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Triatoma infestans Calreticulin: Gene Cloning and Expression of a Main Domain That Interacts with the Host Complement System

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Abstract. *Triatoma infestans* is an important hematophagous vector of Chagas disease, a neglected chronic illness affecting approximately 6 million people in Latin America. Hematophagous insects possess several molecules in their saliva that counteract host defensive responses. Calreticulin (CRT), a multifunctional protein secreted in saliva, contributes to the feeding process in some insects. Human CRT (HuCRT) and *Trypanosoma cruzi* CRT (TcCRT) inhibit the classical pathway of complement activation, mainly by interacting through their central S domain with complement component C1. In previous studies, we have detected CRT in salivary gland extracts from *T. infestans*. We have called this molecule TiCRT. Given that the S domain is responsible for C1 binding, we have tested its role in the classical pathway of complement activation in vertebrate blood. We have cloned and characterized the complete nucleotide sequence of CRT from *T. infestans*, and expressed its S domain. As expected, this S domain binds to human C1 and, as a consequence, it inhibits the classical pathway of complement, at its earliest stage of activation, namely the generation of C4b. Possibly, the presence of TiCRT in the salivary gland represents an evolutionary adaptation in hematophagous insects to control a potential activation of complement proteins, present in the massive blood meal that they ingest, with deleterious consequences at least on the anterior digestive tract of these insects.

INTRODUCTION

Hematophagous insects, such as "kissing bugs," the vectors of Chagas disease, ingest about ten times their body weight in fresh blood. Blood from mammals harbors a remarkable number of molecular and cellular strategies to destroy foreign membranes. The complement system, mainly present in blood, is a central arm of innate and adaptive immune responses. It has several macromolecules specialized in swiftly recognizing a variety of molecular nonself-patterns with subsequent activation of an array of highly efficient destructive mechanisms. It is conceivable that the hematophagous insect saliva contains molecules that counteract the potential deleterious effects that blood complement may have at least on the anterior digestive tissues in these insects. Herein, we propose that salivary TiCRT may provide the insect with such defensive mechanism.

Arthropod vectors of Chagas disease in Chile are *Triatoma infestans*, *Mepraia spinolai*, *Mepraia gajardoi*,[1] and *Mepraia parapatrica*.[2] *Triatoma infestans* is responsible for the domestic cycle of the disease, whereas *M. spinolai*, *M. gajardoi*, and *M. parapatrica* are responsible for the wild cycle. Chagas disease is a zoonosis that affects about 6 million people in Latin America[3] and there is a growing concern because it is emerging in other continents. Thus, only in the United States, 300,000 to 1 million people are now infected.[4] Nonvectorial mechanisms, such as congenital, blood transfusions, organ transplantation from donors infected with *Trypanosoma cruzi*, as well as ingestion of contaminated foods, are considered to be largely responsible.[5]

Although during the last two decades great strides in the study of *T. cruzi* genetics and its relationship with the host immune system have occurred, there is still no effective safe cure for this disease.

Saliva from hematophagous insects possesses different molecules that mediate a successful feeding process. They counteract the hemostatic, inflammatory, and immune processes of the host,[6] such as vasoconstriction, blood coagulation, and platelet aggregation,[7–11] preventing the blood loss that follows tissue injury. However, the success of the hematophagic process also depends on innate and adaptive immune responses and, specially, on the complement system.[12]

The vertebrate complement system is a component of the immune response that has approximately 30 soluble and integral membrane proteins.[13] It has three main macromolecular recognition modules (C1, mannan-binding lectin [MBL], and ficolins) that recognize diverse microorganism-associated molecular patterns ("danger signals"), through three activation routes. The classical pathway can be initiated by the binding of C1 directly to the pathogen surface or during an adaptive immune response, by the binding of C1 to antibody–antigen complexes, being a key link between effector mechanisms of innate and adaptive immunity. The MBL pathway is initiated by the binding of MBL, a serum C1-like molecule, to mannose-containing carbohydrates on bacteria or viruses. The ficolin pathway is initiated by the binding of ficolins, also serum C1-like molecules, to a variety of lipoteichoic acid–like structures on the membrane of aggressor cells. Finally, the alternative pathway is initiated when spontaneously low-rate activated C3b-like molecules bind to permissive membranes. The presence of sialic acid on host cells allows the operation of regulatory molecules that will inactivate these molecules. Since most microbial membranes are devoid of sialic acid, the C3b-like molecules remain capable of recruiting factor B, thus constituting a functional C3-convertase. Derived from these three activation pathways, a cascade of pro-inflammatory, opsonizing, biological membrane–destructive and costimulating of immune response functions, among others, is unleashed.[14]

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Calreticulin (CRT) is a protein that has many functions in mammals.[15,16] Structurally, it is divided in three domains: An N-terminal, the most conserved among CRTs[15]; a P-internal, binds calcium with high affinity; and a C-terminal, highly acidic, binds calcium with high capacity.[17] Of utmost importance in the studies described here, an S domain, located between N and P, binds C1, and neutralizes its capacity to activate C4, an essential step necessary for the initiation of the classical pathway activation.[18] Human and *T. cruzi* CRTs specifically bind C1, inactivating at least two of the three described pathways.[19] On the other hand, salivary gland extracts from hematophagous insects such as *Lutzomyia longipalpis*, *Lutzomyia migonei*, *Panstrongylus megistus*, *Triatoma brasiliensis*, and *Rhodnius prolixus* inhibit the complement system, thus favoring the feeding process.[20] Moreover, it has been probed that *T. brasiliensis* forced to ingest serum in conditions in which the protection of midgut by salivary inhibitors is bypassed, and showed damage by complement in the anterior midgut epithelium and cell death.[12]

To address the role of salivary CRT on the hematophagous feeding process, we have cloned and characterized TiCRT and its S domain (TiS). Herein, we show that recombinant TiS (rTiS) binds human C1 and inhibits the classical pathway of complement activation in vitro. Conceivably, CRT in insect saliva protects the anterior digestive tract of these insects against deleterious effects of the complement system.

METHODS

Experimental insects. Adult live *T. infestans* (Hemiptera: Reduviidae) were reproduced in one of our laboratories (Werner Apt). The insects were maintained at 28°C, 65% relative humidity in a dark room. They were fed weekly on chickens (*Gallus gallus*). The insects were anesthetized and the soft parts were obtained by dissection under 10× magnification and kept on RNA Later (Qiagen, Germantown, MD).

Definition of the functional state of the complement system in blood ingested from *T. infestans*. Blood from *T. infestans* was recovered by introducing a syringe needle into its anterior midgut, immediately after feeding on a chicken. As this blood does not coagulate, plasma was obtained. Microtitration plates (Nunc MaxiSorp; Sigma-Aldrich, St. Louis, MO) were coated with 100 µL of human IgM, at 4 µg/mL (Jackson ImmunoResearch, West Grove, PA) and incubated at 4°C overnight. Nonspecific binding sites were blocked with 250 µL of 3% w/v bovine serum albumin in phosphate-buffered saline (PBS). Then, the following was added: 1) 100 µL of chicken normal serum (CNS) at a 1:320 dilution, as a source of active complement C1. CNS was obtained from the same bird that was used to feed the insects; 2) 100 µL of blood obtained from the insect anterior midgut at a 1:320 dilution; 3) 100 µL of CNS at a 1:320 dilution, plus ethylenediaminetetraacetic acid (EDTA) 10 mM final concentration; and 4) 100 µL of undiluted blood from the insect's anterior midgut plus EDTA 10 mM (3 and 4 were positive inhibition controls). They were incubated for 1 hour at 37°C with human C4 in Veronal Buffer (5,5-diethylpyrimidine-2,4,6[1H,3H,5H]-trione) at pH 7.3. C4b deposits, an indication of complement activation, were detected with an affinity-purified rabbit anti-human C4 antibody (DAKO, CA) at a 1:4,000 dilution, followed by an affinity-purified horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG antibody (DAKO, Carpinteria, CA). HRP activity (measured at 405 nm) was assessed by addition of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) with 0.03% v/v H_2O_2. The results were analyzed by one-way analysis of variance (ANOVA).

Complementary DNA synthesis of TiCRT. Since the sequence of *TiCRT* was unknown, we designed the RACEnest1rev, RACEnest2rev, and CRTfw primers (Table 1) based on an expressed sequence tag from *T. infestans* salivary glands (GenBank: ES597821.1). Total RNA from an insect macerate was extracted with the RNeasy® Mini Kit (Qiagen) and subjected to a reverse transcription polymerase chain reaction (RT-PCR), using the Abridged Anchor Primer and the RACEnest1rev primers (10 µM) (Table 1) with the 5′-RACE® System for Rapid Amplification of cDNA Ends, version 2.0 Kit (Invitrogen, Waltham, MA), according to the manufacturer's instructions. Every primer used in this study was synthesized by Integrated DNA Technologies Inc (San Diego, CA) and is listed in Table 1. The RT-PCR protocol was developed as follows: initial denaturation of 2 minutes at 94°C, 40 cycles each of 45 seconds at 94°C, annealing of 45 seconds at 60°C and extension of 2 minutes at 72°C, and a final extension of 10 minutes at 72°C. An oligo-dC tail was added to the obtained PCR product and this was visualized in an agarose gel stained with ethidium bromide (EthBr) in Tris-borate-EDTA buffer. The band with the expected weight (approximately 1,200 base pairs [bp]) was cut, purified using the Wizard®SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI), and sequenced with the RACEnest1rev primer (Table 1) (Automatic DNA Sequencing Center, Pontificia Universidad Católica de Chile, Santiago, Chile).

TiCRT cloning. The purified PCR product was ligated into the pGEM®-T Easy vector (Promega), according to the manufacturer's instructions. The recombinant vector pGEM-T/TiCRT was transformed by electroporation into competent *Escherichia coli DH5*α cells, plated on Luria Bertani agar/ampicillin with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)

TABLE 1
Primers used in the cloning of TiCRT and its S domain

Primer name	Sequence (5′→3′)	Orientation	Use
RACEnest1REV	GAGTTCGTCGTGTTCGTCTCCTGTTTCTGG	Reverse	5′-RACE
RACEnest2REV	GAGTTCGTCTGTTTCGTCTCCTGTTTCTGG	Reverse	5′-RACE
CRTfor	CGGCACAATATTTGGCAACATGCTGATAAC	Forward	5′-RACE
CRTforATG	ATGTGGGCAACAGTATTAAGTT	Forward	5′-RACE
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	Forward	5′-RACE
pQE_fw_SphI	AAAAAAGCATGCATGTGGGCAACAGTATTAAGTT	Forward	TiCRT subcloning
pQE_rev_XmaI	TGTCCTCTGCTTGTGCTGCTTGAGGGGCCCAAAAAA	Reverse	TiCRT subcloning
TiS_pQE_fw	AAAAAGCATGCATTAATAAAGATATTAGATGCAAGG	Forward	TiS subcloning
TiS_pQE_rev	AAAAAGTCGACATTCCATGGTCCTTTATATTTTG	Reverse	TiS subcloning

and isopropyl-β-D-1-tiogalactopyranoside (IPTG), and cultivated overnight at 37°C. The scrapped colony was amplified by PCR, under standard proceedings, with the CRTforATG and RACEnest2rev primers (10 μM) (Table 1). The product was purified with the Minipreps DNA Purification System Kit (Promega) and sequenced with T7 promoter and SP6 primers. The sequences obtained were analyzed using the BLAST tool from the National Center for Biotechnology Information (NCBI, MD) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The *TiCRT* open reading frame (ORF) was obtained with the tool NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf.html). The TiCRT theoretical molecular weight and isoelectric point were predicted using the ExPASy Computer pI/Mw Tool from the ExPASy Bioinformatics Resources Portal (http://web.expasy.org/compute_pi/). The potential N-glycosylation sites were analyzed with the NetNGlyc 1.0 Server Program (http://www.cbs.dtu.dk/services/NetNGlyc/). The modeling of the tertiary structure of TiCRT was obtained from the primary amino acid structure using the Robetta Beta Full-chain Protein Structure Prediction Server (http://www.robetta.bakerlab.org) and depicted with the UCSF Chimera package (http://www.cgl.ucsf.edu/chimera).

TiCRT subcloning. For TiCRT subcloning, we used the pQE80L vector (Qiagen), containing six histidine residues at the N-terminal end. A PCR amplification of the *TiCRT* coding sequence was performed, using Platinum® Taq DNA Polymerase (Invitrogen) and pQE_fw_SphI and pQE_rev_XmaI primers (Table 1). The amplification was carried out according to the following protocol: 2 minutes at 94°C, 40 cycles each of 45 seconds at 94°C, 45 seconds at 62°C, and 2 minutes at 72°C, followed by 10 minutes at 72°C. After double digestion with Sph I and Xma I, the purified TiCRT DNA was inserted into the pQE80L vector also digested, according to the T4 DNA ligase (New England BioLabs, Ipswich, MA) manufacturer's instructions. The recombinant vector pQE80L/TiCRT was transformed by electroporation into competent *E. coli* BL21(DE3) cells (Novagen, Madison, WI), plated in LB agar/ampicillin and cultured overnight at 37°C. The plasmids were purified and sequenced.

TiCRT expression. A BL21(DE3)/pQE80L/TiCRT recombinant clone was selected, grown in LB medium with 100 mg/mL ampicillin and incubated in agitation at 37°C overnight. This was added to 250 mL LB medium with ampicillin and grown until an OD_{600} of 0.5–0.6. Then, IPTG 1 mM was added and incubated in agitation for three additional hours. The culture was centrifuged for 20 minutes at 2,000 × *g* at 4°C, the supernatant was discarded, and a sample of the pellet was resuspended in 1× PBS and analyzed through denaturating 10% sodium dodecyl sulfate polyacrilamyde gel electrophoresis and Western Blot, under standard conditions.

Sequence analysis and alignment of TiCRT. The *TiCRT* nucleotide sequence was translated with the ExPASy Translate Tool from the ExPASy Bioinformatics Resources Portal (http://web.expasy.org/translate/), and the sequence with the wider ORF was selected. The same procedure was performed with the sequence of CRT from human (NCBI Reference Sequence: NM_004343.3), *Mus musculus* (NCBI Reference Sequence: NM_007591.3), *R. prolixus* (GenBank: FJ196458.1), *Drosophila melanogaster* (GenBank: AB000718.1), *Apis mellifera* (NCBI Reference Sequence: XM_006559506.1), *gambiae* (GenBank: AF457551.1), and *T. cruzi* (GenBank: AF457551.1). Afterward, the amino acid sequences were aligned with the Clustal W Program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A neighbor-joining phylogenetic tree was constructed using the PAUP* 4.b10 Program (http://paup.csit.fsu.edu).

Cloning of the TiCRT S domain. The coding fragment of TiS (463–861 bp from TiCRT, Figure 3) was amplified by PCR using as template TiCRT DNA, Platinum® Taq DNA Polymerase and the TiS_pQE_fw and TiS_pQE_rev primers (Table 1), and was designed based on the S domains of human CRT (HuCRT) and *T. cruzi* CRT (TcCRT). Both recombinant proteins are routinely expressed in our laboratory, using conventional procedures. The PCR protocol was developed according to the 5′-rapid amplification of cDNA ends Kit instructions, and consisted of the following series of incubations: 2 minutes at 94°C, 40 cycles each of 45 seconds at 94°C, 45 seconds at 55°C, and 2 minutes at 72°C, followed by 10 minutes at 72°C. The PCR product was visualized in an agarose gel stained with EthBr.

Subcloning of the TiCRT S domain. The obtained PCR product was extracted from the agarose gel, purified, and ligated into the pQE80L vector. The construct was transformed by electroporation into competent *E. coli* BL21 (DE3) cells, plated on LB agar/ampicillin and cultured overnight at 37°C. A colony PCR was performed to select the recombinant clones that carried TiS.

Expression of the TiCRT S domain. The recombinant clone that carried TiS was inoculated into LB with ampicillin and incubated in agitation at 37°C overnight. The culture was poured on 250 mL LB with ampicillin and incubated in shaker at 200 rpm and 37°C until the OD_{600} reached 0.5–0.6. Then, IPTG 1 mM was added and growing was continued at 150 rpm and 30°C overnight. The next day, the cells were centrifuged for 20 minutes at 2,000 × *g* and 4°C. The supernatant was discarded and the pellet was resuspended in 30 mL of Tris-HCl, NaCl 500 mM at pH 8.0, imidazole 5 mM, 0.1% Triton X-100, and a protease inhibitor (Cocktail Set V, EDTA-free; Calbiochem, Darmstadt, Germany). The sample was sonicated four times on ice for 30 seconds and centrifuged for 12 minutes at 15,600 × *g* and 4°C. The pellet was discarded and the supernatant was filtered, purified, and a sample was analyzed by denaturating 10% SDS-PAGE. The filtered supernatant was passed through a Sepharose column charged with Ni^{2+} 400 mM and eluted with an imidazole gradient. The column was equilibrated with binding buffer (Tris-HCl 160 mM at pH 8.0, NaCl 4 M, and imidazole 40 mM) and the proteins were eluted in a gradient of Tris 80 mM at pH 8.0, NaCl 4 M, and imidazole 4 M. The fractions containing the protein were dialyzed against 1× PBS, analyzed by denaturating SDS-PAGE and Western Blot, using a mouse anti-polyhistidine antibody (Sigma-Aldrich, Saint Louis, MO), followed by a goat alkaline phosphatase–conjugated anti-mouse IgG antibody. Protein concentrations were determined with the Bio-Rad Protein Assay Kit (BioRad, Hercules, CA), according to the manufacturer's indications.

Binding of human C1 to the TiCRT S domain. For simplicity, the complement reagent used will be designed herein as C1. Indeed, the pure reagent obtained from Complement Technology Inc. (Tyler, TX) corresponds to C1q. This is the collectin, devoid of the C1r and C1s serine proteases. By Western Blot, rTiS (12 μg) was incubated with human C1 (100 μg/mL)

and its binding was detected with a goat anti-human C1 antibody followed by a rabbit alkaline phosphatase–conjugated anti-goat IgG antibody. As positive controls, human C1 (200 ng) was detected with a goat anti-human C1q antibody, followed by a rabbit alkaline phosphatase–conjugated anti-goat IgG antibody. We also analyzed the S domain of TcCRT (TcS), previously obtained in our laboratory.[19] TcS was detected with a rabbit anti-TcS polyclonal antibody (previously generated in our laboratory[19]), followed by a goat alkaline phosphatase–conjugated anti-rabbit IgG antibody. As negative control, TiS (12 μg) was incubated with 3% PBS/skimmed milk, followed by a goat anti-human C1 antibody and a rabbit alkaline phosphatase–conjugated anti-goat IgG antibody. They were all revealed with 4-nitroblue tetrazolium chloride solution and 5-bromo-4-chloro-3-indoyl-phosphate. Binding of C1 to rTiS was also demonstrated by Dot Blot, as shown in Supplemental Figure 1.

Inhibition of C4b deposit by the TiCRT S domain. Microtitration plates were coated with 100 μL of human IgM at 4 μg/mL and incubated at 4°C overnight. Nonspecific binding sites were blocked with 250 μL of 2.5% w/v casein in PBS, and the following was added: 1) 100 μL of casein; 2) 100 μL of rTcCRT; 3) 100 μL of rHuCRT; 4) 100 μL of EDTA 10 mM; 5) 100 μL of rTcS; and 6) 100 μL of rTiS. In 1–6, the reagents were added at 2.4, 4.8, and 9.6 μM and were diluted in human normal serum (HNS) as a source of complement human C4. Casein was the negative inhibition control, whereas EDTA was the positive one. They were all incubated for 1 hour at 37°C in Veronal Buffer. C4b deposits were detected with an affinity-purified rabbit anti-human C4 antibody, followed by an affinity-purified goat HRP-conjugated anti-rabbit IgG antibody. HRP activity (measured at 405 nm) was assessed by addition of ABTS with 0.03% v/v H_2O_2. The results were analyzed by two-way ANOVA.

RESULTS

No activation of the classical pathway of complement is detected in blood recovered from the anterior midgut of *T. infestans*. As shown in Figure 1, a drastic decrease in the C4b deposition of the blood recovered from *T. infestans* anterior midgut was observed as compared with the C4b deposition of CNS. This result indicates that in the insect's anterior midgut conditions are such that the classical pathway of the complement system is importantly inactivated. Therefore, the possibility stands that, in agreement with our hypothesis, and as a simple proof-of-concept procedure, salivary CRT may be at least partially involved in such inactivation. We are aware that this experiment will not probe that salivary TiCRT is indeed responsible for complement inactivation. However, should the result be negative (no complement inactivation), it could not be argued that salivary TiCRT is involved.

TiCRT DNA coding sequence identification. The first reported *TiCRT* partial sequence obtained by 5′-RACE, included a segment of 400 bp containing the C-terminal domain, the stop codon and the endoplasmic reticulum (ER) retention signal, HDEL. Based on this, the full-length sequence of the *TiCRT* DNA coding sequence was obtained. This information was also useful for designing the CRTforATG and RACEnest2rev primers (Table 1), since they amplified

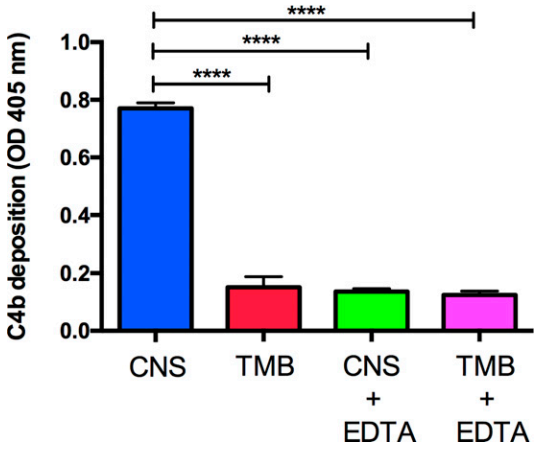

FIGURE 1. C4b deposition is inhibited in blood recovered from the *Triatoma infestans* anterior midgut. Plates were sensitized with IgM and C4b deposition (a direct reflection of C4 activation) was measured in blood recovered from the *T. infestans* anterior midgut (TMB) immediately after feeding. This C4b deposit was compared with chicken normal serum (CNS) obtained from the same bird used to feed the insects. CNS plus ethylenediaminetetraacetic acid (CNS + EDTA) and TMB plus EDTA (TMB + EDTA) were positive inhibition controls. The results correspond to the mean of triplicates and were analyzed by one-way analysis of variance ($P < 0.0001$) using the GraphPad Prism Program version 6.0c.

the complete *TiCRT* ORF (Figure 2A). The *TiCRT* ORF presented 1,212 bp, with a predicted 403-residue amino acid sequence. As shown in Figure 2B, the nucleotide sequence presented the ATG initiation and the TAA stop codons. As expected, we observed the initial methionine and the ER retention signal HDEL. The theoretical molecular weight of TiCRT was 46.17 kDa and the isoelectric point 4.36. In the schematic diagram of the TiCRT deduced protein in Figure 2C, the protein domains and lengths are shown. A three-dimensional model of TiCRT is shown in Figure 2D.

TiCRT shares important primary structure with insect and mammal CRTs, but not with TcCRT. Figure 3A shows the alignment of TiCRT with different CRTs. In all these sequences, the N, P, and C domains, as well as the ER retention signal HDEL (in TiCRT, RpCRT, AmCRT, DmCRT, and AgCRT) or KDEL (in HuCRT, MmCRT, and TcCRT), are detectable. The HDEL retention signal seems to identify insect CRTs. The two characteristic N domain sequences of the CRT-1 and CRT-2 families, KHEQNIDCGGGYIKLF (residues 96–111) and MFGPDICG (residues 129–136); besides the three P domain repeated motifs, PPKKIKDPKAKKPED (residues 202–222), GEWEAPQIDNPEYK (residues 257–270), and PKQIDNP (residues 275–281), are shown (Figure 3A). As shown in Figure 3B, the TiCRT amino acid sequence presents variable identity with CRTs from several species: 78.9% with *R. prolixus* (RpCRT), 71.5% with *A. mellifera* (AmCRT), 69.2% with *D. melanogaster* (DMCRT), 68.7% with *A. gambiae* (AgCRT), 60.8% with *M. musculus* (MmCRT), 59.8% with human (HuCRT), and 38% with *T. cruzi* (TcCRT). This indicates that CRT protein sequences are conserved among species. A neighbor-joining phylogenetic tree showing the CRT relationships in different species is also shown (Figure 3C).

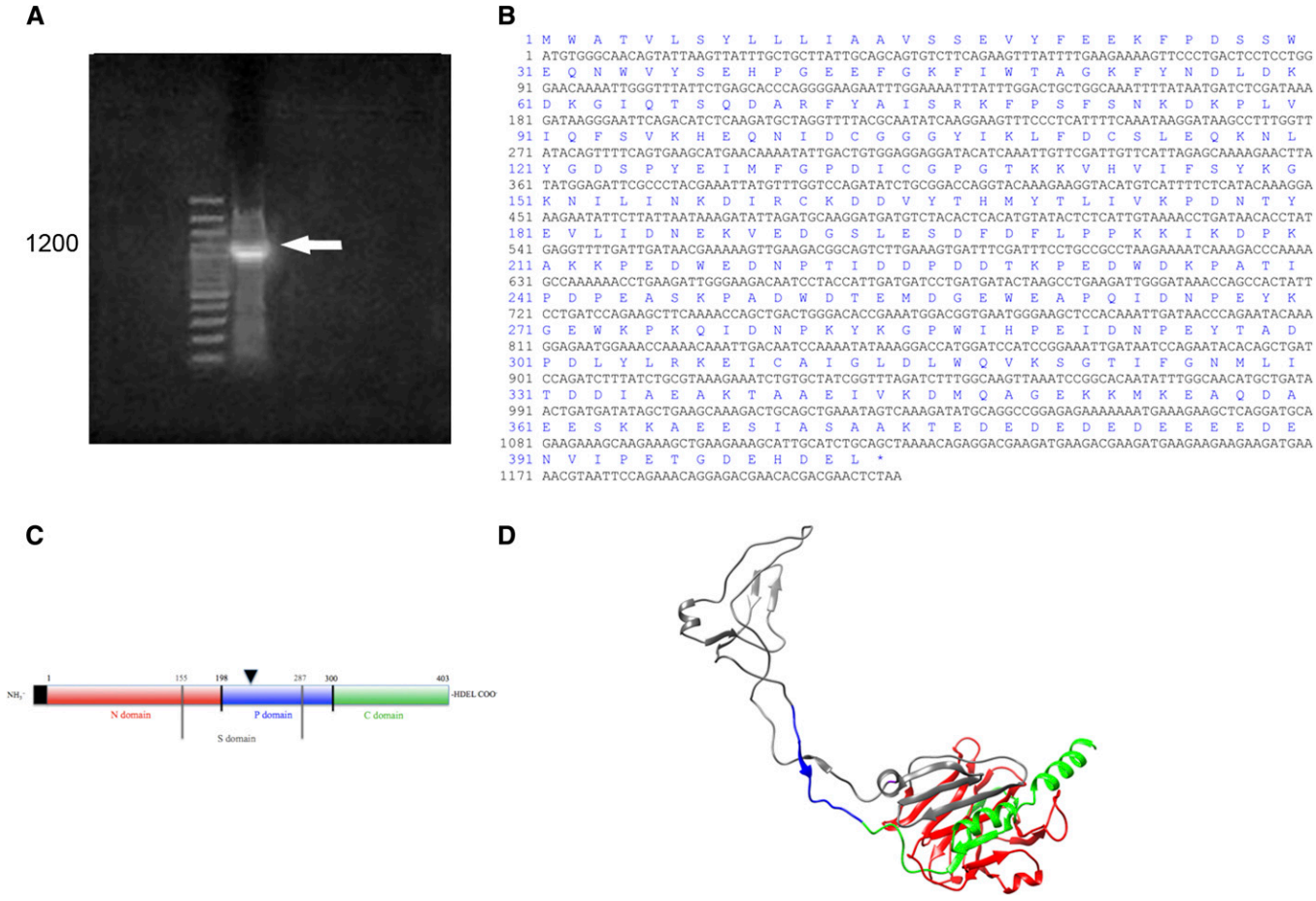

FIGURE 2. TiCRT DNA coding sequence identification. (**A**) 1% agarose gel. Lane 1: 10,000–base pair (bp) ladder; lane 2: nucleotide *TiCRT* 5′-RACE amplification product of 1,200 bp. (**B**) Nucleotide and predicted amino acid sequence of TiCRT. (**C**) Simplified characterization of the TiCRT deduced protein. The N, P, and C domains, residues 1–198, 199–300, and 301–403, are respectively shown. TiS (residues 155–287) is shown between grey lines. The leader sequence is indicated in a black box and the endoplasmic reticulum (ER) retention signal HDEL is shown at the C-terminal end. The glycosylation site is shown with an inverted black triangle (N^{220}). (**D**) Predicted three-dimensional model of the TiCRT deduced protein. Globular N, extended P, S (grey), and C domains, the last one ending in an α-helix, are shown. The color code of Figure 2C is also valid for Figure 2D. It presents a QMEAN4 of −0.41, indicating that the three-dimensional TiCRT model obtained is within the accepted range of closeness to the protein native conformation (–1 to 1).

Recombinant TiCRT S domain expression and purification. As expected, in Figure 4A, we observed the 399-bp band corresponding to the PCR-amplified fragment of TiS. The nucleotide and amino acid sequence of TiS is shown in Figure 4B. Figure 4C shows the denaturating 10% SDS-PAGE patterns of TiS and TcS, that display 30 and 34 kDa, respectively. The expression of TiS was also analyzed by Western Blot using a mouse anti-polyhistidine antibody (Figure 4D).

C1 binds to the TiCRT S domain. CRT from diverse origins binds C1, interfering with the function of this molecule.[21–25] By Western Blot, we showed that rTiS also binds C1, as detected by an anti-C1 antibody (Figures 5A and Supplemental Figure 1).

The TiCRT S domain inhibits the classical pathway of complement. As shown in Figure 6, TiS inhibited the deposit of C4b. Casein, a protein that has no effect on the complement system, was used as a negative control. EDTA, a Ca^{2+} chelator, was the positive inhibition control. HuCRT, TcCRT, and TcS were included also as positive inhibition controls, given their known capacity to inhibit the classical pathway of complement. We observed that TiS also inhibits this pathway of complement, although to a lower grade than complete proteins (HuCRT and TcCRT). TcS showed an inhibition degree similar to that of TiS in HNS.

DISCUSSION

Triatominae insects ingest about ten times their body weight in their blood meals.[26–28] The complement system, mainly present in blood, has at least four macromolecules specialized in the detection of an impressive variety of molecular danger signals. The system can also be activated by default, when it detects the lack of molecular structures normally present in vertebrates, as is the case of the alternative pathway. Thus, membrane-destructive, opsonizing, immune stimulatory, and pro-inflammatory activities are generated.[14] It is conceivable that, given its molecular complexity, the insect's digestive tract may activate the complement system, even in the absence of antibodies. Accordingly, the results presented herein are consistent with the possibility that CRT present in *T. infestans* saliva, by virtue of its capacity to inhibit complement activation, can counteract

A

B

Sequence	Name	Length	Sequence	Name	Length	Identity score
1	TiCRT	403	2	RpCRT	404	78.91
1	TiCRT	403	3	AmCRT	403	71.46
1	TiCRT	403	4	DmCRT	406	69.23
1	TiCRT	403	5	AgCRT	406	68.73
1	TiCRT	403	6	MmCRT	416	60.79
1	TiCRT	403	7	HuCRT	417	59.8
1	TiCRT	403	8	TcCRT	403	37.97

C

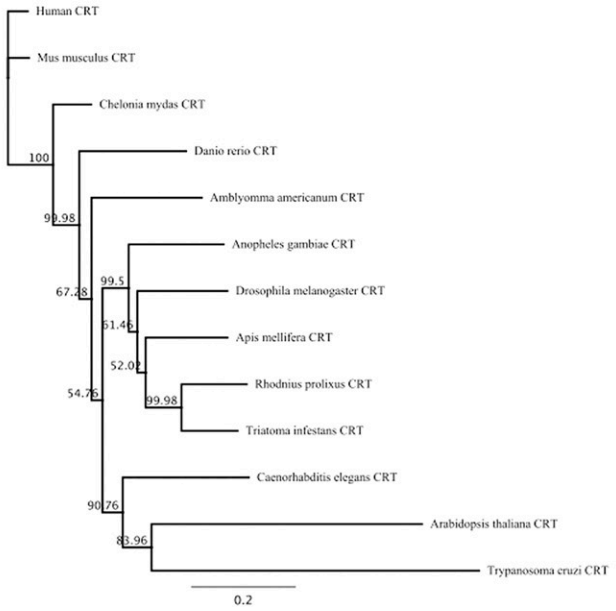

FIGURE 3. The TiCRT deduced protein sequence is conserved among species. (**A**) Alignment of the TiCRT sequence with MmCRT, HuCRT, RpCRT, AmCRT, DmCRT, AgCRT, and TcCRT. The predicted N, P, and C domains are respectively indicated with red, blue, and green lines above the sequence. In both grey boxes, the two characteristic sequences of the CRT N domain (CRT-1 y CRT-2) are shown; in green boxes, the three repeated motifs of the P domain are displayed; and in yellow box, the endoplasmic reticulum (ER) retention signals HDEL or KDEL, are indicated. Conserved residues (*), residues belonging to a same group with strongly conserved properties (:), and residues belonging to a same group with weakly conserved properties (.) are shown. (**B**) Score table showing sequence identity. (**C**) Neighbor-joining tree showing the phylogenetic relationships of CRT amino acid sequences, constructed using the PAUP* 4.b10 Program. Sequences of CRT from *Amblyomma americanum* (GenBank: AAC79094.1), *Arabidopsis thaliana* (GenBank: AAC49697.1), *Danio rerio* (GenBank: AAF13700.1), *Chelonia mydas* (GenBank: EMP37245.1), and *Caenorhabditis elegans* (NCBI Reference Sequence: NP_504575.1) were added to represent other kingdom and classes. Numbers on the branches are bootstrap values of 5,000 replications.

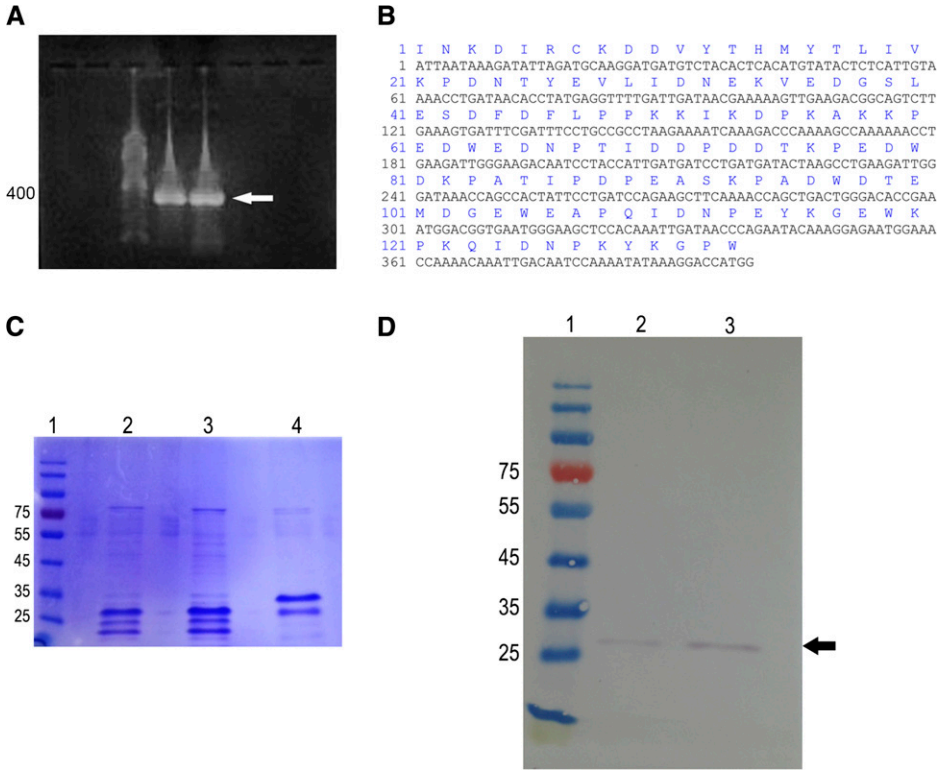

FIGURE 4. TiCRT S domain coding sequence identification and recombinant protein identification. (**A**) TiS nucleotide sequence amplification (1% agarose gel). Lane 1: 100-bp ladder; lanes 2 and 3: *TiS* polymerase chain reaction (PCR) expected product of 399 bp. (**B**) TiS nucleotide and deduced amino acid sequence with 399 bp and 133 residues, respectively, are shown. (**C**) TiS peptide expression (10% denaturant SDS-PAGE). Lane 1: Molecular weight marker; lanes 2 and 3: 6 and 12 µg of purified recombinant TiS, respectively; lane 4: purified recombinant TcS (6 µg). (**D**) Identification of purified TiS by Western Blot. Lane 1: Molecular weight marker; lanes 2 and 3: 40 and 80 ng of purified recombinant TiS, respectively, detected with a mouse anti-polyhistidine antibody, followed by a goat alkaline phosphatase–conjugated anti-mouse IgG antibody.

the complement potential deleterious effects on the digestive tissues in these insects.

Triatoma infestans possesses in its stylet two channels (salivary and alimentary) with afferent and efferent respective uses, connected at the anterior end.[29] It is then possible that saliva, ejected at the bite site, contains molecules that inhibit the complement proteins present in the massive blood meal that floods the first part of the insect's digestive tract. We propose that CRT, a multifunctional molecule, known to be present in salivary gland extracts from insects[30,31] and to inhibit the complement system,[19,32,33] could be responsible at least partly, for this effect (A dynamic view of this functional perception is presented in Supplemental Video).

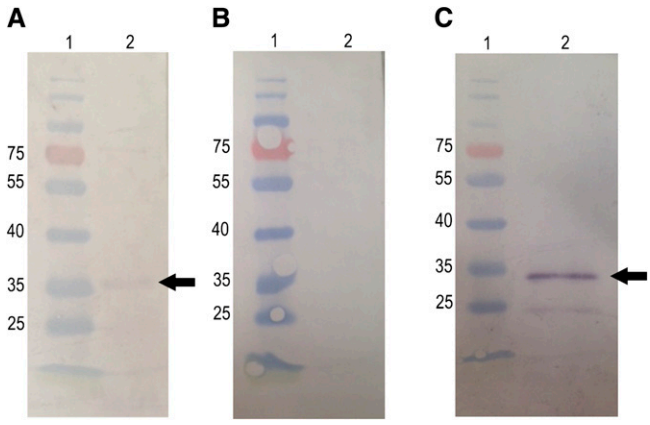

FIGURE 5. Human C1 binds to the TiCRT recombinant S domain. (**A**) rTiS was incubated with human C1, followed by a goat anti-C1 antibody and a rabbit alkaline phosphatase–conjugated anti-goat IgG antibody. (**B**) As a negative control, rTiS was incubated with 3% phosphate-buffered saline (PBS)/skimmed milk, followed by goat anti-C1 antibody and a rabbit alkaline phosphatase–conjugated anti-goat IgG antibody. (**C**) As a positive control, C1 was detected with an anti-C1 antibody, followed by a rabbit alkaline phosphatase–conjugated anti-goat IgG antibody.

We have investigated the complement functional state of the blood contained in the anterior midgut of *T. infestans* immediately after feeding. Complement activity, measured by the C4b deposition, was drastically inhibited in the anterior midgut of the insect, as compared with CNS from the same bird used for feeding purposes (Figure 1). These results are compatible with those of Khattab and others that found C3a and C5b-C9 in the midgut of *Anopheles stephensi* as indicators of complement activation, mainly via the alternative pathway.[34] Our results, by measuring C4b in blood retrieved from the anterior midgut of *T. infestans*, indicate an inhibitory effect on the classical pathway. Whether both protective strategies are operative in *T. infestans*, remains to be determined.

The presence of TiCRT in the *T. infestans* salivary glands, together with a potent C4b inhibition in the anterior midgut of the insect, withstands our hypothesis proposing that the salivary chaperone molecule is involved in this complement inhibition. It was thus necessary to attempt the cloning and

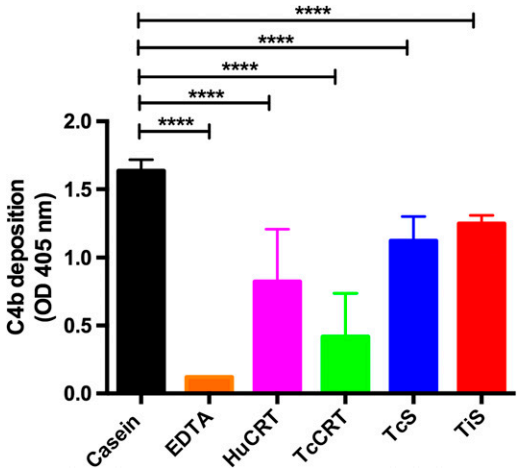

FIGURE 6. TiS inhibits the classical pathway of the complement system. Microtitration plates were sensitized with IgM, and rTiS was added together with human normal serum (HNS), as a source of complement components C1 and C4. As negative and positive inhibition controls, casein and EDTA were respectively used. HuCRT, TcCRT, and TcS were also tested. The results correspond to the mean of triplicates and were analyzed by two-way analysis of variance and Dunnett's multiple comparison test, casein being the control group ($P < 0.0001$), using the GraphPad Prism Program version 6.0c.

characterization of the full-length coding sequence DNA of the *TiCRT* gene.

Our results showed that the coding sequence of the *TiCRT* gene spans 403 amino acids (Figures 2A–C). The modeling of the tertiary structure of TiCRT showed the globular N domain and the central P domain with its characteristic loop (Figure 2D). The calculated theoretical molecular weight of TiCRT was 46 kDa and the isoelectric point 4.4.

The deduced amino acid sequence contained several features common to all CRTs and its three-major domains[15] (N-terminal, central P, and C-terminal). The two potential CRT family signature motifs KHEQNIDCGGGYIKLF and MFGPDICG, which are highly conserved among CRTs, were found in the TiCRT N domain. The P domain included the three repeats CRT motifs, PPKKIKDPKAKKPED, GEWE APQIDNPEYK, and PKQIDNP.

In agreement with the sequence of *R. prolixus*, *A. mellifera*, *D. melanogaster*, and *A. gambiae*, the ER retention sequence HDEL was present at the end of the C-terminal domain (Figure 3A). Alignment of the TiCRT amino acid sequence with *R. prolixus* CRT revealed 79% and approximately 70% identities with CRT from other insects. With mammals, CRT identity was about 60%, whereas with TcCRT, it was 38% (Figure 3B). The relationship of TiCRT is much closer with CRT from insects than with fish, reptile, and mammal CRTs, and the more distant relation is with TcCRT. Surprisingly, the neighbor-joining tree showed that TcCRT is closer to CRT from plants than any other CRT (Figure 3C).

Similar to those of some higher plants and insects,[35] in *TiCRT*, the ER retrieval signal was HDEL, instead of the typical KDEL found in some mammals and parasites. In spite of the presence of ER retention sequences in CRT, in some parasites, the protein is released from the cells. The missing KDEL sequence in CRT from ticks, which is secreted in saliva, could contribute to its routing into a secretory pathway rather than being retained in the ER.[30] In support of this theory, the tick *Amblyomma americanum*, while feeding in their host, secretes CRT, presumably as a mechanism to divert host defensive responses.[36] Moreover, CRTs from several ticks have an ER retrieval signal HEEL,[30,37] whereas two CRTs from *Caenorhabditis elegans* have no ER retrieval signal.[38] These investigations indicate that CRTs from closely related species will likely display the same signal. The biological significance of these differences is unknown.

CRT binds to C1, as shown for HuCRT[21] and CRTs from *Necator americanus*,[22] *Haemonchus contortus*,[23] *Trypanosoma carasii*,[24] *T. cruzi*,[25] *Entamoeba histolytica*, and *Entamoeba dispar*.[32] Resulting inhibition of the classical pathway of the complement system is detectable.[19,32,33,39] Based on this rationale, we asked whether this capacity was present in TiCRT.

Perhaps, poor expression caused by toxicity in the host cell, insolubility, mRNA secondary structure that prevents interactions with cellular machinery, or uncontrolled host cell basal protein expression inhibited the recombinant protein yield, thus explaining our impossibility to express TiCRT.

Given the known relevance of the S domain from other species in complement inhibition,[18] we cloned, sequenced, expressed, and purified the TiCRT S domain (Figures 4A–D). As expected, this domain binds to human C1 (Figures 5A and Supplemental Figure 1) and inhibits the classical pathway of the complement system in HNS (Figure 5A). It is not surprising that the TcS and TiS domains show a lesser inhibitory activity as compared with complete TcCRT and HuCRT, given that this and other functions involve several domains correctly folded in the intact chaperone molecule.

In synthesis, we have:1) identified the entire nucleotide coding sequence of the *TiCRT* gene; 2) deduced the full-length sequence of the protein; 3) established its phylogenetic homology to other CRTs; 4) cloned, sequenced, and expressed the TiCRT S domain; 5) determined the TiS capacity to bind complement component C1 and to inhibit the classical pathway of complement; and 6) proposed that salivary TiCRT could be responsible, at least partly, for the protection of the insect's digestive tract against the damage of complement proteins present in the vertebrate blood. In this scenery, recombinant proteins or relevant domains from hematophagous salivary origins, such as CRT, could be part of vaccines similar to those against the Bm86 polypeptide "hidden antigen" from ticks, used to immunize cattle.

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