Infection, Genetics and Evolution 45 (2016) 320–331

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Cloning, expression, molecular characterization and preliminary studies on immunomodulating properties of recombinant *Trypanosoma congolense* calreticulin

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ARTICLE INFO

Article history:
Received 7 July 2016
Received in revised form 21 September 2016
Accepted 22 September 2016
Available online 26 September 2016

Keywords:
Animal African Trypanosomosis
Calreticulin
Trypanosoma congolense
Antibodies
Immunomodulation

ABSTRACT

Trypanosomes are bloodstream protozoan parasites, which are pathogens of veterinary and medical importance. Several mammalian species, including humans, can be infected by different species of the genus *Trypanosoma* (*T. congolense*, *T. evansi*, *T. brucei*, *T. vivax*) exhibiting more or less virulent and pathogenic phenotypes. A previous screening of the excreted-secreted proteins of *T. congolense* demonstrated an overexpression of several proteins correlated with the virulence and pathogenicity of the strain. Of these proteins, calreticulin (CRT) has shown differential expression between two *T. congolense* strains with opposite infectious behavior and has been selected as a target molecule based on its immune potential functions in parasitic diseases. In this study, we set out to determine the role of *T. congolense* calreticulin as an immune target. Immunization of mice with recombinant *T. congolense* calreticulin induced antibody production, which was associated with delayed parasitemia and increased survival of the challenged animal. These results strongly suggest that some excreted-secreted proteins of *T. congolense* are a worthwhile target candidate to interfere with the infectious process.

1. Introduction

Animal African Trypanosomoses (AAT) are tropical neglected diseases that are widespread throughout the world. In Africa, several species of trypanosomes have been identified, such as *T. congolense*, *T. brucei*, *T. evansi*, *T. vivax*, on different hosts (cattle, sheep, camels, *etc.*). For species and subspecies of the genus *Trypanosoma*, diverse levels of virulence (capacity to multiply inside a host) and pathogenicity (ability to produce a pathology and mortality) have been observed from the clinical viewpoint. To better understand virulence and pathogenicity mechanisms and acquire the means to fight against these parasites, different analytical approaches have been developed and proteomics is among the latest to bring new insights into host-parasite interactions (Geiger et al., 2011). It is known that several microorganisms have the potential to excrete-secrete factors during pathophysiological processes (Dumortier et al., 2008). With a proteomics approach, an association arises between the virulence and pathogenicity phenotype with differential protein expression within the proteome and secretome compartments of trypanosomes (Holzmuller et al., 2008). Such is the case for two strains of *T. congolense*, which exhibit a difference in virulence and pathogenicity profiles associated with differentially expressed excreted-secreted proteins functionally characterized through a bioinformatics data analysis. Among the proteins identified, some have been described as potential therapeutic or diagnostic candidates, in particular calreticulin (Grébaut et al., 2009). Calreticulin is a calcium-binding protein that is highly conserved in trypanosomatids and has multifunctional roles (Michalak et al., 1999). This protein, which exhibit multiple activities, is primarily located in the endoplasmic reticulum where it acts like a chaperone protein (Coe and Michalak, 2009), and it is also found in cytotoxic granules and is implicated in controlling perforin-mediated osmotic lysis (Fraser et al., 2000). Moreover, calreticulin can have a role in apoptotic cell death by binding and activating low density lipoprotein (LDL) receptor-related protein (LRP) (Gardai et al., 2005). The calreticulin from *T. cruzi* was defined as a chaperone (Nauseef et al., 1995; Spiro et al., 1996). Calreticulins from trypanosomatids are

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http://dx.doi.org/10.1016/j.meegid.2016.09.020
1567-1348/

immunogenic, and anti-CRT antibodies are produced during infection in humans (Aguillon et al., 1997; Marcelain et al., 2000) and in mice (Ramos et al., 1991). Calreticulin can be associated with perforins, with a cytotoxic effect (Andrin et al., 1998), can inhibit the classic complement pathway thus improving infection (Ramirez et al., 2011a), can interfere with angiogenesis (Ramirez et al., 2011b), or can activate the host's lymphocytes (Cho et al., 2001). In B-cell leukemia/lymphoma, chaperone proteins, including calreticulin, induce immunoprotective activities against a lethal tumor challenge (Graner et al., 2000). Given the overexpression of the calreticulin of the *T. congolense* strain with high virulence and pathogenicity, and its major potential interactions with host cells or molecules, this protein is well worth further investigation in terms of biological and physiological impact. In this study, we successfully produced the recombinant calreticulin of *T. congolense* and demonstrated its antigenicity and immunogenicity in an *in vivo* immunization-challenge experiment. Immunizations of mice with calreticulin induced high antibody titers, which seemed to neutralize the action of trypanosome excreted-secreted calreticulin on the host immune system, delaying the early development of infection, a subsequent deleterious effect on the host. During infection by *T. congolense*, induced anti-CRT antibodies may act as neutralizing molecules to block the immunomodulating action of CRT against host cells.

2. Materials and methods

2.1. Parasite

The *Trypanosoma* strains of *T. congolense* mostly used in laboratories are of the savannah type, clones IL1180 (Nantulya et al., 1984) and IL3000 (Wellde et al., 1974), the latter being the reference strain for the *T. congolense* genome project (Gibson, 2012). In this study, IL1180 was selected according to two criteria: low parasitemia and induction of chronic disease development, contrary to other strains as the IL3000 strain which was described as developing high parasitemia and a high mortality rate in mice (Grébaut et al., 2009; Bengaly et al., 2002a).

2.2. Construction of T. congolense calreticulin in an expression vector

Complementary DNA (cDNA) was obtained from the *Trypanosoma congolense* IL1180 parasite, by reverse transcription after extraction of messenger RNA (mRNA) with the RNeasy kit (Qiagen). The calreticulin gene (*crt*) was amplified by PCR with the forward primer CAL-F (5′ atgcgcaggggaactatg 3′) and the reverse primer CAL-R (5′ cagatccgccttttcttctt 3′) (designed from AF366570.1, EMBL-EBI) in order to obtain a first construction in the pGEM-T vector (Promega); a second construction was obtained by amplification with primers sp-*Xba*I-CAL-F (5′ *ctctaga*tatgcgcaggggaactattg 3′) and CAL-*Kpn*I-R (5′ *gggtacc*tacagatccgc 3′) where the underlining indicates the position of the start codon and italics indicate the *Xba*I and *Kpn*I restriction sites, respectively, and introduced into the pGEM-T vector. The latter was excised and subcloned into the *Xba*I/*KpnI* pLexsy vector (Jena Bioscience). DNA sequencing was carried out to confirm the identity of all clones obtained (GATC society). Initially, for reasons of post-translational modifications and production in a system homologous to *Trypanosoma*, we chose a protein production system using *Leishmania tarentolae*. Unfortunately, due to very weak CRT production, this system was abandoned for the baculovirus system. This system has several advantages such as continuing to take into account post-translational modifications and the good quality and quantity yield of recombinant protein. Transferral of the insert from the pLexsy vector to the new pAcGP67A (baculovirus vector) was facilitated by the presence of the two restriction sites already introduced in the first construction (*Xba*I and *Not*I) in the polylinker of the pAcGP67A vector (BD Biosciences Pharmingen).

2.3. Expression and purification of calreticulin (HisTcIL1180CRT)

Two million Sf9 insect cells were co-transfected with 2 μg of CRT-pAcGP67A and 0.5 μg of BD BaculoGold Baculovirus linearized DNA according to the manufacturer's instructions (BD Biosciences Pharmingen). The presence of recombinant virus was detected by PCR after DNA virus extraction with QIAmp minielute virus spin (Qiagen). The infected *Sf*9 cells were cultured for 48 h, which corresponded to the optimum time for the best His tagged *T. congolense* calreticulin (His-TcIL1180 CRT) expression level (data not shown). The insect cells were collected, lysed with buffer containing 50 mM TrisHCl, 150 mM NaCl, 1% NP40 at pH 8 and incubated for 30 min on ice, followed by sonication (3 times 10s pulses at 13 watts with 6 s intervals). The homogenate was then centrifuged at 14,000*g* for 10 min at 4 °C and the supernatant was loaded onto a 1 ml Ni-NTA agarose column of (AKTAPRIM system, GE healthcare) pre-equilibrated with buffer A (20 mM Tris HCl buffer pH 7.4; 500 mM NaCl; 40 mM imidazole) at a flow rate of 0.5 ml/min. The column was subsequently washed at 1 ml/min with buffer A until the absorbance at 280 nm returned to basal level. The His-TcIL1180-CRT was eluted with buffer B (20 mM Tris HCl buffer pH 7.4; 500 mM NaCl; 500 mM imidazole) at 1 ml/min. The chromatography procedure was carried out at room temperature. The sample was dialyzed with spectra/Por 3 (Spectrumlabs,) against PBS overnight at 4 °C and concentrated with polyethylene glycol 3350 Da (Sigma aldrich). The concentrated eluate was quantified by the Bradford method (Pierce coomassie protein assay kit, Thermofisher) and analyzed on 10% polyacrylamide SDS-PAGE gels (Schägger and von Jagow, 1987) (Schägger and von Jagow, 1987), stained with page blue staining solution (Thermoscientific).

2.4. Western blot analysis and glycoprotein detection

SDS-PAGE gels were blotted onto 0.45 μm nitrocellulose™ membrane (Biorad). Membranes were saturated in 0.1% BSA (serum albumin bovine, Sigma) and incubated for 1 h at room temperature with shaking. Following five washes in PBS containing 0.05% Tween 20, mouse anti-6His tag antibody conjugated to horseradish peroxidase (Sigma) was added at 1:2000 dilution in PBS containing 0.1% BSA for 1 h at room temperature. Following five washes in PBS containing 0.05% Tween 20, membranes were developed using the Luminata crescendo system (Millipore).

To detect glycolysation on recombinant CRT, the SDS-PAGE gels were stained using a test Glycopro detection kit according to the manufacturer's instructions (Sigma).

2.5. Bioinformatics analysis

We used several bioinformatics tools to investigate the biochemical properties of TcIL1180CRT. The phyML website was used to construct a phenetic tree of CRT among trypanosomatids and corresponding hosts based on the amino acids sequences of the corresponding calreticulin and default parameters of the server. The CBS prediction servers website was used to predict the post translational modifications of CRT, especially N-linked glycosylation (NetNGlyc 1.0 Server (Gupta and E.J.a.S.B., 2004)) and O-glycolysation (NetOGlyc 4.0 Server (Steentoft C et al., 2013)), and phosphorylation sites (NetPhos 2.0 Server (Blom et al., 1999)), but also immunological features such as Linear B-cell epitopes (Bepipred 1.0 Server (Larsen et al., 2006)) and discontinuous B-cell epitopes (DiscoTope 2.0 Server (Kringelum et al., 2012)). The 3D structure was analyzed with Chimera software.

2.6. Immunization schedule in mice and trypanosome challenge

Animals were kept under strict ethical conditions according to the international guidelines for the care and use of laboratory animals. The experiments designed for this study were approved by the regional