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An anionic synthetic sugar containing 6-SO_3-NAcGlc mimics the sulfated cruzipain epitope that plays a central role in immune recognition

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Cruzipain (Cz), the major cysteine proteinase of *Trypanosoma cruzi*, is a glycoprotein that contains sulfated high-mannose-type oligosaccharides. We have previously determined that these sulfate groups are targets of specific immune responses. In order to evaluate the structural requirements for antibody recognition of Cz, a systematic structure–activity study of the chemical characteristics needed for antibody binding to the Cz sulfated epitope was performed by immunoassays. With this aim, different synthesized molecules were coupled to the proteins BSA and aprotinin and confronted with (a) mouse sera specific for Cz and its carboxy-terminal (C-T) domain, (b) antibodies raised in rabbits immunized with Cz and its C-terminal domain and (c) IgGs purified from human Chagas disease sera. Our results indicate that a glucosamine containing an esterifying sulfate group in position O-6 and an N-acetyl group was the preferred epitope for the immune recognition of sera specific for Cz and its C-T domain. Although to a minor extent, other anionic compounds bearing sulfate groups in different positions and number as well as different anionic charged groups including carboxylated or phosphorylated monosaccharides, disaccharides and oligosaccharides were recognized. In conclusion, we found that synthetic anionic sugar conjugates containing *N*-acetyl D-glucosamine-6-sulfate sodium salt (GlcNAc6S) competitively inhibit the binding of affinity purified rabbit anti-C-T IgG to the C-T extension of Cz. Extending these findings to the context of natural infection, immune assays performed with Chagas disease serum confirmed that the structure of synthetic GlcNAc6S mimics the N-glycan-linked sulfated epitope displayed in the C-T domain of Cz.

Introduction

Chagas disease, also known as American trypanosomiasis, is caused by the intracellular parasitic protozoan *Trypanosoma cruzi* and represents an important health problem in Latin America. The Pan American Health Organization estimates that 7.7 million people currently have infection in the 21 endemic countries,

Abbreviations

CP, cysteine proteinase; C-T, carboxy-terminal; Cz, cruzipain; Di 1S, monosulfated disaccharide; Di 2S, disulfated disaccharide; GalA, D-galacturonic acid; Glc6*P*, glucose 6-phosphate; GlcN2S, D-glucosamine 2-sulfate sodium salt; GlcN6S, D-glucosamine 6-sulfate sodium salt; GlcNAc2S, *N*-acetyl D-glucosamine-2-sulfate sodium salt; GlcNAc6S, *N*-acetyl D-glucosamine-6-sulfate sodium salt; Hep, heparin; PGO, non-sulfated oligogalacturonates; PGS, sulfated oligogalacturonates.

which have a total population of 532 million people [1]. Cruzipain (Cz), also known as cruzain or GP57/51 [2], is the major cysteine proteinase (CP) of *T. cruzi*. Natural Cz is expressed as a complex mixture of isoforms by the different developmental stages of the parasite, presenting microheterogeneities [3]. The expression of some Cz isoforms is developmentally regulated [4]. Taking into account that structural diversification of *T. cruzi* proteins is due to gene polymorphism, the changes of a few amino acid residues cause significant differences in their enzymatic specificity, while other substitutions alter the position of N-linked glycosylation sites [5].

This lysosomal enzyme, like all type I CPs from trypanosomatids studied so far, is encoded by multicopy genes arranged in tandem [6] and bears, in addition to a catalytic moiety with high homology to other enzymes belonging to the papain family, a carboxy-terminal (C-T) extension which is retained in the mature protein. This domain contains a number of post-translational modifications [7] and is responsible for the immunodominant antigenic character of the molecule in natural and experimental infections [8–11]. Cz is a glycoprotein which has three potential N-glycosylation sites: the catalytic domain with two N-glycosylation sites, the first bearing high-mannose-type oligosaccharides, and the C-T domain, N-glycosylated at Asn255, carrying high-mannose, hybrid monoantennary or complex biantennary oligosaccharide chains [12–14]. This major CP is a parasite virulence factor [15–17], relevant in the host/parasite relationship which has been studied as a candidate for vaccine development [18] and considered a very promising target for chemotherapy of the disease [19,20]. Early reports showed two types of post-translational modifications involving carbohydrates: a complex N-glycosidic oligosaccharide bearing sialic acid and single *N*-acetyl-glucosamine residues with an O-glycosidic linkage [21]. The use of UV-MALDI-TOF MS analysis allowed us to determine the presence of sulfated high-mannose-type oligosaccharides on the unique N-glycosylation site of the C-T domain as a new striking feature in this glycoprotein. Sulfated $GlcNAc_2Man_3$ to $GlcNAc_2Man_9$ species were identified. The presence of a growing series of sulfated high-mannose species suggested that the sulfate group is located on the chitobiosyl core in accordance with the fact that usually the modifications are present on this unit when the core glycan structure is substituted. These findings evidenced for the first time the presence of sulfated glycoproteins in trypanosomatids [14].

Sulfation is a critical modification in many instances of biological recognition. Sulfated oligosaccharides play diverse relevant roles in development, differentiation and homeostasis [22]. Some of them, such as heparan sulfate or heparan sulfate-like glycans, were shown to play roles in binding of growth factors to receptors [23,24] and viral adhesion to the cell surface [25,26]. In *T. cruzi,* sulfated structures have been described as part of glycolipids representing common antigens on the surface of the parasite and mammalian cells [27–29]. The presence of sulfate groups in N-linked oligosaccharides has been reported in virus [30] and especially in mammalian cells [31,32]. Sulfated N-linked oligosaccharides have been mainly implicated in several molecular recognition processes [32–34]. Interestingly, we have recently reported the antigenic properties of these structures, showing that esterified sulfate groups are absolutely required for eliciting IgG2b responses to Cz in BALB/c mice evidencing that sulfate-bearing glycoproteins in trypanosomatids are targets of specific humoral immune responses. Furthermore, we showed that subjects chronically infected with *T. cruzi* mount specific humoral immune responses to this sulfated glycoprotein [35]. Sulfated high-mannose-type glycans, a component of lysosomal hydrolases from *Dictyostelium discoideum*, have also shown antigenic properties [36,37].

A detailed structural study of the requirements of this peculiar antigenic determinant of the molecule would be of relevance to elucidate the immunogenic relationship between the major CP of the parasite and the host humoral immune responses along *T. cruzi* infection. Thus, the influence of the type, the amount and the localization of the anionic charged group in this particular antigenic oligosaccharide determinant involved in the immune response to Cz was addressed. To achieve this goal, anionic charged oligosaccharides obtained from heparin and pectin, as well as different anionic charged structures (sulfated and/or different selected anionic charged structures including carboxylated or phosphorylated monosaccharides, disaccharides and oligosaccharides), were tested, alone or coupled to a protein moiety by competition assays, dot-blot and ELISA, using as tools mouse and rabbit sera specific for Cz and the C-T domain prior to and after desulfation treatment, human sera from Chagas disease patients and purified IgGs from patients and rabbit immune sera. Taken together, the results obtained clearly demonstrate that a 6-sulfate *N*-acetylglucosamine structure is the best-liked carbohydrate epitope recognized by antibodies specific for Cz and the C-T domain and by IgGs purified from Chagas disease patients' sera.

Results

The structures of sulfated N-linked oligosaccharides have been reported for a few specific proteins. We have already determined that sulfate groups present in the C-T domain of Cz are involved in the antigenicity of the molecule [35]. In order to get deeper into the characteristics of this charged epitope, several structures were synthesized, coupled to a protein carrier and biologically tested with rabbit and mouse polyclonal sera specific for Cz and the C-T domain prior to and after desulfation treatments as well as with Chagas disease patients' sera.

Synthetic sugar derivatives

The synthesis of monosaccharides was performed as described in Doc. S1 [38–40]. The procedure is summarized in Scheme S1.

The monosulfated disaccharide (Di 1S) was prepared as reported by Bultel *et al.* (2009) [41]. A disulfated disaccharide (Di 2S) was synthesized in high yield from methyl maltoside using a classical sequence. The procedure is described and summarized in Scheme S2.

Preparation of sulfated oligosaccharides from heparin

Heparin is a natural highly sulfated polysaccharide. This polysaccharide is composed of units of D-glucuronic acid or L-iduronic acid and *N*-acetyl glucosamine or glucosamine *N*-sulfate. The positions 2 from L-iduronic acids and/or 6 from glucosamines are sulfated. The sulfated oligosaccharides (Scheme S3, DegHep) were obtained by chemical degradation (deamination with nitrous acid and reduction). Nitrous acid chemical degradation of heparin (Hep) and heparan sulfate (HS) has been widely used to produce oligosaccharides. N-sulfated residues are selectively cleaved at pH 1.5 leading to 2,5-anhydromannose terminal units, while N-acetylated domains are preserved and O-sulfate groups are not affected. After reduction, the 2,5-anhydromannoses were converted to 2,5-anhydromannitol units. The oligosaccharides were fractionated according to chain length and charge (high pH anion exchange chromatography with pulsed amperometric detection) as previously reported [42].

Preparation of oligogalacturonates (PGOs) and sulfated oligogalacturonates (PGSs)

A mixture of PGOs was obtained by acid hydrolysis of pectin followed by precipitation in isopropanol–water. PGOs were converted to their *N*,*N*-diisopropylammonium salts and sulfated with $SO_3.NMe_3$ complex in dimethylformamide. Three different proportions of the sulfating reagent (equiv./free OH) were used in order to obtain different sulfate contents: 0.4, 0.6 and 1 sulfate per monosaccharide unit (Scheme S3). These compounds were used to study whether different sulfated structures were also recognized. The resulting oligosaccharides are only characterized in terms of average sulfate content per sugar unit; nevertheless they allow the influence of the total sulfate content on the recognition to be analyzed.

Synthesis of sugar–protein conjugates

Different proteins bearing different numbers of sugar residues and of different molecular weights were used and different reaction conditions were tried in order to obtain appropriate sugar–protein conjugates. Analysis by UV-MALDI-TOF MS was performed to determine the conjugate molecular weights in order to calculate the number of sugar units linked to the protein. In Fig. 1, UV-MALDI-TOF mass spectra performed with methyl 6-amino-6-deoxy-α-D-glucopyranoside coupled to BSA using different sugar : protein relationships (Fig. 1A) and different reaction times (Fig. 1B) are shown. Figure 2 shows UV-MALDI-TOF mass spectra of different sugars coupled to aprotinin under the best conditions found.

Evaluation of specificity in the recognition of different sulfated epitopes

Immune reactivity of sulfated (PGS) and non-sulfated (PGO) oligogalacturonates

Dot-blot assays showed that polyclonal mouse serum specific for the C-T domain was able to react with PGS in a dose-dependent way (Fig. 3A). Anti-C-T sera showed very low antibody binding to PGO. Similar results were obtained when serum specific for Cz was used (data not shown). It is worth noting that PGS, PGO and maltotetraose (a non-charged oligosaccharide) bear α-linkages; however, the latter presented total absence of recognition under the same conditions, indicating that at least a charged group (–COO^-, $-SO_3^-$) is required.

Immune recognition of heparin, partially degraded heparin and monosulfated and disulfated disaccharides

No cross-reactivity was found between Cz and heparin (Fig. 3B). However, when a sample of partially degraded heparin was tested with polyclonal mouse sera specific for Cz by dot-blot assays, some reactivity was observed. The structures of Di 1S, Di 2S and PGS are very different, however, all the them were recognized by mouse sera but to different degrees; all of

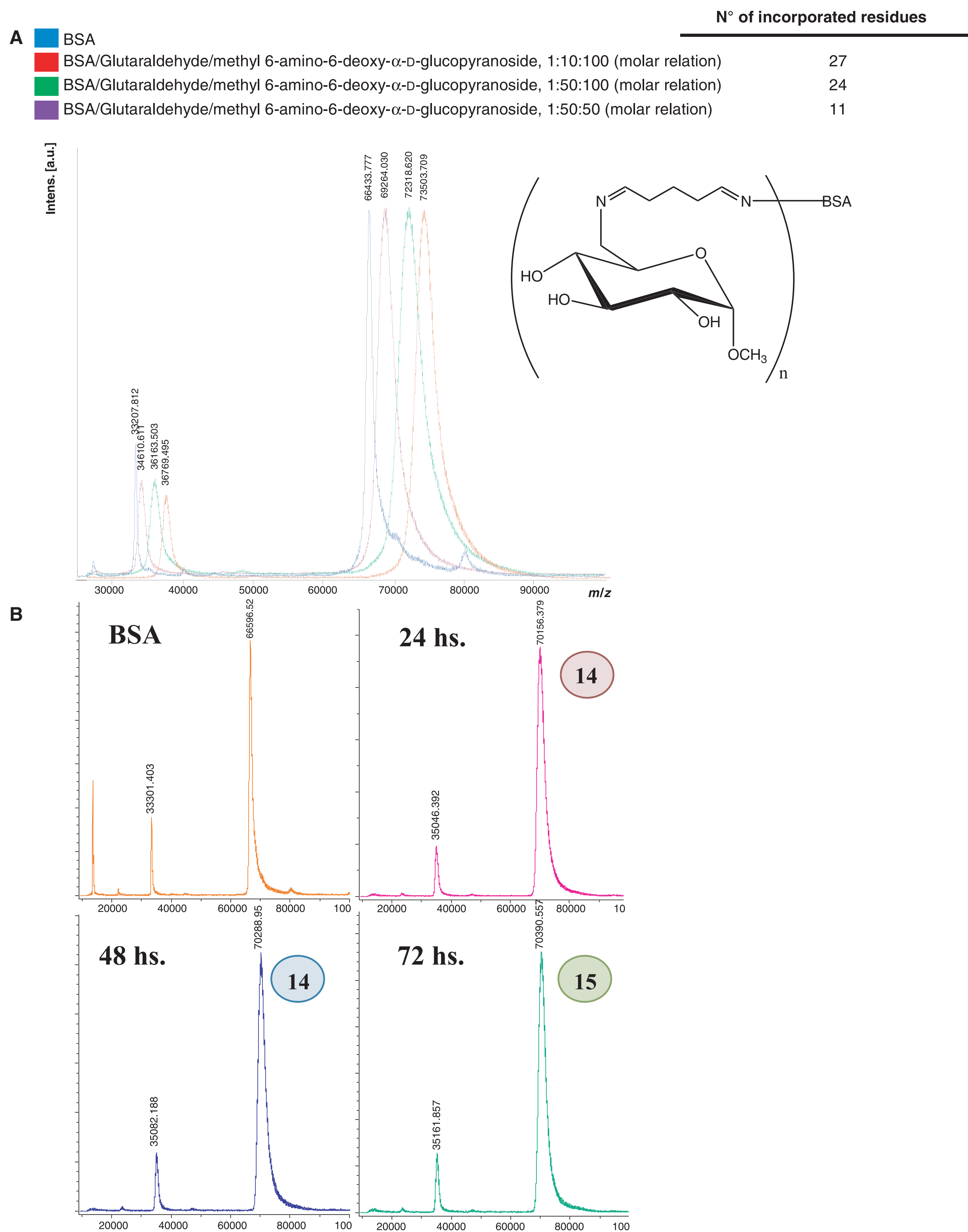

Fig. 1. UV-MALDI-TOF MS analysis of the sugar–BSA conjugates. (A) Methyl 6-amino-6-deoxy-α-D-glucopyranoside linked to BSA using different sugar/glutaraldehyde/protein ratios. (B) Methyl 6-amino-6-deoxy-α-D-glucopyranoside linked to BSA using a 1 : 50 : 50 BSA/glutaraldehyde/sugar molar ratio at different reaction times. The number of sugar residues attached per molecule BSA is given in the circles. Untreated BSA was used as negative control.

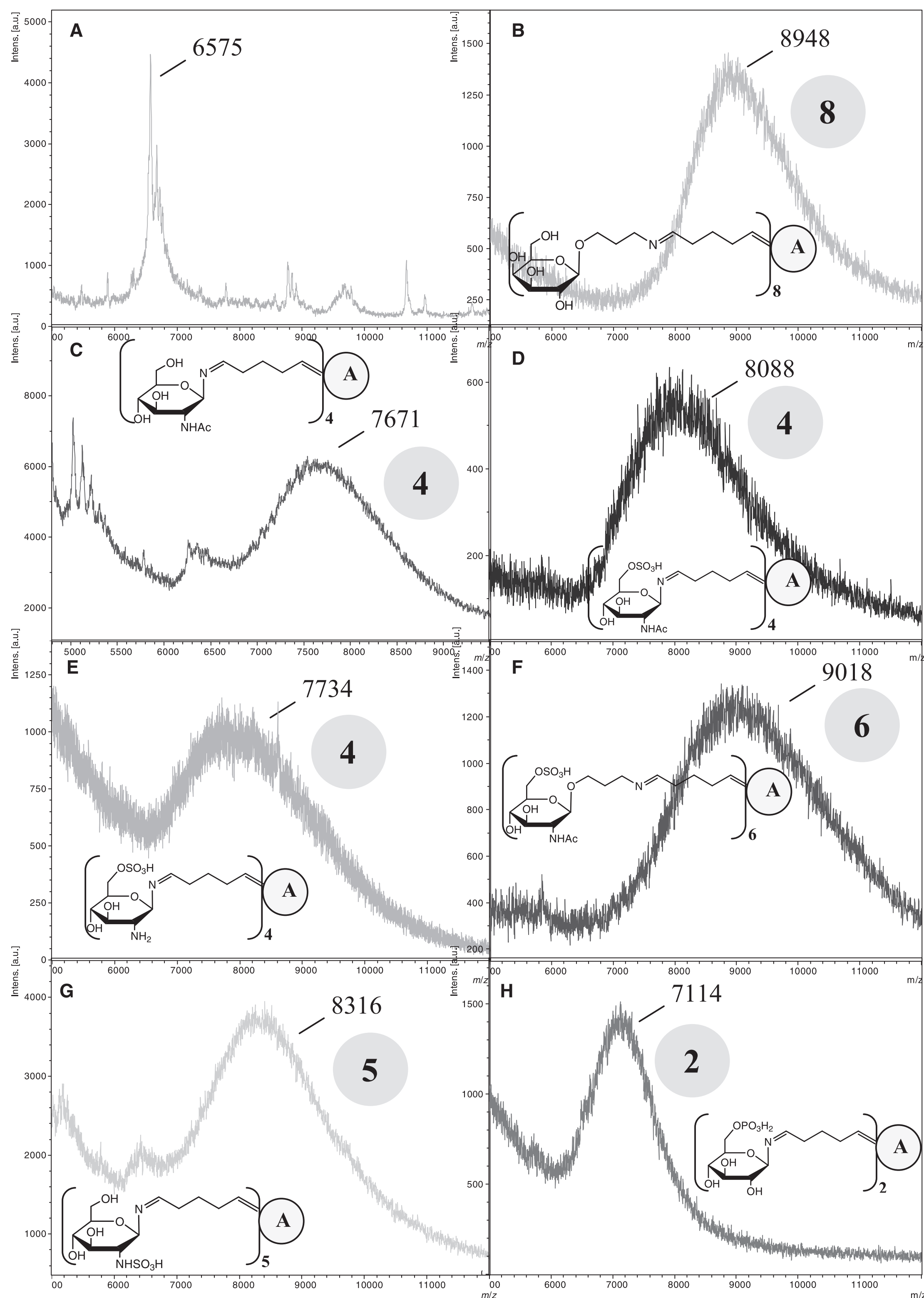

Fig. 2. UV-MALDI-TOF MS analysis of the sugar–aprotinin conjugates: (A) aprotinin; (B) GalA + aprotinin; (C) *N*-acetyl D-glucosamine + aprotinin; (D) GlcNAc6S + aprotinin; (E) D-glucosamine 6-sulfate (GlcN6S) + aprotinin; (F) 3-aminopropyl 2-acetoamido-2-deoxy-6-O-sodium sulfonato-beta-D-glucopyranoside (compound 3) + aprotinin; (G) GlcN2S + aprotinin; (H) Glc6*P* + aprotinin. The number of sugar residues attached per molecule is given in the circles. Aprotinin was used as negative control.

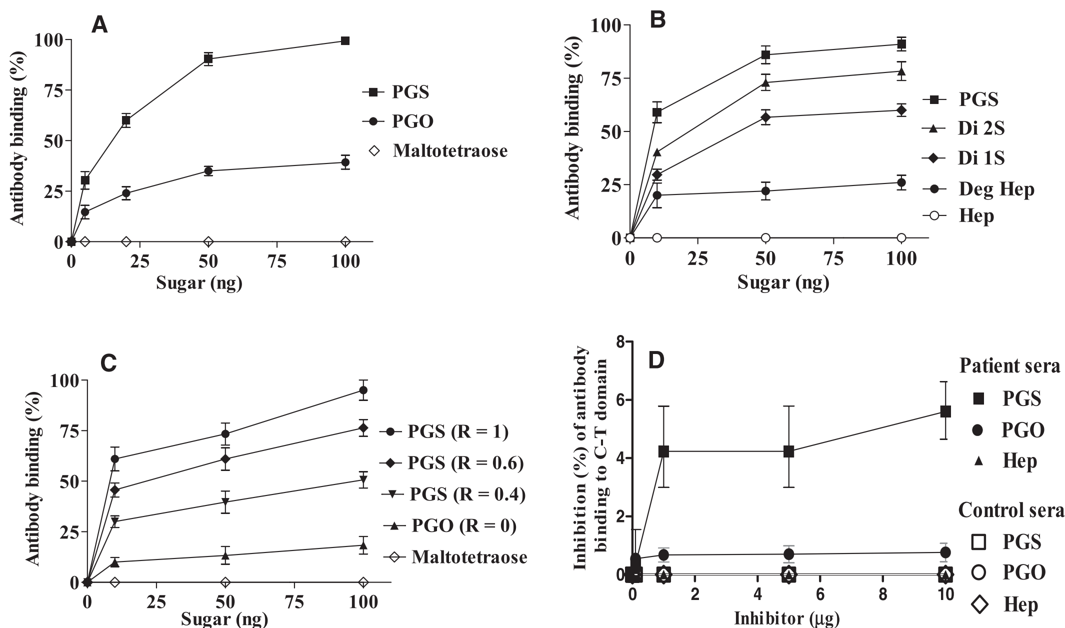

Fig. 3. Evaluation of different sulfated structures as immunoreactive epitopes using either specific polyclonal mouse sera or human chronic Chagas disease patients' sera. (A) Antibody binding (%) with polyclonal mouse sera specific for the C-T domain of PGO (•) and PGS (R = 0.6) (■). Maltotetraose (◇) was used as a non-charged control by dot-blot assays. (B) Antibody binding (%) of heparin (o), partially degraded heparin (•), Di 1S (♦) and Di 2S (▲) with mouse sera specific for Cz by dot-blot assays. (C) Antibody binding (%) of oligogalacturonates bearing different sulfate/carboxylate ratios (PGS R = 1 (•), PGS R = 0.6 (♦), PGS R = 0.4 (▼), PGO R = 0 (▲)) with mouse sera specific for Cz by dot-blot assays. Sera from BALB/c mice immunized with complete Freund adjuvant (CFA) in the absence of any source of protein antigen were used as control. Values obtained were discounted D-Inhibition of antibody binding to C-T extension (%), using this domain as antigen and a pool of chronic Chagas disease patients (N = 6) sera after adsorption with increasing amounts (0.01, 0.1, 1, 5 and 10 μg) of PGO, PGS and heparin by ELISA. In all cases, data are expressed as mean ± SD. Each experiment was repeated at least three times and each point represents duplicates. In (A), (B) and (C) the value corresponding to the maximum reactivity of the sera on PGS was considered 100%. In (D) 100% corresponds to the DO490 value obtained by antibody binding to purified C-T domain used as control.

them were recognized by the mouse sera. Therefore, charged compounds are recognized independently of the saccharidic structure involved. Interestingly, recognition of mouse sera specific for Cz increased concomitantly with the increasing number of charged groups in the sugar antigen tested: the Di 1S showed lower signals than the Di 2S and the latter was less recognized than PGS (R = 0.6). Noticeably, Di 2S carries one sulfate group per monosaccharide, while PGS bears two sulfates per three sugar units (Scheme S3); however, the higher recognition of the latter may be ascribed to the additional carboxylic groups present in the polygalacturonic compound. As expected, in all cases recognition was abolished when mouse sera specific for desulfated Cz was used (data not shown).

Immune recognition of oligogalacturonates bearing different sulfate/carboxylate ratios

Knowing that carboxylated oligosaccharides were partially recognized by polyclonal mouse sera specific for Cz, compounds bearing different sulfate/carboxylate ratios (0.4, 0.6 and 1 sulfate per monosaccharide unit) were tested. Recognition of sera specific for Cz increased concomitantly with the increase of sulfate/carboxylate ratio groups in the oligogalacturonates used (Fig. 3C), showing once more the relevance of the presence of sulfate groups for immune recognition. Maltotetraose, a neutral oligosaccharide, was used as negative control. In all cases recognition was abolished when mouse sera specific for desulfated Cz was used (not shown).

Inhibition of C-T immune recognition by Chagas disease patient sera using sulfated oligalacturonates

Taking into account that a dose-dependent linear correlation was obtained between the anti-Cz/anti-C-T serum immune reactivity and PGSs, in order to increase the strength of experimental evidence showing that sulfated epitopes are immunodominant, inhibition of Chagas disease patients' sera with synthetic polysulfated oligogalacturonate versus normal human sera was evaluated; it was found that antibody binding

to the C-T domain of patients' sera reached a plateau at about 5–6% inhibition on using increasing amounts of a polysulfated structure (Fig. 3D). Heparin is a densely charged structure which may be poorly adsorbed to the dot-blot membrane. However, when it was used as inhibitor of patients' sera antibody binding to the C-T domain by ELISA, the binding was not modified (Fig. 3D), confirming the absence of cross-reactivity between the two molecules.

Evaluation of different sulfated sugars as immunoreactive epitopes by dot-blot assays

The preceding data indicated that sulfated sugars are dominant epitopes in the molecular antigenicity of Cz. Therefore, in a next step, the importance of the sugar nature of these sulfated structures was evaluated. Different synthetic or commercial sulfated monosaccharides, coupled to BSA in order to ameliorate the membrane binding, were confronted with polyclonal rabbit sera specific for Cz (Fig. 4A) and the C-T domain (Fig. 4B). The highest reactivity was obtained with *N*-acetyl D-glucosamine-6-sulfate sodium salt (GlcNAc6S) (Scheme S3), in accordance with the structure present in natural Cz (Fig. 4A). Sera specific for Cz did not allow us to discriminate between position 2 or 6 of the sulfate group present in the glucosamine unit. Although to a lower degree, compounds bearing fewer acidic groups such as phosphate and carboxylate were also recognized, suggesting that the immunoreactivity is dependent upon the electronic charge of the anionic group present. Similar results were obtained by using polyclonal rabbit sera specific for the C-T domain (Fig. 4B). Accordingly, GlcNAc6S showed the highest recognition of all compounds tested. The preference for the sulfate group in position 6 rather than in position 2 of glucosamine was confirmed ($P < 0.01$).

Evaluation of different sulfated sugars as immunoreactive epitopes by ELISA and inhibition ELISA assays

In order to confirm the different antibody binding to sulfated oligosaccharides of immune sera antibodies specific for Cz/C-T domain, direct ELISA assays were performed. In this case, sugars coupled to aprotinin were used. A small molecule was selected as carrier in order to favor recognition of the sugar moiety. Taking into account that the number of sugar residues linked to the protein varied depending on the sugar used, as determined by MALDI-TOF MS (Fig. 2), the sugar–protein conjugates were compared on the basis of the same nanomoles of sugar residues (Fig. 5A). As expected, the results clearly showed that glucosamine (a basic sugar) as well as glucose 6-phosphate (Glc6*P*) and D-galacturonic acid (GalA) conjugates (Scheme S3) presented only 5% recognition, in accordance with the preceding data. Interestingly, polyclonal rabbit serum specific for Cz was not able to differentiate between D-glucosamine 2-sulfate sodium salt (GlcN2S) and D-glucosamine 6-sulfate sodium salt GlcN6S by direct assay, but undoubtedly this serum showed a higher preference for GlcNAc6S as antigen than the non-acetylated molecules ($P < 0.05$). Furthermore, the latter sugar–aprotinin conjugate was synthesized in

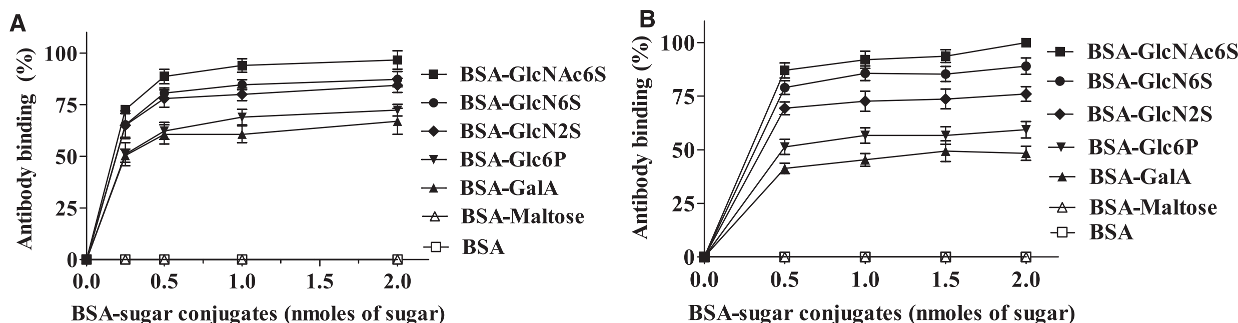

Fig. 4. Evaluation of different sulfated sugars coupled to BSA as immunoreactive epitopes by dot-blot assays using specific polyclonal rabbit sera for Cz and C-T domain. Antibody binding (%) with (A) rabbit sera specific for Cz or (B) rabbit sera specific for C-T domain by quantitative dot-blot assays using different sugar–BSA conjugates: maltose (△), GalA (▲), Glc6*P* (▼), GlcN2S (♦), GlcN6S (•), GlcNAc6S (■). The sugar–protein conjugates were compared on the basis of the same nanomoles of sugar residues. In both cases, data are expressed as mean ± sd. Each experiment was repeated at least three times and each point represents duplicates. 100% corresponds to the maximum reactivity of each sera to BSA coupled to GlcNAc6S. Statistical analysis in (B): BSA–GlcNAc6S versus BSA–GlcN2S ($P < 0.05$); BSA–GlcNAc6S versus BSA–GlcN2S ($P < 0.01$); BSA–GlcN6S versus BSA–GlcN2S ($P < 0.05$).

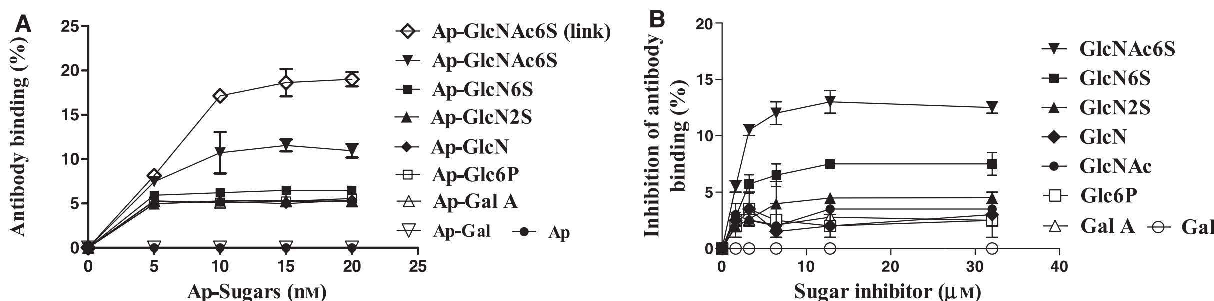

Fig. 5. Evaluation of different sulfated sugars coupled to aprotinin as immunoreactive epitopes by ELISA and inhibition ELISA assays using specific polyclonal rabbit sera for Cz. Polyclonal rabbit sera specific for Cz were confronted with different monosaccharides coupled to aprotinin (A) or with different sulfated proteins (B) by direct ELISA assay. For direct assays, aprotinin was coupled to Gal (▽), GalA (△), Glc6*P* (□), GlcN2S (▲), GlcN6S (■), GlcNAc6S (▼), GlcNAc6S with linker (◇), and the different sugar–protein conjugates were compared on the basis of the same nanomoles of sugar residues. 100% corresponds to antibody binding of purified Cz used as control (1 $\mu g \cdot well^{-1}$). For competitive inhibition ELISA, polyclonal rabbit sera specific for Cz, previously adsorbed with increasing amounts (nmoles) of different sulfated structures [Gal (o), GalA (△), GlcNAc (•), Glc6*P* (□), GlcN2S (▲), GlcN6S (■), GlcNAc6S (▼)] was tested with Cz as antigen (1 $\mu g \cdot well^{-1}$). 2β-Mercaptoethanol [56,59] was added at 1% to the NaCl/P_i–milk solution containing increasing nmoles of the sugars. The percentage of inhibition was determined by comparing serum reactivity in the presence and absence of the inhibitor. The results represent means ± SD of duplicate determinations from at least three independent experiments (B). Statistical analysis in (A): GlcNAc6S (link) versus GlcNAc6S ($P < 0.001$); GlcNAc6S (link) versus GlcN6S ($P < 0.001$); GlcNAc6S (link) versus GlcN2S ($P < 0.001$); GlcNAc6S versus GlcN6S ($P < 0.05$); GlcNAc6S versus GlcN2S ($P < 0.01$). Statistical analysis in (B): GlcNAc6S versus GlcN6S ($P < 0.05$); GlcNAc6S versus GlcN2S ($P < 0.01$); GlcN6S versus GlcN2S ($P < 0.05$).

two different forms, bearing or not a three-carbon linker (see Fig. 2D,F), and ELISA showed that when both of them were compared the molecule containing the hydrocarbon linker (Scheme S3) was preferred ($P < 0.001$), probably due to a higher exposure of the sulfated sugar ascribed to the flexibility of the hydrocarbon linker chain.

In addition, polyclonal sera from rabbit immunized with Cz were adsorbed with increasing amounts of the different structures and used in competitive inhibition ELISA with Cz as antigen (Fig. 5B). When GlcN6S was tested as inhibitor, the percentage reached 7%. This percentage was slightly higher than that obtained with the other molecules tested: non-acetylated, non-sulfated or sulfated in another position (2–3%). However, when the sera was pre-adsorbed with GlcNAc6S, inhibition reached 13%, showing that GlcNAc6S potently inhibits (in a dose-dependent manner) the binding of rabbit antibodies to Cz. The 8% inhibition difference (5% with GlcN6S versus 13% with GlcNAc6S) is statistically significant, confirming that the presence of an N-acetyl group in addition to a 6-sulfate group is relevant, increasing the antigenicity of the epitope ($P < 0.05$). In contrast, the experiments in Figs 5 and 6 suggest that, although the acetyl group contributes to the antigenicity of the epitope, both rabbit and mouse antisera also recognize the GlcN6S quite effectively, indicating that acetylation is not essential as non-acetylated molecules showed quite efficient inhibition. On the other hand, the preference for GlcN6S instead of GlcN2S (Scheme S3) was significant by ELISA inhibition assay ($P < 0.05$). Finally, in order to test whether any natural sulfated sugar containing glycoprotein could be immune recognized by sera specific for Cz, ovalbumin bearing hybrid-type oligosaccharides containing Man-4-sulfate groups [43] was used as antigen (data not shown). Noticeably, the big difference in the recognition of the two glycoproteins detected confirms the high specificity of the sulfated sugar structure required for the immune interaction. In order to obtain a stringent test of our working hypothesis to confirm that the sulfated moiety linked to high-mannose-type oligosaccharides is indeed a dominant epitope displayed on the C-T domain of Cz, assays using purified IgGs from immune animal and patient sera were performed. To achieve this goal, inhibition of binding to the C-T domain with specific IgGs purified from rabbit sera specific for C-T domain after adsorption with increasing amounts of GlcNAc6S was performed, finding a 10% inhibition. Interestingly a similar percentage of inhibition was obtained when IgGs purified from Chagas disease patients' sera were pre-adsorbed with GlcNAc6S, in comparison with those purified from human control sera pre-adsorbed with increasing amounts of the sulfated epitope (Fig. 6). A similar inhibition percentage was found when Cz was used as antigen, confirming the above results (data not shown).

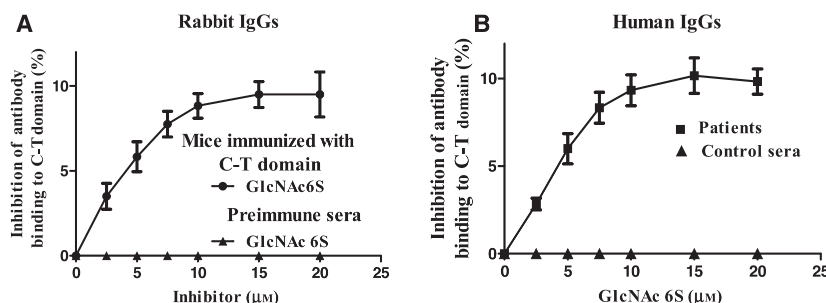

Fig. 6. Inhibition of binding to C-T domain of IgGs purified from rabbit polyclonal sera specific for C-T domain and Chagas disease patients' sera by increasing amounts of GlcNAc6S in ELISA inhibition assays. IgGs were purified from pre-immune and C-T domain immunized rabbit sera and from human control and patient sera. ELISA inhibition assays were performed using C-T domain (1 $\mu g \cdot well^{-1}$) and purified IgGs post-adsorption with increasing amounts of GlcNAc6S (2.5, 5, 7.5, 10, 15, 20 μM). The percentage of inhibition was determined by comparing IgG reactivity in the presence and absence of the inhibitor. The results represent means ± SD of duplicate determinations from at least three independent experiments.

Discussion

Post-translational modifications of *T. cruzi* glycoproteins have been studied in the last few years [13,14,21,44]. In particular, we have determined for the first time the presence of sulfated glycoproteins in trypanosomatids. It is well known that human and murine infections with *T. cruzi* elicit a strong humoral and cellular immune response to Cz [8–11,45,46]. Additionally, we showed that the severity of the disease in chronic Chagas disease patients was associated with high levels of Cz-specific antibodies. Progress in structural characterization by UV-MALDI-TOF MS analysis revealed the presence of sulfated high-mannose-type oligosaccharides in the C-T domain of Cz, which are essential for Cz recognition by IgG antibodies from the serum of Chagas disease patients [35]. Considering that the involvement of the substituted glycans of this protein in the molecule antigenicity has been scarcely explored, a detailed study of the requirements of this particular antigenic determinant was missing. The present experiments were designed to further analyze the influence of the type, the amount and the location of the anionic charged group in the oligosaccharide structure involved in the immune recognition of the Cz molecule.

In a first step, we provided evidence of the presence of an anionic group as an essential requisite for the recognition of Cz by its specific sera. Even more, the recognition enhancement when sera specific for the C-T domain was used in addition to the use of this domain as antigen confirmed that the anionic substituent is located in this part of the molecule. In this context, although other anionic structures, phosphate and carboxylic groups, were recognized by antibodies specific for the Cz/C-T domain, sulfate groups showed the highest antibody binding. It is worth mentioning that the presence of phosphate groups in natural Cz has been discarded [12]. As expected, neither antibodies specific for sulfate-depleted Cz nor those specific for sulfate-depleted C-T domain were reactive with the mentioned anionic groups. It must be noted that the binding between antibodies specific for C-T domain and oligogalacturonates increased concomitantly with the growing number of sulfate groups in the oligosaccharidic molecule. Furthermore, the reaction of disaccharides bearing different numbers of sulfate groups with sera specific for Cz was compared, showing that the recognition increased with the number of anionic substituents.

Various types of macromolecules are sulfated in mammalian cells. These include lipids [47], steroid sulfates [48], proteins (tyrosine *O*-sulfate) [49], proteoglycans [50] and glycoproteins. Sulfated high-mannose-type glycans have been described as components of glycoproteins from *D. discoideum*. However, at difference from Cz, Man-6-sulfate accounts for the majority of the sulfated sugar [37]. Moreover, it has been shown that dextran sulfate and heparin interfere with the recognition of monoclonal antibodies for a common sulfated antigenic determinant from these mold lysosomal proteins [51]. Lysosomal enzymes from *D. discoideum* contain sulfated N-linked oligosaccharides, whose synthesis has been well studied *in vivo* [52]. However, little is known about the properties of the pertinent sulfotransferases. In particular, no sulfotransferases have been reported in *T. cruzi*, so far. Ongoing studies on these enzymes are being performed in our laboratory.

In order to test antibody binding to a highly sulfated molecule, heparin was confronted with sera specific for Cz. Noticeably, no cross-reactivity was observed. In addition, patients' sera antibody binding to the C-T domain was not inhibited by heparin (see Fig. 3).

However, low recognition was observed when low molecular weight heparin was used as antigen. It may be suggested that the highly polyanionic charged epitope contained in this GAG structure may be hindering the antibody binding.

In a next step, to go deeper into the specific binding of sera IgG antibodies to Cz molecules, different sulfated sugars were synthesized and coupled to non-antigenic proteins to mimic the natural environment. Although the O-3 and O-6 are the only potential glycosylation sites on the chitobiose core of natural glycoprotein, the 4-positions are involved in the glycosidic linkages and the 2-positions are N-acetylated, it is well known that sulfation at O-3 is not an easy task. Therefore, we have synthesized six sulfated structures and we have taken advantage of 2-(N) sulfated structures, representative of a secondary substituted position, as alternative structures for immune recognition assays. The importance of the sulfate position was first evidenced by using GlcN2S and GlcN6S coupled to BSA in dot-blot assays; and the specificity of the recognition was shown using GlcNAc6S–BSA as antigen. The results obtained were validated using maltose–BSA, GalA–BSA and Glc6*P*–BSA conjugates as controls.

Finally, competitive ELISAs using the mentioned free sugars as inhibitors were used to demonstrate the preference of an N-acetyl group and a sulfate in position O-6 in this relevant glucosamine epitope. Furthermore, when a new set of carbohydrates linked to aprotinin were used as antigens in ELISAs, the mentioned preference was undoubtedly confirmed. Although sialic acid is present in the N-oligosaccharidic chains of the C-T domain of Cz, it is worth noting that recognition of Cz or the C-T domain by specific antibodies was abolished after desulfation treatments under conditions where sialic acid is preserved [14,21,35].

It should be noted that at plateau levels the percentage of binding of rabbit anti-Cz serum to aprotinin–GlcNAc6S conjugates did not exceed $\sim 17\%$ (Fig. 5A). This value is fairly close to the 13% inhibition induced by soluble GlcNAc6S in the competitive ELISA format (Fig. 5B). Interestingly, addition of the GlcNAc6S ligand (molar excess) inhibits up to 10% of the binding to the C-T domain of IgG antibodies purified from immune rabbits, allowing a more stringent test of our working hypothesis and confirming that the sulfated moiety linked to high-mannose-type oligosaccharides is indeed the dominant epitope displayed on the C-T domain of Cz (Fig. 6A). Moreover a similar inhibition value was also obtained with patient sera (Fig. 6B). Our findings provide evidence that binding of purified IgG antibodies either from serum specific for the C-T or from Chagas disease patients' sera to the C-T domain is inhibited by the anionic synthetic sugar GlcNAc6S mocking the sulfated Cz epitope located in the C-T extension of the molecule.

In the last 20 years major efforts have been made to obtain the three-dimensional structure of the complete mature Cz. However, although the expression of an active, complete mature Cz in an insect cell/baculovirus system could be obtained [53], so far only the X-ray crystal structure of a recombinant truncated molecule lacking the C-T domain has been determined [54]. Herein, a model of the C-T domain of natural Cz was obtained by comparison of sequence homology with other proteins by using the PHIRE server. It can be noted that the Asn255 of the C-T domain, corresponding to the N-linked consensus sequence, is located in a random coil structure favoring the exposure of the sulfated oligosaccharide as an antigenic epitope (Fig. 7). In addition, Asn255 belongs to one of four antigenic determinants present in the C-T domain

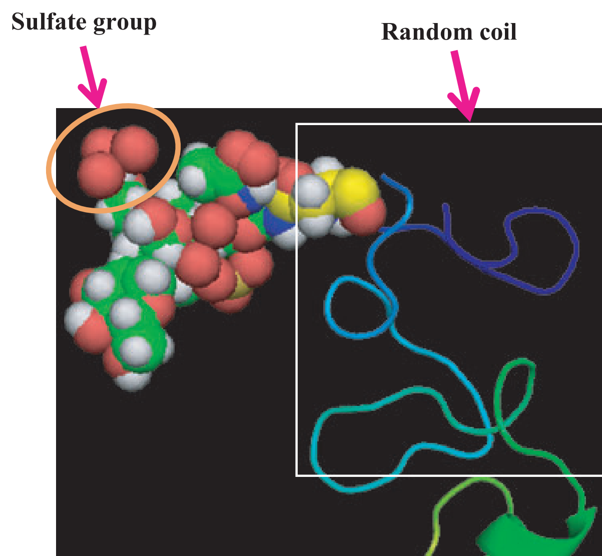

Fig. 7. Modeling showing the exposure of sulfated epitope in the C-T domain of Cz. The present model shows the sulfate group (marked with an orange circle) in the *N*- oligosaccharide linked to Asn255 from the C-T extension (Gen Bank AAB41119.1) of natural Cz. The predicted X-ray crystal structures of the C-T domain were obtained through the automated comparative modeling program PHIRE sequence server, analyzed in the automated mode, and the Protein Data Bank showing the closest match template was selected to create a sugar-containing model. The localization of the Asn255 in a random coil secondary structure favors the exposure of the sulfated oligosaccharide as an antigenic epitope. Homology-based modeling was then analyzed using MACPYMOL to create this figure (PDB c1autl). The figure shows the oligosaccharides GlcNAc-GlcNAc-Man with sulfate group (carbon is yellow, hydrogen white, nitrogen blue, oxygen red, sulfate brown; sphere model) linked to the Asn (sphere model), contained in a random coil secondary structure part of the C-T extension.

as predicted by using the EXPASSY server. Moreover, previous studies, based on the composition of the secondary structure elements calculated using the CONTIN algorithm and the number of amino acid residues of each domain [55], have shown that the C-T domain consists of 46% β structure (60 amino acids) and 54% random coil structure (70 amino acids).

In summary, the fact that sulfated *N*-acetyl D-glucosamine units eliciting the best recognition are present on N-glycans of the C-T domain from natural Cz and that this sulfated carbohydrate epitope presents a predicted favored exposition due to its linkage to an Asn of an antigenic determinant located in a random coil structure indicate that these sulfated sugars play a central role in the binding of this major antigen to specific antibodies from Chagas disease patients' sera. In addition, the fact that other anionic charged structures are also recognized, although to a lesser extent, may help to explain the wide cross-reactivity between host self-proteins and parasite antigens displayed in the immunopathogenesis of Chagas disease.

Experimental procedures

General

All purchased materials were used without further purification. Dichloromethane was distilled from calcium hydride and tetrahydrofuran over sodium and benzophenone. Analytical TLC was carried out on a Merck D.C.-Alufolien Kieselgel 60 F254. Flash chromatography was performed on a Geduran SI 60, 0.040–0.060 mm pore size, using distilled solvents. For ^{1}H, ^{13}C NMR spectra, chemical shifts are reported in parts per million relative to tetramethylsilane or a residual solvent peak ($CHCl_3$: ^{1}H $\delta = 7.26$ p.p.m., ^{13}C $\delta = 77.2$ p.p.m.). Assignments of ^{1}H and ^{13}C were assisted by 2D ^{1}H COSY and 2D ^{1}H–^{13}C CORR experiments. High resolution electrospray experiments and MS-MS were performed on a Waters-Micromass Q-TOF Ultima Global hybrid quadrupole time-of-flight instrument, equipped with an electrospray (Z-spray) ion source (Waters-Micromass, Manchester, UK). Optical rotations were measured in a 1 cm cell in the stated solvent; $[\alpha]_D$ values are given in 10^{-1} deg·cm^{2}·g^{-1} [concentration given as g·(100 mL)$^{-1}$]. Maltose, maltotetraose, D-glucosamine, *N*-acetyl-D-glucosamine, methyl 6-amino-6-deoxy-α-D-glucopyranoside, Glc6*P*, GlcN2S, GlcN6S, GalA, heparin and ovalbumin were purchased from Sigma.

UV-MALDI-TOF MS analysis

Measurements were performed using an Ultraflex II TOF/TOF mass spectrometer equipped with a high performance solid-state laser (λ = 355 nm) and a reflector. The system is operated by the FLEXCONTROL 2.4 software package (Bruker Daltonics GmbH, Bremen, Germany).

All samples were irradiated with a laser power of 60% and were measured in the linear and reflection modes, in positive mode. The mass spectra reported are the result of 1000 laser shots. Sinapinic acid was used as matrix (200 mM in 66 : 33 acetonitrile–trifluoroacetic acid 0.1%), 2 mg·mL^{-1}. Matrices and calibrating chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). The samples were loaded onto a ground steel sample plate (MTP 384 ground steel; Bruker Daltonics GmsH) using the sandwich method or by the classic dried drop method: a sample/matrix solution mixture 1 μL, 1 : 1 (v/v), was deposited on the target plate and left to dry at room temperature.

Antigen purification

Cz purification was carried out from epimastigotes as described previously [56] with some modifications [35]. To obtain the purified C-T domain from Cz, autoproteolysis of the enzyme followed by gel filtration in a Bio Gel P-30 column was performed [21]. Desulfation treatments were performed as described elsewhere [14,36].

Anti-Cz and anti-C-T antibodies

Immunization of BALB/c female mice was performed as described earlier [35]. Rabbit sera were also obtained after immunization with Cz extracted from SDS/PAGE gel bands or with purified C-T domain, both prior to and after desulfation treatment. Three boosters of 50 μg of each antigen by an intradermal route were performed. All experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals of the Instituto Nacional de Parasitología, (INP) Dr 'Mario Fatala Chaben', Ministerio de Salud, Argentina. The precipitation of antibodies from rabbit sera was done with ammonium sulfate, and IgG was purified by passing over a Protein A Sepharose column and eluting the adsorbed IgG with 0.1 M citrate buffer at pH 3.5 according to Ey *et al.* [57].

Patients' sera

Sera from control subjects and chronic Chagas disease patients aged 18–58 years were provided by the Diagnostic Clinical Department, National Institute of Parasitology. The uninfected control group was found to be negative for *T. cruzi* by serological testing. Individuals with hypertension, vascular, ischemic or congenital heart disease, cancer, syphilis, HIV, diabetes, arthritis or serious allergies were excluded from the present study. Human stored sera used from Chagas and non-Chagas disease patients were always codified and anonymized. Blood to be used for serum component analysis was obtained from patients and control subjects by venipuncture, allowed to coagulate at 4 °C and

centrifuged at 1000 ***g*** for 15 min. Non-hemolyzed serum was separated, and aliquots were stored at 70 °C until use. The study was evaluated and approved by the Ethical Committee of the Institute Fatala Chaben. Informed consent was signed for all enrolled patients. For inhibition ELISA a patients' pool of sera ($n = 6$) was used. IgGs from the Chagas disease patients' pool ($n = 20$) sera were purified according to Ey *et al.* [57] as described above.

Quantitative dot-blot assay

Dot-blot assays were performed using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad Laboratories) following the manufacturer's instructions. Briefly, PGO, PGS, Di 1S, Di 2S, heparin, partially degraded heparin (12.5–100 ng) and BSA–sugar conjugates (0.5–2 nmol sugar) were seeded on the nitrocellulose membranes in duplicate in 200 μL of NaCl/Tris (20 mM Tris/HCl pH 7.5, 500 mM NaCl) per spot. Samples were slowly filtered through the membrane and washed with gentle vacuum. Membranes were blocked with NaCl/Tris with 3% non-fat dry milk for 45 min. Samples were confronted with sera from mice immunized with Cz (1 : 3000) and C-T domain (1 : 1000) followed by incubation with a peroxidase conjugated AffiniPure goat anti-rabbit IgG (H + L) (Jackson ImmunoResearch Labs Inc.) (1 : 2000) and visualized using ECL (Amersham) enhanced chemiluminescence reagent. Spots were quantified using a FujiLAS1000 densitometer equipped with IMAGE GAUGE 3.122 software (Fuji Film, Tokyo, Japan). In each experiment, the value corresponding to the highest antibody binding obtained among the tested structures was considered to be 100%.

Enzyme-linked immunosorbent assay (ELISA)

The presence of anti-Cz whole IgG antibodies in serum obtained from immunized rabbit were quantified by ELISA [58] after adsorption treatment with chemically synthesized structures as described in Doc. S1. Briefly, flat bottom (96 well) plates were coated with 1 $\mu g \cdot well^{-1}$ of Cz diluted in 50 μL NaCl/P_i. Plates were blocked in NaCl/P_i, washed three times with NaCl/P_i–0.05% Tween 20, and incubated for 90 min at 37 °C with absorbed anti-Cz rabbit sera. After washing, plates were incubated with anti-rabbit peroxidase. Then *o*-phenylenediamine dihydrochloride was used and optical density (A) was read at 490 nm with an ELISA microplate reader. Absorption of pre-immune and immunized sera in NaCl/P_i with 1% dried powder milk (1 : 1000) with growing amounts of the different inhibitors in the presence of β-mercaptoethanol 1% was performed by incubation for 2 h at 37 °C [59]. In each case, the percentage of inhibition of the recognition of Cz was calculated as {[$A_{490\ nm}$ to Cz (without inhibitor) − $A_{490\ nm}$ to Cz (with inhibitor)]/$A_{490\ nm}$ to Cz (without inhibitor)} × 100.

Data analysis

Values are given as means ± SD of duplicate determinations from a minimum of three separate experiments. Statistical analysis was performed by one-way ANOVA, followed by the non-parametric multiple comparison Tukey test. Differences were considered statistically significant at $P < 0.05$.

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Supporting information

The following supplementary material is available:
Doc. S1. Synthesis of sulfated compounds.
Scheme S1. Synthesis of compounds **3** and **6**.
Scheme S2. Synthesis of compounds **11** and **14**.
Scheme S3. Anionic structures used to mimic sulfated Cz epitope.

This supplementary material can be found in the online version of this article.

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