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Molecular and functional characterization of a *Schistosoma bovis* annexin: Fibrinolytic and anticoagulant activity

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ABSTRACT

Annexins belong to an evolutionarily conserved multigene family of proteins expressed throughout the animal and plant kingdoms. Although they are soluble cytosolic proteins that lack signal sequences, they have also been detected in extracellular fluids and have been associated with cell surface membranes, where they could be involved in anti-haemostatic and anti-inflammatory functions. Schistosome annexins have been identified on the parasite's tegument surface and excretory/secretory products, but their functions are still unknown. Here we report the cloning, sequencing, *in silico* analysis, and functional characterization of a *Schistosoma bovis* annexin. The predicted protein has typical annexin secondary and tertiary structures. Bioassays with the recombinant protein revealed that the protein is biologically active *in vitro*, showing fibrinolytic and anticoagulant properties. Finally, the expression of the native protein on the tegument surface of *S. bovis* schistosomula and adult worms is demonstrated, revealing the possibility of exposure to the host's immune system and thus offering a potential vaccine target for the control of schistosomiasis in ruminants.

1. Introduction

Schistosomiasis is a parasitic disease affecting man and domestic and wild animals that represents an important health and veterinary problem in many tropical and subtropical areas of the world. *Schistosoma bovis* is a cosmopolitan trematode of ruminants that can produce significant economic losses in endemic areas (Vercruysse and Gabriel, 2005). In addition, studies on *S. bovis* are interesting from the perspectives of both veterinary and human medicine because this species represents the genetic and immunological analogue of the important human pathogen *Schistosoma haematobium* (Agnew et al., 1989).

Like all schistosome species, *S. bovis* adult worms can survive in host blood vessels for many years. In order to achieve such long survival times, parasites have molecules that allow them to modulate immune and haemostatic host responses to their own benefit (Pearce and MacDonald, 2002; Mountford, 2005; Secor, 2005; Ramajo-Hernández et al., 2007; De la Torre-Escudero et al., 2010). It is well known that a significant part of schistosome evasion mechanisms are achieved by the parasite's inner and outer tegument surface (Abath and Werkhauser, 1996). The tegument, besides the gut, constitutes one of the most important host–parasite interchange surface, playing a role in the parasite's nutrient uptake, excretion, osmoregulation, sensory reception, signal transduction, and interaction with the host's immune and haemostatic systems (Jones et al., 2004; van Hellemond et al., 2006; Ramajo-Hernández et al., 2007; De la Torre-Escudero et al.,

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2010). Thus, the tegument is a key parasite compartment to be mined for target molecules in the development of new anti-schistosome vaccines and drugs. With this aim, in the last years numerous investigations have focused on the identification and characterization of molecules expressed by schistosomes in their tegument and surface membranes (van Balkom et al., 2005; Braschi et al., 2006; Braschi and Wilson, 2006; Pérez-Sánchez et al., 2006, 2008; Skelly and Wilson, 2006; Mulvenna et al., 2010a; Castro-Borges et al., 2011). As a result, annexins have been one of the molecules frequently identified in the tegument of schistosomes.

Annexins are Ca^{2+}- and phospholipid-binding proteins that form an evolutionarily conserved multigene family, its members being expressed throughout the animal and plant kingdoms. Structurally, annexins are characterized by a highly α-helical and tightly packed protein core domain, considered to represent a Ca^{2+}-regulated membrane-binding module. Human annexins, designated A1–A13 (except annexin A12, which is unassigned), have been implicated in a broad range of biological processes such as membrane trafficking and fusion, plasma membrane repair, anticoagulation, interaction with cytoskeletal proteins and signal transduction (Gerke and Moss, 2002; Moss and Morgan, 2004; Draeger et al., 2011). These soluble cytosolic proteins lack signal sequences that direct them to the classical secretory pathway. Nevertheless, some members of the family have consistently been identified in extracellular fluids. Binding sites for extracellular annexins exist on the cell surface and several possible functions for these proteins have been proposed. They include a role of annexin A5 as an anticoagulant protein, a function of annexin A2 as an endothelial cell-surface receptor for plasminogen and tissue-type plasminogen activator (t-PA), and the anti-inflammatory activities of annexin A1 (Hajjar and Krishnan, 1999; Gerke and Moss, 2002; Cederholm and Frostegård, 2007).

Regarding schistosome annexins, several proteomic studies have identified them on the tegument surface of *Schistosoma mansoni* (Braschi and Wilson, 2006; Castro-Borges et al., 2011) and *Schistosoma japonicum* (Mulvenna et al., 2010a), as well as in the excretion-secretion products of *S. bovis* (Pérez-Sánchez et al., 2006). Moreover, in a recent survey of draft genomes for *S. mansoni* and *S. japonicum* (http://www.schistodb.org), Hofmann et al. (2010) identified 14 annexins from *S. mansoni*, 6 from *S. japonicum* and 1 from *S. haematobium*. Of these, it is known that schistosome annexins 1, 3 and 5 are expressed on the tegument of the adult parasite (Braschi and Wilson, 2006; Mulvenna et al., 2010a; Castro-Borges et al., 2011). Hofmann et al. (2010) reported that annexins are particularly noteworthy as surface-associated molecules of adult schistosomes and are likely to be an abundant surface-related molecule of digeneans such as the human liver fluke, *Opistorchis viverrini*, which also expresses abundant surface annexins (Mulvenna et al., 2010b). The physiological roles of these schistosome annexins are still unknown although it has been speculated that they could play important roles in surface maintenance, such as by ensuring the integrity of the membrane-membranocalyx complex (Braschi and Wilson, 2006).

Unveiling the functions of schistosome annexins, and particularly whether they exhibit extracellular activities such as those reported for the annexins of other organisms (i.e., anticoagulant and fibrinolytic activities), is important as these extracellular activities could be vital for schistosome development and survival. Accordingly, the aims of the present work were to determine the physiological role of the annexin of *S. bovis* adult worms regarding its potential fibrinolytic and anticoagulant activities and to demonstrate its expression on the parasite surface at the host–parasite interface.

2. Material and methods

2.1. Parasite material

The life cycle of *S. bovis* was maintained at the laboratory by routine passage through sheep, golden hamsters, and the intermediate snail host *Planorbarius metidjensis*. Adult worms and lung schistosomula were recovered, respectively, from infected sheep and hamsters as described in De la Torre-Escudero et al. (2010). Briefly, lambs were infected with 2000 cercariae and 4 months later they were sedated with 10 mg of ketamine per kg of live weight and sacrificed by bleeding through the jugular vein.

Adult worms were recovered from mesenteric veins and washed in warm phosphate buffered saline (PBS) pH 7.2 at 37 °C. Worms were inspected microscopically to verify their integrity and vitality, and immediately processed for RNA extraction, the collection of a tegument extract (TG), and immunolocalization studies. The tegument extract was obtained as described by Ramajo-Hernández et al. (2007).

Lung schistosomula were obtained from hamsters, following the method of Gui et al. (1995). The animals were infected through the skin with 1000 cercariae by bathing them individually (in a solution containing the cercariae) for 1 h. Six days after infection, the hamsters were euthanatized and their lungs were removed, minced and incubated in RPMI medium at 37 °C for 2 h on a rocker-shaker. The suspension was sieved and live, intact schistosomula were collected with a 20 μl pipette. After three washes in warm PBS, they were fixed in 4% formalin and stored at 4 °C until use.

Animal experimentation was done according to the rules from the ethical and animal welfare committee from the institution where the experiments were conducted (IRNASA, CSIC), following the corresponding EU rules and regulations.

2.2. RNA isolation, RT-PCR and cloning

Total RNA from adult worms was isolated using the NucleoSpin RNA II kit (Macherey-Nagel), following the manufacturer's instruction, and preserved at −80 °C. Reverse transcription was performed from total RNA using the first Strand cDNA Synthesis kit (Roche). For PCR amplification of *S. bovis* annexin cDNA, primers were designed from the *S. mansoni* and *S. japonicum* annexin sequences (GenBank AF065599 and AY813612, respectively). The forward primer (ANXFw, 5′-ATGGCYAAWRTTTCTGRATTTGG) was designed from the *S. mansoni* and *S. japonicum* annexin