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Schistosoma mansoni venom allergen-like protein 18 (SmVAL18) is a plasminogen-binding protein secreted during the early stages of mammalian-host infection

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ABSTRACT

Schistosomiasis is a neglected tropical disease caused by trematodes of the genus *Schistosoma* which have a complex life cycle characterized by an asexual multiplication phase in the snail intermediate host and a sexual reproduction phase in the mammalian definitive host. The initial steps of the human host infection involve the secretion of proteins contained in the acetabular glands of cercariae that promote parasite adhesion and proteolysis of the skin layers. Herein, we performed a functional analysis of SmVAL18, identified as one of the three SCP/TAPS proteins constituent of cercarial secretions. We evaluated the SmVAL18 binding to immobilized macromolecules of the extracellular matrix (ECM) and to plasma components. Recombinant protein, expressed in *E. coli,* was found to maintain an ordered secondary structure typical of the SCP/TAPS domain after purification. Expression of native SmVAL18 protein was verified to be restricted to cercariae and 3-h schistosomula stages; furthermore, the protein was observed in the corresponding secretions, confirming that SmVAL18 is secreted during the first 3 h of *in vitro* culture. rSmVAL18 was able to interact specifically with plasminogen (PLG) and enhance its conversion into plasmin in the presence of the urokinase-type plasminogen activator (uPA). Protein homology modelling suggested that the PLG-rSmVAL18 interaction was mediated by lysine residues of the protein. This was supported by *in vitro* data using the lysine analogue, 6-aminocaproic acid (ACA), which abolished the interaction. Finally, our results showed that both cercariae and 3-h schistosomula, as well as their corresponding secretions, exhibited the capacity to bind PLG and enhance its conversion into plasmin *in vitro* in the same way as observed for the recombinant protein. In conclusion, our findings show that SmVAL18 is a novel PLG-binding protein secreted during the early stages of the mammalian-host infection.

1. Introduction

Schistosomiasis is a neglected tropical disease that affects approximately 230 million people worldwide with close to 800 million living at risk of infection. *Schistosoma mansoni,* one of the three major causing agents of the disease, has a complex life cycle, which involves an asexual multiplication phase in the snail intermediate host and a sexual reproduction phase in the mammalian definitive host [1,2]. Adult worms living within the mesenteric veins are constantly producing eggs that either become trapped in the tissues inducing an inflammatory response or are released into the environment by the feces. The eggs reach the freshwater supply and hatch releasing the ciliated miracidia, the larval stage that infects a suitable snail host. In the intermediate host, the parasite undergoes asexual replication through mother and daughter sporocysts within which germballs develop culminating with the shedding of thousands of cercariae into the water [2,3].

The human infection is initiated by the contact of cercariae, the infective larval stage, with the lipids on the surface of the skin

Abbreviations: SmVAL, *Schistosoma mansoni* venom allergen-like; SCP/TAPS, sperm coat protein/Tpx-1/Ag5/PR-1/Sc7; ECM, extracellular matrix; BM, basement membrane; PLG, plasminogen; uPA, urokinase-type plasminogen activator

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triggering the mechanical entry into the cornified superficial epidermal layer. However, further penetration demands the degradation of both the basement membrane (BM) and the extracellular matrix (ECM) of the dermis [4]. All invasion processes are assisted by the secretion of proteins contained in the acetabular glands of cercariae that promote parasite adhesion to the skin and proteolysis of the intracellular bridges between epidermal/dermal cells [4–8].

The major components of the cercarial secretions were identified as isoforms of the serine protease cercarial elastase, capable of digesting elastin, and the metalloprotease SmPepM8, likely to play a role in the degradation of skin BM and ECM [4,5]. The minor components released, on the other hand, were all proposed to be immunomodulators. Three novel proteins containing the SCP (Sperm Coat Protein) domain, that have since been classified as members of the SmVAL family (*Schistosoma mansoni* Venom Allergen Like-Protein), SmVAL4, 10 and 18, were identified representing ~3% of the released content [5,9,10].

Of the total 29 SmVAL genes identified to date (SmVAL1-29), 24 encode proteins with a secretion signal peptide (group 1), while only 5 encode intracellular proteins (group 2). Some of those were identified to have a stage specific expression with up-regulation in germballs (the precursor stage of mature cercariae – SmVAL4, 18, 19, 20 and 24), cercariae (SmVAL1, 2, 16, 17 and 21) and 3-day schistosomula (SmVAL7 and 13), raising questions on the involvement of SmVAL proteins with the mammalian host invasion [9,11–13].

In the last four years, we have focused on studying the tissue expression patterns of SmVAL genes as a way to infer possible functions to the respective proteins. We have previously described the localization of group 1 SmVAL7 and group 2 SmVAL6 transcripts in the posterior esophageal gland and in the suckers (oral and ventral) of adult worms, respectively [14]. Recently, we identified SmVAL13 transcript in the anterior esophageal gland of adult worms and localized SmVAL1, 4, 10, 18, 19 and 24 in the acetabular glands of germballs ([12] and Farias, personal communication). Furthermore, the expression profiles of SmVAL21, 22 and 25 suggest the same localization (Farias, personal communication). Thus, the high number of different SmVALs putatively expressed in the acetabular glands draws attention to a hotspot for this gene family expression in this structure.

To date, only two group 1 SmVAL proteins were associated with specific functions. SmVAL9, secreted during miracidia to sporocyst transformation *in vitro*, was found to stimulate transcription of genes involved in host ECM remodeling [13]. Native SmVAL4 was identified among the proteins secreted in the tunnels formed by destruction of epidermal cells by cercariae, during the first two hours of penetration of human skin [15]. Later, the protein was found to have the ability to bind lipids *in vitro* and complement the sterol export phenotype of yeasts *in vivo* [15,16]. So far, with the exception of these studies, no functional data have been associated to any other SmVALs.

Aiming to shed light on the role of other SmVALs secreted during the first hours of the mammalian-host invasion, herein we evaluated the SmVAL18 binding to immobilized macromolecules of the extracellular matrix (ECM), the tissue barrier that migratory larvae must overcome in order to reach a blood vessel, and to plasma components that could impair parasite migration. The data presented demonstrate the ability of SmVAL18 to bind plasminogen and increase its conversion into plasmin in the presence of urokinase-type plasminogen activator (uPA). Data are discussed in the light of the predicted functions of acetabular secretions in the parasite penetration process.

2. Materials and methods

2.1. Ethics statement

All procedures involving animals were carried out in compliance with the Brazilian legislation (decree no. 11790/2008). All animals were handled in strict accordance with good animal practices, and the study protocol received approval by the Institutional Review Board for Animal Research of the Butantan Institute (CEUAIB, São Paulo, Brazil) under license number 799/11.

2.2. Parasites

Schistosoma mansoni (BH strain) cercariae were first obtained by exposure of infected *Biomphalaria glabrata* snails to bright light and schistosomula were obtained by the mechanical transformation of cercariae followed by *in vitro* cultivation for 3 h and 3, 5 and 7 days as previously described [12].

2.3. Cloning, expression and purification of recombinant SmVAL18

After several unsuccessful attempts to obtain the full-length of SmVAL18 cDNA, a fragment corresponding to 84% of the mature protein sequence (from Pro55 to Tyr194), kindly provided by Dr. Iain Chalmers (Aberystwyth University), was used in the production of the recombinant protein. The cDNA sequence was cloned into pGEM®-T Easy vector (Promega), and then amplified by conventional PCR using the following primers: forward (F) 5′ TACGGATCCCCAAAACAACCTC CAGCA 3′; reverse (R) 5′ TACGAATTCTTAATATTCTGCATCATCAAC 3′. This fragment was then subcloned into pAE vector [17] at the EcoRI and BamHI restriction sites for expression in *E. coli*. Plasmid pAE-SmVAL18 containing the confirmed DNA sequence was used to transform *E. coli* BL21 (DE3). For protein expression, a pre-inoculum of 3 mL was first grown overnight and inoculated into 300 mL of LB (Lysogeny broth) medium containing 50 µg/mL of ampicillin. The culture was incubated under continuous shaking (200 RPM) at 37 °C to reach an optical density of 0.6–0.8 at 600 nm ($O.D._{600}$). Protein expression was induced for 20 h at 18 °C with 1.0 mM IPTG (isopropyl-β-D-1-galactopyranoside) under constant agitation.

Cells were harvested from the medium by centrifugation, resuspended in lysis buffer (50 mM Tris pH 8.8; 300 mM NaCl) and lysed by sonication (60 Hz, 1.0 s pulse, 10 min) in an ice bath. Following separation of the soluble and insoluble (inclusion bodies) fractions by centrifugation, rSmVAL18 was purified from the soluble fraction by nickel-affinity chromatography using a 5 mL His-Trap™ HP column (GE Healthcare), then dialyzed in PBS (phosphate buffered-saline) at 4 °C for 48 h. Protein purity was verified by 15% SDS-PAGE and the final rSmVAL18 concentration was determined by Lowry's method (DC Protein Assay – Bio-Rad) using bovine serum albumin as a standard.

2.4. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy measurements were performed at 20 °C in a Jasco J-810 Spectropolarimeter (Japan Spectroscopic) equipped with a Peltier unit for temperature control. The far-UV CD spectrum was acquired using a 1.0 mm path length cell at 0.5 nm intervals along wavelengths ranging from 185 to 260 nm. Each scan was recorded as the average of five scans, and then subtracted from the average blank spectra. Protein concentration was maintained at 10 µM in 10 mM sodium phosphate buffer pH 8.0.

2.5. Polyclonal antibody production

BALB/c mice were subcutaneously immunized with 15 µg of recombinant protein formulated with aluminum hydroxide at a 1:10 ratio (protein: adjuvant). Each animal received a total of three doses of this formulation at 14-day intervals. Retro-orbital bleedings were performed prior to each immunization and a final bleeding was conducted two weeks after the last immunization. Antibody titers were assessed by ELISA.

2.6. Protein expression throughout larval stages in culture

Total protein extracts of cercariae, as well as 3-h, 3-, 5- and 7-day

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