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Trypanosoma carassii calreticulin binds host complement component C1q and inhibits classical complement pathway-mediated lysis

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ABSTRACT

Trypanosoma carassii is an extracellular parasite of economically important fish species that has evolved several strategies to circumvent host immune responses. Proteomic analysis of the excreted/secreted (ES) and surface molecules of the parasite has revealed a number of proteins that may be involved in host–parasite interactions. Among the parasite molecules identified in the ES of *T. carassii* was calreticulin. We cloned and produced *T. carassii* calreticulin (rTcaCRT), and generated a rabbit polyclonal antibody to the recombinant protein. The incubation of parasites with rabbit anti-rTcaCRT affinity-purified IgG antibody indicated substantial CRT levels on the surface of trypanosomes, as well as internal structures of permeabilized organisms. Recombinant parasite calreticulin bound several molecules in host serum including the first complement component, C1q. The host C1q specifically interacted with parasite CRT since the C1q-dependent lysis of sensitized sheep erythrocytes was inhibited by rTcaCRT. Our findings suggest that CRT may be used by the parasite to inhibit hosts' classical complement pathway.

1. Introduction

Calreticulin (CRT) is a highly conserved multifunctional protein originally identified as a major calcium binding protein of the endo/sarcoplasmic reticulum [1]. CRT has since been reported in every cell of the higher organisms with exception of the erythrocytes. CRTs possess lectin-like chaperone activity, participate in lytic activity of perforins from T and natural killer cells, modulate gene expression, enhance phagocytosis of apoptotic cells, inhibit tumor growth, mediate autoimmunity and inhibit C1q-dependent complement activation [2,3]. All CRT proteins contain three structural domains: a globular N-terminal domain, a proline rich P domain and an acidic C-terminal domain. The N-terminal domain is involved in protein–protein interactions, RNA and autoantibody binding ([4–6]. The proline rich P domain binds Ca^{2+} with high affinity and low capacity while the C-terminal domain, which is the least conserved domain among CRTs, binds Ca^{2+} with low affinity [1,7]. The CRT sequence starts with a signal peptide and ends with KDEL or related endoplasmic reticulum retention sequence [3,5,8].

The role of CRT in host–parasite interactions has recently become an important area of research. CRT molecules from a number of human parasites have been cloned and these sequences display about 50% identity with human CRT [9]. Parasite CRTs bind host C1q and inhibit C1q-dependent complement activation [9–12]. It has been reported that *Haemonchus contortus* CRT binds host C-reactive protein (CRP) and is present in the ES molecules of the adult worms [11,13]. These studies indicate parasite CRT may inhibit activation of the classical pathway of complement by binding C1q or CRP. CRP has anti-parasite as well as procoagulant activities and CRT binding to this molecule may prevent blood clotting and allow the parasite to feed longer on its host [11]. The ectoparasite *Amblyomma americanum* secretes CRT during feeding [14] suggesting that CRT may use its anti-coagulant ability to prevent blood clotting and allow the parasite to feed longer on the host or divert host anti-parasite responses. The presence of CRT in the penetration gland cells of schistosome cercariae suggests that this molecule may be important during penetration of host skin [15]. Parasite CRT is important in worm infection and has been reported to induce Th2 type immune response in mice infected with *Heligmosomoides polygyrus* [16]. Experimental vaccination with recombinant *Necator americanus* CRT in absence of adjuvant reduced worm burden by 43–49% in lungs of infected mice compared to control mice [17]. Among protozoan parasites, *T. cruzi* calreticulin (TcCRT) has been characterized. TcCRT binds human C1q and specifically inhibits classical pathway of complement

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[8,9]. TcCRT co-localized with human C1q on parasite surface and antibody against TcCRT inhibited its interaction with human C1q [9,18]. Taken together, these studies highlight the importance of parasite CRT during infection.

We reported previously that *Trypanosoma carassii* is highly resistant to complement-mediated lysis, suggesting that the parasite has evolved potent mechanisms to evade complement-mediated lysis [19]. However, the removal of parasite surface proteins using trypsin increases their susceptibility to complement-mediated lysis *in vitro*, suggesting that molecules expressed on the surface may interfere with binding of complement to the parasite. Parasites regained resistance to lysis 6–24 h following cultivation in the absence of trypsin and resistance to lysis was abrogated with a protein synthesis inhibitor, puromycin, suggesting that parasite proteins mediate the resistance to complement-mediated lysis. [19]. Although *T. carassii* shares similar surface architecture with *T. cruzi*, this fish parasite is also comparable to *T. brucei* because of its extracellular existence. The extracellular nature of *T. carassii* suggests that host humoral responses may also play a role in parasite lysis *in vivo* through the classical pathway of complement. Thus, there is need to further investigate the importance of this pathway in control of this infection and how the parasite may evade it. This is the first report on the molecular characterization of CRT molecule of *T. carassii* (TcaCRT). We show that TcaCRT is located on parasite surface and is present in the supernatants of cultured parasites. Recombinant TcaCRT binds goldfish C1q *in vitro* and inhibits classical complement pathway-mediated lysis of sensitized sheep erythrocytes.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus* L.) and carp (*Cyprinus carpio*) were purchased from Aquatic Imports (Calgary, Alberta, Canada) and maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were kept at 20 °C in a flow-through water system on a simulated natural photoperiod, and fed to satiation daily with trout pellets. The fish were acclimated to this environment for at least 3 weeks prior to use in the experiments. All of the fish ranged from 10 to 15 cm in length and whenever possible an equal number of both sexes were used. Fish were anaesthetized by immersion in tricaine methane sulfonate solution before bleeding.

2.2. Fish serum

Blood was collected through caudal vein of anaesthetized fish using a 23-guage needle fitted to a syringe. Blood was allowed to clot for 4 h at 4 °C and serum was isolated by centrifugation at 3000 × *g* for 30 min.

2.3. Parasites

The parasite used in this study was bloodstream form *T. carassii* strain TrCa (=*T. danilewskyi*; Laveran and Mesnil, 1904). Isolation and cultivation of this trypanosome strain has been previously described [20,21]. Parasites used for preparation of ES products and membrane protein isolation were cultured *in vitro* at 20 °C in TDL-15 medium [22] supplemented with 10% heat-inactivated goldfish serum. Parasites were harvested from 5- to 7-day-old cultures.

2.4. Preparation of parasite excretory/secretory (ES) products

Trypanosomes were washed twice at 400 × *g* for 10 min at 4 °C in serum-free culture medium and re-suspended to a density of 4.3×10^8/mL. Parasites were incubated at 20 °C for 1 h and then centrifuged at 12,000 × *g* for 10 min. Supernatants containing ES products were removed, filtered (0.22 μm filter, Millipore) and concentrated 10-fold using dialysis tubing (MWCO, 3 kDa) and polyethylene glycol flakes. ES products were stored at −20 °C until used.

2.5. SDS-PAGE and Western blot analysis

Proteins were separated by SDS-PAGE under reducing conditions. Briefly, samples were dissolved in an equal volume of Laemmli sample buffer (BioRad), heated at 95 °C for 5 min and electrophoresed through polyacrylamide gels (4% stacking, 12% separating) at 100 V for 15 min followed by 185 V for 45 min. Proteins were transferred to 0.2 μm nitrocellulose membranes (BioRad) at 100 V for 1 h in blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes containing the transferred proteins were treated in the following manner. The nitrocellulose was first incubated in blocking solution consisting of 5% skimmed milk in PBS for 30 min at room temperature. The membranes were then incubated with primary antibody (mouse monoclonal anti-polyhistidine antibody 1:5000 for detection of recombinant TcaCRT or rabbit anti-carp C1q-A antiserum 1:1500 for detection of carp and goldfish C1q) diluted in PBS and incubated overnight at 4 °C. Membranes were washed 3× in TTBS and 3× in TBS for 5 min each, prior to incubation with secondary antibody (alkaline phosphatase-labelled goat anti-mouse IgG 1:1500 or goat IgG 1:3000) diluted in PBS for 1 h at room temperature. Following incubation with the secondary antibody, the blots were washed 3× in TTBS and 3× in TBS for 5 min each. Protein bands were visualized using the chromogenic BCIP/NBT development kit according to the manufacturer's instructions (BioRad). Far Western blotting was performed using a native gel. Native gels were prepared in the absence of SDS by lowering acrylamide concentration to 8% in separating and 3% in stacking gels. Protein samples were dissolved in native sample buffer (0.125 M Tris pH 6.5, 10% glycerol, 0.005% bromophenol blue) and proteins were electrophoresed using native running buffer (25 mM Tris–HCl pH 8.8, 250 mM glycine). Following electrophoresis, proteins were transferred to nitrocellulose membrane as described above. Nitrocellulose strips were incubated with PBS alone or with 200 μg of recombinant TcaCRT (rTcaCRT) diluted in PBS for 3 h at room temperature. Blots were washed and then incubated with rabbit anti-rTcaCRT antibody (1:1500) for 3 h at room temperature. Following washes with TTBS and TBS, blots were incubated with alkaline phosphatase-labelled goat anti-rabbit IgG (1:3000) for 1 h at room temperature. Protein bands were visualized with BCIP/NBT development kit.

2.6. Preparation of whole cell lysate and detection of native CRT (nTcaCRT) by immunoblotting

Whole cell lysates of *T. carassii* were prepared from *in vitro* cultured parasites. Cells were washed twice with TDL-15 medium without serum at 400 × *g* for 10 min and re-suspended in culture medium containing protease inhibitors (Calbiochem). Cells were then subjected to 3 cycles of freeze-thaw. Ten micrograms of ES products and cell lysate were separated by SDS-PAGE under reducing condition. Following gel electrophoresis, proteins were transferred to nitrocellulose membranes and immunoblotted. Membrane strips containing ES products or whole cell lysate proteins were blocked with 5% skimmed milk in PBS for 30 min at room temperature. Membranes were then incubated with polyclonal rabbit anti-rTcaCRT affinity-purified IgG antibodies diluted in PBS (1:1500) overnight at 4 °C. Membranes were washed 3× in TTBS and 3× in TBS for 5 min each before incubation with alkaline