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Exogenous Calreticulin, incorporated onto non-infective *Trypanosoma cruzi* epimastigotes, promotes their internalization into mammal host cells

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ABSTRACT

Chagas disease is an endemic pathology in Latin America, now emerging in developed countries, caused by the intracellular protozoan *Trypanosoma cruzi*, whose life cycle involves three stages: amastigotes, epimastigotes, and trypomastigotes. *T. cruzi* Calreticulin (TcCRT), an endoplasmic reticulum resident chaperone, translocates to the external cellular membrane, where it captures complement component C1, ficolins and MBL, thus inactivating the classical and lectin pathways. Trypomastigote-bound C1 is detected as an "eat me" signal by macrophages and promotes the infective process. Unlike infective trypomastigotes, non-infective epimastigotes either do not express or express only marginal levels of TcCRT on their external membrane. We show that epimastigotes bind exogenous rTcCRT to their cellular membrane and, in the presence of C1q, this parasite form is internalized into normal fibroblasts. On the other hand, Calreticulin (CRT)-deficient fibroblasts show impaired parasite internalization. In synthesis, CRT from both parasite and host cell origin is important in the establishment of C1q-dependent first contacts between parasites and host cells.

1. Introduction

Chagas disease is a chronic ailment, described by Carlos Chagas in 1909 (Chagas, 1909). It is endemic in Latin America, with approximately 8 to 9 million seropositive humans and an undetermined number of domestic and wild animal reservoirs. Available treatments are modestly efficacious and with serious side effects. Due to migratory processes, this disease is now increasingly being reported outside the endemic zone. Thus, it is estimated that there are near to one million infected patients in the USA, and approximately one hundred thousand in the rest of the world (Coura and Vinas, 2010). This disease has acute and chronic stages (Chagas, 1911). The acute stage starts a few days after the infection and fever, headache, muscle pain and shivers are common symptoms. This stage lasts nearly 30 days, where high parasitemia and cellular parasitism are evident. The chronic stage appears in about 30% of the infected patients, having variable symptomatology and duration.

The etiologic agent is the hemiflagellated intracellular protozoan *Trypanosoma cruzi*, transmitted to mammal hosts through the feces of haematophagous arthropods from the *Triatominae* family, such as "kissing bugs" (Canals et al., 1998). *T. cruzi* is a unicellular organism, with characteristic structures of eukaryotic cells. The parasite has a life cycle consisting of 3 stages. Infective, non-replicative trypomastigotes are present in the feces of the arthropod vector, and penetrate mammal cells near the bite site (Coura and Vinas, 2010; Brener, 1973). There, trypomastigotes transform into amastigotes that, after several replication cycles, transform back into infective trypomastigotes that disseminate *via* lymph and blood. In the arthropod vector, infective trypomastigotes and non-infective replicative epimastigotes are present. Epimastigotes, such as those used in this study, were obtained *in vitro*, under special culture conditions (Maya et al., 1997).

Several *T. cruzi* surface molecules have been shown to promote infectivity. Among them gp30, gp35/50, gp82, gp85, trans-sialidase, and calcineurin B, are present in both metacyclic and tissue

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culture-derived trypomastigotes, with Ca^{2+} signal-inducing activities, playing important variable roles in the parasite attachment to host cells and invasion (Yoshida and Cortez, 2008; Araya et al., 2008).

T. cruzi Calreticulin (TcCRT) is also an important *T. cruzi* virulence factor. It is a 45-kDa protein (Ramos et al., 1991), isolated, cloned and characterized in our laboratory and others (Ramos et al., 1991; Aguillon et al., 1995, 2000, 1997; Labriola et al., 1999). TcCRT has the same domains as in other species (N, P, C and S), with similar functions to human CRT such a potent antiangiogenic effect (Molina et al., 2005), participation in the ER quality control for glycoprotein folding and interaction with Cruzipain (Caramelo and Parodi, 2008), and among its infection-promotion properties, it can bind Thrombospondin 1 (TSP-1), thus enhancing the invasion of mouse embryonic fibroblasts (Johnson et al., 2012). *In vitro*, TcCRT is translocated from the endoplasmic reticulum (ER) to the trypomastigote flagellum emergence zone where, through its S domain, it captures C1 (Ferreira et al., 2004), the first component of the classical pathway of the complement system. The interaction of TcCRT with the collagenous tails of C1q inhibits the activation of C1, followed by downregulation of the whole classical pathway (Ferreira et al., 2004; Valck et al., 2010). This mechanism is used by trypomastigotes to resist complement-mediated lysis. Additionally, C1q binding to TcCRT on trypomastigotes promotes increased infectivity (Ramirez et al., 2011a,b), since it mimics a physiological apoptotic cell removal signal. Apoptotic cells translocate CRT to the external membrane. When this molecule binds C1q, an "eat me" signal is generated which, among other possibilities, is recognized by macrophages through their own CRT (cC1qR, also present on the cellular macrophage membrane) (Eggleton et al., 2000; Coppolino and Dedhar, 1998; Basu et al., 2001; Ogden et al., 2001). As a consequence, C1q binding to TcCRT on trypomastigotes allows the parasite to evade complement-mediated lysis and acts as a potent virulence factor (Ferreira et al., 2004). Once the parasite is phagocytized, it escapes the parasitophorous vacuole and continues its infective cycle. TcCRT virulence is confirmed by blocking its capacity to bind C1q with specific anti – rTcCRT $F(ab')_2$ antibody fragments (devoid of their C1-binding capacity, located in the Fc portion) leading to a considerable decrease in parasite internalization by macrophages (Ogden et al., 2001; Ramirez et al., 2011a,b). A second confirmation of TcCRT as a potent virulence factor emerges from our recent obtainment of epimastigotes where one of the two alleles coding for TcCRT was inactivated. These parasites show decreased resistance to complement lysis, impaired replication levels and marginal capacity to transform into the trypomastigote stage (metacyclogenesis), using conventional culture media (Sanchez Valdez et al., 2013; Sanchez-Valdez et al., 2014).

Not much is described about TcCRT in the epimastigote context. Lack of TcCRT on their surface (Sosoniuk et al., 2014) may explain, at least partially, why epimastigotes are non infective, both *in vivo* and *in vitro*. Moreover, *in vivo*, epimastigotes should have two related disadvantages (*i.e.*: incapacity to inactivate, *via* TcCRT, the classical pathway of the complement system, with consequent susceptibility to lysis, and inability to bind C1q, thus resulting in impaired infectivity). Epimastigotes lack TcCRT expression on their surface, however, intracellular expression of the protein, seems to be normal. In this context, TcCRT participates in ER quality control of glycoprotein folding (Caramelo and Parodi, 2008), and is translocated to the cytosol, in an ER Calcium-dependent manner (Labriola et al., 2010).

Hence, the *T. cruzi* infective process is largely C1–mediated, but TcCRT dependent. Specifically, TcCRT is responsible for at least 40% of the trypomastigote infectivity *in vitro* (the parasite capacity to contact and subsequently to infect host mammal cells) (Ramirez et al., 2011a,b), and about 80% in an *ex vivo* system (Castillo et al., 2013). For these reasons, we hypothesize that the binding of recombinant TcCRT (rTcCRT) to the epimastigote cellular membrane, promotes its internalization into host cells. Here we show that epimastigotes did bind exogenous rTcCRT to their cellular membrane and, in the presence of C1q, these parasite forms were internalized into CRT-sufficient (normal) fibroblasts and only marginally into their hemiallelic CRT-deficient counterpart.

2. Materials and methods

2.1. Epimastigotes

T. cruzi Dm28c epimastigotes were obtained from an axenic culture (Laboratory of Drug Biochemistry, Metabolism and Resistance, ICBM, Faculty of Medicine, University of Chile). The parasites were grown at 28 °C in modified Diamond's medium as previously described (Maya et al., 1997).

2.2. Quantification of exogenously bound rTcCRT to epimastigotes' cellular membrane

2.2.1. Functional evaluation of rTcCRT

Microtitration plates (Nunc MaxiSorp, USA) were coated with 100 μl/well of rTcCRT (5 μg/ml). Nonspecific binding sites were blocked with 3% w/v Bovine Serum Albumine (BSA) in Phosphate Buffer Saline (PBS). Then, 0–4 μg/ml of pure human C1q were added in a final volume of 100 μl in Veronal Buffer (5,5-diethylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione). Bound C1q, as an evaluation of rTcCRT function, was detected with an affinity-purified rabbit anti-human C1q antiserum (DAKO, Carpinteria, CA), followed by affinity-purified Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit Ig antibody (DAKO). HRP (405 nm) activity was assessed by addition of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) with H_2O_2. Washing with PBS/0.05% Tween 20 was performed following each step. As controls, microtitration wells were coated as follows: (i) without rTcCRT, (ii) with rTcCRT detected with a rabbit anti-rTcCRT antibody followed by a goat anti-rabbit IgG (DAKO), (iii) with C1q detected with an affinity-purified rabbit anti-human C1q antiserum (DAKO, Carpinteria, CA), followed by an affinity-purified HRP-conjugated goat anti-rabbit Ig antibody (DAKO).

2.2.2. Functional evaluation of fluorescein isothiocyanate (FITC) labeled rTcCRT (F-rTcCRT)

rTcCRT was labeled with FITC using a commercial kit (FluoReporter® FITC Protein labeling kit, Invitrogen, USA). Microtitration plates were coated with 100 μl/well of F-rTcCRT (5 μg/ml). Nonspecific binding sites were blocked with 3% w/v BSA in PBS. Then, 0–4 μg/ml pure human C1q was added in a final volume of 100 μl in Veronal Buffer. Bound C1q was detected with an affinity-purified rabbit anti-human C1q antiserum (DAKO, Carpinteria, CA), followed by affinity-purified HRP-conjugated goat anti-rabbit Ig antibody (DAKO). HRP (405 nm) activity was assessed by addition of ABTS with H_2O_2. Washing with PBS/0.05% Tween 20 was performed following each step. Controls were designed as described above.

2.2.3. Binding of F-rTcCRT to Dm28c epimastigotes

These parasites were obtained from axenic cultures. Binding of F-rTcCRT to the cellular membrane of epimastigotes was detected using flow cytometry (FACScan, BD Biosciences). The parasites were washed twice in 2% w/v BSA in PBS by centrifugation at 450*g*, 5 m at 4 °C, suspended in 2% w/v BSA in PBS and treated with 3–12 μg of F-rTcCRT per 5×10^5 parasites for 30 m at room temperature (final volume 50 μl), followed by 3 additional washes. As control, the capacity of 3–12 μg of unlabeled rTcCRT per 5×10^5 parasites to inhibit the binding of 3 μg F-rTcCRT to parasites was tested. After