Medical Microbiology and Immunology
https://doi.org/10.1007/s00430-017-0529-7

ORIGINAL INVESTIGATION

Trypanosoma cruzi serinecarboxipeptidase is a sulfated glycoprotein and a minor antigen in human Chagas disease infection

Luciana L. Soprano[1] · Juliana E. Parente[2] · Malena Landoni[2] · Alicia S. Couto[2] · Vilma G. Duschak[1]

Received: 13 May 2016 / Accepted: 5 December 2017

Abstract
In this work, the presence of sulfated *N*-glycans was studied in a high-mannose-type glycoprotein of *Trypanosoma cruzi* with serinecarboxipeptidase (*Tc*SCP) activity. The immune cross-reactivity between purified SCP and Cruzipain (Cz) was evidenced using rabbit sera specific for both glycoproteins. Taking advantage that SCP co-purifies with Cz from Concanavalin-A affinity columns, the Cz–SCP mixture was desulfated, ascribing the cross-reactivity to the presence of sulfate groups in both molecules. Therefore, knowing that Cz is a sulfated glycoprotein, with antigenic sulfated epitopes (sulfotopes), SCP was excised from SDS-PAGE and the *N*-glycosydic chains were analyzed by UV–MALDI–TOF-MS, confirming the presence of short-sulfated high-mannose-type oligosaccharidic chains. Besides, the presence of sulfotopes was analyzed in lysates of the different parasite stages demonstrating that a band with apparent molecular weight similar to SCP was highly recognized in trypomastigotes. In addition, SCP was confronted with sera of infected people with different degrees of cardiac dysfunction. Although most sera recognized it in different groups, no statistical association was found between sera antibodies specific for SCP and the severity of the disease. In summary, our findings demonstrate (1) the presence of sulfate groups in the *N*-glycosidic short chains of native *Tc*SCP, (2) the existence of immune cross-reactivity between Cz and SCP, purified from epimastigotes, (3) the presence of common sulfotopes between both parasite glycoproteins, and (4) the enhanced presence of sulfotopes in trypomastigotes, probably involved in parasite–host relationship and/or infection. Interestingly, we show for the first time that SCP is a minor antigen recognized by most of chronic Chagas disease patient's sera.

Keywords *Trypanosoma cruzi* · Serinecarboxypeptidase · Glycomics · Sulfate groups · Sulfotopes · Chagas' disease · Antigenicity

Introduction

American Trypanosomiasis or Chagas disease, caused by the parasitic protozoan *Trypanosoma cruzi* represents a major health problem and continues to be endemic in large areas of Latin America. *T. cruzi* has a complex life cycle with proliferative stages in the vector (epimastigotes) and the vertebrate (intracellular amastigotes), as well as non-proliferative infectious stages (trypomastigotes) in both hosts. Currently, the number of infected people globally estimated by the World Health Organization amounts to 7–8 million, and more than 10,000 deaths are thought to occur annually [1]. The disease has also emerged as a public health problem elsewhere in non-endemic countries, such as USA, Europe, Australia, and Japan, due to transmission of *T. cruzi* by people migrating to other regions of the world [2, 3].

Proteinases have been involved in multiple roles making them attractive potential targets for the development of new

✉ Vilma G. Duschak
vduschak@conicet.gov.ar

[1] Area de Bioquímica de Proteínas y Glicobiología de Parásitos, Consejo Nacional de Investigaciones Científicas y Tecnicas (CONICET), Departamento de Investigación, Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben", ANLIS-Malbrán, Ministerio de Salud de la Nación, CONICET, Av. Paseo Colon 568 (1063), Buenos Aires, Argentina

[2] Centro de Investigación en Hidratos de Carbono (CIHIDECAR), Departamento de Química Orgánica-Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Intendente Güiraldes 2160, C1428GA Buenos Aires, Argentina

Published online: 22 December 2017

drugs against Chagas disease [4–6]. The complete sequence from *T. cruzi* clone CL Brener genome has allowed the prediction of cysteine peptidases (CPs), serine peptidases (SPs), metallopeptidases (MPs), threonine peptidases, and only two aspartic peptidases [7]. Cruzipain, expressed as a mixture of isoforms, is the most abundant CP of the parasite which was also studied as antigen and glycoprotein [8]. Among the SPs described in the parasite, it can be mentioned oligopeptidase B, a member of the prolyl oligopeptidase family involved in Ca^{2+}-signaling during mammalian cell invasion [9, 10]; a prolyl endopeptidase (*Tc*80), also purified and partially characterized from *T. cruzi* [11, 12]; and a lysosomal serine carboxypeptidase, purified, and characterized [13]. In addition, a putative SP, a secreted 75 kDa *T. cruzi* serine oligopeptidase, was purified and the subcellular localization was restricted to intracellular structures, including the flagellar pocket, plasma membrane, and cytoplasmic vesicles resembling reservosomes [14]. On the other hand, some activities such as a dipeptidyl aminopeptidase and other aminopeptidases were detected in cell-free extracts, and a lysosomal serine carboxypeptidase was identified in *T. cruzi* [13].

Carboxypeptidases are peptidases that cleave the C-terminal amino acid residue from peptides and proteins. Based on their catalytic mechanism, they are classified into two major classes, MPs and SPs. Serine carboxypeptidases (SCPs) show C-terminal proteolytic activity under acid conditions, in addition to esterase and deamidase activities [15, 16]. They belong to the S10 family, Clan SC, of SPs [17], among which the best studied is the SCP Y from *Saccharomyces cerevisiae*. The sequences surrounding the active site serine and histidine residues are highly conserved in all the serine carboxypeptidases. SCPs share several structural features including a signal sequence for intracellular trafficking and/or secretion, multiple *N*-linked glycosylation sites, and four evolutionarily conserved regions involved in substrate binding and catalysis [18]. Regarding trypanosomatids, primers, which can specifically detect *T. cruzi* or *T. rangeli* genomic DNA, were recently characterized. The use of intergenic regions, generally divergent in these organisms, and the SCP gene were successful to evaluate potential species-specific markers for the development of new strategies for the accurate diagnosis of infections [19].

Sulfation, widely observed from bacteria to humans, plays a key role in various biological processes such as cell communication, growth, and development [20] and sulfated *N*-linked oligosaccharides have been mainly implicated in several specific molecular recognition processes [21, 22]. Sulfation reaction occurs via the transfer of an activated sulfate group from the donor, 3-phosphoadenosine 5-phosphosulfate (PAPS) [23] to a specific position on a variety of acceptor sugar residues on numerous substrates [24, 25]. ATP sulfurylase, a key enzyme in the synthesis of the high-energy sulfate donor PAPS is known to be inhibited by chlorate treatment [26]. Recent results suggested that *T. cruzi* sulfation occurs via PAPS, evidenced the presence of sulfated epitopes in trypomastigote and amastigote forms and demonstrated their presence on trypomastigotes surface [27].

In the present work, mass spectrometry analysis of *T. cruzi SCP* was performed, confirming the presence of sulfated groups in *N*-glycans of this enzyme. Therefore, considering the antigenicity of these groups [28, 29], the immune cross-reactivity between SCP and Cz, the first sulfated glycoprotein described from this parasite, was studied. In addition, we desulfated the Cz–SCP mixture finding that the cross-reactivity is ascribed to the presence of sulfate groups in both molecules. In addition, the enhanced presence of sulfated epitopes in trypomastigotes was evidenced. On the other hand, when SCP was confronted with sera of infected patients with mild and severe degrees of cardiac dysfunction, although most sera recognized the protein in the different groups, no statistical association could be determined between the presence of sera antibodies specific for SCP and the severity of the disease.

Methods

Parasites

Epimastigotes from Tulahuen strain (stock Tul 2) of *T. cruzi* were used for protein purification and lysates obtainment. Epimastigotes were grown in axenic Brain Heart Tryptose (BHT) medium [30]. Trypomastigote and amastigote forms of the parasite were maintained in Vero cell monolayers. Trypomastigotes were obtained from the culture medium of infected Vero cells free of cellular debris by leaving them to swim off the centrifuged pellet for 1 h at 37 °C and then collected by centrifugation for 10 min at 2300 rpm in a Sorvall SS34 rotor. Amastigotes were obtained from infected Vero cells treated with RPMI 5% FCS and 0.06% SDS for 30 s. The resulting suspension was centrifuged twice, first at 800 rpm to discard cellular debris and then at 3200 rpm to collect the amastigotes. Both steps were performed for 10 min in an Eppendorf 5804R centrifuge [31].

Preparation of parasite lysates

After three-controlled cycles of freezing and thawing of known amounts of different parasite stage forms, lysates were obtained, suspended in Tris ClH 10 mM pH 7.6, mixed with sample buffer for SDS-PAGE, and kept at 20 °C before loading a relationship of E/A/T:1/5/10, in accordance with load similar amounts of proteins.

Cz and SCP purification

The mix Cz–SCP was purified in accordance with [32]. Cz was purified to homogeneity from epimastigotes of Tulahuen strain by affinity chromatography on Concanavalin-A-Sepharose followed by Mono-Q chromatography step [29]. *T. cruzi* SCP was purified by Mono-Q followed by chromatofocusing ion exchange chromatography as described by Parussini et al. [13]. Protein purifications were followed by 10% SDS-PAGE stained with silver nitrate. Protein content was measured by Bradford's method [33].

Desulfation treatment

After desalting and dialysis against water, chemical desulfation of the mix Cz–SCP purified by ConA-Sepharose was achieved with the addition of pyridine (0.015 ml) over the lyophilized sample, which was then dissolved in dimethyl sulfoxide: methanol [9:1 (v/v), 0.2 ml], adjusted to pH 4 with diluted HCl, heated at 100 °C for 2 h, and freeze dried [34]. 1 mg of a mix containing Cz plus SCP was subjected to desulfation as above described.

Glycoprotein digestion

The mix containing Cz plus SCP was submitted to SDS-PAGE (10%) and stained with colloidal Coomassie Brilliant Blue G-250. The protein band corresponding to the SCP was cut out from the gel and washed with acetonitrile. The gel pieces were reduced with 10 mM DTT in 50 mM NH_4HCO_3 at 55 °C for 30 min. They were further washed with acetonitrile and alkylated with 55 mM IAA in 50 mM NH_4HCO_3 for 20 min at room temperature in darkness. After washing with 50 mM NH_4HCO_3 for 10 min and with acetonitrile for 5 min, they were dried in a Speed Vac. The gel slices were rehydrated with 20 ng μl^{-1} trypsin (Sigma) in 40 mM NH_4HCO_3, 9% acetonitrile and incubated at 37 °C overnight. The peptides were extracted by sonication with 50% acetonitrile in 1% TFA, and supernatant was taken to dryness.

Deglycosylation of SCP

The protein band corresponding to the SCP was cut out from the gel, frozen for 3 h, and washed (mixing for 30 min) with (*a*) acetonitrile, (*b*) 20 mM $NaHCO_3$, pH 7, and (*c*) acetonitrile. The gel pieces were dried, and the *N*-glycans were released by incubation with PNGase F (20 milliunits) (New England Biolabs Inc., Beverly, MA, USA) overnight at 37 °C in 20 mM $NaHCO_3$, pH 7 (30 µl). The gel pieces were thoroughly washed, and supernatants were removed and dried. Glycans were filtered through an Ultrafree McFilter (M_r 5000), dried, suspended in 0.1% (v/v) formic acid (20 µl), and left at room temperature for 40 min. Finally, the sample was dried and suspended in water.

Mass spectrometry analysis

Matrices and calibrating chemicals were purchased from Sigma-Aldrich. 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 version 2.4 software package (Bruker Daltonics GmbH, Bremen, Germany). Samples were irradiated with a laser power of 25–50% and measured in the linear and the reflectron modes, in positive and negative ion modes.

Laser-induced dissociation tandem mass spectrometry (LID-MS/MS)

Analysis in the MALDI-TOF/TOF-MS/MS instrument The Ultraflex II MALDI-TOF/TOF-MS spectrometer was used. For all experiments using the tandem time-of-flight LIFT mode, the ion source voltage was set at 8.0 kV with a precursor ion mass window of 3 Da. Precursor ions generated by LID were accelerated at 19.0 kV in the LIFT cell. The reflector voltage was set at 29.5 kV.

Sample preparation Prior to the analysis of the oligosaccharides by UV–MALDI–TOF-MS, remnants of impurities were removed using a biphasic microcolumn consisting of C-18 phase and Dowex 50X-H^+ resin. The sample was incubated on the column for 15 min and eluted with 500 µl of water and dried.

For peptide analysis, the samples were loaded onto an AnchorChip target (Bruker Daltonics GmbH) as 50% mixtures with (3 mg ml^{-1}) α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% TFA.

For glycomic analysis, 2,5-dihydroxybenzoic acid was used as matrix. The samples were loaded onto a ground steel plate (Bruker Daltonics GmbH) using the sandwich method. Mass spectra were the sum of 100–300 single laser shots, depending on the sample conditions.

Immunization protocol and purification of IgGs

Obtainment of sera specific for Cz and SCP

Rabbits (New Zealand white lineage) were immunized with Cz and SCP extracted from polyacrylamide gels (three doses of 50 µg protein intradermal via, in each case). Experimental procedures conducted in the INP, "Dr M. Fatala Chaben", ANLIS-Malbrán, Ministerio de Salud,

Argentina, in accordance with the ethical legislations and regulatory entities, established in Argentina and International Guides for care and use of laboratory animals [29].

Adsorption treatment of sera specific for glycoproteins: obtainment of SCP-adsorbed anti-Cz and Cz-adsorbed anti-SCP sera

The optimal dilutions to be used for sera specific for Cz and SCP were obtained by dot blot (anti-Cz 1/5000 and anti-SCP 1/400). Specific sera were incubated during 2 h at 37 °C with fragments of nitrocellulose sheet containing different amounts of the glycoproteins used as adsorbent. After the incubation, the fragments were taken out and the adsorbed sera were further used as primary antibody in western blot assays. Bands with 0.5, 1, and 2 µg of glycoproteins used as adsorbent were tested by western blot after the adsorption procedure with their cognate's specific antibodies with the aim to evaluate whether the technique was effective.

Purification of IgGs from rabbit sera anti-Cz and anti-desulfated Cz (anti-dCz)

Precipitation of antibodies from rabbit sera was done with ammonium sulfate, and afterwards, pre-immune, anti-Cz, and anti-dCz. IgGs were purified by passing over a Protein A-Sepharose column and eluting the adsorbed IgGs with 0.1 M citrate buffer at pH 3.5 in accordance with Ey et al. [35].

Preparation of the anti-sulfate (AS)-enriched IgG fraction and evaluation by ELISA

Enrichment in antibodies specific for sulfated epitopes

With the aim of having a tool to study sulfated epitopes in *T. cruzi*, purified anti-Cz IgGs were adsorbed with desulfated Cz (dCz). Adsorption protocol with dCz was performed as previously described [36]. Briefly, dCz (3 µg/µl) in PBS were adsorbed onto a nitrocellulose membrane and next blocked with PBS-3% skimmed milk. After blocking, anti-Cz IgGs (0.03 µg/µl in PBS-1% skimmed milk) were incubated with the membrane for 1 h at 37 °C. Anti-Cz IgGs were incubated sequentially with two nitrocellulose-containing dCz. An ELISA using Cz and dCz as antigens was performed to evaluate whether the IgG fraction obtained after adsorption was enriched in anti-sulfated epitopes in accordance with conditions previously described, demonstrating that the anti-Cz IgG fraction adsorbed with dCz is enriched in anti-sulfates (AS) antibodies, so it was called AS-enriched IgG [27].

Study population

The diagnosis of Chagas disease is currently assessed by indirect hemagglutination, indirect immunofluorescence, and enzyme immunoassay using whole homogenates of the epimastigote forms of *T. cruzi* as antigen [37]. An individual is considered infected if at least two out of the three tests are positive. Sera from chronic Chagas disease patients aged from 28 to 58 with different degrees of cardiac dysfunction as determined by Kuschnir system [38] were provided by the Diagnostic Clinical Department, National Institute of Parasitology. Group 0 (G0, $n=9$) comprised seropositive individuals showing a normal chest X-ray and a normal electrocardiography (ECG); group 1 (G1, $n=9$) seropositive patients had a normal chest X-ray with abnormalities in the ECG (being G0 plus G1 patients with mild disease); and group 3 (G3, $n=9$) seropositive patients had ECG abnormalities, heart enlargement, and clinical signs of heart failure, representing patients with severe disease. The uninfected control group was found to be negative for *T. cruzi* by serologic testing as well as for Cz/SCP recognition. Chagas disease patients and uninfected 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 anonymous. The experiments were undertaken with the understanding and written consent of each subject; the study methodologies conformed to the standards set by the Declaration of Helsinki; and the study methodologies were approved by the local ethics committee.

Clinical classification of subjects studied according to Kuschnir, included in this study.

	Control serum (CS)	Patient groups		
		G0	G1	G3
Serology for *T. cruzi*	−	+	+	+
ECG abnormalities	−	−	+	+++
Cardiothoracic ratio	0.50	0.50	0.50	Cardiomegaly
Cardiac Insufficiency	−	−	−	+

ECG electrocardiographic, *CS* control serum of healthy subject

Patient's sera

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.

SDS-PAGE and western blotting

For evaluation of cross-reactivity between Cz and SCP

A 10% SDS-PAGE was loaded with 2 µg of purified SCP or 0.5 µg of purified Cz in accordance with Laemmli [39] and electro-transferred to nitrocellulose membranes at 30V ON. After blotting, nitrocellulose sheets were cut into strips, post-coated with Tris-buffered saline solution containing 3% non-fat power milk (TBS-M), and were probed with rabbit sera specific for Cz (1/5000) and for SCP (1/400) adsorbed with SCP and Cz (0.5, 1, and 2 µg, respectively) as described above, during 90 min. After proper washing, it was followed by incubation with secondary antibody, goat anti-rabbit IgG (H+L) HRP (Jackson Laboratories).

For participation of sulfates in the cross-reactivity between Cz and SCP

Two and five micrograms of the mix Cz plus SCP, prior and after desulfation treatment, respectively, were submitted to SDS-PAGE, 10% gel, and electro-transferred to nitrocellulose membranes at 30V ON. After blotting, nitrocellulose sheets were coated with Tris-buffered saline solution containing 3% non-fat power milk (TBS-M). Rabbit sera specific for Cz and SCP were diluted (1/5000 and 1/400, respectively). After washing, the protocol was followed by incubation with secondary antibody as above described.

For the presence of sulfated molecules in different parasite forms

Western blot analysis with epimastigotes (4×10^5/lane), amastigotes (2×10^6/lane) and trypomastigotes (4×10^6/lane) lysates, 0.5 and 1 µg of purified Cz and SCP, respectively, as control was subjected to 10% SDS-PAGE and electro-transferred to nitrocellulose membranes for 2 h at 200 mA. After blotting, nitrocellulose sheets were coated with Tris-buffered saline solution containing 3% skimmed milk (TBS-M). The transferred proteins were incubated with anti-Cz IgGs or AS-enriched IgGs (diluted 1/500 in TBS-M) for 1 h at room temperature and, afterwards, incubated with peroxidase mouse anti-rabbit IgG HRP (Jackson) (diluted 1/2000 in TBS-M) for 1 h at room temperature. Detection of immune reactive protein bands was performed with the Enhanced chemiluminescence reagent (ECL) (Amersham Biosciences, Cambridge, UK).

For evaluation of the immune recognition of SCP by sera of chronic Chagas disease patients

Purified SCP (1 µg) was loaded in various lanes, electrophoresed in SDS-PAGE (10%), electro-transferred to nitrocellulose, cut into strips and revealed with sera from patients with different degrees of cardiac dysfunctions, mild (G0/G1) and severe (G3), in accordance with the Kuschnir classification, followed by incubation with HRP-goat anti-human IgG, diluted 1/2000, and immune reactive protein bands were developed by means of 4-chloro-naftol reaction. The data analysis was performed by quantitation of signal intensity with software Image J, NIH, USA.

Statistical analysis

Results are presented as the mean plus standard deviation (SD). Each experiment was performed independently three times. Statistical analysis was performed using the PRISM software (Graph Pad Software, San Diego, CA, USA). The level of significance was set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. One-way ANOVA followed by Tukey's multiple comparison test was used to compare both immune cross-reactivity between Cz and SCP with the corresponding adsorbed sera, and for Cz, dCz, SCP, and dSCP detection in WB. Non-parametric one-way ANOVA test followed by Dunn's multiple comparisons test was used for patient's sera assays.

Results

Purification of *T. cruzi* Cz and SCP glycoproteins

It is already known that SCP co-elutes from Con-A-Sepharose affinity columns with Cz, suggesting that it also contains high-mannose oligosaccharides [40]. To purify each glycoprotein, a Mono-Q step was included [29, 41]. The peaks PIa, Ib, and PII obtained from this column correspond to SCP, the mixture of SCP-Cz, and highly purified Cz (Fig. 1, lines 2, 3, and 4), respectively. Purification of *T. cruzi* SCP was achieved by Mono-Q followed by chromatofocusing ion exchange chromatography as described by Parussini et al. (Fig. 1, line 5) [13]. Purified Cz, purified SCP, and /or the mix Cz–SCP obtained from ConA-Sepharose columns were used in different experiments through this work with the aim to study the immunogenic character and acidic post-translational modifications of the glycoprotein *Tc*SCP as well as to demonstrate the cross-reactivity with Cz and to evaluate the relevance of SCP in natural infection.

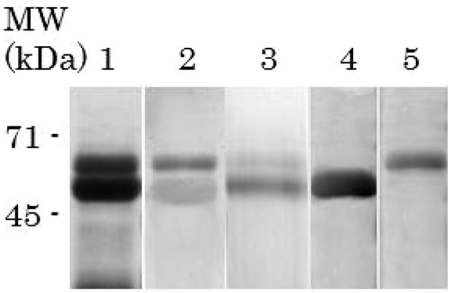


Fig. 1 SDS-PAGE of purification of Cz and SCP from epimastigotes of *T. cruzi*. Samples obtained from different steps of the purification procedure from Cz in accordance with Acosta et al. [29] were analyzed on 10% silver-stained SDS-PAGE: 5 µg Con-A-Sepharose eluate (1); 3 µg from Mono-Q Peak Ia (2); 3 µg Mono-Q fraction Ib (3); 3 µg from Mono-Q, pool II Peak, showing purified Cz (4). *T. cruzi* SCP was purified by Mono-Q followed by chromatofocusing ion exchange chromatography as described by Parussini et al. [13] (2 µg SCP, line 5). Molecular weight markers (kDa values) are shown in the left

Immune cross-reactivity between *T. cruzi* SCP and Cz glycoproteins and involvement of their sulfated moieties in shared epitopes between both molecules

Common epitopes between SCP and Cz

Sera specific for SCP and Cz, respectively, were obtained after immunizations with each of the *T. cruzi* cognate glycoproteins. With the aim to evidence whether both molecules showed immune cross-reactivity, several lanes of purified SCP and Cz (2 and 0.5 µg, respectively) were loaded in polyacrylamide gels, transferred and proved with either anti-SCP or anti-Cz sera, previously adsorbed with growing amounts of Cz and SCP, respectively. As expected, when the SCP and Cz were confronted with their respective specific sera without pre-adsorption treatment, both molecules were recognized (100%). When serum specific for Cz previously adsorbed with growing amounts of SCP was tested, a slight decrease in the immune recognition of Cz was observed. Similarly, the recognition of SCP decreased significantly with anti-SCP serum previously adsorbed with growing amounts of Cz up to disappearance when higher amounts than 5 µg of Cz were used as adsorbent (data not shown). The results obtained indicated the presence of common epitopes between both molecules. The gel bands intensities were monitored (Fig. 2A) and graph bars revealed that anti-Cz serum adsorbed with SCP reached to a reduction of 15% of Cz recognition as adsorption with SCP increased. By contrast, although the decrease in SCP recognition reached at a similar value after adsorption with growing amounts of Cz (18%) (Fig. 2B), the whole SCP recognition was lower than that of Cz (Fig. 2A). These results confirm that independently of the differential immunogenicity of both molecules, there are common epitopes between them.

Involvement of sulfates in the immune cross-reactivity between SCP and Cz

Previously, the presence of sulfate groups in the *N*-linked oligosaccharide chains of the C–T from Cz has been reported [28]. Later, we showed that sulfate-bearing glycoproteins from trypanosomatids can elicit specific immune responses [29]. Considering that both glycoproteins showed a differential immunoreactivity, indicating an immunodominance relationship of Cz on SCP and most likely a difference in sulfation grade, we took advantage of the fact that the two native proteins co-elute from ConA-Sepharose columns to evaluate them together prior and after desulfation treatment followed by immune recognition with anti-Cz and anti-SCP, respectively, to study whether sulfate groups contribute to the immune cross-reactivity above described. SDS-PAGE was loaded with different amounts of mixture of Cz–SCP and desulfated Cz–SCP followed by western blot probed with both sera specific for Cz and for SCP (Fig. 3A). The results showed that the recognition of desulfated Cz–SCP by sera specific for Cz was lower than the observed with the untreated protein mix and this effect was confirmed with the disappearance of the SCP band when 2 µg of desulfated sample were loaded. In addition, a significant decrease in the immune recognition of the desulfated forms by sera specific for SCP, a less immunogenic molecule, was observed with 5 µg and almost a completely disappearance when the minor amount of sample (2 µg) was loaded. Densitometry of the gel bands represented by bar graphs confirmed the results, with either 2 µg (Fig. 3B) or 5 µg (Fig. 3C) of sample demonstrating that the sulfate groups are involved in the observed cross-reactivity between both glycoproteins (Fig. 3C). In addition, these results evidence the relevance of sulfated epitopes in the molecules antigenicity.

Glycomic confirmation of the presence of sulfate groups in SCP

SCP-Cz sample purified by Con-A-Sepharose was analyzed by SDS-PAGE. The upper gel band was excised from the gel (Fig. 1, line 2), reduced, alkylated, and subjected to trypsin digestion. Peptides were extracted with acetonitrile/TFA and analyzed by MALDI-TOF m.s. in the reflectron positive mode using CHCA as matrix. Precursor ions *m/z* 1343.80; *m/z* 1349.86; *m/z* 1773.03; *m/z* 2352.39 y *m/z* 2386.43 were further subjected to LID fragmentation and all the informations were searched in Mascot confirming the identity of

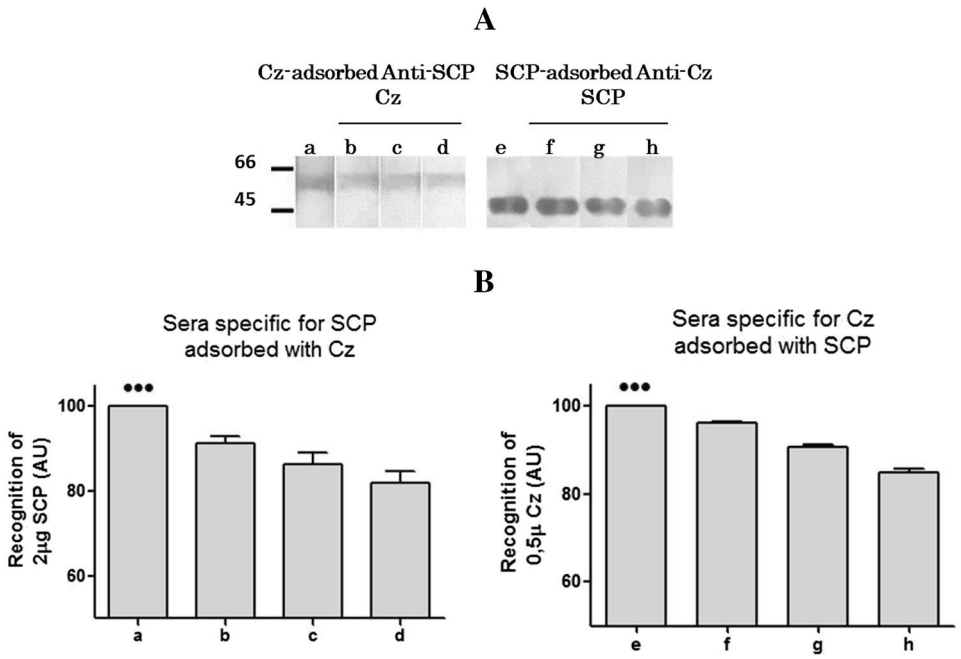


Fig. 2 Immune cross-reactivity between Cz and SCP. **A** SDS-PAGE (10%) was performed loading 2 µg of purified SCP and 0.5 µg of purified Cz in each lane and electro-transferred to nitrocellulose strips. Western blot was revealed with rabbit polyclonal serum anti-SCP adsorbed with Cz a, b, c, and d (0, 0.5, 1, and 2 µg, respectively), and rabbit polyclonal serum anti-Cz adsorbed with growing amounts of SCP: e, f, g, and h (0, 0.5, 1, and 2 µg, respectively). Anti-SCP and anti-Cz were adsorbed with Cz or SCP, respectively, using nitrocellulose as support during 2 h at 37 °C. Later, the cross-reactive antibodies fixed to the membrane are extracted from the solution corresponding to the adsorbed sera. Densitometry analysis of each band was performed considering 100% to the highest recognition value obtained with each non-adsorbed serum, in the different conditions tested. Immunereactive bands were developed by means of 4-chloronaphtol reaction. Quantitation of signal intensity was performed with a software Image J, NIH, USA and the histograms were shown as bar graphs. **B** SCP recognized by anti-SCP serum adsorbed with Cz: a vs d $^{\bullet\bullet\bullet}p < 0.001$; **C** Cz recognized by serum anti-Cz adsorbed with SCP: e vs h $^{\bullet\bullet\bullet}p < 0.001$

the serinecarboxypeptidase of *T. cruzi* (gil407841469, Mass 51726 Score: 239 Expect: 9.2e−17 Matches: 5).

In addition, knowing that SCP is a glycoprotein containing four potential *N*-glycosylation sites, after SDS-PAGE analysis, another sample, was treated with PNgase F, oligosaccharides were separated by Ultrafree McFilters (MW 5000) and freeze–dried. MALDI-TOF mass spectrometry analysis using DHB as matrix showed the presence of several high-mannose-type oligosaccharides (Fig. 4). Table 1 summarizes calculated and measured *m/z* values of each signal, the proposed composition, and structure of the released oligosaccharides. As expected, most ions corresponded to structures substituted with a sulfate group.

Enhanced presence of sulfated epitopes in the trypomastigote parasite stage

AS-enriched IgGs were obtained to evaluate the presence of sulfated epitopes in the three different developmental forms of *T. cruzi*. When western blot assays of epimastigote (Epi), amastigote (Ama), and trypomastigote (Try) lysates of Tulahuen, strain, and stock Tul 2 were confronted with anti-Cz IgGs (Fig. 5, line 1) and AS-enriched IgGs (Fig. 5, line 2), differences in the immune recognition among the different stages of the parasite were evidenced. While in epimastigotes, amastigotes, and trypomastigotes, different Cz- or Cz-like isoforms were revealed with anti-Cz IgGs when AS-enriched IgGs were probed on lysates of the mentioned parasite forms, a similar pattern recognition was observed, although with low intensity. Interestingly, when trypomastigote lysates were probed with AS-enriched IgGs, the main Cz band was not observed, indicating the absence of sulfated epitopes in this Cz isoform. By contrast, an upper band with apparent molecular weight similar to SCP was highly recognized.

Antigenicity of SCP in human chronic Chagas disease patients with different degrees of cardiac dysfunction

Antibodies against the SCP were detected in human sera obtained from patients with chronic Chagas' disease grouped according to Kurchnir classification. Figure 6 shows SCP immune recognition with sera from patients corresponding to G0/G1 and G3 groups, evidencing differences in degree

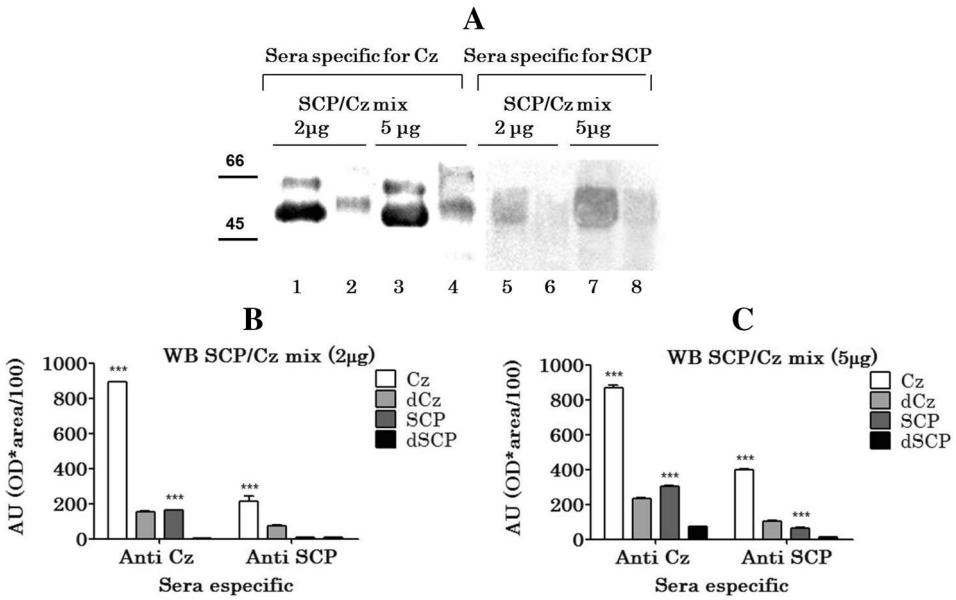


Fig. 3 Involvement of sulfate groups in the immune cross-reactivity between SCP and Cz: **A** 2 and 5 µg of mix Cz + SCP (obtained by Con-A-Sepharose affinity column) without (1, 3, 5, and 7) and with (2, 4, 6, and 8) desulfation treatment were loaded, electrotransferred and probed with sera specific for Cz (1, 2, 3, and 4) and specific for SCP (5, 6, 7, and 8); each bar represents the mean (optical density × area/100) ± SD of the recognition of Cz/dCz or SCP/dSCP by their specific serums. Detection of immunereactive bands was performed by Enhanced Chemiluminiscence (ECL), Amersham Biosciences, Cambridge, UK). Quantitation was performed with a FujiLAS1000 densitometer equipped with IMAGE GAUGE 3.122 software (Fuji Film, Tokyo, Japan). The bands corresponding to 5 µg (**B**) and 2 µg (**C**) of Cz and SCP with and without desulfation treatment after scanning were expressed in graph bars. **B** When anti-Cz was used: Cz vs dCz, $***p < 0.001$; SCP vs dSCP, $***p < 0.001$. When anti-SCP was used Cz vs dCz, $***p < 0.001$. **C** When anti-Cz was used: Cz vs dCz, $***p < 0.001$; SCP vs dSCP, $***p < 0.001$. When anti-SCP was used Cz vs dCz, $***p < 0.001$; SCP vs dSCP, $***p < 0.01$

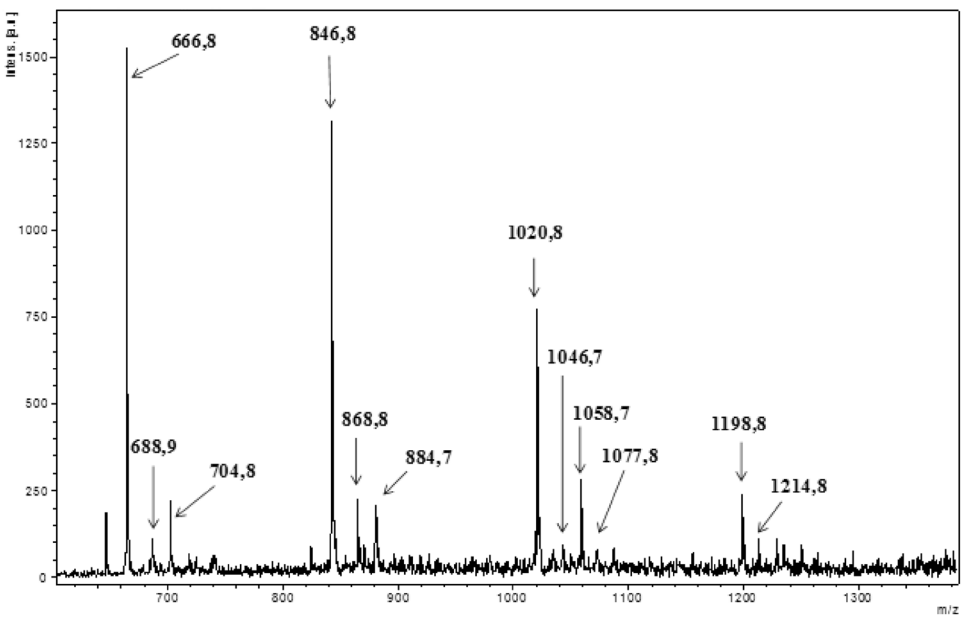
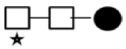
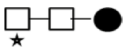
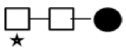
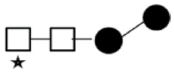
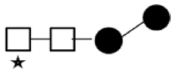
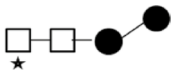
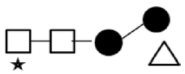
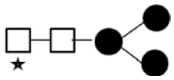
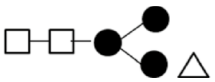
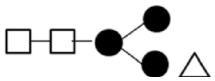
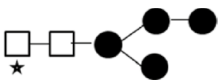
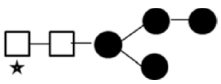


Fig. 4 UV–MALDI-TOF mass spectrum in the lineal positive mode of the oligosaccharides hydrolysed by PNGase F from SCP of *Trypanosoma cruzi*. Structures are detailed in Table 1

Table 1 *m/z* values, proposed composition, and structure of the oligosaccharides released by PNGase F

Ion	m/z [a]calculated	m/z observed	Proposed composition	Structure
$[M(OSO_3H)+H]^+$	667,2	666,8	$NacHex_2Man+ SO_4$	
$[M(OSO_3H)+Na]^+$	689,1	688,9	$NacHex_2Man+ SO_4$	
$[M(OSO_3H)+K]^+$	705,1	704,8	$NacHex_2Man + SO_4$	
$[M(OSO_3H)+H_2O+H]^+$	847,2	846,8	$NacHex_2Man_2 + SO_4$	
$[M(OSO_3H) +H_2O+Na]^+$	869,2	868,8	$NacHex_2Man_2 + SO_4$	
$[M(OSO_3H)+H_2O+K]^+$	885,2	884,7	$NacHex_2Man_2 +$	 SO_4
$[M(OSO_3H)+Na]^+$	1019,2	1020,8	$NacHex_2Man_2Fuc + SO_4$	
$[M(OSO_3H)+H_2O+H]^+$	1047,2	1046,7	$NacHex_2Man_3 + SO_4$	
$[M+H]^+$	1057,4	1058,6	$NacHex_2Man_3Fuc$	
$[M+Na]^+$	1079,4	1077,8	$NacHex_2Man_3Fuc$	
$[M(OSO_3Na)+Na]^+$	1197,3	1198,8	$NacHex_2Man_4 + SO_4$	
$[M(OSO_3Na)+H_2O+Na]^+$	1215,3	1214,8	$NacHex_2Man_4 + SO_4$	

[a]Numbers indicate monoisotopic mass. □ N-Aceylglucosamine, ● mannose, △fucose, ☆-SO_4

of cardiac dysfunction (mild vs severe) between them. Both presented a significant variable recognition of SCP in comparison with sera from healthy subjects, which showed no recognition. The low recognition observed in most patients tested, in conjunction with the low titers measured when the glycoprotein is used as immunogen in rabbits, suggests that SCP is a minor immunogen/antigen in experimental and natural infections.

Discussion

Sulfation is a post-translational modification that plays a key role in various biological processes such as ligand–receptor recognition, receptor-mediated signaling, cellular adhesion, and structural maintenance [20, 24, 42]. In *T. cruzi*, the agent of Chagas' disease, sulfated structures have been described as part of glycolipids [43, 44]. Furthermore, the

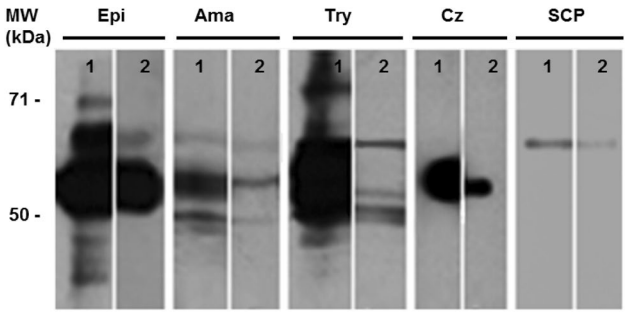


Fig. 5 Presence of sulfate moieties in Cz-like isoforms and/or another parasite sulfated glycoprotein in the three developmental stages from *Trypanosoma cruzi*. Immune recognition of sulfated epitopes by western blot of lysates from *T. cruzi*, Tulahuen strain (stock Tul 2): *Epi* epimastigote; *Ama* amastigotes; *Try* trypomastigote forms (obtained from 4×10^5, 2×10^6, and 4×10^6 parasite number, respectively), and purified Cz (0.5 µg) and SCP (1 µg), as controls were confronted with anti-Cz IgGs (1) and AS-enriched IgGs (2). Detection of the immune reactive bands was performed by Enhanced chemiluminescence reagent (ECL) (Amersham Biosciences, Cambridge, UK)

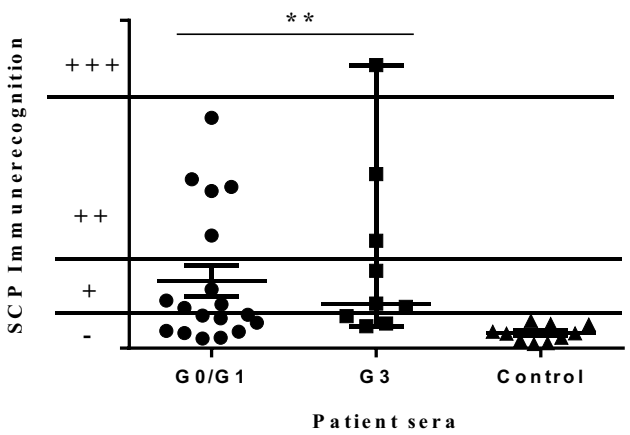


Fig. 6 Antigenicity of SCP in human chronic Chagas disease. The immune recognition of *T. cruzi* SCP by sera from chronic Chagas disease patients, grouped in accordance with Kurchnir classification, was determined by western blot. SCP-containing nitrocellulose strips (1 µg) were incubated with patient's sera (1/400). G0, G1, and G3 correspond to sera from patient's groups with different cardiac dysfunction degrees, as described in "Methods". Controls sera correspond to healthy and serological negative Chagas disease patients. In all cases, each point represents the mean of three independent determinations per patient. After incubation with HRP-goat anti-human IgG (1/2000), immune reactive protein bands were developed by means of 4-chloro-naftol reaction. The data analysis was performed by quantitation of signal intensity with software Image J, NIH, USA. Densitometry analysis of band recognition was performed considering 100% to the highest value obtained with patient sera. The immune recognition intensity was arbitrarily classified in accordance with differences in percentages as: (−) = ≤ 10%; (+) = 10–25%; (++) = 25–75%; and (+++) = ≥ 75%. Non-parametric one-way ANOVA test followed by Dunn's multiple comparisons test was used. G0/1 vs control **($p<0.05$); G3 vs control **($p<0.05$)

sulfoglycolipid structures in epimastigotes have been studied [45]. On the other hand, we have reported sulfated high-mannose-type oligosaccharides in the C–T domain of the cruzipain glycoprotein [28]. Moreover, we demonstrated that sulfates are main targets of immunological responses against Cz and mount humoral responses in patients [29]. In this sense, we have demonstrated that sulfated epitopes are shared between Cz and sulfatides of *T. cruzi* and this cross-reactivity maps to the C-terminal domain of the molecule [45]. Chlorate is known as a universal inhibitor of sulfation pathway. Recently, chlorate treatment of *T. cruzi* was used as a first approach to study the biosynthetic route of sulfates in this parasite, suggesting that *T. cruzi* sulfation occurs via PAPS [27].

In the present work, a new sulfated glycoprotein, SCP, is described in epimastigote forms of the parasite. SCP is known to be in hydrolase-rich compartments of acidic nature as Cz [46] and, therefore, might be sulfated by the same biochemical via [27]. The presence of common epitopes between Cz and SCP has been also determined, attributing them to sulfated moieties. Interestingly, in this case, short-chain-sulfated high-mannose-type oligosaccharides were determined by MALDI-TOF m.s. By contrast, Cz presents besides sulfated $GlcNAc_2Man_3$ to $GlcNAc_2Man_9$ structures, a wide variety of lactosaminic glycans, some of them bearing fucose and sialic acid [28]. It must be noted that, although several SCPs obtained from different organisms, such as *Pseudomonas* sp. B13, *Rhizomucor miehel, Monascus pilosus*, and different fungi [16, 47, 48], have been described, this constitutes the first report of a SCP substituted with sulfated glycans.

In addition, the presence of sulfated glycoproteins in the different developmental parasite stages was addressed using IgGs specific for sulfates, revealing a sulfated molecule of apparent molecular weight similar to SCP in epimastigote, amastigote, and trypomastigote forms, although enhanced in trypomastigotes. The increase observed in trypomastigote forms may be ascribed to the SCP which might be involved in *T. cruzi*–host cell interaction via mannose receptor [27, 49]. It was shown that SCP and Cz present similar sulfate linkages but differences in glycan chain length. A different exposure of the epitopes may explain the low antigenicity as well as to the titer values determined for SCP, which resulted too much lower than those from Cz.

Herein, we report that the SCP from *T. cruzi* is antigenic in the natural course of infection. When SCP was confronted with sera of infected patients with different degrees of cardiac dysfunction, although most sera recognized the protein in the mild and severe disease patient's groups, no association could be determined between the presence of sera antibodies specific for SCP and the severity of the disease. This fact may also be due to the low immunogenicity of this parasite glycoprotein, in line with the low titers observed

when used as immunogen in rabbits. Further assays will allow to verify whether antibody titers specific for sulfates from SCP are also inversely related with the severity of the disease as previously described in other sulfated glycoconjugates (Cz and sulfatides) from *T. cruzi* [29, 45]. Ongoing assays will allow to verify whether these moieties might be used as biomarkers of disease progression.

Altogether, our findings demonstrate the presence of sulfated glycoproteins in the three major stages of *T. cruzi*. Structures of two of them, SCP and Cz, have been studied, and immune cross-reactivity between *T. cruzi* SCP and Cz glycoproteins was demonstrated, involving to their sulfated moieties in shared epitopes between both molecules. In addition, the increased presence of sulfotopes in trypomastigotes suggests their possible involvement in host–parasite relationship and/or infection.

Conclusions

These findings demonstrate: (1) the presence of sulfate groups in the *N*-glycosidic short chains of the native SCP from *T. cruzi* epimastigotes (*Tc*SCP); (2) the existence of immune cross-reactivity between Cz and SCP, purified from epimastigotes; and (3) the involvement of the sulfated moieties from Cz and SCP in the shared sulfate containing epitopes or sulfotopes between both glycoproteins. In addition, we have demonstrated the enhanced presence of sulfotopes in trypomastigotes, probably involved in parasite–host relationship and/or infection. Interestingly, our results showed for the first time that SCP is a minor antigen recognized by most of chronic Chagas disease patient's sera.

Acknowledgements The authors thank to the Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, (PIP 11220110100660; PIP 07912012-2014), Universidad de Buenos Aires, UBA, (20020130100476BA) and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) PICT-2013-0736 grants as well as to the Instituto Nacional de Parasitología "Dr M. Fatala Chaben", ANLIS-Malbrán, Ministerio de Salud de la Nación, Argentina. The Ultraflex II (Bruker) TOF/TOF mass spectrometer was supported by the ANPCyT, (Grant PME 125, CEQUIBIEM).

References

1. World Health Organization (2014) WHO Fact Sheet No 340. http://www.who.int/mediacentre/factsheets/fs340/en/. Accessed 29 May 2014
2. Gascon J, Bern, Pinazo MJ (2010) Chagas disease in Spain, the United States and other non-endemic countries. Acta Trop 115(1–2):22–27
3. Pérez-Molina JA, Norman, López-Vélez R (2012) Chagas disease in non-endemic countries: epidemiology, clinical presentation and treatment. Curr Infect Dis Rep 14(3):263–274
4. Cazzulo JJ (2002) Proteinases of *Trypanosoma cruzi*: potential targets for the chemotherapy of Chagas disease. Curr Topics Med Chem 2:1257–1267
5. Duschak VG (2011) A decade of targets and patented drugs for chemotherapy of Chagas disease. Recent Pat Antiinfect Drug Discov 6:216–259
6. Duschak VG (2016) Targets and patented drugs for chemotherapy of chagas disease in the last 15 years-period, review article. Recent Pat Antiinfect Drug Discov 11:74–173
7. El Sayed NM, Myler PJ, Bartholomeu DC et al (2005) The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science 309(5733):409–415
8. Duschak VG, Couto AS (2009) Cruzipain, the major cysteine protease of *Trypanosoma cruzi*: a sulfated glycoprotein antigen as relevant candidate for vaccine development and drug target. A review. Curr Med Chem 16:3174–3202
9. Burleigh BA, Andrews NW (1995) A 120-kDa alkaline peptidase from *Trypanosoma cruzi* is involved in the generation of a novel $Ca^{(2+)}$-signaling factor for mammalian cells. J Biol Chem 270(10):5172–5180
10. Burleigh BA, Caler EV, Webster I, Andrews NW (1997) A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca^{2+} signaling in mammalian cells. J Cell Biol 136(3):609–620
11. Santana JM, Grellier P, Schrevel J, Teixeira A (1997) A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV. Biochem J 325(1):129–137
12. Bastos IM, Grellier P, Martins NF, Cadavid-Restrepo G, de Souza-Ault MR, Augustyns K, Teixeira AR, Schrevel J, Maigret B, da Silveira JF, Santana JM (2005) Molecular, functional and structural properties of the prolyl oligopeptidase of *Trypanosoma cruzi* (POP Tc80), which is required for parasite entry into mammalian cells. Biochem J 388:29–38
13. Parussini F, García M, Mucci J, Agüero F, Sánchez D, Hellman U, Aslund L. Cazzulo JJ (2003) Characterization of a lysosomal serine carboxypeptidase from *Trypanosoma cruzi*. Mol Biochem Parasitol 131(1):11–23
14. da Silva-Lopez RE, Morgado-Díaz JA, dos Santos PT, Giovanni-De-Simone S (2008) Purification and subcellular localization of a secreted 75 kDa *Trypanosoma cruzi* serine oligopeptidase. Acta Trop 107(2):159–167
15. Remington SJ, Breddam K (1994) Carboxypeptidases C and D. Meth Enzymol 244:231–248
16. Breddam K (1986) Serine carboxypeptidases. A review. Carlsberg Res Commun 51:83–128
17. Barrett A, Rawlings N, Woessner J (2004) Handbook of proteolytic enzymes. Elsevier Academic Press, London
18. Vendrell J, Avilés FX (1999) Carboxypeptidases. In: Turk V (ed) Proteases: new perspectives, vol 2. Birkhauser, Basel, pp 13–34
19. Ferreira KA, Fajardo EF, Baptista RP, Macedo AM, Lages-Silva E, Ramírez LE, Pedrosa AL (2014) Species-specific markers for the differential diagnosis of *Trypanosoma cruzi* and *Trypanosoma rangeli* and polymorphisms detection in *Trypanosoma rangeli*. Parasitol Res 113(6):2199–2207
20. Kovensky J (2009) Sulfated oligosaccharides: new targets for drug development? Curr Med Chem 16:2338–2344
21. Kawasaki N, Ohta M, Hyuga S, Hyuga M, Hayakawa T (2000) Application of liquid chromatography mass spectrometry and liquid chromatography with tandem mass spectrometry to the analysis of the site specific carbohydrate heterogeneity in erythropoietin. Anal Biochem 285:82–91
22. Honke K, Taniguchi N (2002) Sulfotransferases and sulfated oligosaccharides. Med Res Rev 22:637–654
23. Klaassen CD, Boles JW (1997) Sulfation and sulfotransferases 5: the importance of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) in the regulation of sulfation. FASEB J 11(6):404–418

24. Fukuda M, Hiraoka N, Akama TO, Fukuda MN (2001) Carbohydrate-modifyingsulfotransferases: structure, function, and pathophysiology. J Biol Chem 276:47747–47750
25. Esko JD, Lindahl U (2001) Molecular diversity of heparan sulfate. J Clin Invest 108(2):169–173
26. Baeuerle PA, Huttner WB (1986) Chlorate. A potent inhibitor of protein sulfation in intact cells. Biochem Biophys Res Commun 141(2):870–877
27. Ferrero MR, Soprano LL, Acosta DM, García GA, Esteva MI, Couto AS, Duschak VG (2014) Effects of chlorate on the sulfation process of *Trypanosoma cruzi* glycoconjugates. Implication of parasite sulfates in cellular invasion. Acta Trop 137:161–173
28. Barboza M, Duschak VG, Fukuyama Y, Nonami H, Erra-Balsells R, Cazzulo JJ, Couto AS (2005) Structural analysis of the *N*-glycans of the major cysteine proteinase of *Trypanosoma cruzi*. Identification of sulfated high-mannose type oligosaccharides. FEBS J 272(15):3803–3815
29. Acosta DM, Arnaiz MR, Esteva MI, Barboza M, Stivale D, Orlando UD, Torres S, Laucella SA, Couto AS, Duschak VG (2008) Sulfates are main targets of immune responses to cruzipain and are involved in heart damage in BALB/c immunized mice. Int Immunol 20(4):461–470
30. Cazzulo JJ, Franke de Cazzulo BM, Engel JC, Cannata JJ (1985) End products and enzyme levels of aerobic glucose fermentation in trypanosomatids. Mol Biochem Parasitol 16:329–343
31. Andrews NW, Colli W (1982) Adhesion and interiorization of *Trypanosoma cruzi* in mammalian cells. J Protozool 29(2):264–269
32. Labriola C, Sousa M, Cazzulo JJ (1993) Purification of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* by affinity chromatography. Biol Res 26(1–2):101–107
33. Bradford J (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254
34. Freeze HH, Mierendorf RC, Wunderlich R, Dimond RL (1984) Sulfated oligosaccharides block antibodies to many *Dictyostelium discoideum* acid hydrolases. J Biol Chem 259(16):10641–10643
35. Ey PL, Prowse SJ, Jenkin CR (1978) Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-Sepharose. Immunochem 15:429–436
36. Acosta DM, Soprano LL, Ferrero M, Landoni M, Esteva MI, Couto AS, Duschak VG (2011) A striking common *O*-linked *N*-acetylglucosaminyl moiety between cruzipain and myosin. Parasite Immunol 33(7):363–370
37. World Health Organization (1991) Control of Chagas disease. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser 811:1–95
38. Kuschnir E, Sgammini H, Castro R, Evequoz C, Ledesma R, Brunetto J (1985) Evaluation of cardiac function by radioisotopic angiography, in patients with chronic Chagas cardiopathy. Arq Bras Cardiol 45:249–256
39. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685
40. Duschak VG, Couto AS (2009) Cruzipain, the major cysteine protease of *Trypanosoma cruzi*: a sulfated glycoprotein antigen as relevant candidate for vaccine development and drug target. A review. Curr Med Chem 16(24):3174–3202
41. Duschak VG, Riarte A, Segura EL, Laucella SA (2001) Humoral immune response to cruzipain and cardiac dysfunction in chronic Chagas disease. Immunol Lett 78(3):135–142
42. Rapraeger AC, Krufka A, Olwin BB (1991) Requirement of heparan sulfate for ßFGF-mediated fibroblast growth and myoblast differentiation. Science 252:1705–1708
43. Petry K, Nudelman E, Eisen H, Hakomori S (1988) Sulfated lipids represent common antigens on the surface of *Trypanosoma cruzi* and mammalian tissues. Mol Biochem Parasitol 30:113–121
44. Uhrig ML, Couto AS, Colli W, Lederkremer RM (1996) Characterization of inositolphospholipids in *Trypanosoma cruzi* trypomastigote forms. Biochim Biophys Acta 1300:233–239
45. Acosta DM, Soprano LL, Ferrero MR, Esteva MI, Riarte A, Couto AS, Duschak VG (2012) Structural and immunological characterization of sulfatides: relevance of sulfate moieties in *Trypanosoma cruzi* glycoconjugates. Parasite Immunol 34(11):499–510
46. Sant'Anna C, Parussini F, Lourenço D, de Souza W, Cazzulo JJ, Cunha-e-Silva NL (2008) All *Trypanosoma cruzi* developmental forms present lysosome-related organelles. Histochem Cell Biol 130(6):1187–1198
47. Liu F, Tachibana S, Taira T, Ishihara M, Kato F, Yasuda M (2004) Purification and characterization of a high molecular mass serine carboxypeptidase from *Monascus pilosus*. J Ind Microbiol Biotechnol 31:572–580
48. Larriba E, Martín-Nieto J, Lopez-Llorca LV (2012) Gene cloning, molecular modeling, and phylogenetics of serine protease P32 and serine carboxypeptidase SCP1 from nematophagous fungi *Pochonia rubescens* and *Pochonia chlamydosporia*. Can J Microbiol 58(7):815–827
49. Garrido VV, Dulgerian LR, Stempin CC, Cerbán FM (2011) The increase in mannose receptor recycling favors arginase induction and *Trypanosoma cruzi* survival in macrophages. Int J Biol Sci 7(9):1257–1272