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Molecular mechanisms involved in the inactivation of the first component of human complement by *Trypanosoma cruzi* calreticulin

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ABSTRACT

Trypanosoma cruzi (*T. cruzi*), the agent of Chagas' disease, the sixth most important neglected tropical disease worldwide, causes 50,000 deaths per year in Latin America. *T. cruzi* calreticulin (TcCRT), a highly pleiotropic chaperone molecule, plays important roles in several host/parasite interactions. Among other functions, we have previously shown that TcCRT, translocated from the endoplasmic reticulum to the area of flagellar emergence, binds human C1q and inhibits activation of the classical pathway *in vitro*. Based on a series of *in vitro* experiments, we propose here two mechanisms to explain how TcCRT inhibits the classical pathway at the initial stages of C1 (q, r, s) activation. First, TcCRT interacts *in vitro* with both solid phase bound active C1s and C1, but impairment of C4 activating capacity is evident only when the serine proteases are within the structural context of the macromolecular first component. Although C1s activity, in this context, is inhibited by TcCRT, the serine protease is not displaced from the C1 complex. Second, TcCRT prevents C1 formation, by interfering with the ability of the $(C1r–C1s)_2$ tetramer to bind C1q. These complement inhibitory effects are better explained by direct interaction of the parasite protein with C1, rather than by the TcCRT capacity to bind calcium, an essential element for the functional integrity of C1.

1. Introduction

Trypanosoma cruzi (*T. cruzi*) is the agent of Chagas' disease (Chagas, 1909), an acute and chronic illness affecting 20 million people in Latin America (Moncayo, 2003), causing 50,000 deaths per year (Dias et al., 2002; WHO, 2002) and considered the sixth most important neglected tropical disease worldwide. It causes 0.7 million disability-adjusted life-years (Hotez et al., 2006).

In humans, the first component of the complement system, C1, is composed by C1q, the recognition module, and the two associated dimeric serine proteases C1r and C1s, thus generating the $(C1r–C1s)_2$ tetramer. Normally, activation of the classical complement pathway starts when the C1 globular heads recognize "danger signals" represented by the antigen aggregated immunoglobulin Fc domains. Alternatively, activation may also be directly promoted by some pathogen associated molecular patterns (PAMPS). Sequential activation of the C1q-bound C1r and C1s serine proteases will follow (Arlaud et al., 2002). Then, activated C1s will promote proteolysis and activation of C4, an essential step in the generation of C3 and C5 convertases (Gasque, 2004). Finally, the C5 convertase will mediate the generation of the membrane attack complex (Gasque, 2004). Thus, during the activation cascade, peptides with opsonic, anaphylotoxic, immune stimulating and membrane-destructive capacities will be generated. Therefore, any interference with the generation of C4 will result in a longitudinal inhibition of the classical pathway. Detailed knowledge on how *T. cruzi* interferes with the activation of the complement system, a main effector arm of innate immunity, may facilitate the development of strategies to interfere with this aggressor infectivity.

Resistance of infective trypomastigotes to complement activity is mediated by several molecules present on the parasite surface. Among others, the *T. cruzi* complement regulatory protein (CRP) (Norris et al., 1991; Norris and Schrimpf, 1994), the human decay accelerating factor *T. cruzi* homologue (T-DAF) (Norris et al., 1991), sialic acid (Meri and Pangburn, 1990; Tomlinson and Raper, 1998) and specific lipases (with capacity to release complement pro-

Abbreviations: *T. cruzi*, *Trypanosoma cruzi*; TcCRT, *Trypanosoma cruzi* calreticulin; HuCRT, human calreticulin; C1r, C1q-associated serine protease r; C1s, C1q-associated serine protease s; $(C1r–C1s)_2$, C1q-associated tetrameric serine protease complex.

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teins bound to parasite surface components) (Tomlinson and Raper, 1998), have been described.

We have cloned, sequenced and expressed the *TcCRT* gene, which codes for a 45 kDa protein more than 50% identical to the human counterpart (HuCRT) (Aguillon et al., 2000; Ferreira et al., 2004a, 2002). Moreover, a TcCRT region (TcS: amino acids 159–281) is up to 80% identical to certain functional regions of the HuCRT S domain (Ferreira et al., 2002). We have also shown that TcCRT, similar to its human counterpart (Stuart et al., 1997, 1996), inhibits the classical pathway of human complement activation *in vitro*, as a consequence of its capacity to interact with the first component (Ferreira et al., 2005, 2004a,b). TcCRT, translocated from the ER to the trypomastigote area of flagellum emergence, recruits C1q (and possibly C1), an immune evasion strategy, resulting in complement inhibition and increased infectivity (Ferreira et al., 2004b; Rimoldi et al., 1989), two central events in the host/parasite interactions. Because the mechanisms involved in the functional inhibition of the first complement component by TcCRT are still unknown, we address this issue herein. We now show that TcCRT not only competes with the $(C1r–C1s)_2$ tetrameric complex for binding to the collagenous C1q tails, but also operationally interferes with the activity of C1s, in a calcium independent manner, without involving the release of the serine protease from the C1q recognition module. Since these complement evasion strategies result in important decrease in C4b generation, with resulting interference in the activation of the classical pathway, important consequences in the host/parasite relationships are predictable.

2. Materials and methods

In all immunometric enzymatic assays, a goat anti-rabbit peroxidase-bound IgG and ABTS were respectively used as secondary antibody and substrate. The reactions were read at 405 nm. TcCRT binding was detected with a rabbit anti-TcCRT polyclonal antiserum, generated in our laboratory (Aguilar et al., 2005). Human C1q, C1, C1r, C1s and C4 were obtained from Complement Technology, Tyler, TX, USA. Recombinant TcCRT and polyclonal rabbit antisera against the whole molecule and functional domains were generated in our laboratory (Aguilar et al., 2005).

2.1. TcCRT interference with C1 activation

C1 (2 μg/ml in Veronal buffer, VB^{2+} (5,5-diethylbarbituric acid sodium salt 5 mM, NaCl 140 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 0.15 mM, 0.05% Tween-20)) was captured by solid phase-bound human IgG (4 μg/ml). After blocking the remaining active sites with PBS/BSA 3% for 2 h at 37 °C, and washing with TBS/Ca^{2+}/Tween-20 (TBS supplemented with 1 mM calcium and 0.05% Tween-20), increasing TcCRT concentrations in VB^{2+} were added and binding of the parasite protein was detected with a rabbit anti-TcCRT polyclonal antiserum.

C1 (1 μg/ml in VB^{2+}) was captured by solid phase-bound human IgG (4 μg/ml) and incubated with increasing TcCRT concentrations in VB^{2+}, for 1 h at 37 °C. After washing with TBS/Ca^{2+}/Tween-20, human C4 (4 μg/ml in VB^{2+}) was added. After an incubation of 1 h at 37 °C, C1 activation was assessed by measuring the covalently bound C4b deposits with goat anti-human C4 antibodies (Calbiochem). C1 integrity, at the end of the assays, was assessed using goat anti-C1q and goat anti-C1s antibodies.

2.2. TcCRT interaction with solid phase bound C1r and C1s

The solid phase was sensitized with C1r or C1s enzymes (4 μg/ml each, in VB^{2+}) overnight at 4 °C. After blocking the remaining active sites with PBS/BSA 3% for 2 h at 37 °C, TcCRT (4.8 μM in VB^{2+}) was added. After 1 h at 37 °C, the plate was washed with PBS/Tween-20 (PBS with 0.05% Tween-20). TcCRT binding to C1r or C1s was detected with a rabbit anti-TcCRT polyclonal antiserum.

The solid phase was sensitized with C1s-enzyme and C1s-proenzyme (4 μg/ml in VB^{2+}). After blocking, human C4 was added and covalent C4b binding to the solid phase was assessed with goat anti-human C4 antibodies. Alternatively, the assay was performed in the presence of increasing concentrations of TcCRT in VB^{2+} before adding C4. Equimolar concentrations of BSA were used as negative controls.

2.3. TcCRT interference with $(C1r–C1s)_2$/C1q interaction

The plastic solid phase was sensitized with IgG (4 μg/ml) overnight, at 4 °C. C1q (1 μg/ml in VB^{2+}) was added and incubated for 1 h at 37 °C. After washing with TBS/Ca^{2+}/Tween-20, different concentrations of TcCRT or HuCRT in VB^{2+} were added and incubated for 1 h at 37 °C. The plate was washed and C1r–C1s (preformed at 2 μg/ml each, in VB^{2+}) was added. After incubation for 1 h at 37 °C, the plate was washed and the binding of the serine protease complex to C1q was detected with a goat anti-human C1s antiserum (Calbiochem).

2.4. TcCRT capacity to release $(C1r–C1s)_2$ from C1

The plate was sensitized with IgG (4 μg/ml) overnight at 4 °C. C1q (1 μg/ml in VB^{2+}) was added followed by incubation for 1 h at 37 °C. After incubation with C1r–C1s (preformed at 2 μg/ml each, in VB^{2+}) for 1 h at 37 °C, the plate was washed with TBS/Ca^{2+}/Tween-20. Different concentrations of TcCRT or HuCRT in VB^{2+} were added. After 1 h incubation at 37 °C, the binding of the serine protease complex to C1q was detected with a goat anti-human C1s antiserum (Calbiochem).

2.5. Relationship between TcCRT capacity to bind calcium and its ability to inhibit C1 function

The solid phase was sensitized with IgG (4 μg/ml) overnight at 4 °C. C1 (1 μg/ml in VB^{2+}) was added and incubated for 1 h at 37 °C. After washing with TBS/Ca^{2+}/Tween-20, the calcium dependence in C1 activation, was assessed adding human C4 (4 μg/ml) in VB^{2+} with different calcium concentrations. The C4b deposit was detected with a goat anti-human C4 antiserum (Calbiochem). The limiting calcium concentration (0.10 mM) for C1 activation was included into the Veronal buffer to pre-incubate TcCRT for 30 min at 37 °C. Then, a centrifugation through a 10 kDa pore membrane (Centricon, Millipore) was performed in order to obtain an TcCRT-free buffer. This buffer was used in the C4 activation assay, as previously described, using pure C1. In addition, the TcCRT (9.6 μM) C1 (1 μg/ml)-inhibitory capacity was tested under saturating calcium conditions (5 mM in VB^{2+}), followed by the addition of C4 (4 μg/ml in VB^{2+}). Then, the protein mixture was added onto immobilized IgG and left at 37 °C for 1 h. The C4b deposit was measured as described above. As a positive inhibition control, the same experimental setup was tested with EDTA 10 mM. BSA (9.6 μM) was used as negative control.

3. Results

3.1. TcCRT binds to immobilized C1 and interferes with its activation

We previously showed that TcCRT inhibits the activation of the classical complement pathway when both TcCRT and C1 are in fluid phase (Ferreira et al., 2004b). In order to determine if this inhibition also occurs once C1 is bound to one of its natural activating

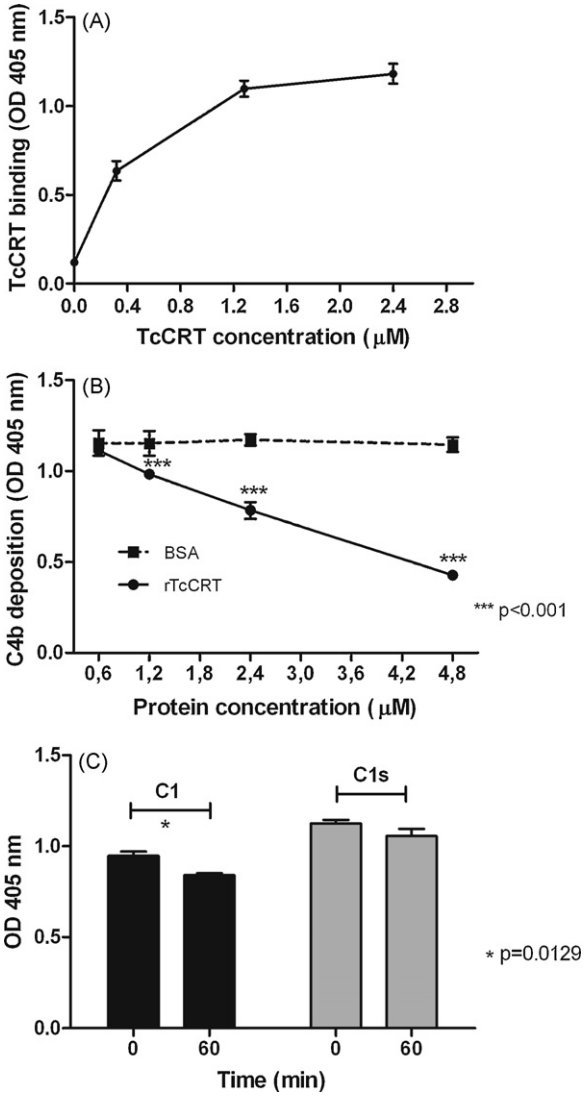

Fig. 1. TcCRT binds to immobilized C1 and interferes with its activation. C1 was captured by solid phase-bound IgG. Increasing TcCRT concentrations were added and (A) its binding to C1 was detected with a rabbit anti-TcCRT polyclonal antiserum, and (B) C1 activation was assessed by addition of human C4, and C4b deposits detected with a goat anti-human C4 antiserum. (C) The C1 integrity and its persistence in the experimental wells at the end of the assay, in the presence of the highest TcCRT concentration, was assessed using goat anti-C1q and goat anti-C1s antibodies. The results in (B) were analyzed by two-way ANOVA test and in (C) by two-tailed non-paired *t*-test. Each assay was performed in triplicates. Bars represent the standard deviations of the means.

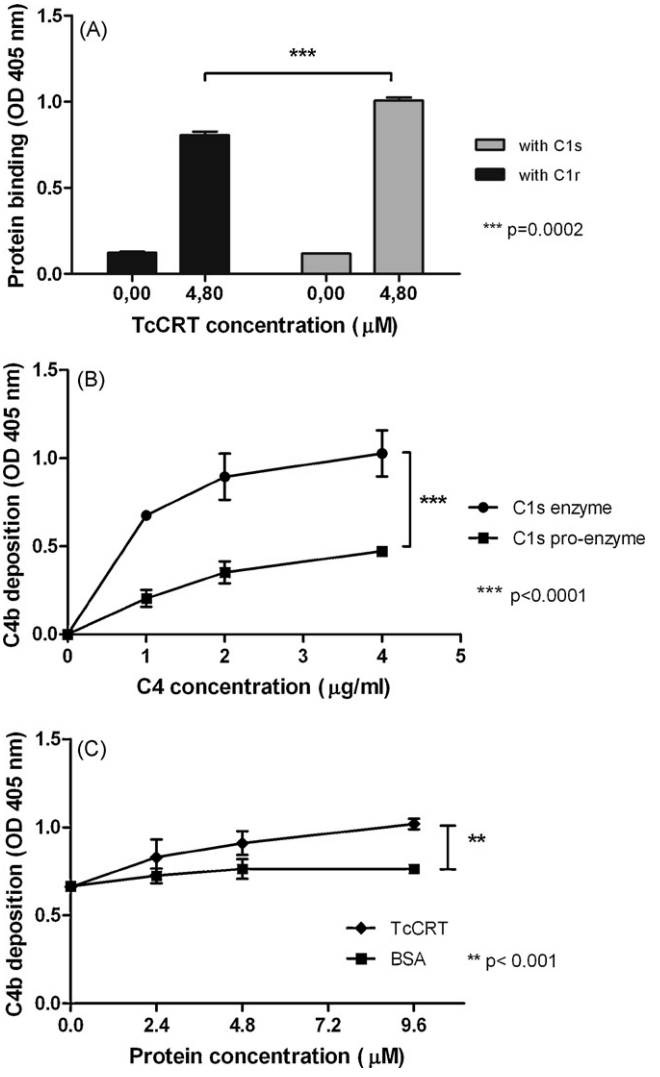

Fig. 2. TcCRT interacts with solid phase bound C1s, but does not inactivate it. (A) The solid phase was sensitized with C1r or C1s enzymes, followed by TcCRT addition. TcCRT binding to C1r or C1s was detected with a rabbit anti-TcCRT polyclonal antiserum. (B) The solid phase was sensitized with C1s enzyme and C1s pro-enzyme, C4 was added and the C4b generation was assessed by anti-human C4 antibody. (C) The solid phase was sensitized with C1s-enzyme, TcCRT was added, and C1s-enzyme activation, in the presence of TcCRT, was assessed adding C4 and detecting C4b generation by anti-human C4 antibody. The results in (A) were analyzed by two-tailed non-paired *t*-test and in (B) and (C) by two-way ANOVA test. Each assay was performed in triplicates. Bars represent the standard deviations of the means.

ligands, the first component was captured by solid phase-bound human IgG, followed by incubation with increasing TcCRT concentrations. Different from HuCRT, TcCRT binds C1 in a dose-dependent manner (Fig. 1A). Also, a significant decrease in C4b deposit was readily detected as a function of TcCRT concentrations (Fig. 1B). Since the immunometric assay used required many washes, it was necessary to confirm that C1 was still present in the experimental wells, at the end of the assay. For this reason, the experiment shown in Fig. 1C was performed before and after 60 min incubation (end of the assay), in parallel wells saved for that purpose. The highest TcCRT concentration was used, and the presence of C1 was detected with both, anti-C1q and anti-C1s antibodies. C1s remained bound to C1q throughout the assay (Fig. 1C), as determined by its reactivity with anti-human C1s antibodies. Thus, close to 90% of C1 was still present. As reported previously, TcCRT and C1q interact in a dose-dependent and saturating way (Ferreira et al., 2004b) and, as expected C1 function was abrogated by EDTA (Zapf and Loos, 1985) (data not shown).

3.2. *TcCRT interacts with solid phase bound C1s, but does not inactivate it*

Since chaperones interact with a variety of molecules, TcCRT does not seem to be an exception to this rule. We asked whether TcCRT capacity to inactivate C1 enzymatic function derives from its direct interaction with the serine proteases. Microtitration plates were sensitized with equimolar C1r or C1s concentrations. TcCRT was then added (in a C1 inhibitory concentration) and its binding to both C1r and C1s was then detected (Fig. 2A). Fig. 2B shows that C4 is activated, in a dose-dependent and saturating manner, by solid phase bound C1s. Equimolar concentrations of the C1s pro-enzyme showed residual C4 activating capacity, due to minor contamination with C1s enzyme. TcCRT, tested in this experimental set up,

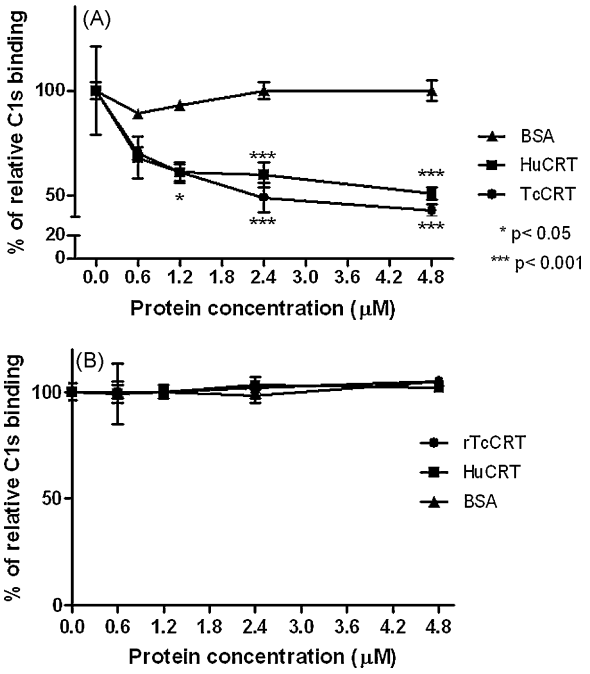

Fig. 3. TcCRT prevents binding of $(C1r–C1s)_2$ to C1q, but does not release the serine proteases from immobilized C1. C1 was captured by solid phase-bound human IgG. C1q was incubated with different TcCRT, HuCRT or BSA concentrations, before (A) or after (B) incubating with C1r–C1s. The binding of the serine proteases to C1q was detected with a goat anti-human C1s antiserum. The results were analyzed by two-way ANOVA. Each assay was performed in triplicates. Bars represent the standard deviations of the means.

under non-saturating C4b generating conditions, did not show C1s inhibitory effects (Fig. 2C). In other words, the TcCRT/C1s enzyme interaction did not affect its enzymatic activity, when the enzyme is bound to the plastic solid phase.

3.3. TcCRT prevents binding of C1r and C1s to C1q, but does not release the serine proteases from preformed immobilized C1

C1q was captured by solid phase-bound human IgG and then incubated with different concentrations of TcCRT, HuCRT or BSA, before (Fig. 3A) or after (Fig. 3B) incubating with C1r and C1s. The binding of the serine proteases to C1q was then measured in immunometric assays. Although both HuCRT and TcCRT prevented the binding of the serine proteases to C1q, in a dose-dependent manner, the chaperones did not displace the serine proteases from the preformed C1 complex, regardless of their parasitic or human origins.

3.4. TcCRT-mediated C1 function inhibition is not related with the capacity of the parasite protein to bind calcium

Since TcCRT is a calcium-binding protein (data not shown) and calcium is essential for C1 function (Gregory et al., 2003; Teillet et al., 2008), the possibility exists that TcCRT sequesters C1-bound calcium, with resulting functional inhibition. To address this question, a non-saturating calcium concentration for C4 activation was determined as shown in Fig. 4A, where the results of a C4b deposition assay performed in the presence of increasing calcium concentrations are summarized. To the buffer containing a limiting calcium concentration (0.1 mM), calcium-free TcCRT was added (to a final concentration of 0, 4.8 and 9.6 μM). The mixtures were centrifuged through a 10 kDa pore membrane, in order to retain TcCRT and newly bound calcium. The flow through, TcCRT-free buffers, were used in the C4 activation assay, in the presence of pure C1 (Fig. 4B).

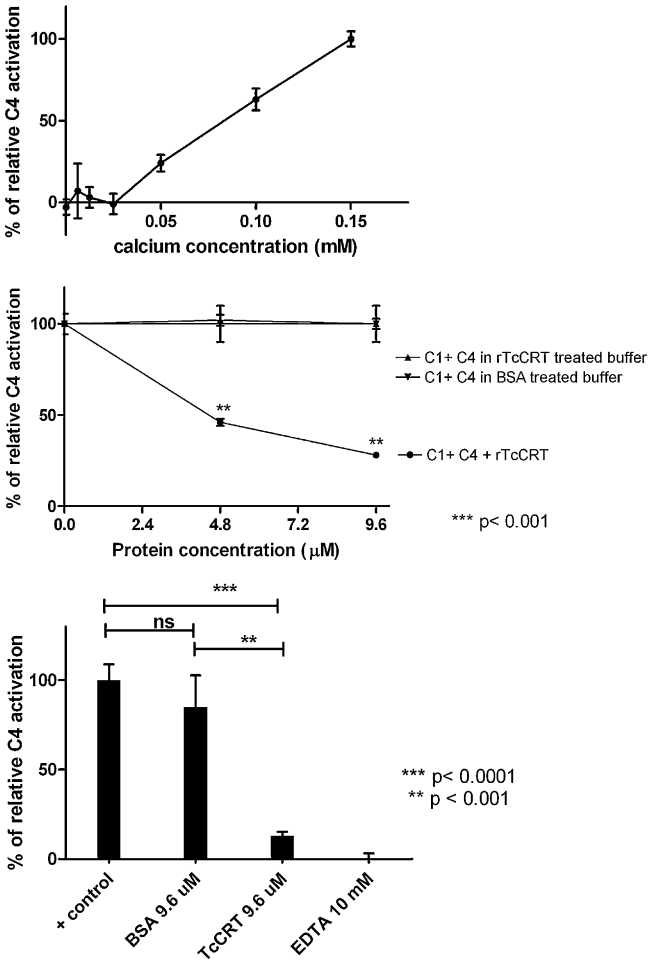

Fig. 4. TcCRT inhibition of C1 function is not a consequence of its calcium-binding capacity. (A) C1 was captured by solid phase-bound IgG and its activation was assessed by its capacity to activate human C4, under different calcium concentrations. (B) TcCRT was incubated in VB^{2+} buffer, under non-saturating Ca^{2+} conditions. TcCRT-free buffer was used in the C4 activation assay, as shown in (A), using pure C1 or human serum as a complement source. (C) TcCRT or BSA was incubated in VB^{2+} buffer under saturating Ca^{2+} conditions. C4 activation was measured as C4b deposits with a goat anti-human C4 antiserum. EDTA was used as positive inhibition control. The results in (B) were analyzed by two-way ANOVA and in (C) by two-tailed non-paired *t*-test. Each assay was performed in triplicates. Bars represent the standard deviations of the means.

No inhibition of C4 activation occurred under these conditions. As a positive control, the inhibition mediated by the presence of the parasite molecule is shown in the same figure. As an alternative approach, we reasoned that if TcCRT-mediated inhibition of C4 activation also occurs under calcium excess conditions, then a role for TcCRT calcium binding in the inhibition process could be ruled out. Thus, TcCRT or BSA was incubated in buffer under saturating calcium conditions and C4 activation was measured. As expected, TcCRT mediated a strong inhibitory effect, in the presence of calcium excess. BSA and EDTA were used as negative and positive controls, respectively (Fig. 4C).

4. Discussion

By interacting with the first complement component *in vitro*, the *T. cruzi* chaperone molecule TcCRT, similar to HuCRT (Stuart et al., 1997, 1996), inhibits the human classical complement pathway (Ferreira et al., 2005, 2004a,b). TcCRT, most likely in a *T. cruzi* immune evasion strategy, is translocated from the parasite ER to the area of flagellum emergence. There, complement sub-

component C1q is swiftly recruited, the system is inhibited and "eat me" signals are sent to host cells, with consequent increases in infectivity (Ferreira et al., 2004b; Rimoldi et al., 1989), after an apoptotic mimicry parasite effort. In separate experiments (results not shown), C1 also binds to the trypomastigote surface.

Therefore, TcCRT seems to play central roles in parasite survival by intervening early in the complement activation cascade. The results presented here contribute to understand how TcCRT/C1q interactions lead to complement inactivation.

C1 was captured by solid phase-bound human IgG, followed by incubation with C4 in the presence of increasing TcCRT concentrations. Since C4b deposits decreased as a function of TcCRT concentrations (Fig. 1B), we then tested if direct effects of the parasite molecule on C1s activity occur. As shown in Fig. 2A, both human serine proteases bound to the parasite molecule, a fact consistent with their important homology, not only in overall amino acid sequence, but also in several functional domains (Gal et al., 2009; Sim et al., 1977). In agreement with these results, TcCRT interaction with solid phase bound C1 occurred in a dose-dependent manner (Fig. 1A), a result paralleled by TcCRT capacity to inhibit the C4 activation by C1 (Fig. 1B).

Fig. 2B shows that C4 is activated, in a dose-dependent and saturating manner, by solid phase bound C1s (most likely the C1s pro-enzyme residual activity is due to contaminating C1s-enzyme, as detected in reducing SDS-PAGE; results not shown). TcCRT, tested in this experimental set up, under non-saturating C4b generating conditions, was ineffective to inhibit the function of solid phase bound C1s, as summarized in Fig. 2C. Curiously, when C1s is bound to a plastic solid phase, TcCRT mediated a moderate but significant ($p < 0.001$) increase in C1s capacity to cleave C4, as compared to the situation when the serine protease is located in its natural C1 microenvironment.

Recent data on the C1 complex crystallographic structure indicate that the C1r–C1s serine proteases locate inside the dome generated by the six collagenous tails (Wallis et al., 2009). This interaction is strong since six binding sites are generated between the tetramer and C1q. Most likely, in order to exert its inhibitory effect, the parasite molecule either accesses this dome in order to interact with the protease or, alternatively, TcCRT interacts with the enzyme catalytic sites when they protrude between the collagenous tails, after C1 interacts with immune complexes or PAMPS (Phillips et al., 2009; Wallis et al., 2009). In both cases, relevant C4 interactions with C1s are prevented.

The fact that TcCRT inactivates C1s only in the C1 context (Fig. 1B) may be explained by its dual capacity to interact both with the collagenous tails, as shown previously (Ferreira et al., 2004b), and also with C1s, as shown here. Thus, in the C1 molecule, adequate structural and affinity conditions are provided for TcCRT C1s inhibition.

We then asked whether TcCRT prevents the binding of C1r and C1s to C1q or releases the serine proteases from immobilized C1. Fig. 3 shows that, although both HuCRT and TcCRT did prevent the binding of the serine proteases to C1q, neither of them displaced the serine proteases from the preformed stabilized C1 complex, during the incubation times tested.

With regard to the TcCRT calcium-binding capacity and its role in the inhibition of C1, as shown in Fig. 4, the parasite molecule inhibited C4 activation both under limiting or excess calcium conditions. Most likely, the TcCRT capacity to inhibit C4 activation is based mainly on the ability of the parasite molecule to directly interact with the serine proteases and C1q.

Of relevance to our findings is the fact that, the CUB domain, present in the HuCRT S domain (aa 160–283), is also present in the serine proteases C1r and C1s (Stuart et al., 1997). The HuCRT S domain binds to the collagenous C1q tails, at the $(C1r–C1s)_2$ binding sites and inhibition of the classical pathway follows (Stuart et al., 1996). Therefore, it has been proposed that soluble HuCRT S domain may also inhibit the binding of C1q (and possibly MBL) to their receptor, cell surface calreticulin (Zhou et al., 1991). In serum, C1q is mainly found as a part of the C1 complex (about 90% of total C1q (Steino et al., 2004)), that does not bind to HuCRT. However, at sites of inflammation, complement activation and local C1q synthesis by macrophages or microglia may result in increased supply of native C1q, which may interact with excess $(C1r–C1s)_2$ tetramer and with C1q receptors (Zhou et al., 1991). In the context of *T. cruzi*, we have previously shown that the parasite translocates its calreticulin from the ER to the area of flagellum emergence, where it interacts with C1q. In agreement with the results reported here, under these conditions, the first complement component should be unable to recruit $(C1r–C1s)_2$ and a privileged microenvironment may be generated around the parasite. Moreover, parasite bound C1q increases its infectivity (Rimoldi et al., 1989) by sending "eat me" signals to a variety of host cells, in a similar way as apoptotic mammal cells do (Fraser et al., 2009). In this regard, recently we have shown that parasite surface TcCRT recruits C1q, probably as a *T. cruzi* apoptotic cell mimicry strategy (unpublished result). Finally, we show here a novel interaction between TcCRT and C1r/C1s.

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