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***Amblyomma americanum* tick calreticulin binds C1q but does not inhibit activation of the classical complement cascade**

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Abstract

In this study we characterized *Amblyomma americanum* (*Aam*) tick calreticulin (CRT) homolog in tick feeding physiology. In nature, different tick species can be found feeding on the same animal host. This suggests that different tick species found feeding on the same host can modulate the same host anti-tick defense pathways to successfully feed. From this perspective it's plausible that different tick species can utilize universally conserved proteins such as CRT to regulate and facilitate feeding. CRT is a multi-functional protein found in most taxa that is injected into the vertebrate host during tick feeding. Apart from it's current use as a biomarker for human tick bites, role(s) of this protein in tick feeding physiology have not been elucidated. Here we show that annotated functional CRT amino acid motifs are well conserved in tick CRT. However our data show that despite high amino acid identity levels to functionally characterized CRT homologs in other organisms, *Aam*CRT is apparently functionally different. *Pichia pastoris* expressed recombinant (r) *Aam*CRT bound C1q, the first component of the classical complement system, but it did not inhibit activation of this pathway. This contrast with reports of other parasite CRT that inhibited activation of the classical complement pathway through sequestration of C1q. Furthermore r*Aam*CRT did not bind factor Xa in contrast to reports of parasite CRT binding factor Xa, an important protease in the blood clotting system. Consistent with this observation, r*Aam*CRT did not affect plasma clotting or platelet aggregation aggregation. We discuss our findings in the context of tick feeding physiology.

Keywords

Amblyomma americanum; tick calreticulin; tick-feeding physiology

Introduction

In nature different tick species can be found feeding on the same animal host simultaneously (Durden et al., 1991, Kollars et al., 1997). This implies that different species of ticks that feed on the same host have to overcome the same host anti-tick defense mechanisms to

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complete feeding. This may raise questions of whether or not different species of ticks that feed on the same animal host can utilize orthologous tick saliva proteins to mediate the evasion of the host's anti-tick feeding defense responses. One such highly conserved tick saliva protein is tick calreticulin (CRT) (Xu et al., 2004, 2005, Gao et al., 2008).

CRT was originally identified as a high calcium (Ca^{2+}) binding protein from the sarcoplasmic reticulum of rabbit muscle (Ostwald and MacLennan, 1974, Fliegel et al., 1989). Coding cDNAs have now been identified in multiple organisms including ticks (Jaworski et al., 1995, Ferreira et al., 2002, Xu et al., 2004, 2005, Kaewhom et al., 2008, Gao et al., 2008), fleas (Jaworski et al., 1996), nematodes (Smith, 1992, Huggins et al., 1995, Tsuji et al., 1998, Scott et al., 1999, Mendlovic et al., 2004, Cabezón et al., 2008, Li et al., 2011), protozoan parasites (Aguillón et al., 2000, Marcelain et al., 2000, González et al., 2002), fish (Kales et al., 2004, Bai et al., 2012) and plants (Jia et al., 2008, Jin et al., 2009, An et al., 2011). Many more CRT sequences have been deposited in GenBank unpublished as direct submissions. For example, at the time of this write up, searching the NCBI sequence database with the key word "calreticulin and Ixodidae" retrieved 92 records.

In mammals where the bulk of data has been collected, CRT has been linked to both intracellular and extracellular functions, in health and disease (Holoshitz et al., 2010, Martins et al., 2010, Gold et al., 2010, Wang et al., 2012). The role of CRT in intracellular Ca^{2+} homeostasis associates this protein with multiple functions including heart muscle function, adipocyte differentiation and cellular stress responses (Tarr et al., 2010, Van Duyn Graham et al., 2010 Wang et al., 2012). Likewise at the extracellular level, CRT is linked to multiple functions such as in wound healing, the immune response, cell adhesion and migration, fibrosis, cancer pathogenesis, and anti-thrombotic functions (Gold et al., 2006, Nanney et al., 2008). In plants, CRT proteins have been linked to regulating plant growth and mediating the plant response to biotic and abiotic stress and plant defense mechanisms (Qiu et al., 2012a, 2012b). In hematophagous parasites, CRT proteins are thought to play role(s) in mediation of evasion of host defense mechanisms by invertebrate, protozoan and helminth parasites (Schroeder et al., 2009). In *Trypanosoma carassii* (Oladiran and Belosevic 2010), *T. cruzi* (Ferreira et al., 2004a, 2004b, Ramírez et al., 2011), *Entomoeba histolytica* (Vaithilingam et al., 2012) and helminthes, *Hemonchus contortus* (Naresha et al., 2009), and *Nector americanus* (Kasper et al., 2001) have been shown to interfere with activation of the classical complement pathway by binding and sequestering the C1q protein, the initiator component of this pathway. Additionally *H. contortus* CRT has also been implicated in mediating the anticoagulant function of this parasite by sequestering blood coagulation factors, factor Xa and the C-reactive protein (Suchitra and Joshi, 2005, Suchitra et al., 2008). Likewise, the rodent intestinal nematode, *Heligmosomoides polygyrus*, CRT protein is important for Th-2 skewing and it binds to murine scavenger receptor A (Rzepecka et al., 2009). In arthropods, an endoparasitoid wasp, *Cortesia rubercula*, was demonstrated to inject a CRT-like protein to interfere with the host immune system to enhance survival of their progeny (Zhang et al., 2006). In the *T. solium* model, recombinant CRT conferred protective immunity in mice (Leon-Cabrera et al., 2012, Fonseca-Coronado et al., 2011). In DNA vaccine development, high CRT immunogenicity has been exploited to investigate the potential of CRT proteins as molecular adjuvants (Park et al., 2008).

In tick biology, functional analysis data on CRT is limited to being a validated tick saliva protein used as the biomarker for human tick bites (Sanders et al., 1998, Malouin et al., 2003, Alarcon-Chaidez et al., 2006). Sanders et al., (1998) observed that repeated *Ixodes scapularis* infestations in humans conferred an anamnestic antibody response further confirming that tick CRT is injected into the host during tick feeding. A limited number of tick CRT sequences that have been characterized show amino acid identity levels ranging from 77–98% among ticks (Xu et al., 2004, Kaewhom et al., 2008), and ~70% when compared to mammalian CRT sequences (Kaewhom et al., 2008). In the tick *Amblyomma americanum* (*Aam*), CRT was previously found in dopamine/theophylline-induced saliva (Jaworski et al., 1995). Given that *Aam*CRT is present in saliva and injected into the host during tick feeding, our goal was to investigate the role(s) of *Aam*CRT protein in tick feeding physiology against host defense mechanisms.

Materials and Methods

Bioinformatics and phylogeny analyses

To determine if annotated calreticulin (CRT) functional amino acid motifs are conserved in ticks, we aligned *A. americanum* (AY395246) CRT sequence with human (NP_004334) and other previously functionally characterized parasite CRT proteins including, *T. solium* (AF340232, Fonseca-Coronado et al., 2011, Leon-Cabrera et al., 2012), *N. americanus* (AJ006790, Kasper et al., 2001), *H. contortus* (AAR99585, Naresha et al., 2009), *T. cruzi* (EKG06053, Ferreira et al., 2004a, 2004b, Ramírez et al., 2011), *T. carrasi* (ACV30040, Oladiran and Belosevic 2010), and *H. polygyrus* (CAL30086, Rzepecka et al., 2009) using ClustalW multiple sequence alignment provided in MacVector software (MacVector Inc., Cary, NC). The aligned sequences were screened for three CRT characteristic domains: (1) the globular N- domain, (2) the proline rich P-domain containing low capacity, high affinity Ca^{2+} binding site (Baksh and Michalak, 1991), a nuclear localization signal [NLS] (PPKKIKDPD motif) and three calreticulin P domain repeated motif [CPRM] KPEDWD (Fliegel et. al. 1989), and (3) the C- domain containing the high capacity low affinity Ca^{2+} binding site (Baksh and Michalak, 1991), glycosylation site, and 17–56 acidic amino acid sequence ending with an endoplasmic reticulum retention signal [ERRS] (K/H)(D/E)EL (Munro and Pelham, 1987, Fliegel et. al. 1989, Smith and Koch, 1989). Additionally, we also inspected tick CRT sequences for C1q (DEEKDKG, KDIRCKDD, GEWKPKQ, WEREYIDD, FNYKGKN, and IESKHKSD) and Zn^{2+} (histidine amino acid residues) (Baksh et al., 1995, Stuart et al., 1996, Kovacs et al., 1998, Naresha et al., 2009) binding site amino acid motifs. To predict N- and O- linked glycosylation sites, *Aam*CRT amino acid sequence (GenBank: AY395246) was scanned against the NetOGlyc 4.0 and NetNGlyc 1.0 CBS Servers (www.cbs.dtu.dk/services/) to predict only two O-linked glycosylation sites.

To gain insight into the sequence relationships among tick CRT sequences, functionally annotated amino acid motifs found in *Aam*CRT were compared to 34 other tick CRT sequences. Subsequently a phylogenetic tree of tick, human (NP_004334.1), and other hemoparasites CRT sequences tree out-rooted from *Homo sapiens* calnexin (GenBank: AAA21013.1) was constructed using Molecular Evolutionary Genetics Analysis (MEGA) 5.2.2 online software (<http://www.megasoftware.net>). CRT amino acid sequences were

aligned using T-coffee in MacVector (MacVector Inc., Cary, NC) under default settings. To estimate bootstrap values, replications were set to 1000. Tick CRT sequences included in the analysis included *A. americanum*_AY395246, *A. scutatum*_AAR29938.1, *A. rotundatum*_AAR29937.1, *A. geayi*_AAR29935.1, *A. cooperi*_AAR29934.1, *A. brasiliense*_AAR29933.1, *A. variegatum*_DAA34570.1, *A. triste*_JAC35302.1, *A. parvum*_JAC26090.1, *A. cajennense*_JAC22751.1, *A. maculatum*_AEO34580.1, *Dermacentor variabilis*_AAR29944.1, *D. occidentalis*_AAR29943.1, *D. andersoni*_AAR29942.1, *D. albipictus*_AAR29941.1, *Hyalomma anatolicum anatolicum*_AFQ98396.1, *H. dromedarii*_AEW43369.1, *H. anatolicum excavatum*_AAR29945.1, *Rhipicephalus microplus*_AAR29940, *R. sanguineus*_AAR29961.1, *Haemaphysalis qinghaiensis*_AAY42204.1, *H. longicornis*_AAQ18695.1, *H. leporispalustris*_AAR29947.1, *Ixodes ricinus*_AAR29958.1, *I. persulcatus*_AAR29957.1, *I. pararicinus*_AAR29956.1, *I. pacificus*_AAR29955.1, *I. pavlovskyi*_AAR29954.1, *I. ovatus*_AAR29953.1, *I. nipponensis*_AAR29952.1, *I. muris*_AAR29951.1, *I. minor*_AAR29950.1, *I. jellisoni*_AAR29949.1, *I. affinis*_AAR29948.1, and *I. scapularis*_XP_002402080.1). Hemoparasite CRT sequences included *Heligmosomoides polygyrus*_CAL30086.1, *Trypanosoma cruzi*_EKG06053.1, *T. carassii*_ACV30040.1, *Haemonchus contortus*_AAR99585.1, *Necator americanus*_AJ006790.1, and *Taenia solium*_AF340232.1.

Expression, affinity purification and characterization of recombinant (r) AamCRT

Expression of rAamCRT was done using pPICZαA yeast expression plasmid and the X-33 *Pichia pastoris* yeast host cell as previously described (Mulenga et al., 2013). AamCRT coding domain was amplified from OligodT primed cDNA template of five day fed ticks using PCR primers (For: **GAATTCGATCCCACCGTTTACTTCAAAG** and Rev: **GCGGCCGCCAGTTCCTCGTGCTTGTTGGTC**) with added enzyme sites (*EcoRI*/*NotI*, bold faced) designed based on NCBI sequence, GenBank: AY395246. The amplified AamCRT coding domain was sub-cloned into pPICZαA plasmid (Life Technologies, Carlsbad, CA) using directional approach. After sequence confirmation, recombinant pPICZαA-AamCRT plasmid was used to transform X-33 *P. pastoris* as previously described (Mulenga et al., 2013). Transformed colonies were selected on Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) agar plates with zeocin (100 µg/ml), and then selected for methanol utilization on Minimal Methanol (MM) agar plates, both incubated at 28°C. Positive transformants from PCR check were inoculated in Buffered Glycerol-complex Medium (BMGY) and grown overnight at 28°C with shaking (230–250 rpm). Subsequently the cells were used to inoculate Buffered Methanol-complex Medium (BMMY) to A₆₀₀ of 1 after which protein expression was induced by adding methanol to 0.5% final concentration every 24 h for five days. Preliminary results showed, cumulative expression of rAamCRT appeared to peak by the third day. Thus, for large-scale expression and affinity purification of rAamCRT, we cultured yeast for up to three days. The pPICZαA plasmid secretes the recombinant protein into media, so the spent media was concentrated using ammonium sulfate precipitation and affinity purified as previously described (Mulenga et al., 2013). The affinity-purified rAamCRT was dialyzed against 10mM HEPES buffer pH 7.4, 1X PBS pH 7.4 or normal saline (0.9% NaCl in sterile molecular grade water) and stored in –80°C until used. Expression and affinity purification of rAamCRT was

confirmed using standard SDS-PAGE with Coomassie blue staining or western blotting analysis using antibodies C-terminus hexa-histidine fusion tag (Life Technologies).

Western blotting analysis

In previous studies tick CRT was validated as a secreted tick saliva protein (Jaworski et al., 1995, Sanders et al., 1998, Malouin et al., 2003, Alarcon-Chaidez et al., 2006, Radulovic et al., 2014). To validate if these data were consistent in this study, affinity purified rAamCRT was subjected to western blotting analysis using antibodies to 48 h *A. americanum* tick saliva proteins. Production of antibodies to 48h *A. americanum* tick saliva proteins used in this study was previously described (Chalaire et al., 2011). Preliminary analysis predicted N- and O-linked glycosylation sites in AamCRT. To confirm post-translational modification, affinity purified rAamCRT was treated with the protein deglycosylation mix (New England Biolabs, Ipswich, MA), which removes O- and N-linked glycosylation sites. The sample treated and non-treated with deglycosylation mix were subjected to western blotting analysis using antibodies to the C-terminus hexa-histidine fusion tag (Life Technologies) on the pPICZA α vector.

Factor Xa and complement C1q protein binding assay

The potential for rAamCRT to bind blood-clotting factor Xa (Sigma-Aldrich, St. Louis, MO) or the complement C1q protein (EMD Millipore, Billerica, MA) was done using ELISA approach as previously described (Suchitra and Joshi, 2005). Different amounts of rAamCRT, 3, 1.5, 0.75, 0.375, and 0 μ M of rAamCRT diluted in 200 μ L ELISA coating buffer (carbonate buffer, pH 9.5) was bound onto ELISA plates overnight at 4°C. Control wells received buffer only or ligand (C1q or factor Xa). Following coating, non-specific binding sites were blocked with 300 μ L 5% blocking buffer (5% skim milk powder dissolved in 1X PBS w/Tween-20 pH 7.4) for 2 h at room temperature. Subsequently 0.5 μ g of the complement activation pathway C1q protein or factor Xa in 5% blocking buffer in 100 μ L was added to each well, with the exception of the ligand (C1q or factor Xa) only control wells and incubated at 4°C overnight. Wells were washed with 300 μ L of 1X PBS w/ Tween-20 pH 7.4 for three times. Subsequently antibodies to human C1q or factor Xa proteins diluted at 1:1000 were added to the wells and the plates incubated for 2 h at room temperature (RT). Following incubation, wells were washed with three changes of 300 μ L of 1X PBS w/Tween-20 pH 7.4. After washing, wells were incubated for 2 h at RT with 100 μ L of goat anti-rabbit peroxidase conjugated IgG secondary antibody (EMD Millipore) diluted to a final 1:2000 concentration in 5% blocking buffer. Following incubation, wells were washed as above. Bound peroxidase activity, proxy for bound factor Xa or the C1q protein was detected by adding 1-Step Ultra TMB-ELISA substrate (Thermo Scientific, Rockford, IL) and stopped with 2 M sulfuric acid upon color change. The intensity of color development, which directly represented the amount of bound C1q or factor Xa was quantified at A_{450} using VersaMax microplate reader (Molecular Devices LLC, Sunnyvale, CA).

Anti-platelet aggregation effects of rAamCRT

The platelet aggregation function assay was done using two methods. In the first method inhibition of platelet aggregation was determined using whole cattle blood (WCB). Cattle blood collected from the TAMU slaughterhouse was mixed with acid citrate dextrose (ACD) solution in 9:1 ratio to prevent clotting. Affinity purified 0.175 and 0.702 μM rAamCRT with adenosine phosphate (ADP) to 20 μM final concentration was pre-incubated for 15 min at 37°C. Following pre-incubation, platelet aggregation was induced by addition of ADP-rAamCRT to pre-warmed citrated WCB (500 μL) diluted with normal saline (0.9% NaCl) in a 1:1 ratio. Platelet aggregation as a function of increased appendence in ohms (Ω) was then monitored and recorded using the Whole blood platelet aggregometer (Chrono-Log Corporation, Havertown, PA).

In the second method anti-platelet aggregation function was determined using platelet rich plasma (PRP) isolated from citrated WCB and analyzed on the VersaMax microplate reader (Molecular Devices) as previously described (Horn et al., 2000). To prepare PRP, fresh citrated WCB was centrifuged at 200 g for 20 min at 18°C. Subsequently the PRP (top layer) was transferred into a new tube and centrifuged at 800 g for 20 min at 18°C. The pellet containing the platelets were washed and diluted with Tyrode Buffer pH 7.4 (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO_3 , 0.42 mM Na_2HPO_4 , 1 mM MgCl_2 , 0.1% glucose, 0.25% BSA) until the $A_{650} = 0.15$. To determine anti-platelet aggregation function of AamCRT, 2.5 and 1.25 μM of rAamCRT was pre-incubated for 15 min at 37°C with 20 μM ADP in a 50 μL reaction. Adding 100 μL of pre-warmed PRP triggered platelet aggregation. Samples were detected every 20 s for 30 min at A_{650} using the VersaMax microplate reader (Molecular Devices LLC).

Effects of rAamCRT on blood clotting

The effect of rAamCRT on the blood clotting system was determined using four approaches: (1) recalcification time (RCT), (2) activated partial thromboplastin time (APPT), (3) prothrombin time (PT), and (4) thrombin time (TT). The RCT assay, which measures the effect on the clotting system in its entirety, was done according to previously published methods (Liao et al., 2009, Gao et al., 2011) that were adopted in our lab (Mulenga et al., 2013) using two approaches. In the first approach, 20 μL of universal coagulation reference human plasma [UCRP] (Thermo Scientific) diluted to 90 μL with 10mM HEPES buffer pH 7.4 was pre-incubated with 3, 1.5, 0.75, and 0.375 μM of affinity purified rAamCRT for 10 min at 37°C. Following pre-incubation, adding 10 μL of 25 mM calcium chloride (CaCl_2) the pre-warmed to 37°C triggered plasma clotting. The basis of the RCT assay is that resupplying of Ca^{2+} to citrated plasma triggers plasma clotting (Spillert and Lazaro, 1995). Given that mammalian CRT is a validated Ca^{2+} binding proteins (Baksh and Michalak, 1991), we wanted to test if pre-incubating rAamCRT with CaCl_2 would sequester Ca^{2+} to affect plasma clotting. Thus in the second approach, similar amounts of rAamCRT that were used in the first approach up to 70 μL with 10mM HEPES buffer pH 7.4 were pre-incubated with 10 μL of 25 mM CaCl_2 for 10 min at 37°C before adding to 20 μL plasma pre-warmed to 37°C. Plasma clotting time was monitored at $A_{650\text{nm}}$ at 20 s intervals over 20 min using VersaMax microplate reader (Molecular Devices LLC) set to 37°C. The interpretation of data was that formation and firming of the clot was directly proportional increase in OD.

In the APPT assay, which measures the effect on the intrinsic blood clotting activation pathway, various amounts of rAamCRT were pre-incubated for 5 min at 37°C with 30 µL UCRP diluted up to 100 µL with 10 mM HEPES buffer, pH 7.4. Subsequently, 100 µL of the APPT reagent (Thermo Scientific) was added to plasma and incubated at 37°C for an additional 5 min to activate the reaction. Addition of 100 µL 25 mM CaCl₂ to the reaction triggered clotting. Alternatively we repeated this experiment by pre-incubating rAamCRT with 100 µL 25 mM CaCl₂ for 5 min at 37°C before adding to activated plasma to trigger clotting.

In the PT assay, which measures the effect on the extrinsic blood clotting activation pathway, various amounts of rAamCRT indicated above were pre-incubated at 37°C for 5 min with 25 µL UCRP diluted up to 100 µL with 10 mM HEPES buffer. Adding 200 µL of the pre-warmed PT reagent (Thermo Scientific) and CaCl₂ mixture triggered plasma clotting.

In the TT assay, to measure effects on the common blood clotting activation pathway, various amounts of rAamCRT indicated above used in the two assays above were pre-incubated at 37°C for 5 min with 25 µL of the TT reagent (Thermo Scientific) and CaCl₂ mixture diluted up to 100 µL with 10 mM HEPES buffer. Adding 200 µL of pre-warmed UCRP started the clotting reaction. For APTT, PT and TT assays, plasma-clotting time was determined using the KC1 DELTA coagulometer (Trinity Biotech, NJ, Parsippany). All assays were done in duplicates and results presented as mean (M).

Anti-complement function

To determine the effect of rAamCRT on the complement activation pathway two methods were used. In the first method the MicroVue CH50 ELISA kit (Quidel Corp., San Diego, CA) was used. Various amounts, 5.6, 2.8, 1.4, 0.7, and 0.35 µM of rAamCRT was pre-incubated at 37°C for 10 min with 14 µL serum. The serum-rAamCRT was then incubated with 86 µL of the complement activator solution for 1 h at 37°C. The end point of CH50 ELISA kit is that it quantifies the amount of terminal complement complexes (TCC) that are formed when the complement system is activated. To quantify the amount of formed TCC, the reaction mix was diluted 1:200. One hundred microliter of the diluted sample was applied to wells that were pre-coated with human antibodies to TCC and then incubated for 1 h at RT. Following appropriate washing, wells were incubated with 100 µL HRP-conjugated goat antibodies to TCC and incubated for 1 h at RT. Subsequently after appropriate washes, incubation with 100 µL of the chromogenic HRP substrate provided with the kit continued at RT for 15 min when the reaction was stopped with 100 µL stop solution (2 M sulfuric acid). The intensity of color change, which directly represented the amount of formed TCC was quantified by reading A_{450} using the VersaMax microplate reader (Molecular Devices LLC).

In the second method we used bacteria to activate the classical complement cascade in serum. The expectation for this assay was that upon inoculation of serum with bacteria, the complement cascade would be triggered and the bacteria would be lysed. On the flip side inoculation of serum depleted of C1q with bacteria, the complement cascade would not be triggered and the bacteria would not be lysed. The idea was supplementing the C1q depleted

serum with exogenous C1q would restore the complement cascade and cause bacteria lysis similar to normal serum.

We used two *Escherichia coli* K-12 strains, DH5 α and BL21 (Life Technologies) transformed with green fluorescent protein (GFP) vector pGlo that conferred ampicillin resistance (Bio-Rad, Hercules, CA). Both DH5 α and BL21 bacteria cells were grown at 37°C with shaking overnight in 7 ml of S.O.B broth with ampicillin (75 μ g/mL). Subsequently, 1 mL of bacteria culture was centrifuged at 5000 g for 10 min and the pellet re-suspended in 1 mL of 1X PBS pH 7.4. A further 10,000 fold dilution of bacteria cells in 1X PBS pH 7.4 was used for the assay. In triplicates various amounts, 4, 2, and 1 μ M, of rAamCRT in 47 μ L were pre-incubated with 3 μ L of C1q (0.2 μ M) at 37°C for 10 min. Subsequently for a final reaction volume of 100 μ L, 30 μ L of diluted bacteria, an empirically determined 20 μ L of C1q depleted human serum (Quidel Corp.), and 1X PBS was added to the rAamCRT-C1q reaction mixture. To trigger the complement cascade the reaction mix was incubated at 37°C for 15 min. Following incubation the 100 μ L reaction mixture was plated on LB agar plates containing ampicillin (75 μ g/mL) and arabinose (6 mg/mL) (Alfa Aesar, Ward Hill, MA) and incubated at 37°C overnight. Arabinose induced expression of the green fluorescent protein (GFP). GFP expressing bacteria colonies were visualized under UV light source to count colonies. Normal serum (positive control: no bacteria growth = activated complement cascade), C1q depleted serum (negative control: bacteria growth = no complement cascade activation), bacteria only in 1X PBS (bacteria growth expected) were included under same conditions as controls.

Statistical analysis

To determine statistical significance between treatments in C1q or Factor Xa binding, anti-haemostasis, and anti-complement function assays were subjected to non-parametric student t-test set to 95% confidence interval. Statistical software packages in PRISM version 6 (GraphPad Software Inc., La Jolla, CA) were used.

RESULTS

Annotated functional CRT amino acid motifs conserved in ticks

Amino acid identity levels among tick CRT sequences, and between tick and human CRT sequences were reported (Kaewhom et al., 2008). In this study, the analysis focused on the possibility that annotated CRT functional amino acid motifs and/or amino acid residues involved in ligand binding were conserved in ticks. Inspection of the multiple sequence alignment reveal that typical CRT features: the globular N-, the proline rich P- (Baksh and Michalak, 1991), and the C- domains are conserved in AamCRT at positions 20–195, 196–305, and 313–410 respectively (Fig. 1). Within the “N” domain, four of the five-histidine [H, marked with asterisks sign (*)] amino residues that in human CRT were verified as Zn²⁺ binding sites (Baksh et al., 1995) are conserved in AamCRT. In the P domain 18 proline (P) amino acid residues are noted with filled circles (●) on top, while the nuclear localization signal [NLS] (PPKKIKDPD motif) and three calreticulin P domain repeated motif [CPRM] KPEDWD (Fliegel et. al. 1989) are boxed with broken lines and noted. In the C- domain, AamCRT contains 43 acidic amino acid residues (Aspartic (D)/Glutamic (E) acid, marked

with filled square (■) on top, an endoplasmic reticulum retention signal, [ERRS] and two possible O-linked glycosylation sites at positions 396 and 397 consistent with a typical CRT protein (Fig. 1). The presence of the NLS and ERRS motifs supports the presence of *AamCRT* both in the nucleus and endoplasmic reticulum consistent with mammals (Opas et al., 1991). The six functionally annotated C1q binding amino acid motifs (boxed with solid lines and marked as C1qBS1-6, (Fig. 1) are conserved to various levels in *AamCRT*.

To gain insight on diversity among ticks and relationship to human and other parasites, we extracted CRT motifs in figure 1 and from other tick CRT amino acid sequences deposited in GenBank and then compared them as summarized in figure 2. Based on multiple sequence alignment, the CRT motifs in ticks are closer to humans than to other parasites (Fig. 2). Of the six C1q binding sites, C1qBS1 (human reference motif, DEEKDKG) is the least conserved between ticks and humans or other parasite CRT sequences. Additionally we noticed that C1qBS1 of other parasite CRT sequences in figure 1 show 66–100% amino acid identity to human CRT (Fig. 2). Notably the C1qBS1 and 2 are the least conserved between metastrata and prostrata tick CRT. Tick C1qBS2-6 (human reference motifs, KDIRCKDD, GEWKPKQ, WEREYIDD, FNYKGKN, and IESKHKSD) sites show high conservation ranging from 71–100% to human and 33–100% to parasite CRT motifs sequences respectively. The other three amino acid motifs, “NLS”, “CPRM” and “ERRS” motifs show high conservation in all organisms overall (Fig. 2).

AamCRT amino acid sequences are highly conserved across tick species

Figure 3 summarizes the phylogeny relationship of *AamCRT* proteins with other ticks, and functionally characterized human and hemoparasite CRTs retrieved from GenBank. As shown in figure 3, CRTs segregated into three clusters labeled as X, Y, and Z, supported by a respective 100, 91 and 97% bootstrap values. Tick CRTs in cluster X further segregated into clusters A-F showing high amino acid identity levels within genera. Pairwise alignment of tick amino acid sequences reveal that within clusters: A for *Amblyomma*, B for *Rhipicephalus*, C for *Hyalomma*, D for *Dermacentor*, E for *Haemaphysalis*, and F for *Ixodes* show a respective range of 92–100, 98, 79–92, 98–99, 85–92, and 92–99% identities (Fig. 3). Additionally, *AamCRT* segregated with other *Amblyomma* tick CRTs showing 92–96% amino acid identity (not shown). Furthermore *AamCRT* showed 89–90% amino acid identity to cluster B, 75–92% to C, 91–92% to D, 83–91% to E, and 77–78% to F (not shown). However when compared to remaining sequences amino acid identity levels decreased to 68% in cluster Y and 45–62% in cluster Z (not shown). It is interesting to note that *AamCRT* amino acid sequence is 69% identical to human, and 45–60% to parasite CRT proteins (not shown)

Expression of recombinant (r) AamCRT in *Pichia pastoris*

In order to characterize the role(s) of tick CRT at tick-feeding site, we thought to express recombinant (r) *A. americanum* tick CRT in *Pichia pastoris* (Fig. 4). In figure 4A, we show that r*AamCRT* was robustly expressed through out the all five days of culture. In figure 4B, we verified the purity of the affinity-purified r*AamCRT* using Coomassie blue staining. The pPICZα vector adds either ~3.5 kDa if the vector's α-factor signal peptide (SP) is cleaved off from the recombinant protein, or ~11.8 kDa if the SP is not cleaved off. On this

basis the expected molecular weight of rAamCRT was ~50 kDa (46 kDa mature tick CRT plus 3.5 kDa vector fusion) if SP is processed out, or ~58/60 kDa (expected ~46 kDa mature AamCRT plus 11.8 kDa fusion tag from vector) if the SP is not cleaved off as observed in figure 4. The increased MW of rAamCRT can also result from post-translational modifications. However, when subjected to N- or O-linked deglycosylation treatment, no molecular weight shift was observed (not shown).

In previous studies tick CRT was demonstrated to be among tick saliva proteins that are injected into the host during tick feeding (Jaworski et al., 1995, Sanders et al., 1998, Malouin et al., 2003, Alarcon-Chaidez et al., 2006, Radulovic et. al., 2014). In this study experiments summarized in figure 5 validate these reports in that rAamCRT specifically reacted with antibodies to 48 h fed *A. americanum* tick saliva proteins.

rAamCRT binds C1q protein, not factor Xa and does not inhibit classical complement cascade

Published studies demonstrated that parasite CRT proteins could bind both C1q and factor Xa (Suchitra and Joshi, 2005). To investigate if this function was conserved in ticks, we subjected rAamCRT to C1q (Fig. 6) and factor Xa (not shown) binding assays. While rAamCRT bound the C1q protein in dose response manner (Fig. 6), it did not bind to factor Xa (not shown). Given the observed rAamCRT binding of C1q, we thought to investigate if this translated to inhibition of the classical complement cascade. Data from two approaches showed that rAamCRT binding of C1q, an important component of the classical complement cascade, did not inhibit this cascade (data not shown). In the first analysis using the MicroVue CH50 ELISA kit we expected that higher concentrations of rAamCRT would lead to reduced production of terminal complement complexes (TCC). To our surprise we observed that increased amounts rAamCRT enhanced production of TCC instead (not shown). In the second approach, we used C1q depleted serum in which exogenous C1q restored the complement cascade and bacteria was lysed. Our expectation was that pre-incubating C1q with rAamCRT prior to adding to C1q depleted serum would sequester C1q, the complement cascade was not activated, and bacteria were not lysed. However, we observed no difference with or without rAamCRT (not shown).

rAamCRT has no apparent anti-hemostatic function

To investigate if tick CRT plays a role(s) in tick modulation of host hemostasis, we subjected rAamCRT to platelet aggregation and blood clotting function assays. Consistent with observations that rAamCRT did not bind factor Xa, pre-incubating rAamCRT with plasma did not affect clotting time (not shown). Likewise pre-incubating rAamCRT with CaCl₂ did not affect plasma clotting. In blood clotting assays we used citrated whole blood or UCRP in which sodium citrate sequestered Ca²⁺ ions, the fourth co-factor of the blood clotting system. Thus resupplying Ca²⁺ ions to citrated blood triggers plasma clotting. Our expectation was that since CRT was a validated Ca²⁺ ion binding protein, pre-incubating rAamCRT with CaCl₂ would sequester Ca²⁺ ions and prevent plasma clotting. However this was not the case in this study (not shown). Additionally in the platelet aggregation function assay, we did not see any inhibitory effects using two sources: (1) citrated whole blood or (2) platelet rich plasma (not shown).

Discussion

This study describes an update on bioinformatics analysis and characterization of role(s) of tick CRT in tick feeding physiology. Previous studies have reported high amino acid identity levels of more than 85% among ticks, and ~60–70% between tick and vertebrate CRT sequences (Kaewhom et al., 2008). Data in this study builds on previous reports and show that tick CRT proteins show high amino acid identity to human than to parasite CRT proteins. Consistent with reported studies (Jaworski et al., 1995, Sanders et al., 1998, Malouin et al., 2003, Alarcon-Chaidez et al., 2006, Radulovic et al., 2014), *Pichia pastoris*-expressed rAamCRT used in this study specifically reacted with antibodies to *A. americanum* tick saliva proteins confirming that AamCRT is injected into the host during tick feeding. Additionally in an ongoing study, we have found a calreticulin in *I. scapularis* tick saliva that shows 78% amino acid identity to AamCRT (*unpublished*) and have also verified the presence of AamCRT in *A. americanum* tick saliva proteomes (*unpublished*). Likewise a recent study by Tirloni et al., (2014) found a calreticulin that shows 89% amino acid identity to AamCRT in saliva of *R. microplus*.

The observed high conservation of annotated functional motifs between ticks and functionally characterized CRT proteins in human and other parasites are suggestive of conservation of function. However data in this study show that this is not the case. Although rAamCRT bound C1q, the first protein in the classical complement activation pathway (Nayak et al., 2010), the progression of the pathway was not affected. This is in contrast to other parasite CRT (Ferreira et al., 2004a, 2004b, Naresha et 2009, Schroeder et al., 2009, Ramírez et al., 2011, Kasper et al., 2001), which bound C1q and stopped the classical complement pathway. Data in this study is not unique. In a personal communication with C.A. Ferreira (Pontifical Catholic University of Rio Grande do Sul, Brazil), *R. microplus* CRT that shows 89% amino acid identity to AamCRT characterized in this study also bound C1q but did not inhibit activation of the classical complement pathway. The lack of effect on the classical complement system despite rAamCRT binding of the C1q protein is intriguing and may be explained by three plausible explanations. The first is that one of the six functionally validated C1q binding amino acid motifs (Stuart et al., 1996, Kovacs et al., 1998, Naresha et al., 2009) is poorly conserved in AamCRT as revealed by sequence analysis in this study. However, whether or not this amino acid motif is critical for sequestering C1q and thus interfering with the classical complement activation pathway has not been determined. The classical complement system is initiated by binding of C1q, C1r and C1s on pathogen surfaces (Nayak et al., 2010). Thus, the second explanation could be that while rAamCRT bound to C1q it did not sequester it from the interaction with C1r and C1s, and complement system was activated. It is also interesting to note that in the terminal complement complex (TCC) production assay, we observed more TCC formed with increased amount of rAamCRT. From this perspective, there is a possibility that tick CRT may enhance the classical complement pathway and contribute to tick feeding success by keeping the tick-feeding site sterile. It is most likely that in addition to preventing blood from clotting, the tick has to keep the feeding-site free of infection from contaminating microbial organisms that may arise from the skin. Alternatively, a remotely possible explanation could be that *in vivo* tick CRT acts in concert with other proteins to inhibit the

complement activation system. In this way, tick CRT acts as scaffold for binding C1q, which is subsequently degraded by yet identified protein inhibitors of the complement activation system. Given that tick CRT is at the tick-feeding site, it is conceivable that tick CRT is involved in other functions that contribute to the outcome of tick and host interactions. The nematode *H. polygyrus* CRT protein that show 64% amino acid identity to *AamCRT* was linked to skewing of Th-2 response in infested rodents (Rzepecka et al., 2009). Several studies have shown that yet to be identified tick saliva protein factor(s) skews a Th-2 host response to tick feeding (Schoeler et al., 1999, Castagnolli et al., 2008, Menten-Dedoyart et al., 2008). It would be interesting to determine if tick *AamCRT* is responsible for the observed skewing of a Th-2 response in tick infestations of animals.

Another observation in this study is that *rAamCRT* neither bound factor Xa nor affected plasma-clotting time. This was particularly interesting given that recombinant *H. contortus* CRT, which in this study showed 64% amino acid identity to *AamCRT* bound both factor Xa and delayed plasma clotting (Suchitra and Joshi, 2005, Suchitra et al., 2008). There is also a possibility that *A. americanum* ticks may not need CRT to bind factor Xa because they have alternatives. Previous studies have reported tick salivary gland anticoagulants that target multiple hemostatic factors including factor Xa (Wang et al., 1996, Mans et al., 2002, Ková , 2004, Waxman et al., 1990, Limo et al., 1991, Ibrahim et al., 2001, Francischetti et al., 2002). In both of the plasma clotting assays (APPT and RCT) used in this study plasma clotting was triggered by addition of CaCl_2 . Given that CRT is a validated Ca^{2+} binding protein (Ostwald and MacLennan, 1974, Gelebart et al., 2005), we expected a delay in plasma clotting time when *rAamCRT* was pre-incubated with CaCl_2 before adding to plasma. However we did not observe any effects in plasma clotting time. This finding may be explained by two possibilities: (1) either *AamCRT* is not a functional Ca^{2+} binding protein or (2) that it does bind but it does not necessary sequester Ca^{2+} ions from the reaction. To resolve this question, further experiments are needed to determine if *rAamCRT* can bind to radio-labeled Ca^{2+} using blot overlay assays (Naik et al., 1997, Naaby-Hansen et al., 2010).

Given the high amino acid sequence identity between ticks and vertebrate CRT as reported in this study and others (Kaewhom et al., 2008), it is interesting that in these studies a strong host antibody response to tick and other parasite CRTs was observed. The expectation would be that an antigen that is highly homologous to the vertebrate protein might be a poor antigen in that it might be recognized as self and thus not stimulate a robust antibody response as is observed with tick and other parasite CRT proteins.. It is also notable that human CRT acts as an auto-antigen in autoimmune diseases such as rheumatoid arthritis, complete congenital heart block, and coeliac disease (Holoshitz et al., 2010, Ling et al., 2010). In mice, cross-reactivity of antibodies to *T. cruzi* CRT with the mouse CRT protein was advanced as a possible explanation for the observed chagasic autoimmunity (Teixeira et al., 2011, 2012, Machado et al., 2012). The possibility of antibodies to tick CRT cross-reacting with host CRT has the potential to provide ticks with means to evade host defense mechanisms to tick feeding activity. Human CRT was demonstrated to enhance healing of difficult diabetic wounds (Nanney et al., 2008). To successfully feed, ticks have to stop the wound healing response by the host, which would otherwise lead to blood clotting and

thereby denying ticks a full blood meal. Thus, the potential for antibodies to tick CRT cross-reacting with host CRT raises the possibility for tick antibodies to bind host CRT and delay wound healing, and other yet unknown anti-tick feeding functions of host CRT.

In conclusion, data in this study demonstrates that *AamCRT* is not apparently involved in mediating tick anti-hemostasis and anti-complement functions. The high amino acid conservation among tick CRT proteins suggests the yet to be fully resolved functions of this protein in ticks are conserved in all tick species. Given that *AamCRT* is injected into the host, it is most likely that this protein is associated with regulating important tick functions, and thus follow up experiments are warranted to understand role(s) of tick CRT in tick feeding physiology.

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Figure 1. Multiple sequence alignment of *Amblyomma americanum* (Aam) CRT and amino acid sequences of functionally characterized CRT in human and other parasites

Downloaded CRT amino acid (AA) sequences of *A. americanum* (GenBank:AY395246), human (NP_004334) and other parasite CRT, *T. cruzi* (EKG06053), *T. carrasi* (ACV30040), *T. solium* (AF340232), *H. contortus* (AAR99585), *N. americanus* (AJ006790) and *H. polygyrus* (CAL30086) were subjected to multiple sequence alignment using ClustalW in MacVector. The aligned sequences were visually inspected for, globular N-domain, the proline rich P-domain and the C- domain noted in figure 1. The P- domain contains the nuclear localization signal [NLS] (solid box and noted) and three calreticulin P domain repeated motifs [CPRM1-3] (dotted box and noted). The C-domain contains the endoplasmic reticulum retention signal [ERRS, boxed and noted]. The six C1q binding site AA motifs [C1qBS1-6] are noted and boxed with solid lines. CRT conserved histidine (H) AA residues are marked with an asterisks (*) sign above, the proline (P) AA residues are noted with filled circles (●) above, and the characteristic acidic AA residues in *Aam*CRT c-domain are marked with filled squares (■).

Figure 2. Multiple sequence alignment of functionally annotated amino acid motifs of *Amblyomma americanum* and other parasite CRT proteins

Thirty four tick CRT amino acid sequences from NCBI consisting of Metastriata: *A. americanum* (GenBank:AY395246), *A. scutatum*_AAR29938.1, *A. rotundatum*_AAR29937.1, *A. geayi*_AAR29935.1, *A. cooperi*_AAR29934.1, *A. brasiliense*_AAR29933.1, *A. variegatum*_DAA34570.1, *A. triste*_JAC35302.1, *A. parvum*_JAC26090.1, *A. cajennense*_JAC22751.1, *A. maculatum*_AEO34580.1), *Rhipicephalus* (*R. microplus*_AAR29940, *R. sanguineus*_AAR29961.1), *Hyalomma* (*H. anatolicum*_AFQ98396.1, *H. dromedarii*_AEW43369.1, *H. anatolicum excavatum*_AAR29945.1), *Dermacentor* (*D. variabilis*_AAR29944.1, *D. occidentalis*_AAR29943.1, *D. andersoni*_AAR29942.1, *D. albipictus*_AAR29941.1), *Haemaphysalis* (*H. qinghaiensis*_AAY42204.1, *H. longicornis*_AAQ18695.1, *H. leporispalustris*_AAR29947.1), and Prosthiata ticks: *Ixodes* (*I. ricinus*_AAR29958.1, *I. persulcatus*_AAR29957.1, *I. parvicornis*_AAR29956.1, *I. pacificus*_AAR29955.1, *I. pavlovskyi*_AAR29954.1, *I. ovatus*_AAR29953.1, *I. nipponensis*_AAR29952.1, *I. muris*_AAR29951.1, *I. minor*_AAR29950.1, *I. jellisoni*_AAR29949.1, *I. affinis*_AAR29948.1, *I. scapularis*_XP_002402080.1), six other parasites: *T. cruzi* (EKG06053), *T. carrasi* (ACV30040), *T. solium* (AF340232), *H. contortus* (AAR99585), *N. americanus* (AJ006790) and *H. polygyrus* (CAL30086) and human: *H. sapiens* (NP_004334) CRTs, were subjected to multiple sequence alignment using T-coffee in MacVector software. The aligned sequences were visually inspected for presence of the six C1q binding site amino acid motifs (C1qBS1-6), the nuclear localization signal (NLS), three calreticulin P domain repeated motifs (CPRM1-3), and the endoplasmic reticulum retention signal [ERRS].

Figure 3. Phylogeny analysis of *Amblyomma americanum*, other parasites, and human CRT amino acid sequences

A guide phylogeny tree of *AamCRT* protein sequence with other tick, parasite, and human CRT sequences was constructed using the Maximum Likelihood method with bootstrap replicates set to 1000. Number at each node represents bootstrap values that signify the level of confidence in the branch. Three main clusters: X (tick CRTs), Y (*Homo sapiens* [GenBank:NP004334.1]), and Z other parasite and an outgroup of human calnexin (GenBank:AAA2103.1) noted with asterisk (*). The overall amino acid identity percentage levels are noted for cluster A–F.

Figure 4. Expression and affinity purification of recombinant *Amblyomma americanum* CRT in *Pichia pastoris*

The coding domain for mature *Aam*CRT amplified as described in materials and methods was sub-cloned into pPICZαA yeast expression plasmid using routine directional approach. Following confirmation of sequence, recombinant pPICZαA-*Aam*CRT was expressed and affinity purified as described in materials and methods. Expression and affinity purification of r*Aam*CRT was respectively confirmed using routine western blotting analysis using antibodies C-terminus hexa-histidine tag and detected with chemiluminescent substrates (A) and SDS-PAGE with Coomassie blue staining (B). Lanes 1–5 in “4A” = daily expression levels of r*Aam*CRT. In “4B”, AP1 and AP2 = affinity purified fractions 1 and 2.

Figure 5. Western blotting analysis of rAamCRT using antibodies to 48h *Amblyomma americanum* tick saliva proteins

Affinity purified rAamCRT loaded into triplicate lanes was electrophoresed on a 10% Tris-glycine acrylamide gel and electroblotted onto PVDF membranes using routine methods. The three lanes were cut into individual strips, which were exposed to three different concentrations (noted in Fig. 5) of pre-immune and antibodies to 48h *A. americanum* tick saliva proteins as indicated.

Figure 6. Recombinant *AamCRT* binding of the C1q protein

Different amounts of r*AamCRT* (indicated) were diluted in 200μL ELISA coating buffer (carbonate buffer, pH 9.5) and bound onto ELISA plates overnight at 4°C. Control wells received buffer only or ligand (C1q or factor Xa). Following coating, wells were blocked as described in materials and methods and subsequently incubated with 100μL (0.5μg) of C1q protein with the exception of the ligand (C1q) only control wells, overnight at 4°C. Following appropriate washing, wells were incubated with antibodies to human C1q for 2h at room temperature (RT). Following incubation and appropriate washing, plates were incubated for 2h at RT with 100μL of anti-rabbit peroxidase conjugated IgG. Following appropriate washing bound peroxidase activity, proxy for bound C1q protein was detected by adding OPD substrate as described and level of bound C1q quantified at A₄₅₀ using the VersaMax microplate reader. Shown in the figure is bound C1q OD with background removed.