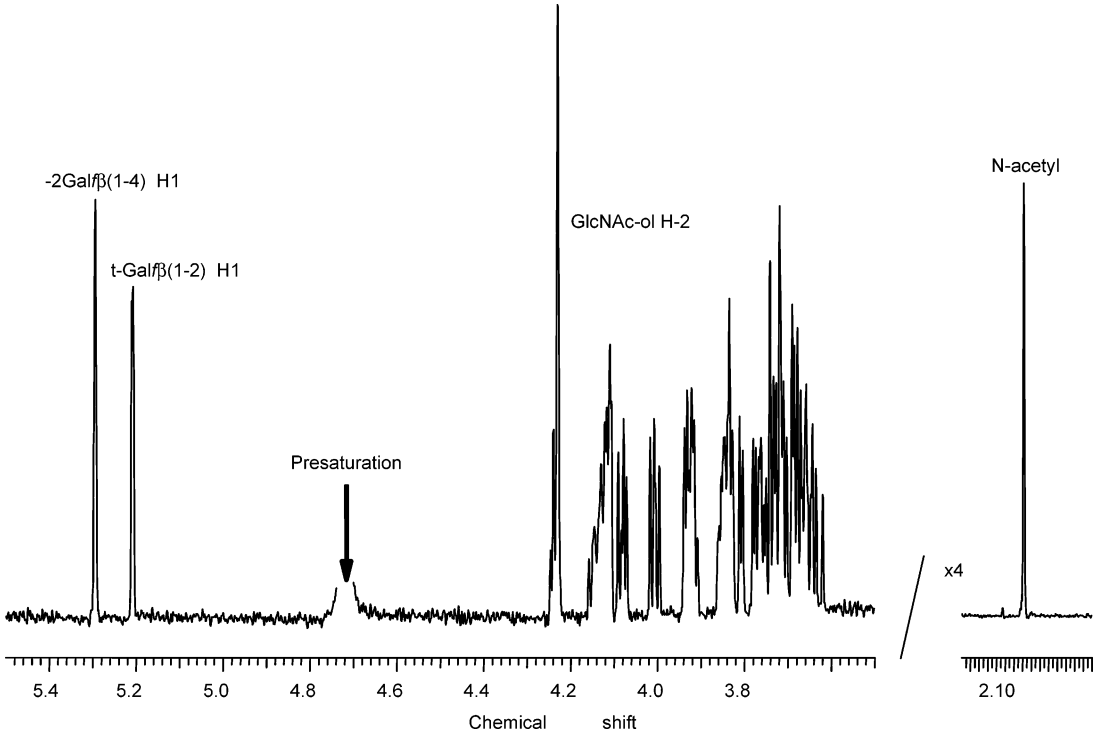

FIGURE 2: One-dimensional 500 MHz ^{1}H NMR spectrum of the novel alditol Gal*f*-β-(1−2)-Gal*f*-β-(1−4)-GlcNAc-ol. The key resonances are labeled.

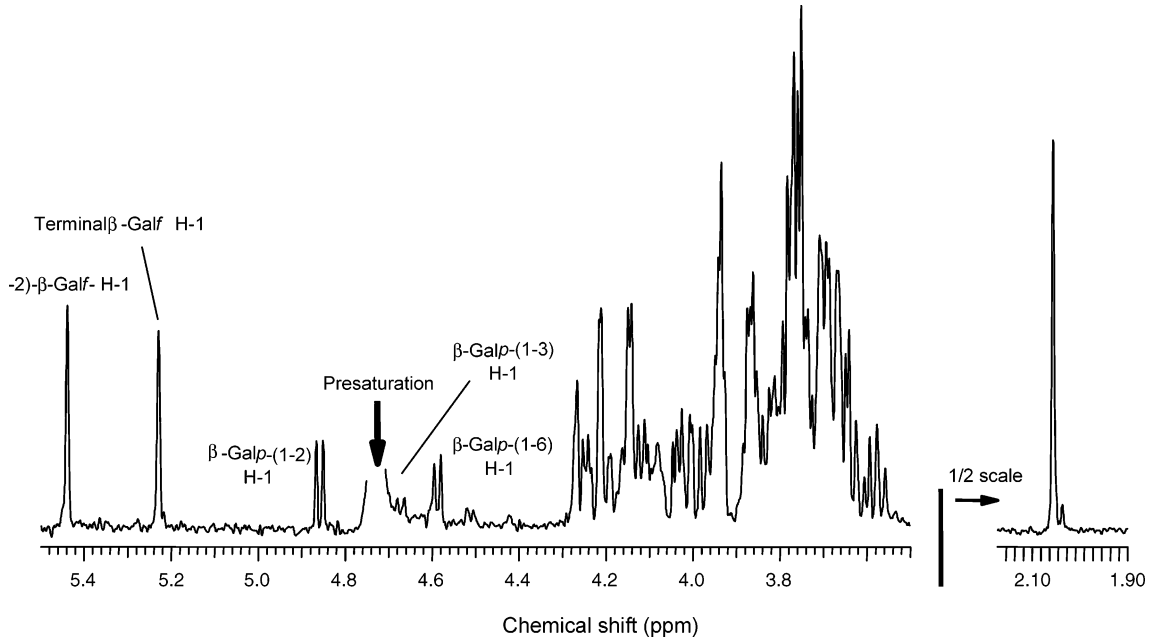

FIGURE 3: One-dimensional 500 MHz ^{1}H NMR spectrum of the novel alditol Gal*f*-β-(1−2)-Gal*f*-β-(1−4)-[Gal*p*-β-(1−2){Gal*p*-β-(1−3)}-Gal*p*-β-(1−6)]GlcNAc-ol. Key resonances are labeled.

addition of one or more Gal*p*-β-(1→2) residues, while in the G strain the next Gal*p* added is Gal*p*-β-(1→3), leading to a branched Gal*p*-β-(1→3)[Gal*p*-β-(1→2)]Gal*p*-β-(1→6) substructure. The O-glycosylation pattern of the mucin from the Tulahuen strain is much more complex than in the other *T. cruzi* strains, forming both core families with substitution of the GlcNAc-ol O-4 by either β-Gal*p* (Figure 5A) or β-Gal*f* (Figure 5B). The Gal*f*-β-(1→4)-GlcNAc-ol core is further elaborated by the action of galatopyranosyl or galactofuranosyl transferases, yielding three series of structures containing unsubstituted Gal*f*, Gal*p*-β-(1→2)-Gal*f*-β-(1→4), or Gal*f*-β-(1→2)-Gal*f*-β-(1→4) (Figure 5B). These substructures have not previously been observed. Further elaboration at the GlcNAc-ol O-6 (Figure 5) can occur.

These structural studies point to the participation of a large number of glycosyltransferases in *O*-glycan biosynthesis in *T. cruzi*. The high *O*-glycan diversity found in these mucins may involve either competition among glycosyltransferases for the same substrate, the concentration of donor substrates, or modulation of transferase activity due to neighboring glycosylation sites. In this context, the length (*31*) and the structure of core I glycans from porcine submaxillary mucin

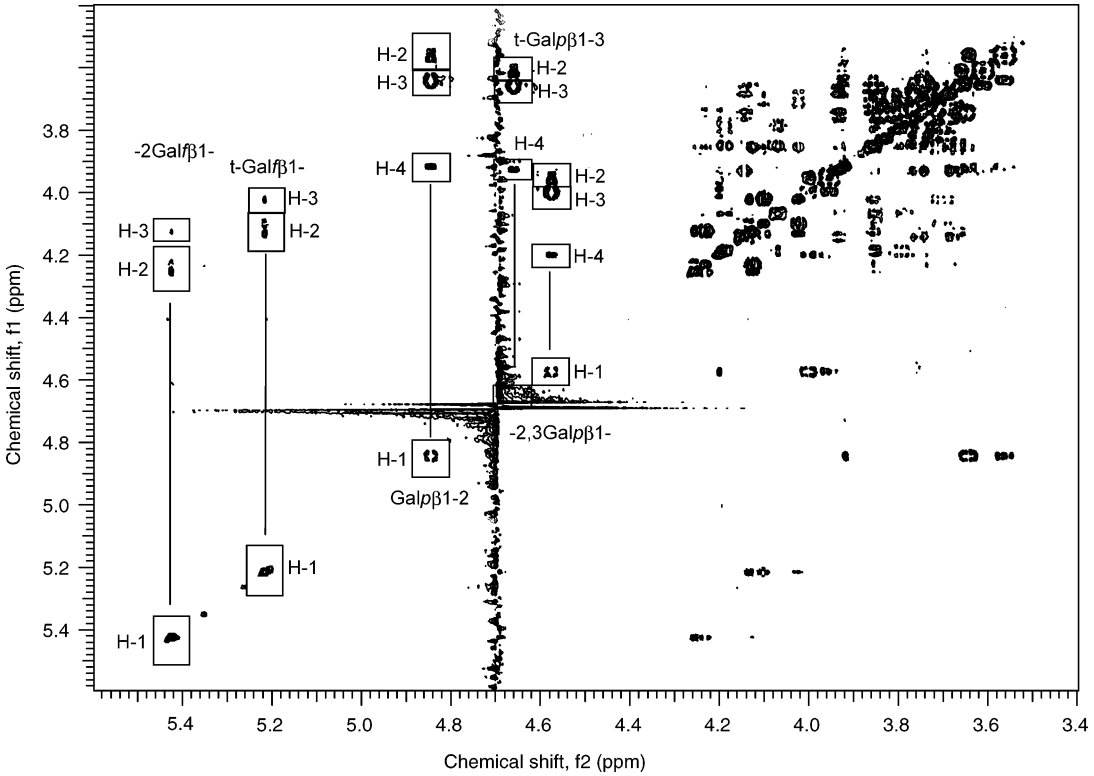

FIGURE 4: Partial 500 MHz TOCSY spectrum of the novel alditol Gal*f*-β-(1−2)-Gal*f*- β-(1−4)-[Gal*p*-β-(1−2){Gal*p*-β-(1−3)}Gal*p*-β-(1−6)]GlcNAc-ol. The spin systems from the two β-Gal*f*, three β-Gal*p*, and the GlcNAc-ol residue are labeled.

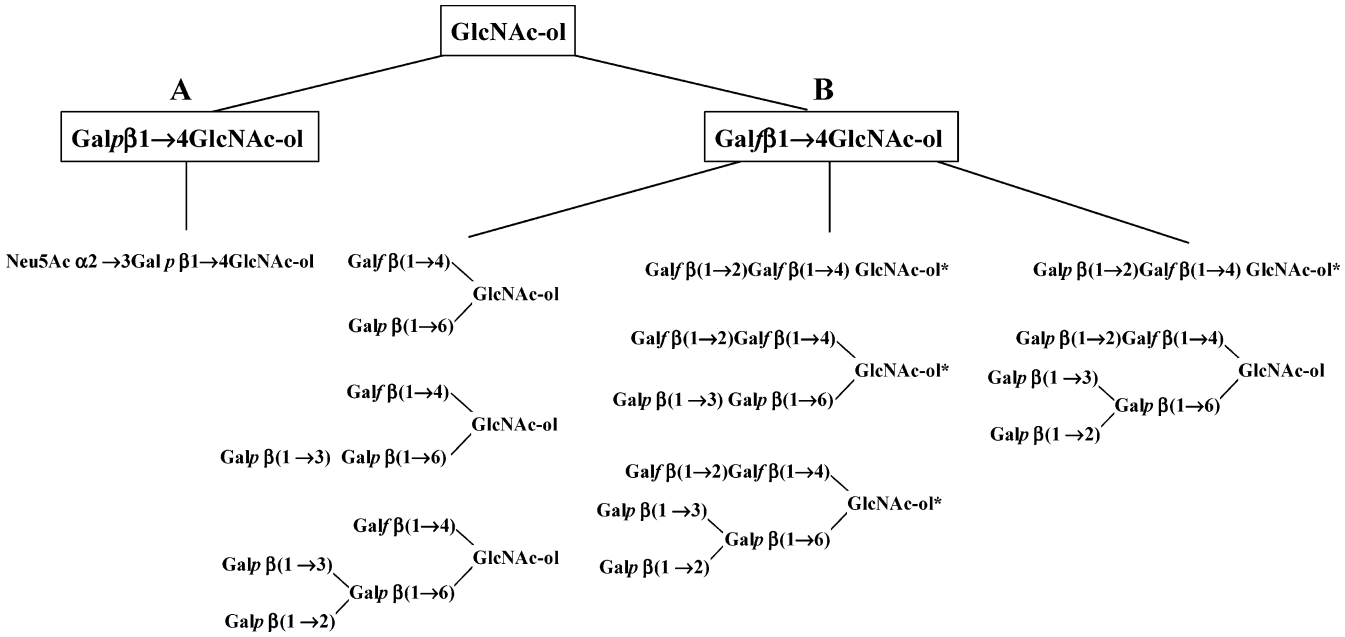

FIGURE 5: Diagrammatic representation of the oligosaccharide alditol structures isolated from the *T. cruzi* Tulahuen strain. (A) shows the oligosaccharides derived from the Gal*p*-β-(1−4)-GlcNAc-ol core and (B) the oligosaccharides derived from the Gal*f*-β-(1−4)-GlcNAc-ol core. Asterisks indicate new *O*-glycan structures.

have been suggested to be modulated by the peptide/glycopeptide sensitivities (*32*, *33*), a feature not yet explored in *T. cruzi* mucins. However, these observations could, in part, explain the high abundance of unsubstituted GlcNAc, Gal*p*-β-(1→4)GlcNAc, and Gal*f*-β-(1→4)GlcNAc (Figure 1, peaks 1, 2, and 5, respectively) in mucins from the Tulahuen strain.

The biosynthesis of Gal*f* in parasites deserves further attention since mammalian hosts do not produce glycoconjugates containing this residue. On account of this, exposed Gal*f* residues are highly immunogenic (*34*). It was found that chronic chagasic sera recognize Gal*f*-containing glycoproteins (*35*, *36*). Furthermore, antibodies against terminal β-Gal*f* epitopes inhibit *T. cruzi* internalization into mammalian cells (*37*). These findings point to Gal*f* as an antigenic epitope involved in parasite inactivation by immune mammalian hosts. As environmental and immunological factors are likely to influence the survival of strains after infection, the presence of β-Gal*f*-containing mucins may correlate with the epidemiological studies suggesting an association of the

T. cruzi II group with human infection, while *T. cruzi* I strains are rarely pathogenic and usually asymptomatic in humans (*10*, *11*). Interestingly, as suggested by Di Noia et al. (*38*), recognition of *T. cruzi* mucins by the monoclonal antibody 10D8 correlates with the presence of a β-Gal*f* epitope in *T. cruzi* mucin since it reacts with Tulahuen 35−50 kDa bands and failed to detect Y- and CL-Brener strains.

The metabolic pathway involved in the transference of the Gal*f* unit to *T. cruzi* glycoconjugates has not been elucidated. Recently, Mikusova et al. (*39*) demonstrated that transfer of Gal*f* to galactan from the mycobacterial cell wall is catalyzed by a galactofuranosyl transferase which uses UDP-Gal*f* as donor. The UDP-Gal*f* is generated from UDP-Gal*p* by a UDP-Gal*p* mutase. This area is of growing interest since UDP-Gal*p* mutase is not present in host cells and can be a target for specific inhibitors. The mutase and transferase enzymes involved in the biosynthesis of Gal*f*-containing glycoconjugates from *T. cruzi* and their importance for the establishment of infection deserve further study.

A β-galactopyranosyl transferase that specifically catalyzes the addition of β-Gal*p* to β-GlcNAc O-4 to give rise to the Gal*p*-β-(1→4)-GlcNAc core was characterized in the *T. cruzi* Y strain (*40*). In the Tulahuen strain, the Gal*p*-β-(1→4)-GlcNAc core is the only characterized acceptor for sialic acid. Neu5Ac-α-(2→3)-Gal*p*-β-(1→4)-GlcNAc-ol was the major sialylated product isolated in this work, while a trisaccharide alditol Gal*f*-β-(1→4)[Neu5Ac-α-(2→3)Gal*p*-β-(1→6)]GlcNAc-ol was isolated from the Dm28c strain (*18*). The digalactopyranosyl parent of the monosialylated glycans Gal*p*-β-(1→4)-[Neu5Ac-α-(2→3)-Gal*p*-β-(1→6)]-GlcNAc-ol and Gal*p*-β-(1→6)[Neu5Ac-α-(2→3)Gal*p*-β-(1→4)]GlcNAc-ol obtained from the CL strain (*17*) was not observed in the Tulahuen strain. The concomitant presence of both α-2→3-linked sialic acid and terminal β-Gal*p* residues may have biological implications in the *T. cruzi*−host cell interaction. Recently, we demonstrated that the inactive *trans*-sialidase also expressed on the *T. cruzi* surface (*41*) is a lectin containing two sugar binding sites, one for α-2,3-sialic acid-containing molecules (42) and a second for β-galactosides (*43*). Thus exposure of β-Gal*p* residues would favor enzymatically inactive *trans*-sialidase-mediated interaction with both host sialoglycoconjugates and terminal β-Gal*p*-containing glycoproteins on the parasite surface, thereby enhancing *T. cruzi*−host adhesion. In this scenario, the presence of terminal β-Gal*f* would hamper lectin−*trans*-sialidase-mediated interaction. This hypothesis is in agreement with the results observed by Ruiz et al. (*44*), who found that *T. cruzi* II strains, CL and Y, are more infective, in vitro, than *T. cruzi* I strains, such as Tulahuen, G, and DM28c.

In conclusion, in this work we demonstrate that structural analysis of the *O*-glycan chains from mucin-like molecules of different *T. cruzi* strains and consequently differences in the expression and activity of glycosyltransferases that are involved in the generation of distinct carbohydrate epitopes may provide a link between genetic diversity and the ability of these strains to infect different hosts and cause disease.

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