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The Classical Activation Pathway of the Human Complement System Is Specifically Inhibited by Calreticulin from Trypanosoma cruzi

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The high resistance of Trypanosoma cruzi trypanomastigotes, the causal agent of Chagas’ disease, to complement involves several parasite strategies. In these in vitro studies, we show that T. cruzi calreticulin (TcCRT) and two subfragments thereof (TcCRT S and TcCRT R domains) bind specifically to recognition subcomponents of the classical and lectin activation pathways (i.e., to collagenous tails of C1q and to mannan-binding lectin) of the human complement system. As a consequence of this binding, specific functional inhibition of the classical pathway and impaired mannan-binding lectin to mannosme were observed. By flow cytometry, TcCRT was detected on the surface of viable trypanomastigotes and, by confocal microscopy, colocalization of human C1q with surface TcCRT of infective trypanomastigotes was visualized. Taken together, these findings imply that TcCRT may be a critical factor contributing to the ability of trypanomastigotes to interfere at the earliest stages of complement activation. The Journal of Immunology, 2004, 172: 3042–3050.

C hagas’ disease, an endemic, normally incurable ailment, caused by the Trypanosoma cruzi protozoan, is a major health problem in Latin America, where 18 million people are infected and 90 million are at risk (1, 2). Although T. cruzi is normally transmitted by infected triatomid insects, cases associated with blood transfusion, organ transplantation, and congenital infection have been described, even in nonendemic countries (3–5).

T. cruzi infection results in a generally self-limiting acute parasitemic phase, followed by an indeterminate stage in which parasitemia is commonly undetectable and most patients remain asymptomatic. Approximately 30% of individuals in the indeterminate phase progress to a chronic stage with severe cardiomyopathy or pathological enlargement of the digestive tract (megasyndrome). People with untreated lifelong T. cruzi infections have low-level parasitemias and easily detectable Abs to parasite Ags (6, 7).

Calreticulin (CRT) is highly conserved and present in every cell of higher organisms, except erythrocytes. It is associated with lectin-like chaperoning, calcium storage and signaling, modulation of gene expression, induction of phagocytosis of apoptotic cells, mediator of autoimmunity, anti-angiogenesis and inhibition of tumoral growth, participation in the lytic activity of perforins from T and NK cells, and inhibition of C1q-dependent complement activity in vitro (8, 9).

The classical and lectin pathways of complement activation are initiated by related, but distinct molecules, with C1q being the only recognition component of the classical pathway. Mannan-binding lectin (MBL), L-ficolin, and H-ficolin each may serve as carbohydrate recognition components of the lectin pathway (10, 11). MBL, a member of the collectin family (12), is present in serum as a multimer of homotrimeric chains composed of an N-terminal collagen-like region, a neck region, and a globular C-terminal C-type lectin domain (13). The structural and functional properties of both MBL and collectins are strikingly similar to those of C1q, except that the latter has globular Ig binding domains and lacks lectin activity (14, 15). The collagen-like part of C1q and MBL interacts with serine proteases (10, 16–18), which are responsible for the complement-activating properties of these recognition molecules, through the cleavage of C4 and C2 (10, 18, 19).

Human CRT (HuCRT) binds to the collagenous region of C1q and members of the collectin family, such as MBL, lung surfactant protein A, bovine conglutinin, and collectin 43 (20–24). The collagenous tails of C1q and MBL bind to HuCRT, through its 12-kDa S domain (included in N and P domains) (21, 22, 24). Functional consequences in the complement pathway, of the binding of vertebrate CRT to complement components, have been shown only for human C1q (21, 22, 25–27). Physiological consequences of binding of vertebrate CRT to human MBL have not been demonstrated. Likewise, no information is available with regard to a possible role of CRT, from protozoan parasites, in the modulation of human C.

HuCRT is ~50% identical with CRT from Onchocerca volvulus, Schistosoma mansoni, Leishmania donovani, and T. cruzi. The consensus features of all CRT proteins are an acidic C-terminal, a proline-rich P, and a globular N-terminal domain (8, 28, 29). The amino acid sequences of both the N-terminal and P domains of CRT are well conserved among species, suggesting important roles...
in the functions of this protein. The primary sequences of CRT, from several species, initiate with a signal peptide and terminate with Lys, Asp, Glu, Leu or related endoplasmic reticulum (ER) retention sequences (8, 30–33). However, CRT is also present in a wide spectrum of non-ER locations (20, 34–41).

One of the early lines of vertebrate host defenses is the activation of the complement system, which can result in direct killing of microorganisms, their enhanced clearance by phagocytes, and potentiation of acquired immunity (42). As a countermeasure, many pathogens, especially blood-dwelling organisms and those that disseminate from the initial site of infection, have evolved elaborate means to avoid these defensive strategies.

_T. cruzi_ CRT (TcCRT) was first isolated in our laboratory and named Tc45 (43–46). We have cloned, sequenced, and expressed a TcCRT gene (32). TcCRT from another _T. cruzi_ strain has also been characterized (33, 47). TcCRT is a 45-kDa immunodominant (45), dimorphic Ag (different parasite clones present alternative 45- and 43-kDa forms), with multiple gene copies located in a variable number of chromosomes (32, 43). Importantly, certain regions within the TcCRT S (TcS) domain are up to 80% identical with HuCRT. This important degree of similarity, in relevant functional domains, prompted us to propose that TcCRT binds to host C1q and MBL, with possible inhibitory consequences in complement activation.

In _T. cruzi_, important proteins participating directly in the stage-specific inhibition of complement activation, such as complement regulatory protein and decay-accelerating factor-like (48–53), have been described. Thus, _T. cruzi_ trypomastigotes, but not epimastigotes, are normally resistant to complement lytic effects. During the course of chronic infections, however, the vertebrate hosts produce Abs that render trypomastigotes sensitive to lysis, primarily via the alternative complement cascade. These Abs could block the above-mentioned regulatory proteins, among others (54). Complement-activated lysis of trypomastigotes in vitro requires an intact alternative pathway because serum depletion of factor B and P completely abrogates lysis of trypomastigotes precoated with IgG (54, 55). The classical pathway, although unable to efficiently lyse trypomastigotes on its own, serves an enhancing effect on the amplifying properties of the alternative pathway (48). This impairment of the classical pathway suggests the importance of exploring the existence of other parasite complement regulatory proteins. TcCRT may be relevant in this respect.

Native TcCRT is immunogenic in humans (43, 44) and mice (45). Thus, most, if not all, _T. cruzi_-seropositive humans display variable amounts of anti-TcCRT IgGs. This important fact implies that experimental or natural infection with _T. cruzi_ provides TcCRT to immunocompetent cells, via surface expression, secretion, significant parasite attrition in the tissues of the host, or combinations thereof.

In this study, we investigate the ability of TcCRT to specifically interact with human C1q and MBL, and whether these interactions result in functional alterations of the corresponding pathways. Our results show in vitro specific binding of TcCRT to human C1q collagenous tails and to human MBL. As a functional consequence, the C1q-mediated classical pathway is strongly impaired. These findings represent the first study showing the ability of CRT, from an intracellular parasite, to specifically bind to C1q, with important functional consequences in the activation of the vertebrate classical complement system. Because, as also shown in this study, TcCRT is expressed on the surface of infective trypomastigotes and colocalizes with human C1q, its in vivo interaction with C1q may mediate complement inhibition in the microenvironment surrounding the parasite.

### Materials and Methods

#### Generation of rTcCRT and its TcS and TcCRT R domains

The TcCRT gene and fragments coding for the TcS (aa 159–281) and TcCRT R domains (aa 136–281) domains (the latter with an extra 23 N-terminal residues) (Entrez accession AF162779) were amplified by PCR using platinum Taq polymerase (Invitrogen, San Diego, CA). The primers used were: for TcCRT, reverse (5′-gaattaacctagctacaccttctt-3′) and forward (5′-ggaattcctggtttctccacag-3′) (Invitrogen); for the TcS and TcCRT R domains, reverse (5′-gtaaggtgcctctcgcggagtagggactcag-3′) and forward (5′-ataagacatcagctcctcggagtagggactcag-3′) (TAGN, Gatehead, U.K.). The amplified TcCRT DNA was purified and ligated into the EcoRI restriction enzyme site of the pET-28b+ plasmid (Novagen, Madison, WI). DNA of TcS and TcCRT R domains was ligated into the BamHI/NdeI restriction enzyme sites of the pET15b plasmid (Novagen). Competent Escherichia coli TOP10F* bacteria were transformed with the plasmids, plated (Luria-Bertani medium), and selected with kanamycin (50 μg/ml) in the case of TcCRT, or with ampicillin (50 μg/ml) in the case of the TcS and TcCRT R domains. For protein expression, _E. coli_ BL21(DE3)pLysS was transformed with the corresponding plasmids and grown in the presence of chloramphenicol (34 μg/ml), with kanamycin (50 μg/ml) for TcCRT, or with ampicillin (50 μg/ml) for the TcS and TcCRT R domains. After induction with 1 mM isopropyl-β-D-thiogalactoside and 3 h of incubation, the cells were harvested, sonicated on ice, and centrifuged, and the supernatants were filtered. The recombinant proteins were purified using His Bind resin (Novagen), eluted with buffer containing 1 M of imidazole, and dialyzed against 2 mM of Tris-HCl and 150 mM of NaCl, pH 7.4. The TcS and TcCRT R domains were treated with thrombin (Roche, Mannheim, Germany) to remove the His-tag, and further purified by affinity chromatography (UNO Q Biochromatography column; Bio-Rad, Hercules, CA). Purity of rTcCRT and its TcS and TcCRT R domains was assessed by conventional SDS-PAGE.

#### Parasites

MF strain trypomastigotes, from a chronic Chagasic patient, were cultured on a monolayer of 75% confluent Vero cells in RPMI 1640 (Sigma-Aldrich, St. Louis, MO), supplemented with penicillin-streptomycin (100 U/ml, 100 μg/ml), and 5% FCS, all from Life Technologies, in a 5% CO2 atmosphere. Trypomastigotes were harvested from the cell culture supernatant from the fourth day postinfection. The supernatants were centrifuged, and the parasites were resuspended in RPMI 1640 with 10% FCS.

Axenic cultured epimastigotes (Y strain), donated by Y. Repetto (Institute of Biomedical Sciences, Faculty of Medicine, University of Chile), were used to obtain a cell extract for Immuno Western blot (IWB).

#### Production of polyclonal antiserum to purified TcS domain

Two 6-month-old New Zealand White female rabbits (Institute of Public Health, Santiago, Chile) were bled from the central ear artery, as a source of preimmune sera. Four days later, the animals were immunized s.c. with 150 μg of TcS domain in CFA. On two subsequent occasions, 7 days apart, the same volume of CFA was injected with 150 μg of TcS domain in IFA. The animals were bled twice per week, starting 7 days after the third immunization. Reactivity of the two antiseras (Co10 and Co11) and their respective preimmune sera were evaluated by ELISA and IWB against rTcCRT, TcS and TcCRT R domains, and whole epimastigote extract. All immunizations and bleeding procedures were performed under veterinary supervision.

Rabbit IgG F(ab’)2 were generated from anti-TcS and preimmune sera, by standard procedures (56).

#### ELISA

Unless otherwise indicated, ELISAs were performed using Nunc Maxisorp plates (Fisher Scientific, Pittsburgh, PA) coated with 100 μl/well of proteins diluted in carbonate buffer (15 mM of Na2CO3, 35 mM of NaHCO3, pH 9.6). As controls, wells received buffer alone or with 3% v/v BSA. Nonspecific binding sites were blocked with 5% v/v BSA in PBS. Each step was followed by washing with PBS/0.05% Tween 20. HRP (595 nm) and alkaline phosphatase (405 nm) activities were assessed by addition of 2-(1-(4-hydroxymethyl) phenyl)amine]-3-methylbutyldiene]-5,5-dimethyl-1,3-cyclohexanedione, 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate with H2O, and 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich), respectively.

**ELISA to assess the interaction of TcCRT with human C1q and MBL.** Plates were coated with rTcCRT or its TcS or TcCRT R domains or BSA at 0.3 μM. Then 0.04 μg of pure human C1q (Sigma-Aldrich) was added...
in a final volume of 100 μl in PBS/1% w/v BSA/0.05% Tween. Bound C1q was detected with an affinity-purified rabbit anti-human C1q antiserum (DAKO, Carpinteria, CA), followed by affinity-purified HRP-conjugated goat anti-rabbit Ig Abs (DAKO). To detect the interaction of rTcCRT with human MBL, plates were blocked with TBS with 0.1% w/v human serum albumin (TBS/HS/A) and washed with TBS/0.05% Tween5/5 mM of CaCl2. A total of 100 μl of pure human MBL (Statens Serum Institut, Copenhagen, Denmark) (0.8–0.9 μg) was added in MBL-binding buffer (10 mM of Tris-HCl, 10 mM of NaCl, 10 mM of CaCl2, 0.05% Triton X-100, and 0.1% w/v HSA, pH 7.4). Bound MBL was detected with a mouse anti-human MBL mAb (Ab 131-1; Ab Shop, Gentofte, Denmark), followed by an affinity-purified alkaline phosphatase-conjugated goat anti-mouse IgG Ab (DAKO).

ELISA to assess interaction of TcCRT with C1q collagen-like stalks. Plates were coated with 0–50 μg/ml soluble human C1q collagen-like stalks (0–11 μM) with 2% w/v BSA (TBS/BSA). Washes were performed throughout the assay with TBS (10 mM of NaCl)/0.05% Tween. A total of 100 μl of rTcCRT or TeS (1 μM) was added in binding buffer, TBS (10 mM of NaCl)/0.05% Tween 20/2% w/v BSA. Bound TcCRT was detected with a mouse anti-TcCRT mAb (E2G7), generated previously by us (44), followed by an affinity-purified HRP-conjugated goat anti-mouse IgG Ab (Sigma-Aldrich).

ELISA to detect classical pathway complement activation. Plates were coated with purified human IgM (2 μg/ml) and incubated with normal human serum (NHS) in a final 1/60 dilution, in the presence or absence of rTcCRT or its TeS or TcCRT R domains or control proteins (0–8.8 μM), diluted in Veronal buffer20 (VB20) (5 mM of 5,5-diethylbarbituric acid sodium salt, 140 mM of NaCl, 0.5 mM of MgCl2, and 0.15 mM of CaCl2) with 0.05% Tween 20. Deposition of activated human C4 was assessed using a phosphatase-conjugated chicken Ab (Immunosystem AB, Uppsala, Sweden).

ELISA to detect inhibition of human MBL binding to mannan. Wells were coated with 10 μg/ml mannan (from Saccharomyces cerevisiae; M7504; Sigma-Aldrich), blocked with TBS/HS/A, and washed with TBS/0.05% Tween5/5 mM CaCl2. NHS or human C1q-deficient sera (Calbiochem, La Jolla, CA), diluted 1/60, were premixed with the proteins (TeS or TcCRT R domains, recombinant Schistosoma japonicum GST–rGST, or BSA; 0–1.3 μM) in modified VB20 (4 mM of 5,5-diethylbarbituric acid sodium salt, 10 mM of NaCl, 2 mM of CaCl2, and 1 mM of MgCl2, pH 7.4). A total of 100 μl of each mixture was transferred to the blocked mannan-coated plate and further incubated for 1 h, at 37°C. Human MBL binding was detected by using the mAb 131-1, as described above.

ELISA to detect lectin pathway complement activation. Mannan-coated plates were incubated overnight at 4°C, with dilutions of NHS in high salt–MBL-binding buffer (20 mM of Tris-HCl, 1 M NaCl, 0.05% Triton X-100, and 0.1% w/v HSA, pH 7.4), as a source of MBL–MBL-associated serine protease (MASP). Plates were washed with TBS/0.05% Tween5/5 mM of CaCl2, and rTcCRT or TeS was added (5 μM), followed by 1 μg/ml human C4, purified by standard procedures (58), in modified VB20. Activation of human C4 was assessed by measuring depositions of C4b with a specific rabbit polyclonal conjugated chicken Ab (Immunosystem AB). A sub-saturating amount (0.3 μM) of MBL–MASP complex (Statens Serum Institut) was mixed with 1 μM of rTcCRT or TeS in modified VB20 and added to the mannan-coated plates. A total of 0.1 μg/ml human C4 was added immediately and incubated for 1 h at 37°C, and C4b deposition was assessed.

**Hemolytic assays**

For classical pathway complement activation, SRBC was sensitized with rabbit Abs (donated by D. Castillo, Institute of Public Health). A total of 107 Ab-sensitized erythrocytes (EA) was incubated in the presence of NHS diluted 1/100 in VB20 in a 200 μl final volume. In a similar assay, C1q-dependent hemolysis was assessed using C1q-depleted human serum (Calbiochem), diluted 1/40, and a limiting amount (1 μg) of purified human C1q (Sigma-Aldrich). In these assays, rTcCRT, TeS, and TcCRT R domains and controls (BSA and rGST) were tested by premixing appropriate concentrations (0–1.1 μM) with the complement source (100 μl total volume), before addition of the mixture to the EA (107 in 100 μl), followed by a 60-min incubation. After addition of 50 μl of cold VB20, intact cells were centrifuged (1250 × g, 7 min), and hemoglobin was measured at 405 nm in the supernatant. Total hemolysis (100%) was measured by lysing 107 EA with water. Background spontaneous hemolysis (0%) was determined by incubating EA without sera. Hemolytic activity was expressed as a percentage of the total hemolysis.

Assessment of rTcCRT location on the surface of live trypomastigotes: fluorescence immunoassay with confocal immunoassay

A total of 1.5 × 107 live MF trypomastigotes was incubated at 4°C for 30 min with rabbit anti-TcS domain sera (Co10 and Co11), the respective preimmune sera, or rabbit anti-T. cruzi GAPDH serum (donated by J. Carzulo, Fundación Campomar, Buenos Aires, Argentina) (1/200) in RPMI 1640 with 10% FCS, in a final 40 μl. Parasites were then washed with cold RPMI 1640 and incubated with affinity-purified FITC-conjugated goat anti-rabbit Ig Abs (BD Pharmingen, San Diego, CA), at 4°C for 30 min. Parasites were washed and resuspended in RPMI 1640. Propidium iodide (1 μg/ml; Sigma-Aldrich) was added to each sample at the moment of acquiring the data. Stained trypomastigotes were analyzed by flow cytometry using a BD Biosciences (San Diego, CA) FACScan equipped with CellQuest software, and a minimum of 10,000 events per sample was counted. TcCRT on live parasites was expressed as relative fluorescence intensity, gated based on scatter parameters (forward light scatter (FSC) × side scatter (SSC)) and negative staining for propidium iodide (fluorescence 3 (FL3) negative), although the presence of FL3-positive parasites was less than 1%, before gating. The relative FITC fluorescence intensity of the gated events was expressed as overlayed histograms of the results obtained with the TcCRT-specific antisera and the negative controls (preimmune sera and rabbit anti-T. cruzi GAPDH polyclonal antiserum). A control experiment was conducted in parallel to validate the anti-GAPDH Ab (which recognizes the exclusively intracellular protein). The parasites were fixed with 4% paraformaldehyde and processed under the conditions already mentioned. These results were also expressed as an overlayed histogram, but only gating for the FSC × SSC dot-plot distribution (all FL3-positive).

**Results**

**rTcCRT and its TeS and TcCRT R domains bind human C1q, C1q collagen-like stalks, and MBL**

Correct expression and purity of rTcCRT and its TeS and TcCRT R functional domains were verified by SDS-PAGE (data not shown). Because we have observed that the N-terminal region of the HuCRT S domain degrades slowly, we have extended the N-terminal region of the TcCRT S domain. Sequences of TcS and TcCRT R domains with human MBL. A specific, dose-dependent, and saturable binding of C1q to increasing concentrations of rTcCRT and its TeS and TcCRT R domains is observed (Fig. 1. A–C, respectively). Also, as expected, TcCRT S domain interacts with C1q specifically through the C1q collagen-like stalks (Fig. 2).

Fig. 3. A, and B, shows the results of ELISAs detecting interactions of TcCRT and TeS and TcCRT R domains with human MBL. A specific, dose-dependent, and saturable binding of MBL to increasing concentrations of TcCRT (Fig. 3A) and TeS and TcCRT R (Fig. 3B) domains is evident. The control protein rGST showed no activity (Fig. 3C).
rTcCRT and its TcS and TcCRT R domains inhibit the classical complement pathway by blocking the activity of C1q

To study the functional impact of TcCRT binding to C1q, classical pathway complement-dependent hemolytic and C4 activation assays were used.

A C1q-dependent hemolytic assay was used to determine the effect of the TcS and TcCRT R domains on complement activation. The rationale for this assay is based on the observation that HuCRT does not interact with Ig-bound C1q when C1r2 C1s2 are already associated (22, 59). The assay requires purified C1q to be added back to C1q-deficient serum, to reconstitute the C1 complex.

We show in this study that when 11 μM of TcS and TcCRT R domains were incubated with the standard 1/100 NHS dilution, before addition of the mixtures to EA, the complement hemolytic activity was lowered from 80% to below 15% (Fig. 4A).

Because, under physiological conditions, the majority of C1q is associated with high affinity to C1r and C1s, a NHS complement activation assay was also used to determine the effect of rTcCRT proteins on complement activation. A 1/100 dilution of NHS produced 75–80% hemolysis. When 0–11 μM of rTcCRT and its TcS and TcCRT R domains were incubated with the standard 1/100 NHS dilution, before the addition of the mixtures to EA, the complement hemolytic activity was lowered from ~80% to below 15%.
We then aimed at defining at which level the inhibition of the classical complement pathway was occurring. On the one hand, rTcCRT and its TcS and TcCRT R domains bind to C1q in a direct binding assay (Fig. 1, A–C). In contrast, rTcCRT and its domains inhibit the final consequence of the activation of the classical pathway, as shown by the inhibition of EA lysis (Fig. 4). We then tested the ability of rTcCRT and its TcS and TcCRT R domains to inhibit the activation of C4 via the classical pathway (Fig. 5). Incubation of IgM-coated ELISA plates with a 1/80 dilution of NHS resulted in deposition of subsaturating amounts of activated C4 (data not shown). This C4 deposit (considered 100%) was chosen for these series of experiments. Incubation of NHS (Fig. 6A) or C1q-deficient serum (Fig. 6B) with TcS and the plate (data not shown). This dilution was chosen for these series of experiments and was considered 100%. Incubation of NHS (Fig. 6A) or C1q-deficient serum (Fig. 6B) with TcS and

TcS and TcCRT R domains inhibit the binding of human MBL to mannose

To study the functional impact of TcCRT binding to MBL, we standardized an assay to measure the ability of the TcS and TcCRT R domains to inhibit the binding of MBL to mannose, a natural activating ligand of the lectin pathway. Incubation of mannan-coated ELISA plates with a 1/60 dilution of NHS or C1q-deficient serum resulted in deposition of a subsaturating amount of MBL on

**FIGURE 4.** rTcCRT and its TcS and TcCRT R domains inhibit classical pathway-mediated hemolysis. A. A total of 1 μg of pure human C1q in a 1/40 dilution of C1q-deficient sera was added to variable concentrations of the TcS and TcCRT R domains or controls, in a final volume of 100 μl, before adding the mixture to EA (10⁸ cells/ml). The mixtures were incubated for 60 min at 37°C. After centrifugation, the OD of the supernatants was measured. The percentage of lysis was calculated with reference to 100% lysis of the cells in water. B. Different concentrations of the rTcCRT and its TcS and TcCRT R domains or controls were added to a 1/100 NHS dilution, and then added to the EA (10⁸ cells/ml). The rest of the procedure was similar in both assays. Results are representative of three independent experiments, and the data show the mean of duplicate observations, each one differing no more than 5% from the mean.

**FIGURE 5.** rTcCRT and its TcS and TcCRT R domains inhibit classical pathway-mediated C4 activation. Microtitration plates were coated with 2 μg/ml human IgM and incubated with NHS (1/60 dilution), in the presence or absence of different concentrations of rTcCRT or its TcS or TcCRT R domains, BSA, or rGST in VB²⁺ with 0.05% Tween 20. Complement activation was assessed using a phosphatase-conjugated chicken anti-human C4 Ab to detect deposition of activated C4. Results are representative of three independent experiments, and the data show the mean of duplicate observations, each one differing no more than 5% from the mean.

**FIGURE 6.** TcS and TcCRT R domains inhibit the binding of human MBL from NHS and C1q-deficient sera to mannose. Microtitration plates were coated with mannan and blocked with TBS/HSA. NHS (A) or human C1q-deficient sera (B), diluted 1/60, were premixed with different concentrations of TcS or TcCRT R domains, rGST, or BSA, in modified VB²⁺, transferred to the mannan-coated plate, and further incubated for 1 h at 37°C. MBL binding was detected by using a mAb (131-1), followed by an alkaline phosphatase-conjugated goat Ab anti-mouse IgG. Results are representative of three independent experiments, and the data show the mean of duplicate observations, each one differing no more than 5% from the mean.
TcCRT R domains inhibited the binding of MBL to mannan in a dose-dependent manner. Thus, in the presence of 11.3 μM of TcS and TcCRT R domains, binding of MBL to mannan was inhibited ~50%, relative to the sera in the absence of protein. The control protein (BSA) had no effect (Fig. 6). Despite this capacity of TcS and TcCRT R domain to partially inhibit the binding of MBL to mannan, this was not reflected in their capacity to inhibit the activation of human C4, as shown in Fig. 7, A (MBL-MASP complex from NHS captured on mannan-coated plates, before exposure to TcCRT) and B (soluble pure human MBL-MASP complex, preincubated with TcCRT, before addition to mannan-coated plates).

TcCRT is present on the surface of live T. cruzi trypomastigotes and colocalizes with human C1q

If TcCRT is present on the surface of live infective trypomastigotes, as determined by indirect immunofluorescence through FACS analysis, TcCRT could interact with the complement system. Thus, when trypanosomes are exposed to rabbit polyclonal Co11 (Fig. 8A) and Co10 (Fig. 8B) sera directed against TcS domain, a clear shift in fluorescence intensity is observed, as compared with the live parasites incubated with the corresponding preimmune sera, or with rabbit anti-T. cruzi GAPDH antisera.

In the FACS analysis, the anti-T. cruzi GAPDH antisera were used as a negative control (it detects an exclusively intracellular protein). To validate its reactivity, a control experiment using dead, paraformaldehyde-fixed trypomastigotes was conducted (Fig. 8C). The results were expressed only gating for the FSC X SSC dot-plot representation, and show clear recognition of this intracellular protein.

The polyclonal Co11 and Co10 antisera, as well as their F(ab’)_2, specifically recognize, by ELISA and IWB, native TcCRT in epimastigote extract, as well as the recombinant proteins. Importantly, these anti-TcS domain antisera and F(ab’)_2 can also recognize native TcCRT in the context of fixed trypomastigotes and epimastigotes through indirect immunofluorescence microscopy (results not shown).
FIGURE 9. TcCRT on the surface of trypomastigotes colocalizes with human C1q. Four percent of paraformaldehyde-fixed trypomastigotes was sequentially incubated with human C1q, rabbit F(ab’)2 anti-TcS, rhodamine-conjugated goat F(ab’)2 Abs, anti-rabbit F(ab’)2, and FITC-conjugated rabbit anti-human C1q Abs. Parasites were imaged using confocal microscopy. A, Fluorescence image of C1q (67). B, Fluorescence image of TcCRT (red). C, Colocalization of C1q with trypomastigote TcCRT is represented by yellow appearance in the overlay. Preimmune rabbit Abs did not react with the parasites. The figures represent three experiments and the analysis of at least 50 parasites.

Discussion

We have hypothesized that TcCRT, a potentially multifunctional T. cruzi molecule, may be part of a unified multimolecular parasite immune evasion strategy, given the documented ability of its human counterpart (HuCRT) to interact with C1q and MBL, the recognition molecules of the vertebrate complement system (14). The in vitro data presented in this work support this hypothesis.

As shown in Fig. 1, C1q binds to rTcCRT and its TeS and TcCRT R domains in a dose-dependent, specific, and saturable manner, under physiological salt concentrations. In Fig. 2, C1q collagen tails optimally bind TeS under low ionic strength conditions, as observed for HuCRT (21). The S domain of HuCRT contains part of a lectin site (repeats A and part of repeats B), with 77% identity to the homologous region in TcCRT. As hypothesized for HuCRT (21), this lectin region may supplement the protein-protein interaction between TcCRT and the glycosylated collagen stalks of C1q (60). Perhaps the conformation of collagenous tails, in the context of the whole C1q molecule, favors the interaction with TcCRT, under physiological salt concentrations.

Functional consequences of the above interactions resulted in blocking of the activation of the classical pathway, as confirmed by inhibition of hemolytic activity, mediated by rTcCRT and its TeS and TcCRT R domains (Fig. 4). In addition, the same proteins inhibited complement activation even when NHS was added to the EA (Fig. 4B), suggesting that rTcCRT or its domains can bind to fluid-phase C1q or C1. Our results are in agreement with those obtained by Kasper et al. (27), who used CRT from the nematode Necator americanus. Although it has been shown that HuCRT reacts with Ig-bound C1q and not with C1q in the Ig-bound C1(q,r,s) macromolecule (59), our results (Fig. 4) suggest that TcCRT binds to fluid-phase C1q present in the C1 macromolecule, in the context of NHS. It remains to be determined whether TcCRT displaces C1r and/or C1s from fluid-phase C1, generating a non-functional macromolecular complex, unable to reassociate with these serine proteases. This seems feasible, due to the fact that HuCRT S domain, once associated to Ig-bound C1q, inhibits its reassociation with C1r/C1s (22) and to a 50–80% sequence identity between the TcCRT and HuCRT S domains (32).

If the resulting hemolysis inhibition by TcCRT or its domains is due to an interaction with C1q, a previous inhibition of C4 activation should also occur. To detect this inhibition, we coated microtiteration plates with IgM, a substrate for C1q binding, and BSA was used as a saturating agent. After activation of C1r2-C1s2, C4 is cleaved, a metastable thiol-ester bond is generated, and binding of C4b to available hydroxyl or amino groups is promoted (61). As predicted, addition of rTcCRT or its domains resulted in a clear dose-dependent and saturable inhibition of covalent C4b deposition on the solid phase, indicating that rTcCRT or its domains interacted with C1, thus inhibiting its ability to activate C4 (Fig. 5). Therefore, differently from other T. cruzi complement regulators already described, TcCRT acts at the earliest stage of complement activation. Thus, we provide in this work experimental evidence showing that TcCRT, by virtue of its capacity to bind and inhibit the function of C1q, may contribute to the well-known (48) inability of the classical pathway to play a preponderant role in the defense against this parasite.

Binding of MBL to TeS and TcCRT R domains is dose dependent, specific, and saturable, as shown in Fig. 3. In contrast to C1q, and in agreement with HuCRT S domain, the binding of MBL to TeS and TcCRT R domains was found to be ionic strength dependent (results not shown). This could indicate that it is predominantly a protein-protein interaction based upon a region of charged residues on the collagen stalks of MBL, as proposed for HuCRT (21). Interactions of TcCRT with the collagenous tails of both C1q and MBL could mediate a steric change at the globular head level, in the case of C1q, or at the level of the C-type lectin carbohydrate recognition modules, in the case of MBL, resulting in inhibition of their interactions with their natural ligands.

We also show in this study that TeS and TcCRT R domains inhibit the interaction of MBL with mannose, one of the natural activating ligands for the lectin pathway. This inhibition occurs even within the context of NHS, thus indicating that fluid-phase C1q or C1 is unable to efficiently compete with this interaction. This assumption is supported by the fact that when C1q-deficient
The possibility that TcCRT interacts in vivo with the complement system of the hosts is in agreement with our finding that TcCRT is found on the surface of infective trypomastigotes and that it colocalizes with C1q. Comparison of Fig. 8 shows that TcCRT is expressed on the trypomastigote surface. A confocal microscopic approach (Fig. 9) shows colocalization of trypomastigote TcCRT with human C1q, a strong indication that both molecules do interact on the infective parasite surface. Possibly, this fact correlates with our in vitro results, which show that TcCRT strongly inhibits the classical human complement activation (Figs. 4 and 5). In addition, the confocal microscopy assays corroborate the presence of TcCRT on the parasite surface.

Epimastigote TcCRT is found in the microsomal subcellular fraction (33), compatible with the presence of an ER retrieval sequence. In contrast, we have shown that epimastigotes display an important amount of TcCRT in the cytoplasmic compartment (45). Although the presence of TcCRT in supernatants from trypomastigote and epimastigote cultures, on the epimastigote surface, and in sera of T. cruzi-infected individuals and mice, should be addressed, at present it is not possible to estimate the real TcCRT concentrations in the relevant parasite in vivo microenvironment.

Abs present in most infected humans (43, 44), against released or surface-bound TcCRT, may modulate the putative extracellular functions of this molecule in the host, with unknown consequences for parasite infectivity. Our rabbit Abs against rTcCRT did not support complement-mediated lysis of trypomastigotes, when conducted as described (62) (data not shown). Similar results were observed by Sepulveda et al. (52) when Abs against recombinant complement regulatory protein were used. Low immunogenicity of the functionally relevant rTcCRT epitopes could explain our results. For this reason, we have initiated DNA immunization procedures with the TcCRT gene. Experiments in progress aim at determining whether F(ab)_2 rabbit anti-rTcCRT polyclonal fragments revert the observed in vitro complement-inhibitory effects.

Additional consequences of the complement-modulating capacity of TcCRT could be proposed. Thus, TcCRT interference with C1q functions could alter immune complex processing with likely consequences in the pathogenesis of Chagas’ disease (63–66).

In synthesis, we show in this work that rTcCRT and its TcS and TcCRT R domains bind human C1q, through its collagen tails, inhibiting the complement classical pathway. Thus, TcCRT may contribute to the inability of the classical pathway to play a more preponderant role in the defense against this parasite. In agreement with these findings, we showed that TcCRT is present on the surface of live trypomastigotes, where it interacts with human C1q, with predictable complement-inhibitory effects.

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