MAJOR ARTICLE

Complement C2 Receptor Inhibitor Trispanning Confers an Increased Ability to Resist Complement-Mediated Lysis in *Trypanosoma cruzi*

Igor dos S. Cestari,[1] Ingrid Evans-Osses,[1] Juliana C. Freitas,[1] Jameel M. Inal,[2,3,a] and Marcel I. Ramirez[1,a,b]

[1]Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil; [2]Department of Health and Human Sciences and Institute for Health Research and Policy, London Metropolitan University, London, United Kingdom; [3]Department of Research, Immunonephrology, University Hospital Basel, Basel, Switzerland

The ability to resist complement differs between the Y and Colombiana *Trypanosoma cruzi* strains. We found that the Y strain of *T. cruzi* was more able to resist the classical and lectin pathways of complement activation than the Colombiana strain. The complement C2 receptor inhibitor trispanning gene *(CRIT)* is highly conserved in both strains. At the protein level, CRIT is expressed only in stationary-phase epimastigotes of the Y but not the Colombiana strain and is expressed in infectious metacyclic trypomastigotes of both strains. Y strain epimastigotes with an overexpressed *CRIT* gene (pTEX-CRIT) had higher survival in normal human serum (NHS). Overexpression of the Y strain *CRIT* gene in Colombiana epimastigote forms increased the parasite's resistance to lysis mediated by the classical and lectin pathways but not to lysis mediated by alternative pathways. CRIT involvement on the parasite surface was confirmed by showing that the lytic activity of NHS against epimastigotes could be restored by adding excess C2.

Trypanosoma cruzi is a protozoan parasite that causes Chagas disease and that affects 18 million people in Latin America, with 90 million at risk of infection worldwide [1]. Approximately 30% of individuals in the indeterminate stage, in which parasitemia is undetectable and patients remain asymptomatic, progress to the chronic stage with severe sequelae, such as cardiomyopathy or gastrointestinal dysfunction [2, 3].

T. cruzi needs to evade the host innate immune response to establish an infection and initiate disease. The first mechanism of vertebrate host defense against the parasite is the complement (C) system, which is composed of 30 serum proteins activated in a cascade that culminates in the lysis of the parasite. There are 3 C pathways leading to pathogen lysis by the membrane attack complex. The classical pathway (CP), activated by IgG/IgM binding to pathogen, and the alternative pathway (AP), activated through C3b binding to pathogen, have been described. However, there has been little description of lectin pathway (LP) activation in trypanosomatids, via mannan-binding lectin (MBL) binding to carbohydrate on the pathogen surface [4–8].

During its life cycle, the parasite undergoes a series of developmentally regulated morphological and physiological changes to survive within insects and mammalian cells. Thus, vertebrate-stage parasites (blood trypomastigotes and amastigotes) and infective vector-stage parasites (metacyclic trypomastigotes) typically resist direct serum killing and can also evade ingestion or intracellular killing by a host phagocyte cell. In contrast, most noninfective vector-stage parasites (epimastigotes) are susceptible to lysis by serum and to phagocytosis and destruction by polymorphonuclear leukocytes.

The membrane changes that control the transformation of these parasites from being C sensitive to resistant are only partially understood. Pioneering studies of C-mediated lysis of trypanosomatids described antibod-

Received 13 December 2007; accepted 4 May 2008; electronically published 9 September 2008.

Potential conflicts of interest: none reported.

Financial support: Conselho Nacional de Desenvolvimento Cientifico e Tecnologico, Brazil (476737/2004-4); Swiss National Foundation (3200-066708); Council for Assisting Refugee Academics.

[a] J.M.I. and M.I.R. codirected this work and are co–corresponding authors.

[b] M.I.R. is on leave at the Centre for Biomedical Sciences, School of Biological Sciences, Royal Holloway, University of London, Egham, United Kingdom.

Reprints or correspondence: Dr. Marcel Ramirez, Instituto Oswaldo Cruz (Fiocruz), Departamento de Bioquímica e Biologia Molecular, Av. Brasil, 4365, 21040–900, Rio de Janeiro-RJ, Brazil (marcelr@fiocruz.br).

The Journal of Infectious Diseases 2008; 198:1276–83
© 2008 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2008/19809-0005$15.00
DOI: 10.1086/592167

ies of patients in the chronic phase responsible for CP activation and parasite killing [4, 6, 9]. Otherwise, the prevailing explanation for C activation during the infection is that parasites activate lysis through the AP, thereby lacking the involvement of antibodies, despite the deposition of C3b on the trypomastigote parasite surface [8] and parasite lysis occurring in the presence of serum depleted of factors of the AP [9].

The mechanisms displayed by *T. cruzi* for avoiding the C system are refined, specific molecules having been reported as C inhibitors. Trypanosome decay–accelerating factor has been reported to bind to C component C3 and C-regulatory protein to bind to C3 and C4, thereby blocking the CP and AP [10, 11]. Other molecules have been shown to confer resistance to CP- and AP-mediated lysis, such as calreticulin, which interacts with C1q and blocks only the CP [12], as well as gp 58/68, which inhibits the AP [13]. Recently, a receptor responsible for specific inhibition of CP activation in *Schistosoma* species [14] was described, a homologue of which we recently identified in *T. cruzi* [15]. Complement C2 receptor inhibitor trispanning (CRIT) is a transmembrane protein of 32 kDa. The N-terminal first extracellular domain (ed1) of CRIT shows sequence homology with part of the C4 β-chain and competes for binding to C2 [16] via its von Willebrand factor A domain [17]. In binding C2, CRIT inhibits the C1s-mediated cleavage of C2. CRIT is highly conserved in *Schistosoma* species, *T. cruzi* strains, and mammals [15].

To have an overall understanding of the mechanism of C activation in *T. cruzi* infection, we need to consider the wide variety of strains described. On the basis of biological and epidemiological data, *T. cruzi* has been divided into 2 divergent groups or lineages. *T. cruzi* class I is found in the sylvatic transmission cycle, which affects mainly American marsupials and edentates [18, 19]. As an example of a class I strain, *T. cruzi* Colombiana is used in this study. By contrast, *T. cruzi* class II is found in the domestic transmission cycle and affects mainly humans; an example of a class II strain, *T. cruzi* Y strain is used. Several molecular markers allow the classes to be distinguished, such as the 24SαrRNA gene, the intergenic region of tandemly repeated mini-exon gene, microsatellite DNA, and polymerase I promoter activity [19–21]. The physiological relevance of *T. cruzi* divergence was recently shown when it was revealed that >87% of patients with Chagas disease have antibodies against the trypomastigote small surface antigen of *T. cruzi* II [22].

Differential expression of C-regulatory genes within the divergent classes of *T. cruzi* has to date not been linked with the ability to resist C-mediated lysis. First, we show that C activation differs between the *T. cruzi* Y and Colombiana strains, as does the expression of the *CRIT* gene, which is expressed in a class-specific manner. We find CRIT protein expressed in the infective stage of *T. cruzi* and show that epimastigote transgenic parasites expressing CRIT are more resistant to C lysis, blocking the CP and LP.

METHODS

Parasites. *T. cruzi* epimastigote forms of strains Y and Colombiana were cultured in liver infusion tryptose (LIT) medium supplemented with 10% fetal bovine serum at 27°C [23]. Metacyclic forms were obtained and purified as described elsewhere [23, 24].

C-mediated lysis assay. First, 5.0×10^5 logarithmic epimastigote forms of *T. cruzi* were resuspended in 100 μL of fresh RPMI 1640 and mixed with 100 μL of diluted normal human serum (NHS) in RPMI 1640 medium at 37°C in microcentrifuge tubes for 30 min. After incubation (30 s to 30 min), 800 μL of cold RPMI 1640 was added, and the tube was placed on ice. Heat-inactivated NHS (56°C for 30 min) was used as a negative control. Surviving parasites were stained with trypan blue and quantified in a Neubauer chamber under a light microscope. For calculating the serum concentration that causes 50% lysis of parasites (serum lethal concentration [SLC]–50), parasites were incubated in 2-fold serial dilutions, ranging from undiluted to 3.12% NHS.

The kinetics of C-mediated killing was determined by incubating parasites in 25% NHS for 0.5, 1, 2.5, 5, 10, and 30 min. CP and LP inhibition were performed by incubating NHS with 10 mmol/L ethylene glycol tetraacetic acid (EGTA) and 7 mmol/L $MgCl_2$.

C-mediated lysis was assayed using transgenic (pTEX and pTEX-CRIT) and wild-type parasites from the *T. cruzi* Y and Colombiana strains. Parasites growing in LIT medium (with 200 μg/mL G418 added for transgenic parasite culture) at logarithmic phase (3 days) were used in this experiment. *T. cruzi* Y strain parasites were incubated with 25% NHS, whereas Colombiana strain parasites were incubated with 12.5% NHS. In certain experiments in which 0.5 μg of C2 (0.02 μmol/L) was added to restore the lysis of pTEX-CRIT transgenic parasites, kinetics were realized with 25% NHS (CP, LP, and AP activation).

Gene cloning and sequencing. Oligonlucleotides (5'-CCCGGATTCATGTACGGGCCAGATAT-3' [forward] and 5'-CCCAAGCTTTTAATTTCGATAAGCCAGT-3' [reverse]) based on *CRIT* DNA sequences from *Schistosoma mansoni* (GenBank accession number 6650015) were used to amplify a 0.85-kb fragment from *T. cruzi* Y strain genomic DNA by polymerase chain reaction (PCR). The PCR product was cloned into pGEM-T Easy Vector (Promega) and sequenced with T7 and T3 oligonucleotides using an ABI 3100 automated DNA sequencer. The sequence of *T. cruzi* Y strain was deposited in GenBank (accession no. AY464185). The genes of *T. cruzi* Cl Brener, Colombiana, and Dm28c were amplified by PCR from genomic DNA using oligonucleotides (5'-ATGTCTCCAAGTCTAGTG-3' [forward] and 5'-TTAACAAGAAGAGTGAG-3' [reverse]) based on the CRIT sequence of the *T. cruzi* Y strain (GenBank accession nos. AF212030, AF212031, and AF212029, respectively).

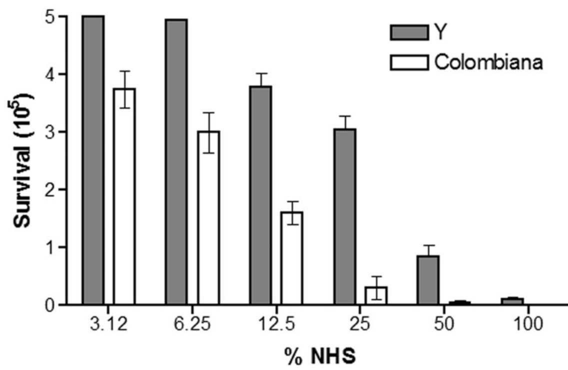


Figure 1. Determination of the serum concentration that causes 50% lysis of parasites. *Trypanosoma cruzi* Y and Colombiana strains were incubated in several concentrations of normal human serum (NHS) for 30 min at 37°C. Bars represent the SDs of 3 individual experiments performed in triplicate. The results are significantly different at 6.25%, 12.5%, 25%, and 50% NHS ($P < .05$).

Constructions and transfections. The *CRIT* gene from the Y strain cloned into pGEM-T Easy Vector was digested with *Eco*RI and subcloned into the trypanosomatid expression vector pTEX, generating the vector pTEX-CRIT. Twenty-five micrograms of pTEX and pTEX-CRIT was used to transfect epimastigote forms of *T. cruzi* Y and Colombiana strains in logarithmic phase by an electroporation method described elsewhere [21].

Western blotting. Parasites were washed in PBS, adjusted to 5.0×10^6 parasites/μL, and lysed with 0.1% Triton X-100/PBS/protease inhibitor cocktails, as described elsewhere [13]. In brief, after SDS-PAGE and transfer to nitrocellulose, the primary antibody used was affinity-purified rabbit anti–CRIT-ed1 (1:100), as described elsewhere [14]. Immunoabsorption was performed by preincubating the anti–CRIT-ed1 with synthetic peptide ed1 [16] before incubation with the membrane.

Fluorescence-activated cell sorter (FACS) analysis. *T. cruzi* metacyclic trypomastigotes (2.0×10^5 parasites) were incubated with 1 μg of C2 for 1 h at 4°C. After that, parasites were washed 2 times with PBS and incubated with fluorescein isothiocyanate (FITC)–conjugated anti-C2 antibody, diluted 1:10 in PBS–1% bovine serum albumin, for 2 h at 4°C. IgG-FITC was used as an isotype control. Parasites were then washed 3 times and analyzed by flow cytometry using the Guava EasyCyte ExpressPlus System (Guava Technologies).

Data presentation and statistical analysis. All data are shown as means ± SDs. Groups were compared by the unpaired *t* test for repeated measures, using Prism software (version 5.0; GraphPad). Differences were considered statistically significant at $P < .05$ with a confidence interval of 99%.

RESULTS

Greater susceptibility of the* T. cruzi *Colombiana strain than the Y strain to C-mediated lysis in the presence of NHS. Assays of C-mediated lysis were performed on epimastigote forms of *T. cruzi* Colombiana and Y strains, grown to the logarithmic phase, with serial dilutions of NHS to determine the serum concentration that causes SLC-50 in 30 min at 37°C.

T. cruzi Y strain epimastigote forms were lysed with NHS at concentrations of 6.25% and above, and the SLC-50 was between 25% and 50%. However, the Colombiana strain needed only as little as 3.12% NHS, and the SLC-50 was between 6.25% and 12.5% (figure 1). *T. cruzi* Colombiana was more susceptible to C lysis than *T. cruzi* Y at all serum concentrations, being 8-fold more lysed than the Y strain at 25% NHS.

The kinetics of C pathway–mediated lysis in* T. cruzi *Colombiana and Y strains. The kinetics of C-mediated lysis with NHS (CP, LP, and AP) and EGTA-treated NHS (evaluating only the AP, a calcium-independent process) showed differing de-

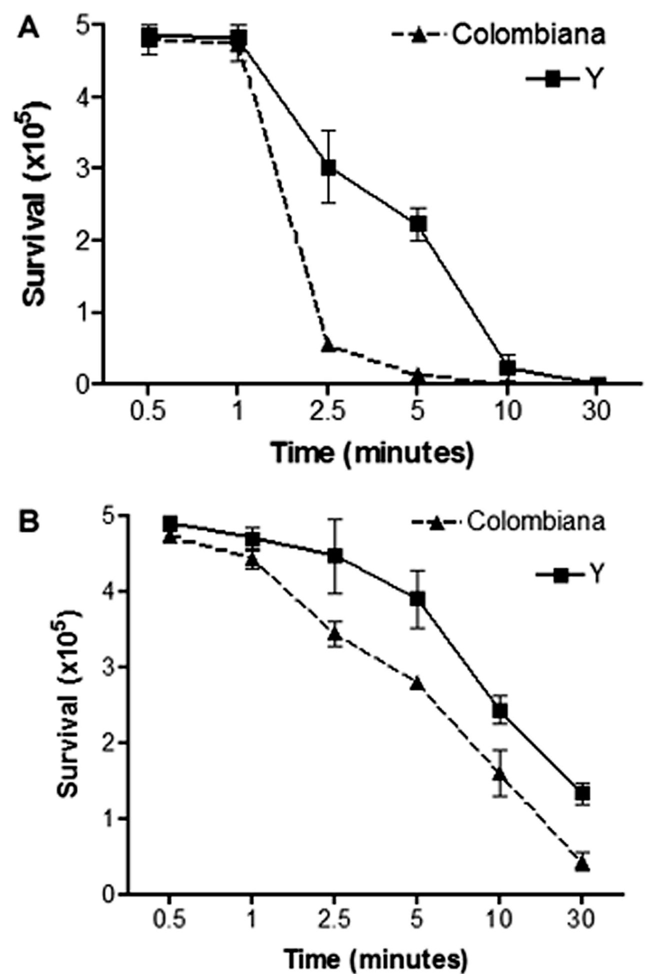
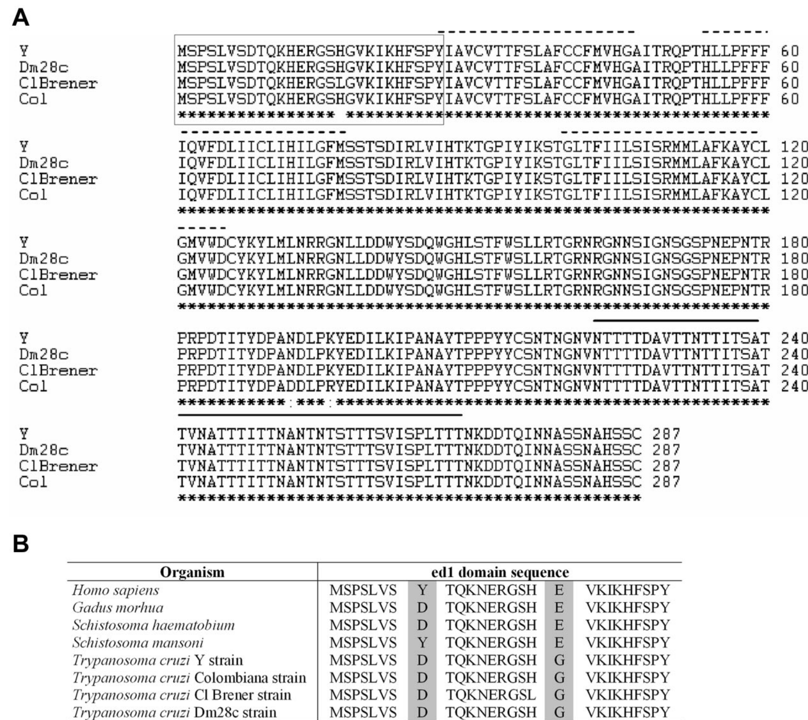


Figure 2. Kinetics of complement activation. *A, Trypanosoma cruzi* Y and Colombiana strains incubated with 25% normal human serum (NHS) at 37°C for 0.5–30 min. *B, T. cruzi* Y and Colombiana strains incubated with 25% NHS treated with 10 mmol/L ethylene glycol tetraacetic acid and 7 mmol/L $MgCl_2$ for 0.5–30 min. Bars represent SDs of 3 individual experiments performed in triplicate. In panel A, results are significantly different at 2.5 and 5 min; in panel B, results are significantly different at 5 and 30 min ($P < .05$).



Organism	ed1 domain sequence				
Homo sapiens	MSPSLVS	Y	TQKNERGSH	E	VKIKHFSPY
Gadus morhua	MSPSLVS	D	TQKNERGSH	E	VKIKHFSPY
Schistosoma haematobium	MSPSLVS	D	TQKNERGSH	E	VKIKHFSPY
Schistosoma mansoni	MSPSLVS	Y	TQKNERGSH	E	VKIKHFSPY
Trypanosoma cruzi Y strain	MSPSLVS	D	TQKNERGSH	G	VKIKHFSPY
Trypanosoma cruzi Colombiana strain	MSPSLVS	D	TQKNERGSH	G	VKIKHFSPY
Trypanosoma cruzi Cl Brener strain	MSPSLVS	D	TQKNERGSL	G	VKIKHFSPY
Trypanosoma cruzi Dm28c strain	MSPSLVS	D	TQKNERGSH	G	VKIKHFSPY

Figure 3. *A,* Similarity analysis of complement C2 receptor inhibitor trispanning (CRIT) amino acid sequences from *Trypanosoma cruzi* Y, Colombiana, Cl Brener, and Dm28c strains. The alignment was done using the ClustalW tool. Asterisks indicate identical amino acids, dots indicate related amino acids, the box indicates the 27-aa sequence of extracellular domain 1 (ed1) in the N-terminal region, bars indicate the serine-threonine–rich region in the C-terminal region (amino acid positions 224–274), and dotted lines indicate hydrophobic regions. *B,* Comparison of the 27 aa that comprise the ed1 of CRIT from various species. Shaded areas indicate where amino acids differ between the species.

grees of resistance in the *T. cruzi* Y and Colombiana strains (figure 2*A* and 2*B*). The *T. cruzi* Colombiana and Y strains exhibited a divergence in lysis after 1 min of incubation with NHS, and at 2.5 and 5 min the survival levels of Colombiana strains were significantly lower (figure 2*A*) than for *T. cruzi* Y strain. After 10 min, the Colombiana strain was totally lysed; with the Y strain, 17.4% of parasites survived, these being totally lysed only after 30 min (figure 2*A*). Alternatively, when kinetic assays were performed in the presence of AP alone, both parasites survived up to 30 min of lysis (figure 2*B*). After 2.5 and 5 min the Colombiana strain showed 68% and 56% survival, respectively, where the Y strain showed significantly higher levels after the same intervals (90% and 78% survival, respectively) (figure 2*B*).

Cloning of CRIT, inhibitor of the CP and LP, in* T. cruzi *strains. Having described a C2 inhibitory receptor, CRIT [13, 15], in *S. mansoni* and *Schistosoma haematobium* parasites, we used oligonucleotides based on *S. haematobium CRIT* sequences to amplify from the genomic DNA of the *T. cruzi* Y strain. The PCR product was amplified and cloned into the pGEM-T Easy Vector for sequencing. The sequence shares a high nucleotide similarity with *CRIT* described in *Schistosoma* species. BLAST analysis at the amino acid level showed similarities of 97% and 96% with *S. mansoni* and *S. haematobium* CRIT, respectively [15].

The *CRIT* gene was also amplified by PCR from genomic DNA of *T. cruzi* strains Cl Brener, Colombiana, and Dm28c. The PCR fragments were sequenced and found to share a high similarity with *CRIT* from the *T. cruzi* Y strain, *Schistosoma* species, and mammals. At the amino acid level, the similarity between CRIT from *T. cruzi* Cl Brener, Colombiana, and Dm28c strains compared with *T. cruzi* Y strains was >90% (figure 3*A*). The CRIT sequences reveal in the N-terminal region an ed1 domain of 27 residues and in the C-terminal region (residues 224–274) a serine-threonine–rich region. The ed1 (figure 3*A*) was shown to bind to C factor C2 and inhibit CP activation [16]. CRIT does not have an N-terminal signal peptide and possesses 3 highly hydrophobic regions predicted to form transmembrane helices that could be integrated into the parasite membrane. The predicted topology of all the CRIT proteins remains that of a type IIIb membrane protein [25] with an extracellular N-terminus and cytoplasmic C-terminal tail. All of the *T. cruzi* strains have an identical ed1 (figure 3*B*). Searching various sequence databases reveals that the cytoplasmic tail of CRIT (mammalian parasite) has homology with similar regions in receptors that are endocytosed or that have a lysosomal targeting sequence, as highlighted elsewhere [26].

Differential expression of CRIT in* T. cruzi *Y and Colombiana strains. The expression of CRIT in *T. cruzi* Y and Co-

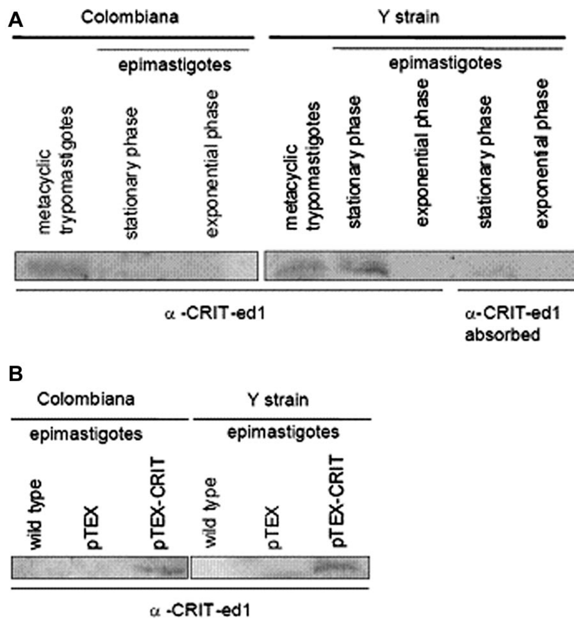


Figure 4. Western blotting of *Trypanosoma cruzi* Y and Colombiana strains. In panel A, total protein extracts were obtained from epimastigotes of logarithmic- and stationary-phase and from metacyclic-form parasites with Triton X-100 (1% in PBS). The membrane was incubated with affinity-purified anti–CRIT-ed1 polyclonal antibody. Immunoabsorption was done with extracellular domain 1 (ed1) peptide to block the binding of the antibody to complement C2 receptor inhibitor trispanning (CRIT) protein. In panel B, total protein extracts from wild-type, pTEX, and pTEX-CRIT transfectants of *T. cruzi* Y and Colombiana strains were obtained similarly and probed with antibody, as in panel A.

lombiana strains was analyzed during the in vitro differentiation from epimastigote to metacyclic trypomastigote forms. Immunoblotting using anti–CRIT-ed1 (which is specific for the N-terminal domain ed1 of CRIT) showed expression in metacyclic trypomastigote forms of both strains and in stationary- but not logarithmic-phase epimastigote forms of *T. cruzi* Y strain (figure 4). CRIT was detected as a protein of 27–32 kDa, compared with the already-described 32-kDa CRIT protein in *Schistosoma* species and mammals, and this signal could be removed by adsorption of the antibody with CRIT-ed1 peptide. Apart from a homology [16] to part of the C4 β-chain, which is 78 kDa, the CRIT-ed1 sequence is quite specific to the CRIT family of proteins.

***Resistance to C-mediated lysis conferred by overexpression of CRIT in the epimastigote form of* T. cruzi.** To understand the role that CRIT plays in providing resistance to C-mediated lysis in *T. cruzi,* the *CRIT* gene of the Y strain was cloned into the pTEX expression vector and overexpressed in logarithmic epimastigote forms of the *T. cruzi* Y and Colombiana strains (figure 4*B*). The overexpressed *CRIT* gene in Y and Colombiana confers an increased resistance to C-mediated lysis in NHS compared with wild-type parasites. The transgenic parasites were assayed for their ability to resist C-mediated lysis by monitoring the kinetics of lysis. *T. cruzi* Y strain was incubated with 25% NHS, and within 5 and 10 min a difference was detected in lysis (figure 5*A*). Transgenic parasites (pTEX-CRIT), after 5 min of incubation with serum, showed equal numbers of survivors, with pTEX and wild-type parasites. After 10 min, wild-type and pTEX transgenic parasites had significantly fewer survivors than pTEX-CRIT transgenic strains. When these parasites were incubated with 25% NHS treated with EGTA and $MgCl_2$ to inhibit the CP and LP (figure 5*B*), the transgenic (pTEX-CRIT), pTEX, and

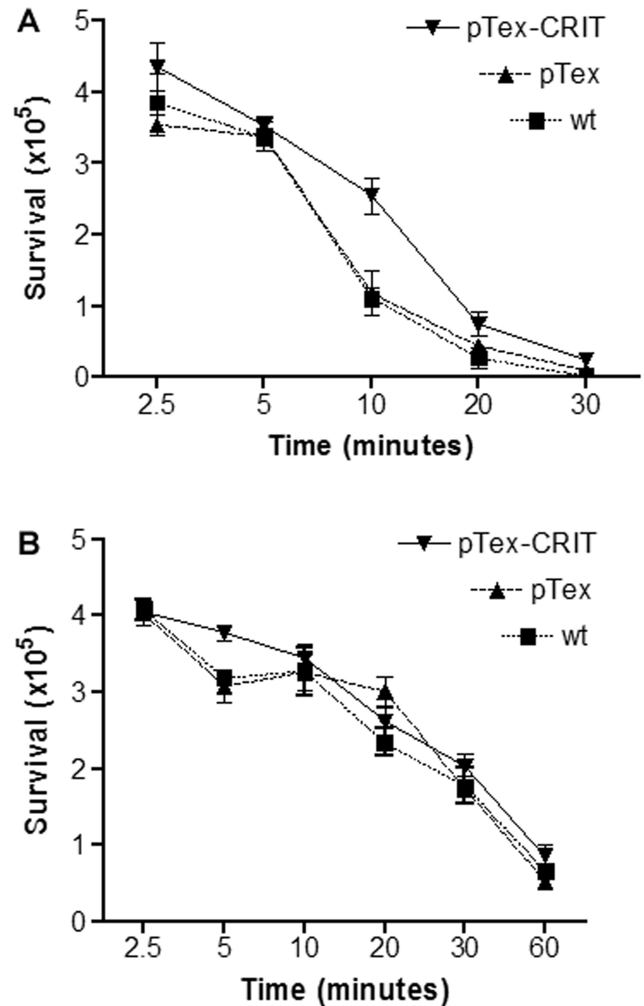


Figure 5. Complement lysis assay comparing the resistance to lysis of *Trypanosoma cruzi* Y strain wild-type (wt), transgenic pTEX, and pTEX–complement C2 receptor inhibitor trispanning (CRIT) parasites. *A,* Kinetics realized with 25% normal human serum (NHS) (classical, lectin, and alternative pathway activation) over a time course of 2.5–30 min at 37°C. *B,* Kinetics realized with 25% NHS treated with 10 mmol/L ethylene glycol tetraacetic acid and 7 mmol/L $MgCl_2$ (alternative pathway activation only) over a time course of 2.5–60 min at 37°C. Bars represent SDs of single individual experiments performed in triplicate. In panel A, results are significantly different at 10 min comparing pTEX-CRIT with wt and with pTEX transgenic parasites ($P < .05$). In panel B, results are not significantly different comparing pTEX-CRIT with wt and with pTEX transgenic parasites.

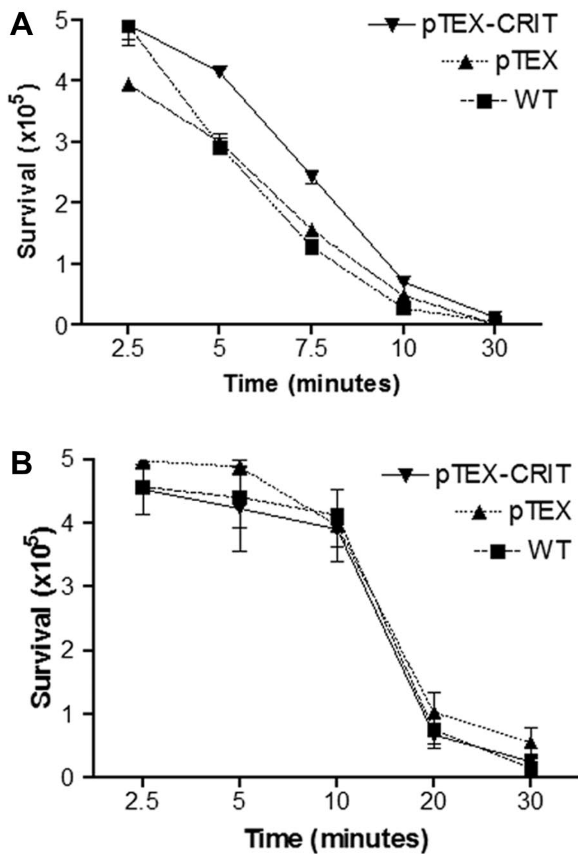


Figure 6. Complement lysis assay comparing the resistance to lysis of *Trypanosoma cruzi* Colombiana strain wild-type (WT), transgenic pTEX, and pTEX–complement C2 receptor inhibitor trispanning (CRIT) parasites. *A,* Kinetics realized with 12.5% normal human serum (NHS) (classical, lectin, and alternative pathway activation) over a time course of 2.5–30 min at 37°C. *B,* Kinetics realized with 12.5% NHS treated with 10 mmol/L ethylene glycol tetraacetic acid and 7 mmol/L $MgCl_2$ (alternative pathway activation only) over a time course of 2.5–30 min at 37°C. Bars represent SDs of single individual experiments performed in triplicate. In panel A, results are significantly different at 5 and 7.5 min comparing pTEX-CRIT with WT and with pTEX transgenic parasites ($P < .05$). In panel B, results are not significantly different comparing pTEX-CRIT with WT and with pTEX transgenic parasites.

wild-type strains were lysed to a similar extent. This indicates that CRIT inhibits the CP and the LP but does not confer resistance to AP-mediated lysis. The overexpression of the *CRIT* gene of the *T. cruzi* Y strain in the *T. cruzi* Colombiana strain was thus able to confer resistance to C-mediated lysis by NHS. Incubation of Colombiana strain expressing pTEX-CRIT with 12.5% NHS after 7.5 min showed that transgenic parasites were 2-fold more resistant (2.43×10^5 surviving parasites [49%]) compared with wild-type parasites (1.28×10^5 surviving parasites [26%]) (figure 6). These data indicate that the CRIT of Y strain was functional in the Colombiana strain and that the difference in the susceptibility to lysis by C may be associated with the differential expression of specific molecules present in each strain.

Restoration of the susceptibility to lysis by exogenous C2 in transgenic parasites overexpressing CRIT. The ability of overexpressed CRIT in epimastigote Y strain to resist C-mediated lysis is directly related to C2 binding to CRIT on the parasite surface and depletion of available C2 from NHS. We performed experiments in which C2 added to NHS restored its ability to lyse epimastigotes. With the addition of 0.5 μg of C2 (0.02 μmol/L), the 65% survival rate of transgenic parasites (pTEX-CRIT) was reduced to 43%, comparable with the survival levels of pTEX epimastigotes (parasites transfected with vector alone) and wild-type parasites (figure 7*A*). Furthermore, C2 binding to the infective metacyclic trypomastigote form of *T. cruzi* was confirmed by FACS analysis (figure 7*B*).

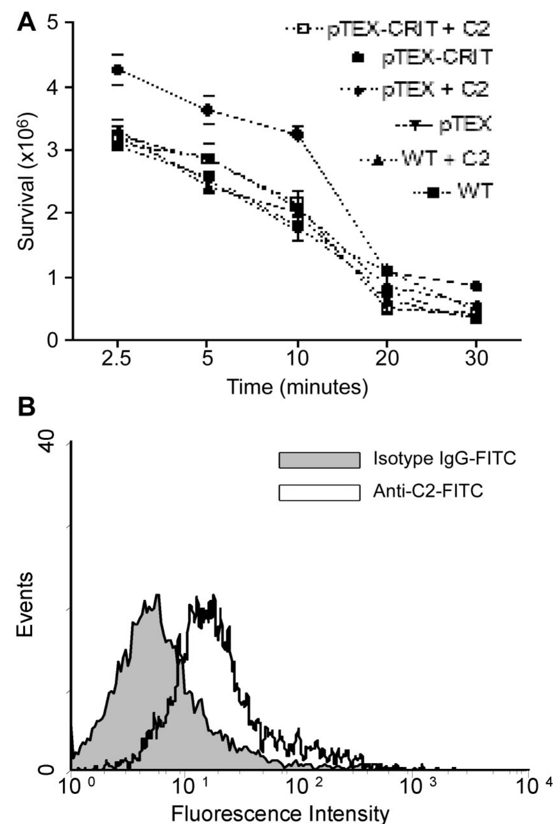


Figure 7. Complement lysis assay showing restoration of the lysis of pTEX–complement C2 receptor inhibitor trispanning (CRIT) transgenic parasites via added C2. *A,* Kinetics realized with 25% normal human serum (NHS) (classical, lectin, and alternative pathway activation) with wild-type (WT), transgenic pTEX, and pTEX-CRIT of *Trypanosoma cruzi* Y strain. The NHS was restored with 0.5 μg of C2. The reaction was incubated at 37°C over a time course of 2.5–30 min. Results are significantly different at 2.5, 5, and 10 min comparing pTEX-CRIT with both WT and pTEX-CRIT transgenic parasites ($P < .05$); results are not significantly different comparing pTEX-CRIT (with added C2) with WT or pTEX transgenic parasites (both with added C2). *B,* Binding of C2 to metacyclic trypomastigote forms of *T. cruzi.* Parasites (2.0×10^5) were incubated with C2 for 1 h at 4°C followed by incubation with fluorescein isothiocyanate (FITC)–conjugated anti-C2 antibody. Analysis was done by flow cytometry using the Guava EasyCyte ExpressPlus System (Guava Technologies). IgG-FITC was used as an isotype control.

DISCUSSION

We have shown that the Colombiana and Y strains of *T. cruzi,* which belong to the 2 phylogenetic lineages class I and class II, respectively, differ in their capacity to resist C-mediated lysis, and we attribute this in part to the expression of CRIT. It was also our aim to investigate the expression of CRIT in epimastigote forms as they differentiate into infective metacyclic trypomastigotes.

Complement lysis assays showed that the *T. cruzi* Colombiana strain is more susceptible to lysis than the *T. cruzi* Y strain at all serum concentrations. Kinetic assays were then performed to ascertain to which pathways strains were resistant. After 2.5 and 5 min in the presence of all 3 pathways, the Colombiana strain showed survival levels of 11% and 2.6%, respectively, whereas the Y strain presented survival levels of 60% and 44.6%, respectively. However, when the kinetic assays were performed with EGTA-treated NHS, which blocked the CP and the LP, lysis was slow to occur and had similar levels between the strains, with survival still being maintained for up to 30 min. Thus, the Colombiana strain's greater susceptibility to C lysis appears to be mainly due to the CP and LP in early activation. These findings are in accordance with those of similar experiments in *Leishmania* species and *Crithidia* species [27], which showed a rapid activation of the CP. Additionally, the involvement of the AP, supposed to be the first mechanism in C-mediated lysis in *T. cruzi* [7, 8, 28], showed a minimum role in immediate lysis.

As a mechanism of C-mediated lysis in *T. cruzi,* which is important during the early activation of C, the LP [29, 30] has been little considered. However, in *Leishmania braziliensis* it was recently shown that MBL binds to surface glycoconjugates and activates the LP [5]. SA85–1, a mannose receptor found in amastigote forms of *T. cruzi,* binds to MBL and is involved in the uptake of the parasites into macrophages, thus increasing infection [31]. Recently, it was reported that MBL deficiency in patients increases susceptibility to infection [32, 33].

The switch by which trypanosomatids have evolved from the epimastigote form, which is sensitive to C-mediated lysis by NHS, to the C-resistant metacyclic form is not well understood. Norris [11] showed that a C3 receptor, C-regulatory protein, was expressed in trypomastigote forms and not in insect-stage epimastigotes. The overexpression of the gene for C-regulatory protein in insect forms through transgenic parasites conferred resistance to C-mediated lysis. The mere expression of C-regulatory genes only in the metacyclic form might explain the acquired resistance to serum lysis, but other mechanisms could be involved [34]. We have found a novel C2 receptor, CRIT, expressed in infective forms of the Y and Colombiana strains of *T. cruzi,* providing them with resistance to C-mediated lysis by NHS. The *CRIT* gene is highly similar to the gene described in *Schistosoma* species and mammals [16]. CRIT in the Y strain possesses the N-terminal ed1, reported to bind to C2 and block the CP and the LP, and 3 transmembrane regions that could be involved in membrane insertion. The gene sequences obtained from the *T. cruzi* Cl Brener, Colombiana, and Dm28c strains are highly homologous to that of the Y strain, with 88%, 90%, and 98% identity, respectively.

Overexpression of the Y strain *CRIT* gene in the C-sensitive epimastigote forms resulted in transgenic epimastigote forms being better able to resist C-mediated lysis. The level of lysis, compared with that for wild-type parasites, decreased within the first minutes, showing that CRIT blocks the propagation of the C cascade. This effect resembles metacyclic trypomastigote resistance to C at the first contact in vertebrate hosts. Interestingly, in the presence of AP alone, the degrees of lysis were similar in epimastigotes overexpressing CRIT and in wild-type parasites, indicating that CRIT inhibits specifically the CP and the LP, as was described in *Schistosoma* species and their hosts. The expression of the class II *CRIT* gene of the Y strain in the *T. cruzi* Colombiana strain conferred resistance to C lysis, indicating that the gene is functional in the class I strain.

A broad set of virulence factors and invasion-associated molecules has been studied by different groups searching for qualitative and/or quantitative differences among *T. cruzi* class I and II strains [18]. The phenomenon of the bipolar biological behavior between classes associated with differentially expressed antigens has not been interpreted convincingly. In this work, we have seen differences at the level of C resistance between Colombiana (a class I strain) and Y (a class II strain) and found CRIT, at the protein level, to be expressed differentially in the Y strain. The lack of an effective vaccine or chemotherapeutic treatment that protects the human host against trypanosomatid invasion makes it especially important to understand the molecular basis of the differences in C activation between class I and II strains and thereby, it is hoped, to find putative new targets to control the disease. The characterization of CRIT, a novel C-inhibiting protein, could be key to elaborating a new chemotherapeutic or vaccine-based strategy and to understanding the biological differences between class I and II strains.

Acknowledgments

Thanks are due to K. M. Hui for helping with the sequencing and J. A. Schifferli for supporting a collaborative visit at the beginning of this study.

References

1. Teixeira A R, Nitz N, Guimaro MC, Gomes C, Santos-Buch CA. Chagas disease. Postgrad Med J **2006**; 82:788–98.
2. Moncayo A, Ortiz Yanine MI. An update on Chagas disease (human American trypaosomiasis). Ann Trop Med Parasitol **2006**; 100:663–77.
3. Dutra WO, Rocha MOC, Teixeira MM. The clinical immunology of human Chagas disease. Trends Parasitol **2005**; 21:581–7.
4. Almeida IC, Ferguson MA, Schenkman S, Travassos LR. Lytic anti-alpha-galactosyl antibodies from patients with chronic Chagas' disease recognize novel O-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of *Trypanosoma cruzi*. Biochem J **1994**; 304:793–802.

5. Ambrosio AR, Messias-Reason LJT. *Leishmania (Viannia) braziliensis:* interaction of mannose-binding lectin with surface glycoconjugates and complement activation: an antibody-independent defence mechanism. Parasite Immunol **2005**; 27:333–40.
6. Zulantay I, Venegas J, Apt W, Solari A. Lytic antibodies in *Trypanosoma cruzi*-infected persons with low parasitemia. Am J Trop Med Hyg **1998**; 58:775–9.
7. Sher A, Hieny S, Joiner K. Evasion of the alternative complement pathway by metacyclic trypomastigotes of *Trypanosoma cruzi:* dependence on the developmentally regulated synthesis of surface protein and N-linked carbohydrate. J Immunol **1986**; 137:2961–7.
8. Krettli AU, Pontes de Carvalho LC. Binding of C3 fragments to the *T. cruzi* surface in the absence of specific antibodies and without activation of the complement cascade. Clin Exp Immunol **1985**; 62:270–7.
9. Krautz GM, Kissinger JC, Krettli AU. The targets of the lytic antibody response against *Trypanosoma cruzi*. Parasitol Today **2000**; 16:31–4.
10. Tambourgi DV. A partial cDNA clone of trypomastigote the decay accelerating factor (T-DAF), a developmentally regulated complement inhibitor of *T. cruzi,* has genetic and functional similarities to the human complement inhibitor DAF. Infect Immun **1993**; 61:3656–63.
11. Norris KA. Stable transfection of *Trypanosoma cruzi* epimastigotes with the trypomastigote-specific complement regulatory protein cDNA confers complement resistance. Infect Immun **1998**; 66:2460–5.
12. FerreiraV, Valck C, Sanchez G, et al. The classical activation pathway of the human complement system is specifically inhibited by calreticulin from *Trypanosoma cruzi*. J Immunol **2004**; 172:3042–50.
13. Fischer EM, Ouassi P, Velge J, Kazatchkine MC. gp58/68, a parasite component that contributes to the escape of the trypomastigote form of *T. cruzi* from the human alternative complement pathway. Immunology **1988**; 65:299–303.
14. Inal JM, Sim RB. A *Schistosoma* protein, Sh-TOR, is a novel inhibitor of complement which binds human C2. FEBS Lett **2000**; 470:131–4.
15. Inal JM, Hui KM, Miot S, et al. Complement C2 receptor inhibitor trispanning: a novel human complement inhibitory receptor. J Immunol **2005**; 174:356–66.
16. Inal JM, Schifferli JA. Complement C2 receptor inhibitor trispanning and the β-chain of C4 share a binding site for complement C2. J Immunol **2002**; 168:5213–21.
17. Hui KM, Orriss GL, Schirmer T, Magnadottir B, Inal JM. Expression of functional recombinant von Willebrand factor-A domain from human complement C2: a potential binding site for C4 and CRIT. Biochem J **2005**; 389:863–8.
18. Buscaglia CA, Di Noia JM. The *Trypanosoma cruzi* clonal diversity and the epidemiology of Chagas' disease. Microbes Infect **2003**; 5:419–27.
19. Briones MR, Souto RP, Stolf BS, Zingales B. The evolution of two *Trypanosoma cruzi* subgroups inferred from rRNA genes can be correlated with the interchange of American mammalian faunas in the Cenozoic and has implications to pathogenicity and host specificity. Mol Biochem Parasitol **1999**; 104:219–32.
20. Fernandes O, Mangia RH, Lisboa CV, et al. The complexity of the sylvatic cycle of *Trypanosoma cruzi* in Rio de Janeiro state (Brazil) revealed by the non-transcribed spacer of the mini-exon gene. Parasitology **1999**; 118:161–6.
21. Ramirez MI, Yamauchi LM, De Freitas Jr LH, Uemura H, Schenkman S. The use of the green fluorescent protein to monitor and improve transfection in *Trypanosoma cruzi*. Mol Biochem Parasitol **2000**; 111:235–40.
22. Di Noia JM, Buscaglia CA, De Marchi CA, Almeida IC, Frasch AC. A *Trypanosoma cruzi* small surface molecule provides the first immunological evidence that Chagas' disease is due to a single parasite lineage. J Exp Med **2002**; 195:401–13.
23. Ramirez MI, Ruiz RC, Araya JE, Da Silveira JF, Yoshida N. Involvement of the stage-specific 82-kilodalton adhesion molecule of *Trypanosoma cruzi* metacyclic trypomastigotes in host cell invasion. Infect Immun **1993**; 61:3636–41.
24. Contreras VT, Salles JM, Thomas N, Morel CM, Goldenberg S. In vitro differentiation of *Trypanosoma cruzi* under chemically defined conditions. Mol Biochem Parasitol **1985**; 16:315–27.
25. Inal JM. Complement C2 receptor inhibitor trispanning: from man to schistosome. Springer Semin Immunopathol **2005**; 27:320–31.
26. Inal JM, Miot S, Schifferli JA. The complement inhibitor, CRIT, undergoes clathrin-dependent endocytosis. Exp Cell Res **2005**; 310:54–65.
27. Dominguez M, Moreno I, Lopez-Trascasa M, Torano A. Complement interaction with trypanosomatid promastigotes in normal human serum. J Exp Med **2002**; 195:451–9.
28. Nogueira N, Bianco C, Cohn Z. Studies on the selective lysis and purification of *Trypanosoma cruzi*. J Exp Med **1975**; 142:224–9.
29. Ji YH, Fujita T, Hatsuse H, Takahashi A, Matsushita M, Kawakami M. Activation of the C4 and C2 components of complement by a proteinase in serum bactericidal factor, Ra reactive factor. J Immunol **1993**; 150: 571–8.
30. Roos A, Bouwman LH, Munoz J, et al. Functional characterization of the lectin pathway of complement in human serum. Mol Immunol **2003**; 39:655–68.
31. Khan S, Wleklinski M, Aruffo A, Farr A, Coder D, Khan M. *Trypanosoma cruzi* amastigote adhesion to macrophages is facilitated by the mannose receptor. J Exp Med **1995**; 182:1243–58.
32. Peterslund NAC, Koch CC, Jensenius JC, Thiel S. Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. Lancet **2001**; 358:637–8.
33. Dumestre-Perard C, Doerr E, Colomb MG, Loos M. Involvement of complement pathways in patients with bacterial septicemia. Mol Immunol **2007**; 44:1631–8.
34. Tomlinson S, Raper J. The lysis of *Trypanosoma brucei brucei* by human serum. Nat Biotechnol **1996**; 14:717–21.