Molecular characterization of *Schistosoma mansoni* tegument annexins and comparative analysis of antibody responses following parasite infection

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**Highlights:**
- Structural and immunobiochemical characterization of four homologous annexins expressed in *Schistosoma mansoni* tegument.
• Gene expression profiles of four homologous annexins throughout the life cycle of the schistosome.

• Expression and immunolocalization of annexin molecules in the tegument of *Schistosoma mansoni*.

• Recombinant annexins are rich in alpha-helices and the proteins are recognized by the sera of mice infected with *S. mansoni*.

**Abstract**

Schistosomes are parasitic blood flukes that infect approximately 250 million people worldwide. The disease known as schistosomiasis, is the second most significant tropical parasitic disease after malaria. Praziquantel is the only effective drug currently licensed for schistosomiasis and there are concerns about resistance to the drug. There has been much effort to develop vaccines against schistosomiasis to produce long-term protection in endemic regions. Surface-associated proteins, and in particular, those expressed in the body wall, or tegument, have been proposed as potential vaccine targets. Of these, annexins are thought to be of integral importance for the stability of this apical membrane system. Here, we present the structural and immunobiochemical characterization of four homologous annexins namely annexin B30, annexin B5a, annexin B7a and annexin B5b from *S. mansoni*. Bioinformatics analysis showed that there was no signal peptide predicted for any annexin in this study. Further analysis showed that each of all four annexin protein possesses a primary structure consisting of a short but variable N-terminal region and a long C-terminal core containing four homologous annexin repeats (I-IV), which contain five alpha-helices. The life cycle expression profile of each annexin was assessed using quantitative PCR. The results showed that the overall transcript levels of the each of four homologous annexins were relatively low in the egg stage, but
increased gradually after the transition of cercariae (the invasive schistosome larvae) to schistosomula (the post-invasive larvae). Circular dichroism (CD) demonstrated that rAnnexin B30, rAnnexin B5a and rAnnexin 7a were folded, showing a secondary structure content rich in alpha-helices. The membrane binding affinity was enhanced when rAnnexin B30, rAnnexin B5a and rAnnexin 7a was incubated in the presence of Ca^{2+}. All annexin members evaluated in this study were immunolocalized to the tegument, with immunoreactivity also occurring in cells and in muscle of adult parasites. All four recombinant annexins were immunoreactive and they were recognized by the sera of mice infected with *S. mansoni*. In conclusion, the overall results present the molecular characterization of annexin B30, annexin B5a, annexin B7a and annexin B5b from *S. mansoni* in host-parasite interactions and strongly suggest that the molecules could be useful candidates for vaccine or diagnostic development.

**Keywords:** annexin; immunoreactivity; parasite–host interactions; *Schistosoma mansoni*; schistosomiasis; immunolocalization

**Introduction**

Schistosomes are parasitic blood flukes that infect approximately 250 million people in 74 countries [1, 2]. The parasites cause the disease schistosomiasis, which is the second most significant tropical parasitic disease after malaria, and is of great public health and socio-economic importance in the developing world [3]. These five species, *Schistosoma mansoni, S. japonicum, S. mekongi, S. intercalatum* and *S. haematobium*, are the main contributors to human schistosomiasis. Severe disease in schistosomiasis is caused by the deposition of eggs in blood vessels surrounding the gut or bladder of infected hosts.
Transmission of the parasite takes place in freshwater, in regions where there is poor sanitation [4, 5]. Current control efforts against schistosomiasis rely on mass chemotherapeutic treatment in the form of the oral drug praziquantel (PZQ), but to sustain the effectiveness of this approach, the drug must be administered for an indefinite period of time [5-7]. Because PZQ does not confer protection against re-infection, and has short plasma half-life, vaccination is considered to be an important alternative in controlling and reducing morbidity of schistosomiasis [6-9].

Annexins are calcium-dependent phospholipid binding proteins. All known annexins contain an N-terminal head region and four highly conserved core domains or repeats at their C-terminal tail region [10-13]. The N-terminus, located on the concave side of the folded molecule, is diverse in its sequence and is potentially the key factor in distinguishing among the otherwise highly conserved annexins [14]. This head region is reported to play a vital role in regulation of annexin-membrane associations [14]. The four conserved annexin repeats occur on the convex side of the molecule. Each repeat is composed of approximately 70 amino acids containing a calcium-binding motif [11, 15].

Annexins are expressed in a wide range of parasites, including Giardia lamblia [16, 17], the tapeworm Taenia solium (the larval stage is also called Cysticercus cellulosae) [18] [19, 20] and the monogenean Microcotyle sebastis [21]. Recent proteomic studies show that there are several annexins present in the tegument layer of the blood flukes S. mansoni [22-25], S. japonicum [26] and S. bovis [27]. In preliminary investigation, Hofmann and colleagues ascertained that 13 annexins are present in the S. mansoni
genome, while six are known for *S. japonicum* and one for a third schistosome, *S. haematobium* [28]. Of these annexins, Anx(\(Sm\))3 (Smp_077720) from *S. mansoni* and its orthologue, Anx(\(Sj\))3 from *S. japonicum*, are the most abundantly expressed in the tegument [22, 26]. The single annexin known for *Schistosoma bovis* is homologous to Anx(\(Sm\))1 (Smp_074150) of *S. mansoni* and is expressed in the tegument of the parasites [27].

Annexins are multifunctional proteins that function in cell physiology and cell adaptation [29, 30]. In parasites, annexins such as Anx(\(Sm\))3 (Smp_077720) identified in *S. mansoni* is proposed to have a role as a “molecular glue” by holding the plasma membrane and membranocalyx together through calcium-dependent phospholipid binding property. The *S. bovis* tegument annexin (ACC78610) has fibrinolytic and anticoagulant properties [27]. Annexins of *Giardia* (known as alpha giardins) are proposed to stabilise the membrane of parasites in host intestine [16]. Mammalian annexins such as annexin A1 and A2 are reported to promote cell membrane repair or remodeling [31-33], while, annexins A2, A5 and A6 are known as major components in chondrocyte differentiation which is essential for mammalian musculoskeletal development [34-37]. Annexin A4 functions as a regulator of membrane protein mobility and may play a role in modulation of the membrane channel activity via the inhibition of \(\text{Ca}^{2+}\)-dependent chloride ion conductance [38]. Annexin A1 and annexin A2 are thought to be involved in regulating redox reactions and reactive oxygen molecules [39-41]. A similar role for redox activity has also been proposed for plant annexins [42, 43].
Although proteomics analyses and localisation have shown that some of the annexins are present in the tegument of schistosomes [25, 27, 44, 45], their physiological roles in the parasite tegument requires further elucidation. A recent study from Leow et. al. revealed that *S. mansoni* annexin B22 is localized in the parasite tegument. The protein structural data supports the hypothesis that annexin B22 providing structural integrity in the tegument which is responsible for the immune evasion strategies of schistosomes [46]. Technically, the apical membrane complex of the schistosome tegument is a major protective barrier for the parasite. If this layer could be disrupted, through blockade of function of molecules, like the annexins, then novel control strategies could be uncovered. In order to unravel further information of other annexin members in the tegument of parasitic schistosomes, insights into the functional biology of four homologues annexins identified from the *S. mansoni* proteome as being resident in the tegument of adult parasites were provided in this study. Four homologues annexins identified from *S. mansoni* designated as annexin B30, annexin B5a, annexin B7a and annexin B5b were cloned for molecular characterisation, identification and immunolocalization. The work presented here also examined the binding behaviour of recombinant annexins to phospholipid vesicles in a calcium-dependent manner. The immunogenicity analysis of recombinant annexins was also discussed in the present study.

**Materials and methods**

**Ethics statement**

All experiments involving animals were approved by the QIMR Berghofer Medical Research Institute Animal Ethics committee, project number P1289.
Parasites

The Puerto Rican strain of *S. mansoni* was maintained at the QIMR Berghofer Medical Research Institute. To maintain the life cycle, female ARC Swiss mice (3 weeks old) were used as the definitive host for *S. mansoni* and *Biomphalaria glabrata* snails as the intermediate host. Cercariae for animal challenge were collected from the snails 4 - 6 weeks following miracidia infection. Adult worm pairs were perfused from the mice six weeks after cercariae infection using warm RPMI 1640 as perfusion buffer [47].

Gene retrieval and bioinformatics analysis

Initially, both nucleic acid and amino acid sequences encoding Open Reading Frame (ORF) of annexin B30, annexin B5a, annexin B7a and annexin B5b were searched and retrieved in FASTA format from SchistoDB (http://www.genedb.org/Homepage/Smansoni), a public domain sequence database. Amino acid sequences of each protein were submitted to the Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and SMART (http://smart.embl-heidelberg.de/) to identify their conserved domains. Physico-chemical properties of the gene, including theoretical isoelectric point (pI), molecular weight (Mw), and grand average hydropathy (GRAVY) were predicted using the Expasy ProtParam server (http://web.expasy.org/protparam/). Predictions for both signal peptide and transmembrane helices were performed individually in SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM Server V 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. Protein phosphorylation sites
were predicted by NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) whereas NMT (http://mendel.imp.ac.at/myristate/SUPLpredictor.htm) was used to predict the myristoylation sites in the N-terminal side of the proteins. Secondary structure protein sequence alignment, SBAL (http://www.structuralchemistry.org/pcs/#sbal), an online tool designed to generate and edit secondary-based sequence alignments [48], was employed for calculating the secondary structural features of the predicted protein sequences. Alignments of protein sequences were done using the Clustal W2 algorithm on the EBI Server (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A minimum evolution phylogenetic tree was constructed using MEGA version 6.0 software [49] with the Neighbour Joining method. The data set was bootstrapped 1000 times and uniform rates of evolution among sites were assumed.

**Total RNA extraction and cDNA synthesis**

Total RNA from 20 – 30 adult worm pairs was extracted using Trizol/RNeasy Mini Kit (Qiagen) method as described [50]. The quantity of purified total RNA was measured using Nanodrop ND-1000 spectrophotometer (Thermo). First strand cDNA was synthesised using Superscript III Reverse Transcriptase Kit (Invitrogen) as described previously [51]. The concentration of cDNA was measured using Nanodrop ND-1000 spectrophotometer (Thermo). The resulting cDNA was aliquoted and stored at -20°C until use.

**Quantitative PCR**
Transcription profiles of these four homologues annexin B30, annexin B5a, annexin B7a and annexin B5b genes from the different life stages of the parasite were determined by quantitative PCR (qPCR). Primers for the four homologues annexin genes were designed using Primer3 (http://frodo.wi.mit.edu/) (Table 1). Each qPCR sample was prepared to a final volume of 20 µL, containing 5 µL (5 ng/µL) of cDNA, 10 µl of SYBER® Green (Applied Biosystems), 3 µl of nuclease-free water and 1µl (5 pmol/µL) each of forward and reverse primers. qPCR was performed and analysed using previously described protocols [52] using DNA segregation ATPase (Accession no. Smp_176580) [50] as normalising housekeeping gene. Data was analyzed and quantified using the Rotor Gene 6 software (Corbett Research).

**Gene cloning**

Primers targeting full length sequences of each *S. mansoni* annexin assessed in this study were designed from the sequences of the corresponding annexin genes retrieved from SchistoDB (http://www.genedb.org/Homepage/Smanson) using Primer3 (http://frodo.wi.mit.edu/). The primers used for full length gene cloning are listed in Table 2. The gene construct was amplified by PCR in 50 µL according to the manufacturer’s protocol. Briefly, the PCR cocktail consisted of 1X *Pfu* PCR buffer, 1 mM dNTP mix, 0.25 µM of each forward and reverse primer, 100 ng of *S. mansoni* adult worm pairs first strand cDNA mix template and 5U of *Pfu* DNA polymerase (Stratagene). The PCR reaction was performed with the following cycling conditions: an initial template denaturing step of 1 minute at 95°C followed by 30 cycles of denaturing at 95°C for 10 seconds, annealing at 53°C for 30 seconds and extension at 68°C for 3 minutes.
The final extension step was set at 68°C for 7 minutes. Purified PCR product corresponding to each gene was cloned into pET28a (Novagen) in a double digest as described previously [53]. Clones were sequenced using Big Dye Terminator v3.1 (Amersham Bioscience) sequencing protocol. Identity and sequence of gene fragment in the recombinant plasmids were confirmed through nucleotide sequencing as previously described [54].

**Recombinant protein production and purification**

Recombinant protein expression was performed using previously described protocol [46]. Briefly, recombinant plasmid containing corresponding gene construct was transformed in chemically competent *E. coli* BL21(DE3). The cells were grown in 2YT medium at 37°C with agitation at 250 rpm. When 0.6 of optical density achieved, 0.5 mM isopropylthio-β-galactoside (IPTG) was added and the induced cells were shaken continuously at 200 rpm overnight at 18°C. The cells were then harvested at 6693 x g for 20 minutes at 4°C using rotor JLA 10.500 in Beckman Centrifuge (Beckman). The cell pellet was resuspended in 30 mL of Buffer 1 (50 mM Tris, 3 mM MgCl₂, 300 mM NaCl; pH 8.0) and stored at -80°C. His-tagged recombinant proteins were purified under native conditions. Cell pellets were thawed on ice-water and disrupted using a French Press (Amicon). The soluble cell lysate was centrifuged at 10,000 x g for 30 minutes to remove cell debris. The his-tagged recombinant proteins were purified by affinity chromatography using Ni-NTA agarose (Sigma) according to the manufacturer’s instructions. The soluble recombinant protein was dialyzed at 4°C for 24 hours against 20 mM Tris buffer (pH 8.0). Purified recombinant protein was identified 12% SDS-PAGE
and confirmed by Western blot with anti-His antibodies. Quantification of the concentrations of purified recombinant proteins was performed using a Pierce BCA Protein Assay Kit (Thermo) according to manufacturer instruction.

**Circular dichroism (CD) spectroscopy**

Each of the four purified recombinant annexins (5 mg/mL) was prepared in standard buffer (100 mM NaCl; 20 mM HEPES; pH 7.5) as described previously [46]. All samples were subjected respectively to far-UV CD spectra analysis using a J-810 spectrophotometer (Jasco) at 20°C. An average spectrum was recorded following three scans between 190 nm and 250 nm. Experiments in the presence of 1 mM CaCl$_2$ were also performed under the same conditions as described above. All acquired spectra data were calculated for their secondary structure using ACDP ver2.9 prediction algorithm [55].

**Liposome-based copelleting assay**

Different ratios of phospholipid vesicles were prepared from 1,2-dioleoyl-sn-glycero-3-phosphoserine (PS) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC) (Avanti Polar Lipids) according to the protocol of Reeves & Dowben (1969) [56]. The vesicles were converted into large unilamellar vesicles (LUV) using five freeze–thaw cycles and subsequent extrusion (11 times) through 0.1 µm filter membranes using an extruder (Avanti Polar Lipids) at 37°C. In order to assess the annexin–membrane binding behaviour, a copelleting assay was conducted [57]. A total of 0.2 µmol phospholipids was used for each individual sample (500 µL) composed of 0.5 nmol protein in liposome
buffer (180 mM sucrose, 50 mM NaCl, 10 mM HEPES, pH 7.5) and varying amounts of calcium (ranging from 0, 1, 2, 10 and 20 mM). As a control, a sample of 0.1 nmol protein in 100 µL of 10% SDS was prepared. All samples were centrifuged (45 minutes, 13,000 rpm, 4°C), the pellets were resuspended with 50 µL of 10% SDS and separated by 12% SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and analysed densitometrically using the program ImageJ Analysis Software (National Institute of Health). Each calcium concentration was independently assessed three times.

**Antibody production**

Polyclonal antiserum was raised against each of the four recombinant annexins in 3-week-old female ARC Swiss mice. The mice were subcutaneously administered with 25 µg of each recombinant protein emulsified with Complete Freund's Adjuvant (CFA) (Sigma). The mice were boosted twice at two weekly intervals via intraperitoneal injection with 25 µg recombinant protein emulsified in Freund’s Incomplete Adjuvant (FIA) (Sigma). Mice were bled 10 days following the third immunization by tail-tip sampling. The serum was stored at -20 °C until required.

**Immunolocalization**

**(I) Immunofluorescence**

Freshly perfused adult *S. mansoni* were washed in PBS and then fixed with cold (- 20°C) 100% methanol (Chem-Supply). The fixed parasites were embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (ProSciTech) and sectioned at 7.0 µm thickness using a cryostat. Immunofluorescence assays of adult parasite sections were
conducted following Gobert et al [58]. Sections were blocked overnight with blocking solution (5% low fat skim milk; 1% BSA; 0.02% Tween-20) at 4°C with gentle shaking. After three washes with 0.05% PBST (5 minutes each), sections were incubated for one hour at room temperature with either anti-annexin B30, anti-annexin B5a, anti-annexin B7a or anti-annexin B5b mouse sera diluted in 0.05% PBST (ranging from 1:10 to 1:100, depending on the end titer of each sera). Normal mouse serum was used as a negative control. The sections were washed three times with 0.05% PBST for 5 minutes each, and incubated with 1:500 PBST-diluted Alexa Fluor 488® goat anti-mouse IgG (H+L) (Life Technologies, USA) for 30 minutes at room temperature. Sections were washed three times with 0.05% PBST, the slides were air-dried briefly and mounted with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratory). The mounted sections were examined using an EVOS AMG fluorescence microscope (Olympus).

(II) Immunoelectron microscopy

High-pressure frozen adult males were cryo-substituted in 0.2% uranyl acetate and 5% water in acetone and embedded in Lowicryl HM20 resin for immunocytochemistry as described previously [59]. Ultrathin sections mounted onto copper grids were washed three times in PBS (five minutes each) followed by blocking for 15 minutes at room temperature in blocking solution (0.2% BSA; 0.2% FSG; 20 mM glycine; 20% PBS). After blocking, the ultrathin sections were incubated respectively for 30 minutes at room temperature with anti-annexin B30, anti-annexin B5a, anti-annexin B7a or anti-annexin B5b mouse serum diluted 1:50 in blocking solution. Following four washes in PBS of 5 minutes duration each, the ultrathin sections were incubated for 30 minutes at room
temperature with 10 nm Protein-A Gold (Sigma) (1:60 dilution in blocking solution). Normal mouse serum was used as a negative control for immunolabeling. Following four washes each in PBS and MilliQ water (2 minutes each), the grids were dried briefly on blotting paper. Ultrathin sections were contrasted in uranyl acetate and lead citrate and examined and photographed using a JEM 1011 transmission electron microscope (JEOL) operated at 80 kV.

**Preparation of native *S. mansoni* extracts**

Approximately one hundred pairs of *S. mansoni* adults (6 weeks old) freshly perfused from Swiss mice were used for adult worm protein extraction. Following perfusion, the worms were washed five times with RPMI 1640 (Invitrogen) to remove contaminating host components. The worms were homogenised on ice in 20 mM Tris-buffer (pH7.4) containing 1 mM EDTA and a protease inhibitor cocktail (Roche). After incubation for 10 minutes at 4°C, the homogenate was centrifuged at 16,000 x g for 1 hour at 4°C. The supernatant containing parasites soluble proteins was collected as Soluble adult Worm Antigen Preparation (SWAP) and stored at –80°C. The insoluble pellet was resuspended and washed twice with 20 mM Tris-buffer (pH7.4). The clean pellet was dissolved in 1% SDS (Sigma) for 2 hours with rotation at 4°C. The insoluble protein was then pelleted by centrifugation at 16,000 x g for 1 hour at 25°C. The supernatant containing parasites insoluble proteins was collected as Insoluble adult Worm Antigen Preparation (IWAP) and stored at -80°C.

**Detection of native annexins in schistosome extracts**
Five µg of adult worms extracts (SWAP and IWAP) and 100 ng of purified recombinant proteins corresponding either to annexin B30, annexin B5a, annexin B7a or annexin B5b were run in a 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated respectively with anti-annexin B30, anti-annexin B5a, anti-annexin B7a or anti-annexin B5b mouse sera (1:1000 dilution in 0.1% PBST) for 1 hour at room temperature. The membrane was incubated with secondary antibody was goat anti-mouse IgG (H+L) HRP conjugated (Invitrogen) (1:2000 dilution in 0.1% PBST) for 1 hour at room temperature. One mL of chemiluminescent substrate solution (ECL, Amersham Pharmacia) was added onto the membrane for development. The detection of the Western reaction was carried out by exposing film (Fuji) to the blot in a film cassette prepared in a darkroom and developed through a Kodak X-Omat imaging machine (Kodak). Normal mouse serum was used as negative control.

**Immunogenicity assay of recombinant annexins**

The immunoreactivity level of annexin B30, annexin B5a, annexin B7a and annexin B5b was analysed by Western blot using *S. mansoni* experimental infected mice sera. Briefly, five µg of each recombinant protein was separated by 12% SDS-PAGE. The details of the blotting method is described above, with the exception that the primary antibody used here was *S. mansoni*-infected mouse serum (1:100 dilution in 0.1% PBST). For quantitative assay, an ELISA was used to evaluate the immunological recognition of recombinant proteins by mouse sera infected experimentally with *S. mansoni* or *S. japonicum*. Nunc Maxisorp 96-well plates were coated overnight respectively with 100 µL of 5 µg/mL of recombinant annexin B30, annexin B5a, annexin B7a or annexin B5b in PBS at 4°C. The
plates were blocked with 300 µL of 2% skim milk in 0.1% PBST for one hour at room temperature with gentle shaking. The plates were washed three times with 0.1% PBST at 5 minutes intervals. 100 µL of mouse serum (1:100 dilution in 0.1% PBST) was added to each well. The plates were incubated in the dark at room temperature with gentle shaking for an hour. Following a final wash step with 0.1% PBST, 100 µL goat anti-mouse IgG-HRP (horseradish peroxidase-conjugated) (Invitrogen) (1:2000 dilution in 0.1% PBST) was added to each well. The plates were incubated in the dark for an hour at room temperature with gentle shaking. Following a final wash step with 0.1% PBST, 100 µL water-dissolved OPD (o-phenylenediamine dihydrochloride) tablet (Sigma) was added to each well and the plates were incubated in the dark for 20 minutes at room temperature. The titration endpoints were read and measured calorimetrically at an absorbance of 450 nm in a Biotek Synergy H4 Microplate reader (Biotek).

Results

Cloning and in silico characterization of S. mansoni annexins

In order to gain a better knowledge of the distribution and molecular characteristics of annexins of S. mansoni, four homologues genes, encoding annexins predicted from proteomic analyses to be present in the tegument of schistosomes were selected and amplified from a cDNA library of mixed adult stage of S. mansoni. The selected genes were cloned into bacterial cells. Detailed information of the gene structure for these sequences, designated as annexin B30, annexin B5a, annexin B7a and annexin B5b, are summarized in Table 3. DNA sequencing was performed to verify the sequence of each corresponding S. mansoni annexin amplicon. Sequencing of the Open Reading Frame
(ORF) revealed that the lengths of *S. mansoni* annexin B30, annexin B5a, annexin B7a and annexin B5b was 1023 bp, 996 bp, 1065 bp and 1464 bp, respectively. These annexins were predicted to encode polypeptides consisting of 340 aa, 331 aa, 354 aa and 487 aa (amino acids) residues, respectively, with total predicted molecular masses of 39.3, 36.9, 40.0 and 53.7 kDa, and predicted pI values of 7.58, 5.96, 4.86 and 6.58, respectively. With respect to the post-translational modifications, the N-terminal regions of annexin B30, annexin B5a and annexin B7a were predicted to harbor one phosphorylation site, whereas fifteen phosphorylation sites were predicted in annexin B5b. In contrast to human annexins, all four homologous *S. mansoni* annexins selected in this study did not contain a signal peptide and were not predicted to be transmembrane proteins.

In order to understand the degree of homology between annexin members, amino acid sequences of four *S. mansoni* homologues annexins selected in this study were aligned with two annexins from *Homo sapiens* (AnxA2 and AnxA5), *Schistosoma mansoni* annexin B22, *Schistosoma bovis* annexin, *Taenia solium* annexin B1, *Microcotyle sebastis* annexin and the plant *Arabidopsis thaliana* annexin (Figure 1). The alignment result showed that the *S. mansoni* annexins shared a well conserved type II calcium binding site (G-X-G-T-(38 residues)-(D/E) within each repeat, with the exception of annexin B30, which lacks two calcium binding sites in repeats II and III. The calcium binding site of repeat III in annexin B30 was substituted with a KGD motif. *S. mansoni* annexins also possessed an arginine residue in repeat I, repeat III and repeat IV while an IRI motif was seen in the fourth repeat domain of annexin B30, annexin B5a and annexin
B5b. This motif has been known to play a crucial role in membrane cytoskeleton dynamics in some of the vertebrate annexins [11]. Glutamic acid and arginine residues, considered important in ion channel activity, were present in repeats II and IV of annexin B5a, annexin B7a and annexin B5b. It has been reported that the annexin-like protein Oxy5, from Arabidopsis thaliana, contains an unusual cluster formed by a methionine and two cysteines in repeats II and III, respectively [60, 61]. Annexin B5a and annexin B5b have a similar cluster, but the methionine residue in repeat II was substituted by isoleucine in schistosome annexins. Furthermore, a heme-binding domain (located in repeat I of Oxy5 of A. thaliana) was also detected in annexin B30, annexin B5a and annexin B5b. These two motifs are involved in plant antioxidant defense moderated through peroxidase activity [60, 61].

**Phylogenetic analysis of S. mansoni annexins**

In order to obtain better insights into evolutionary relationships, 60 annexins from a variety of organisms were selected (Figure 2). A phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap replicates. To increase the reliability of the analysis, the phylogenetic tree was constructed using C-terminal region of the annexins which were conserved across the protein family. The phylogenetic tree classified the 60 deduced protein sequences into eleven groups of related protein clades designated I to XI. The majority of platyhelminth annexins grouped in Clade I which also included annexin B22, annexin B30 and annexin B7a. Other S. mansoni annexins such as Anx(Sm)10 was found in clade X whilst annexin B39a in Clade XI. annexin B5a and annexin B5b, clustered into group III, which was found to form a bifurcate root node with
vertebrate annexin A13 (clade IV). Annexin B5a, orthologous with Anx(Sj)4, is a likely parologue of annexin B13, annexin B31 and annexin B5b. *Caenorhabditis elegans* annexin shares little homology with schistosome annexins and was clustered in group V and group VII. The tree shows that the schistosomes and the mammalian homologs form distinguishable subclusters. Plant annexins were segmented into group VIII. Two annexins from *Giardia* were represented in cluster IX but one was assigned to cluster XI.

**Stage-specific expression of *S. mansoni* tegument annexins**

The expression changes of a gene are usually associated with the physiological alterations in every living organism. In order to understand the importance roles of annexins in schistosome development, the life cycle expression profiles of the four homologous annexins studied were ascertained using qPCR. Relative levels of annexin gene transcripts in different stages are represented in Figure 3. Steady-state mRNA levels of the five annexins were low in eggs. The transcript levels of each annexin increased gradually after the transition of cercariae to schistosomula. Among the four annexins, annexin B5a and annexin B5b were expressed abundantly in adult males, while annexin B30 and annexin B7a were shown to have relatively high abundance during schistosomulum stage. The abundance of annexins found in infective stages, particularly in schistosomula and in adults, indicating the crucial roles of these molecules towards the parasite development and host-parasite interactions [20, 46, 62].

**Expression and protein fold characterisation of soluble recombinant annexins**
In order to examine the biochemical characteristics of four homologous schistosome annexins, the full length ORF encoding annexin B30, annexin B5a, annexin B7a and annexin B5b were expressed in *E. coli* strain BL21(DE3) as soluble His-tagged protein. Due to the purified recombinant annexin B5b was not being stable, only recombinant annexin B30, recombinant annexin B5a and recombinant annexin B7a were used for secondary structure assessment and calcium-dependent membrane binding assay. The Circular Dichroism (CD) spectrum demonstrated by each recombinant protein showed an average peak rising to the maximum of around 196 nm and a broad negative band rounding 215 nm (Figure 4), indicating the proteins were structurally folded along with alpha-helical secondary structure content as expected for annexins [20]. The presence of 1 mM calcium resulted in slight reduction of mean residue ellipticities. As seen in Figure 4, small gradual changes in the UV CD spectrum band shape were observed with the addition of 1 mM calcium to each of the three recombinant annexins.

**Calcium-dependent membrane binding assay**

The calcium-dependent membrane binding of annexin was assessed using large unilamellar vesicles (LUVs) at two different phosphatidylserine (PS) to phosphatidylcholine (PC) ratios. Recombinant annexin B30, recombinant annexin B5a and recombinant annexin B7a displayed calcium-dependent binding behavior to the lipid vesicles as showed in Figure 5. Annexins had an enhanced binding affinity to vesicles that contained higher concentrations of PS. Maximal binding degree displayed for annexin B5a and annexin B7a was 90% at 5 mM Ca\(^{2+}\), while annexin B30 was 80% at 2
mM Ca$^{2+}$. Taken together, each of three *S. mansoni* homologous annexins exhibited calcium-dependent behavior when binding to lipid vesicles.

**Detection of native annexins in schistosome extracts**

An immunoassay is a biochemical test that measures the presence of a protein or peptide in a solution through the use of an antibody. In this section, native schistosome annexins in extracts of adult parasites were examined using mouse sera raised respectively against rAnnexin B30, rAnnexin B5a, rAnnexin B7a and rAnnexin B5b. As a control, the respective antibody was used to probe the purified recombinant annexins and native annexins in adult worms extracts. Immunized mice antisera were able to identity each of the recombinant annexins and the specific native annexin members in the SWAP (Soluble Worms Antigen Proteins) portions but not IWAP (Insoluble Worms Antigen Proteins), of whole adult worm extracts (Figure 6). No bands were seen when adult worm extracts were probed with mouse pre-immune sera. Recombinant annexins generated in this study shared similar antigenic structure with their native molecules and confirms they are immunogenic and applicable for immunolocalization use.

**Immunolocalization of annexins in adult *S. mansoni***

Immunolocalization is widely used to identify the location of a protein in a cell or tissue using the immunological techniques. In this study, mice antisera raised against each of four homologous annexins were used respectively to localize the native annexins in tissues of adult male *S. mansoni* given that the high expression of mRNA transcript at this stage. The localization was performed because the tegument of schistosomes has multiple
membrane-bound components. It is important to identify which annexins are linked with specific schistosomes membranes. By immunofluorescence, four homologous annexin members investigated in this study were shown to be localized in the tegument and in cellular region basal to the tegument (Figure 7). Furthermore, annexin B5a, annexin B7a and annexin B5b were also detected in the lining of the gastrodermis (digestive tissues) of the adult parasites. No fluorescence staining was detected when pre-immune mouse serum was used or in the absence of primary antibodies.

To further confirm the localization of each corresponding annexin in the outer membrane of adult parasites, a higher resolution localization of the annexins was obtained by immunogold labelling and electron microscopy (Figure 8). Immunolabeling of annexins in adult parasites was detected in regions corresponding to the tegument. The mouse serum raised against annexin B30, annexin B5a and annexin B7a showed positive reactivity over the tegument outer layer and diffuse reactivity over the muscle region. Annexin B5a gave a weakly positive label that was associated with internal membrane structures, but not the surface membrane. Positive immunoreactivity was associated with the basal membrane, annexin B30 and annexin B7a immunoreactivity was more clearly associated with the apical membrane as well as internal membrane components. In contrast, only a few randomly associated gold particles were observed in the outer layer of tegument when the grid sections were labelled with the anti-annexin B5b mouse serum. Negative controls using PBS as substitution to primary antibody did not detect any reactivity in the outer membrane region of the adult parasites, and only diffuse label throughout the matrix. The presence of annexins in the tegument and some in
gastrodermis of the parasite indicates that the annexins may play different roles in tegument \[27, 45, 46, 62\] and gut-specific tissues \[63\].

**Immunogenicity analysis of recombinant S. mansoni annexins with sera from experimentally infected mice**

Immunogenicity is the ability of an antigen to trigger host immune response. For a vaccine target selection, understanding of the immunogenicity of an antigen is important. In this study, the evaluation of the immunogenicity of each of the recombinant annexin was performed by probing the corresponding recombinant proteins with sera from mice infected with either *S. mansoni* or *S. japonicum*. Sera from uninfected mice were used as negative controls. The results showed that sera from *S. mansoni* infected mice recognized all recombinant annexins albeit with different degrees of intensity. rAnnexin B5a and rAnnexin B5b were faintly recognized by pre-immune mouse serum. Quantification by ELISA assay of immunoreactivity of each recombinant annexin against *S. mansoni* infected mice sera showed that rAnnexin B7a was strongly recognized by sera, 2.68-fold higher than the control group. Reactivity of rAnnexin B30 and rAnnexin B5a were 1.76-fold and 1.77-fold, respectively, higher than the control group while rAnnexin B5b was the least reactive recombinant antigen, only 1.43-fold higher compared to the control group (Figure 9).

**Evaluation cross-reactivity recombinant annexins with S. japonicum Chinese strain**

Each of the recombinant *S. mansoni* annexins were also evaluated for their cross-reactivity response with *S. japonicum* Chinese strain using ELISA assay. As illustrated in
Figure 10, it is shown that all recombinant annexins tested herein were recognized by sera of mice infected with *S. japonicum* Chinese strain, indicating that the recombinant annexins designed specifically for *S. mansoni* were also immunogenic to other orthologues species. Of these, rAnnexin B7a was still the most reactive antigen with a fold change of 2.39 higher than the control group. rAnnexin B5a was shown to be less reactive than the *S. japonicum* infected mice sera with about 1.58-fold higher than the control group. Reactivity responses demonstrated by rAnnexin B30 and rAnnexin B5b were close to each other with a range of 1.26-fold and 1.25-fold slightly higher than the uninfected mice sera. Taken together, the results obtained herein indicates that recombinant annexins generated in this study may share a similar conformational structure to their corresponding native annexins in the parasites.

**Discussion**

Many remarkable morphological and molecular adaptations have accompanied the adoption of parasitism in schistosomes to ensure survival of the parasites in the hostile host environment [64]. Some essential molecules expressed on the surface of the parasites have evolved to mimic host proteins [64], leading to a high sequence similarity between parasitic group and those expressed in the host [65]. Annexins, one of the most abundantly transcribed genes in both vertebrates and invertebrates, have duplicated extensively and independently during evolution [13]. The wide distribution of annexin members in schistosomes lends an importance of these molecules in playing their roles in cellular physiology which may be important for parasitism. It remains a major interest as to how these molecules have diversified with the adaption to parasitism.
Previously we have reported the crystal structure and immunological properties of annexin B22. It was hypothesized that the protein may potentially play a vital role in maintaining the structure integrity of schistosomal tegument [46]. In this study, in order to further explore the molecular functions of other annexin members identified in schistosome tegument, four homologues schistosome annexins known as annexin B30, annexin B5a, annexin B7aand annexin B5b present in tegument were cloned and characterized.

Schistosome annexins were shown to be made up of a diversified amino terminus and a conserved core domain containing four repeat domains (also known as tetra core) comprise of approximately of 70 amino acid residues at their C-terminal. Each schistosome annexin showed a well conserved type II calcium binding site, suggesting schistosome annexins repeats contain the conserved calcium ion binding sites as observed in other published annexins [13, 66]. Post-translational modification prediction indicated that there is at least one phosphorylation site found in the N-terminal region of each schistosome annexins. Tyrosine phosphorylation and PKC phosphorylation are the most common phosphorylation sites in vertebrate annexins and these sites are vital in modulating the annexin interactions with other proteins [67]. The absence of this region in annexins of Giardia is thought to reflect the functional restrictions of the alpha-giardins [68]. Unlike the core domain, the phosphorylated N-terminal region is variable in schistosome annexins. This region is postulated to be the regulatory domain of the protein as it contains major sites for proteolysis, phosphorylation and interaction with
other molecules [13]. Further investigations on the core domain of *S. mansoni* annexins showed that there is a Lys-Gly-Asp motif (KGD) found in repeat III of annexin B30. The substitution of a calcium ion binding site with this motif indicates that annexin B30 may be a potential ligand for membrane receptors as observed for other annexins with this motif [69]. The possession of KGD motif in human annexin A5 repeat IV was reported to be crucial in the anticoagulation process by acting as glycoprotein (GP) IIb/IIIa antagonist [70]. 11% of all annexin repeat domains contained a novel K/H/RGD motif consistent with the functional loss of type II calcium ion binding sites [71]. In annexin B22, a L\textsubscript{62}CQL/SL\textsubscript{77} motif in the core domain was found to be identical with that reported for *S. bovis* annexin, a protein postulated to have a role in profibrinolytic activity [27]. This motif, with the sequence of L\textsubscript{8}CKL/SL\textsubscript{13}, has also been identified at the N-terminal end of human annexin A2. The motif has been reported to serve as a platform for the interaction between annexin A2 and tissue-type plasminogen activator (t-PA) for fibrinolytic activity through plasminogen binding [72]. The presence of motif associated with fibrinolytic and anticoagulant activity of the annexins that are associated with the schistosome tegument membrane may indicate a strong role for these molecules in presenting coagulation in the blood stream [27]. Arginine residues, which are involved in structural stability of the annexin tertiary structure [11, 73], were found to be conserved in repeats I, III and IV of the C-terminal core domain of *S. mansoni* annexins.

Phylogenetic tree construction of annexins shows that the proteins were grouped into eleven distinct clades. Schistosome annexins were grouped into clade I and clade IV. Clade I is apparently dominated by annexins derived from platyhelminth parasites.
Notably, many annexins clustered in this clade are known to possess a unique long linker region between repeat II and repeat III [28, 74]. Annexin B22 is placed nearest to both *S. bovis* and Anx(*Sj*)1 while it forms different branches with Anx(*Ts*)B1 and Anx(*Ts*)B2 from *Taenia solium*. *S. bovis* annexin was identified on the tegument surface [27, 75], supporting annexin B22 may share a similar role in parasite-host interaction [46].

Although both annexin B30 and annexin B7a grouped in clade I, they form distinct subclusters from each other. Annexin B30 is closely related to Anx(*Sj*)3. Both molecules are among the most abundant proteins found in surface membrane extracts of schistosomes [26]. An annexin from monogenean *Microcotyle sebastis*, which was identified in fish gill fluke [21] also grouped within this cluster. Furthermore, annexin B7a, an orthologue of Anx(*Sj*)5, forms a bifurcate node with Anx(*Ts*)3. Given that they are branched from the same cluster node, all annexin members grouped within this cluster may probably share a common ancestor, as shown for annexins of vertebrates [11, 76]. Interestingly, annexin B5a and annexin B5b are clustered altogether in clade III and close to annexin A13 in clade IV, revealing that the annexins in this group have undergone duplication events from a common ancestor. Annexin A13 has been known as the original founder gene of 12 members in the vertebrate annexin family. It is the earliest branching subfamily in vertebrates and is physically non-syntenic from other human annexins [77]. Annexin A13 is an intestine-specific expression associated with a highly differentiated intracellular transport function [77].

Transcriptional data presented here indicated that transcripts of each annexin were less abundant in the egg but transcript abundance increased gradually after the transition of
cercariae stage to schistosomulum and into the adult stage. This observation is in agreement with stage-specific profiling of schistosomes using microarray analyses, which demonstrated the up-regulation of an annexin in the lung schistosomulum and adult male of *S. japonicum* [78]. Furthermore, high levels of annexin B30 transcript detected in the schistosomulum of *S. mansoni* in our study is similar to the data reported previously [45]. The up-regulation of annexins in both schistosomulum and adult stages is probably associated with the substantial remodelling of the schistosome tegument that occurs after cercariae transformation [79]. Parasite annexins have been known to have a role in anti-inflammatory responses and protection against membrane degeneration [28]. A general potential role for the annexins may be for tegument structural maintenance [22, 80]. Annexin B30 is present abundantly in the schistosome tegument. Its location has been confirmed by proteomics [25] and immunofluorescence [45]. Preliminary proteomic data indicates the presence of other annexins in the tegument of schistosomes (Dr. Jason Mulvenna, QIMR Berghofer Medical Research Institute, unpublished data). To confirm the presence of these annexins in the tegument, antisera against all five annexins predicted to have a tegumentary location were raised in this study. All four homologues annexins in *S. mansoni* were primarily localized in the tegument and in cells within the parenchyma. Such a pattern is consistent with localization occurring in the tegument and the tegument cell bodies that synthesize molecules for the tegument [46, 81, 82]. Of note, some annexins were found by immuno-electron microscopy (Immuno-EM) appear to be strongly associated with the apical plasma membrane of the tegument. These calcium-dependent phospholipid binding molecules are potentially required for promoting the adhesion of the membranocalyx to plasma membrane via the interactions between
phospholipids on the membrane and the calcium binding domains in the annexins [23]. Nevertheless, detection of distribution of annexin molecules in *S. mansoni* muscle region indicates that they could be essential for actin dynamics and parasitic motility. Several studies have revealed that annexins are membrane F-actin binding proteins which are important in maintaining plasticity of the dynamic membrane-associated actin vertebrate cytoskeleton through calcium ion transportation [83-85]. This includes the observation that annexin A2 is well known for its capability of interacting with polymerised actin through the mapping of the F-binding-site to the core domain of the annexin molecule [11]. This data presented here strongly supports that annexins may play a role in dynamic membrane-associated tegument maintenance through calcium ion regulation. In addition, annexin B5a, annexin B7a and annexin B5b were detected in the gut lining of schistosome. The gastrodermis (lining of the gut) is a syncytial epithelium involved in digestion and absorption of undigested nutrients. It is not surprising that annexins are also found in this tissue. Similarly, annexin found in *Microcotyle sebastis* [21] and annexin A13 from human are reported to be associated with the intestinal cells [77]. Previous finding showed that annexin A13 plays a role in lipid-mediated delivery of apical proteins [86]. Phylogenetic tree presented here also indicates Anx(*Sm*)4 and Anx(*Sm*)12 are related to vertebrate annexin A13, suggesting a similar function is shared among of these annexins.

Data from this current study indicates that schistosome annexins exhibited the feature of calcium-dependent binding to acidic phospholipid membranes. This property has been shown for annexin B22 from *S. mansoni* [46] and annexin B1 from *Cysticercus*
It has also been demonstrated that Annexin B22 [46] and annexin B30 [45] were enriched in the membrane fraction of *S. mansoni* adult tegument in the presence of calcium, suggesting schistosome annexins are calcium-dependent proteins associated with the apical membrane, supporting the data in the present studies. Furthermore, the schistosome annexins exhibited different calcium binding affinity among different members, indicating each individual annexin repeat domain has preferential effects on the binding activities of annexins [46, 87].

Tegument proteins that can be recognized by host immune systems and elicit strong host immune responses have potentially value for vaccine and immunodiagnostics development against schistosomes [9, 88]. Data from this study shows that the sera from mice immunized with corresponding recombinant antigens were able to recognize native schistosome proteins in the SWAP extracts, suggesting that the recombinant annexins contain conformational epitopes that are similar to their corresponding natural antigen. Antigen cross-reactivity assay further revealed that recombinant annexins used in this study were recognized by mice sera infected between *S. mansoni* and *S. japonicum* species. These observations have important implications for development of treatments using schistosome annexins. First, any drug or vaccine could be used against multiple schistosome species. Secondly, schistosome annexins, being recognized by the hosts, are possibly involved in host-interaction. Therefore, care need to be taken in the use of these molecules as sera from uninfected mice showed light reactivity against some schistosome annexins such as annexin B5a and annexin B5b.
Conclusion

In conclusion, the full-length cDNAs encoding four homologous annexins made up of annexin B30, annexin B5a, annexin B7a and annexin B5b identified from the dataset in the tegument *S. mansoni* were cloned and characterized for their structural characteristics, gene expression patterns and tissue-specific distributions. The data obtained in this study sheds light on the biological functions of these molecules in host-parasite interaction. Furthermore, the results presented herein demonstrate that four homologues annexins of *S. mansoni* assessed in this study induced high levels of specific antibody production, suggesting that the proteins are immunogenic. Collectively, *S. mansoni* tegument annexins could potentially be used as effective targets for the development of sensitive diagnostic or protective immunoprophylactic agents against schistosome infection.

Competing interests

The authors declare that they have no competing interests.

Acknowledgement

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Centre for Microscopy and Microanalysis, University of Queensland. We thank Mary Duke for assistance with the schistosome life cycle and animal maintenance at QIMR Berghofer Medical Research Institute.
References


Figure Legends

Figure 1: Multiple sequence alignment of four homologous *Schistosoma mansoni* (*Sm*) annexin C-terminal core amino acid sequences in comparison to annexins selected from *Homo sapiens* (*Hu*), *Schistosoma bovis* (*Sb*), *Taenia solium* (*Ts*), *Microcotyle sebastis* (*Ms*) and *Arabidopsis thaliana* (*At*). Sequence alignment was performed in ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Asterisks mark conserved amino acids, a colon indicates a conservative substitution of amino acid and full stop indicates a semi-conservative substitution of amino acids. The annexin repeats I, II, III and IV are highlighted in purple, yellow, green and light blue, respectively. Type II calcium binding sites (G-X-G-T-(38 residues)-(D/E)) are coloured with grey. KGD motifs identified in repeat III of annexin B30 and repeat IV of human annexin A5, are coloured with red. Arginine residues identified in repeat I, repeat III and repeat IV are coloured with white letter filled in brown background. “IRI” motif identified in repeat IV is underlined. Heme-binding domain in repeat I is highlighted with light blue in italics font. Both glutamic acid and arginine residues observed in repeat II and repeat IV are coloured with white letter filled in dark blue background. An unusual cluster formed by a methionine and two cysteines in repeats II and III, respectively is highlighted with white letter filled in dark background. A schistosome-specific linker region spanning repeat II and repeat III is boxed with red dashed line. The dotted red-coloured box showing the linker region spanned within II – III repeats of each annexin.
Figure 2: Minimum evolution phylogenetic tree showing the relationship of the 60 deduced C-terminal amino acid sequences of annexins from a variety of organisms. Species name and published accession numbers are displayed in each annexin sequence. *S. mansoni* annexins used in this study are marked with black box. Clade number is represented in italic numbers. The phylogenetic tree was generated using MEGA version 6.0 software performed with Neighbour Joining method. The dataset was bootstrapped 1000 times and uniform rates among sites were assumed.
Figure 3: Analysis of stage-specific mRNA expression of five *S. mansoni* annexins using quantitative PCR. (A) Annexin B30 (B) Annexin B5a (C) Annexin B7a and (D) Annexin B5b. 4 H NTS, 4 hours newly-transformed schistosomula; 4 D NTS, 4 days newly-transformed schistosomula; 4 D NTS + RBC, 4 D NTS, 4 days newly-transformed schistosomula supplemented with human red blood cells; 22 D mixed worms, 22 days mature schistosomula. Standard error values are displayed on the graph.
Figure 4: Far-UV (190 – 260nm) CD spectra of purified soluble recombinant (A) Annexin B30, (B) Annexin B5a and (C) Annexin B7a. All tested recombinant *S. mansoni* annexins were structurally folded and showed alpha-helical secondary structure content. The presence of 1 mM calcium (red line) resulted in slight reduction of mean residue ellipticities compared with calcium-free aqueous solution.
Figure 5: Calcium-dependent binding of *S. mansoni* recombinant annexins. A distinct calcium-dependent binding behavior of (A) Annexin B30, (B) Annexin B5a and (C) Annexin B7a to PS/PC (3:1; blue box) and PS/PC (1:3 red circle) is shown in each panel. The membrane binding affinity of each recombinant annexins was enhanced when the recombinant annexins were treated with higher contents of PtdSer (phosphatidylserine) / PtdCho (phosphatidycholine) (PS:PC = 3:1) in the presence of Ca$^{2+}$. Standard error values are displayed on the graph.
Figure 6: Detection of native annexin proteins in *S. mansoni* adult worm extract using Western blot. Parasite extract was probed with (A) pre-immune mouse serum, (B) anti-annexin B30 mouse serum, (C) anti-annexin B5a mouse serum, (D) anti-annexin B7a mouse serum and (E) anti-annexin B5b mouse serum. For each panel, Lane 1 is Soluble Worms Antigen Proteins (SWAP). Lane 2 is Insoluble Worms Antigen Proteins (IWAP). Lane 3 is purified *S. mansoni* recombinant annexin.
Figure 7: Immunofluorescence labeling of *S. mansoni* adult annexins. The adult worms section were stained with corresponding mouse anti-\textit{Sm}-annexin serum and goat anti-mouse IgG-Cy3 with DAPI counterstain. The localization of each annexin member in adult worms is represented by (A) annexin B30, (B) annexin B5a, (C) annexin B7a and (D) annexin B5b. Negative control images are represented by (E) and (F) where sections were probed using pre-immune mouse sera and secondary antibody only, respectively. Immunofluorescence labelling showed the green fluorescence signal corresponding to each annexin was observed in the tegument and in epithelial tissues surrounding the gastrodermis of the adult parasites (red arrowheads). Magnification bar (200 µm) is shown in each panel.
Figure 8: Transmission electron micrographs of *S. mansoni* male adult outer membrane following anti-Sm-annexin immunogold labeling. The distribution of localisation of each annexin member is represented by A) annexin B30, (B) annexin B5a, (C) annexin B7a and (D) annexin B5b. Negative control images are represented by (E) and (F) where the sections were probed with pre-immune mice sera and secondary antibody (Protein A Gold) only, respectively. Gold labeling was observed within tegument (T) basal membrane (BM) and muscle region (M) as shown by the arrows. Magnification bar (1 µm) is shown in each panel.
Figure 9: Immunogenicity analysis of the four homologous recombinant *S. mansoni* annexins with the mice sera infected experimentally with *S. mansoni*. Panel A showing the detection of corresponded recombinant *S. mansoni* annexin by the infected mice sera as indicated by red arrow. Lane P, purified recombinant annexin; Lane 1, uninfected mice sera; Lane 2, infected mice sera. Panel B showing the ELISA quantification of reactivity of each recombinant annexin against both *Sm*-uninfected and *Sm*-infected mice sera. Data shown are means ± standard error mean from 6 mice. Statistical significance was determined with two-tailed Student’s t-test. *P<0.05; **P<0.001.
Figure 10: Cross-reactivity analysis of the four homologous recombinant *S. mansoni* annexins with murine sera infected experimentally with *S. mansoni* or *S. japonicum* (Chinese strain). Quantification for reactivity of each recombinant annexin against *Sm*-infected and *SjC*-infected mice sera was performed using ELISA method. Data output is expressed as fold-change relative to value of uninfected mice sera ± standard error mean from 6 mice per group. Legend represents types of infected mice sera challenged with *S. mansoni* (*Sm*) and *S. japonicum* Chinese strain (*SjC*).
Table 1
Primer sequences used for quantitative PCR

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<th>Amplicon Size</th>
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<td>Annexin B30</td>
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Table 2: Forward and reverse primers used to amplify each homologous full length annexin sequence. Each primer incorporates a specific restriction site (underlined).

Primers containing specific restriction sites at the 5’ and 3’ of the genes were used for the cloning of each *S. mansoni* annexin into pET28a following PCR amplification.

<table>
<thead>
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<th>Gene ID</th>
<th>Accession Number</th>
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<th>Amplicon Size</th>
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Table 3
Summary of *Schistosoma mansoni* homologus annexins characterised in this study.

Four genes encoding annexin expressed in the tegument of *S. mansoni* are designated as annexin B30, annexin B5a, annexin B7a and annexin B5b. The physical and chemical parameters for each protein, as predicted by ProtParam server are shown. GenBank and SchistoDB accession numbers corresponding to *S. mansoni* annexins are listed accordingly. Tyrosine and threonine phosphorylation sites predicted in the N-terminal region of each schistosome annexins are highlighted in dark background.

<table>
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<th>Length (aa)</th>
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<th>pI</th>
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<th>SchistoDB accession no.</th>
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