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Schistosoma mansoni Annexin 2: Molecular characterization and immunolocalization

Cibele Aparecida Tararam [a], Leonardo Paiva Farias [a], R. Alan Wilson [b], Luciana Cezar de Cerqueira Leite [a,*]

[a] Centro de Biotecnologia, Instituto Butantan, Av. Vital Brasil, 1500, São Paulo, SP, Brazil
[b] Department of Biology, University of York, PO Box 373, York YO10 5YW, UK

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ABSTRACT

We here describe the cloning and characterization of the *Schistosoma mansoni* Annexin 2, previously identified in the tegument by proteomic studies, and as an up-regulated gene in schistosomulum stage by microarray data. *In silico* analysis predicts a conserved core containing four repeat domains of Annexin (ANX) and a variable N-terminal region similar to that described for mammalian isoforms. Real-time RT-PCR and Western blot analysis determined that *S. mansoni* Annexin 2 is significantly up-regulated in the transition from free-living cercaria to schistosomulum and adult worm parasitic stages. Immunolocalization experiments and tegument membrane preparations confirmed Annexin 2 as a protein mainly localized in the tegument of schistosomula and adult worms. Furthermore, it binds to the tegument surface membranes in a calcium-dependent manner. These results suggest that *S. mansoni* Annexin 2 is closely associated to the tegument arrangement, being a potential target for immune intervention.

1. Introduction

Schistosomiasis is an important public health problem in developing countries; approximately 200 million individuals are infected with *Schistosoma mansoni*, with 250,000 fatalities per year (McManus and Loukas, 2008; WHO, 2002). The granulomatous reaction around eggs within the liver and other organs characterize the pathology of this disease, resulting in the morbidity of infected individuals or even death, in case of severe infections (King, 2001). Chemotherapy with praziquantel is the main control strategy used; however, mass treatment does not prevent reinfection and its cumulative effects (Wilson and Coulson, 1999). Furthermore, drug resistant parasites are also concerns that have to be considered (Fallon et al., 1995; Ismail et al., 1999). Thus, the development of a defined vaccine against schistosomiasis combined with chemotherapy would contribute to the current control strategy (Bergquist, 2002).

One of the major challenges in the search for new vaccine candidates is the identification of surface-exposed molecules, mainly those up-regulated in the transition from cercaria to schistosomula stage, which is believed to be the main target of immune attack in the highly protective cercariae attenuated vaccine (Coulson, 1997). Recently, the transcriptome (Verjovski-Almeida et al., 2003) and the whole-genome sequence projects of *S. mansoni* (Berriman et al., 2009; Verjovski-Almeida et al., 2003) and *S. japonicum* (Hu et al., 2003; Liu et al., 2009), provided a large repository of schistosome genes and opened the opportunity for proteomics and microarray analysis. As a result, a remarkable understanding of the protein composition of the schistosome tegument and its surface membranes has been achieved, enabling the search for new vaccine candidates (Braschi et al., 2006a; Braschi and Wilson, 2006; van Balkom et al., 2005). In these proteomic studies, a member of the Annexin protein family was identified as a component of the plasma membrane structure (Braschi et al., 2006a) and biotinylation labeling indicated it to be a tegument surface-exposed protein (Braschi and Wilson, 2006). Concomitantly, the corresponding Annexin mRNA was identified as a gene preferentially expressed in the lung schistosomulum through microarray analysis (Dillon et al., 2006). Interestingly, a *S. bovis* Annexin was also identified by proteomic analysis in the excretory/secretory products from adult worms (Perez-Sanchez et al., 2006). All these data support the rational of investigating Annexins as potential vaccine candidates for schistosomiasis. Additionally, Annexins have shown protective effect as vaccine antigens against *Giardia lamblia* (Weiland et al., 2003) and *Cysticercus cellulosae* (Guo et al., 2004).

Annexins are a large family of Ca^{+2}-dependent phospholipid-binding proteins widely distributed in eukaryotes and plants. They have been implicated in a broad range of important biological processes such as membrane trafficking and fusion, anticoagulation, interaction with cytoskeletal proteins and signal transduction (Gerke et al., 2005; Gerke and Moss, 2002; Hayes et al., 2006). These functions would suggest they could be important for tegument development, and therefore, survival of the parasite in their

* Corresponding author. Fax: +55 11 3726 9150.
E-mail address: lccleite@butantan.gov.br (L.C. de C. Leite).

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hosts (Perez-Sanchez et al., 2006). A typical Annexin consists of a core domain of 4 or 6 homologous repeats (ANX), which can display type II or III Ca^{+2}-binding sites and an N-terminal tail, variable in length and sequence, that is responsible for the functional diversity of Annexins (Gerke and Moss, 2002).

In the present study, we describe several *S. mansoni* Annexin putative genes from genome assembly version 4 (www.SchistoDB.org) and characterize one of them, which has been previously identified in the above-mentioned microarray and tegument proteomic studies. The protein was named Annexin 2 and showed Ca^{+2}-dependent membrane-binding properties. Data provided here confirmed its surface exposure and up-regulation in schistosomula, indicating it should be evaluated as a potential vaccine candidate.

2. Materials and methods

2.1. Parasite material and ethics statement

The life cycle of *S. mansoni* (BH strain) was maintained in the laboratory by routine passage through mice and the intermediate snail host *Biomphalaria glabrata*. *S. mansoni* eggs were extracted from infected mouse livers and miracidia were hatched from *S. mansoni* eggs, as previously described (Dillon et al., 2006; Dalton et al., 1997). Schistosomula were cultivated in culture medium after transformation of cercariae, as previously described (Basch, 1981). Adult worms were obtained by perfusion from hamsters, 7–8 weeks after infection with subcutaneous injection of approximately 100 cercariae.

All animals were handled in strict accordance with good animal practice as defined by Animals Use Ethics Committee of Instituto Butantan (São Paulo, Brazil), and the study was conducted adhering to the institution's guidelines for animal husbandry.

2.2. Molecular characterization

The Annexin nucleotide sequences were identified searching the v4.0 of *S. mansoni* genome assembly (GeneDB). The search for conserved domains was performed using SMART (http://smart.embl-heidelberg.de/). The molecular weight (MW) and isoelectric point (pI) were calculated with the Compute pI/Mw tool (http://www.expasy.ch/tools/pi_tool.html). Protein sequences alignments were performed using the ClustalX 1.83 software. The gene structure (exons and introns positions) of the Annexin 2 was investigated by aligning the cDNA sequence with the gene sequence from the genomic database.

2.3. Real-time RT-PCR

Total RNA was extracted from 7-day-old schistosomula and adult worms using TRIzol (Invitrogen), and from cercariae using the RNAeasy Protect Kit (Qiagen), as per the manufacturer's recommendations. The RNA was quantified by spectrophotometry (NanoDrop instrument) and the quality was analyzed in the Agilent 2100 Bioanalyzer. The cDNA synthesis and the real-time RT-PCR reactions were performed according to (Rofatto et al., 2009). The primers were designed in the software Primer Express (Applied Biosystems) (Supplementary Table 1) and as internal control we choose primers targeting *S. mansoni* alpha tubulin (Accession: M80214), a gene product used extensively as a reference for quantitative reverse transcription PCR analyses (Chalmers et al., 2008; Faghiri and Skelly, 2009; Fitzpatrick et al., 2008). Quantitation of relative differences in expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. Statistical comparisons were performed with *t*-Student or one-way ANOVA followed by a Tukey pairwise comparison, as required. A p value < 0.05 was considered statistically significant.

2.4. Cloning

The 5′ and 3′ oligonucleotides were designed using the *S. mansoni* genome assembly sequence Smp_077720 (EST Sm03987). The SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen) was used to amplify the complete open reading frame of Annexin 2. The obtained PCR fragment was cloned into pAE-6His vector (Ramos et al., 2004) and sequenced to confirm its identity. Specific primers were used to re-amplify fragments containing the following domains of the gene, AnneI–II, AnneII–III, AnneIII–IV and AnneI–III (Supplementary Table 1). The PCR reactions were performed using Platinum *Pfx* enzyme (Invitrogen) as per the manufacturer's recommendations with 55 °C annealing temperature. PCR products were purified from agarose gel electrophoresis and digested with *Xho*I and *Kpn*I to be cloned into pAE-6His vector.

2.5. Expression of recombinant protein fragments and polyclonal antibody production

The plasmids containing the inserts were transformed into BL21 (SI) (Invitrogen) and the transformed cells were grown in 300 ml LB ON plus ampicillin (100 μg/ml) until they reached an OD 600 = 0.7. Sodium chloride (NaCl) was added to the culture to a final concentration of 300 mM, and cells were incubated for 4 h at 30 °C. Cells were harvested by centrifugation and resuspended in 30 ml of lysis buffer (20 mM Tris pH 8.8, 150 mM NaCl). The cell suspension was passed twice (2000 psi) through a French Press and the crude homogenate was centrifuged at 20,000*g* for 30 min. The pelleted inclusion bodies were washed twice with wash buffer (lysis buffer, 2% Triton X-100, 2 M urea), and finally resuspended in solubilization buffer (lysis buffer, 10 mM imidazole, 8 M urea). The recombinant protein was refolded from the inclusion bodies by diluting 200-fold into equilibration buffer (solubilization buffer without urea). The recombinant protein was then purified by metal affinity chromatography using the Akta-Prime system (Amersham Biosciences) under native conditions. Briefly, the sample was loaded onto a Ni^{2+}–NTA column pre-equilibrated with equilibration buffer. The column was washed with 10 bed volumes of the equilibration buffer and then eluted with 10–500 mM imidazole linear gradient. The main peak was pooled and the protein purity of fractions was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Further, the elution buffer was exchanged for Tris 20 mM NaCl 150 mM pH 8.8 before use of the protein.

Polyclonal rat anti-serum was produced against the preparation of recombinant protein fragment rAnneI-II. Rodents were inoculated four times, at 15-day intervals with 100 μg of protein mixed with TiterMax adjuvant (CytRx Corporation; first dose) or PBS (in subsequent doses). Fifteen days after the last inoculation, rodents were exsanguinated. The anti-serum was negatively purified by four washing steps using BL21 (DE3) *Escherichia coli* extract in PBS at 5 °C for 16 h. The anti-rAnneI-II serum was used at a dilution of 1:1000 for Western blots and 1:40 for indirect immunofluorescence assays.

2.6. Protein expression profile

Total protein extracts from eggs, miracidia, cercariae, 7 days-old schistosomula and adult worms of *S. mansoni* were prepared as described in (Rofatto et al., 2009). The tegument extract was obtained by a freeze/thaw/vortex procedure, as previously described (Roberts et al., 1983). Briefly, frozen worms (1000) were thawed on ice in the presence of 1 ml ice-cold RPMI medium plus protease inhibitors (Protease inhibitor cocktail; Sigma), with 10 vortex pulses at maximum speed duration 1 s to detach the tegument. After the stripped worms had settled, the supernatant was