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.

Keywords: Annexin; *Schistosoma bovis*; Plasminogen; Anticoagulant activity; Tegument.

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 *S. 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 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, 1997). 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 et al., 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, 2009; 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 to 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 et al., 2007).

Regarding schistosome annexins, several proteomic studies have identified them on the tegument surface of *S. mansoni* (Braschi and Wilson, 2006; Castro-Borges et al., 2011) and *S. 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), Hoffmann 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; Catro-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 at al. (2010). Briefly, lambs were infected with 2,000 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 37ºC. 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 1,000 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 37ºC for two hours 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 -80ºC.

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 consensus sequence. This primer was used in two amplifications with two different reverse primers designed, respectively, from the *S. mansoni* and *S. japonicum* annexin sequences (ANXRev1, 5'-TTATTGTTCTTCATTATATATTTC; ANXRev2, 5'- TTATTCACCTAGACCACATAATG).

PCR amplifications were performed in 35 cycles of 94ºC for 40 s, 42ºC for 40 s, and 72ºC for 1 min 30 s for the 5 first cycles, and 94ºC for 40 s, 48ºC for 40 s, and 72ºC for 1 min 30 s for the remaining 30 cycles. PCR products were electrophoresed in agarose gel and the cDNA band corresponding to the ANXFw/ANXRev2 amplification was purified from the gel using the StrataPrep DNA Gel Extraction kit (Stratagene). This PCR product was cloned into the pSC-A vector using the StrataClone PCR Cloning kit (Stratagene), following the manufacturer's instruction, and sequenced on both strands. At least three different clones of the insert were sequenced to check for errors caused by PCR amplification.

2.3. Bioinformatic analysis

The deduced amino-acid sequence of *S. bovis* annexin (SbANX) was analyzed as follows: BLAST searching of the most similar sequences were conducted in the Swissprot/Uniprot and SchistoDB databases (http://www.uniprot.org/ and

http://www.genedb.org/Homepage/Smansoni); analysis of conserved domains was performed using SMART at http://smart.embl-heidelberg.de; theoretical isoelectric point (pI) and molecular weight (MW) calculations at http://www.expasy.org/tools/pi_tool.html; prediction of transmembrane helices using the TMHMM Server v. 2.0 at http://www.cbs.dtu.dk/services/TMHMM-2.0; prediction of signal peptides with SignalP 3.0 (Bendtsen et al., 2004) at http://www.cbs.dtu.dk/services/SignalP, and search for glycosyl-phosphatidyl anchors in the sequence with the big-PI Predictor (Eisenhaber et al., 2000) at http://mendel.imp.ac.at/sat/gpi/gpi_server.html.

The amino acid sequence and secondary structure of the SbANX were compared to those of four parasite and human annexins known to possess fibrinolytic or anticoagulant activities, namely, the *Taenia solium* B1 and B2 (ANX B1, ANX B2) and the human A2 and A5 (ANX A2, ANX A5). Multiple sequence alignment of all these five annexins was done with ClustalW 2.1 at http://www.ebi.ac.uk/Tools/msa/clustalw2/. For the prediction of the secondary structures and three-dimensional modelling, the amino acid sequences were submitted to the Swiss-Model server (Arnold et al., 2006) at http://swissmodel.expasy.org/. The 3-D models were visualized using the Pymol package (DeLano, 2002).

2.4. Expression and purification of recombinant annexin and polyclonal antibody production

The full-length cDNA sequence of SbANX was subcloned into the expression vector pQE-30 (Qiagen) and expressed in *Escherichia coli* M15 cells (Qiagen). For this, two new primers were designed from the *S. bovis* complete annexin sequence that contained KpnI adapters to assist in the subclonning (sense primer; 5´-

193 GGGGTACCATGGCTAATGTTTCTGAATTTGG; antisense primer, 5´-
194 GGGGTACCTTATTCACCAAGTAGAACAC) (underlined letters represent the
195 restriction enzyme sites). The complete cDNA coding sequence of *S. bovis* annexin was
196 amplified from the SbANX-pSC-A construction. PCR amplification was accomplished
197 under the following conditions: 35 cycles of 94ºC for 15 s, 64ºC for 30 s and, 72ºC for
198 40 s. The amplified product was purified using the DNA Gel extraction kit (Stratagene)
199 and digested with KpnI restriction endonuclease (Roche). After digestion, the PCR
200 product was purified again from agarose gel and ligated to the KpnI predigested pQE-30
201 vector. This construct was used to transform *E. coli* M15 cells. Single recombinant
202 clones were selected and plasmid DNA extracted and sequenced to confirm the presence
203 and the correct orientation of the SbANX cDNA insert.

204 Expression was then induced in correctly transformed *E. coli* M15 cells by
205 adding isopropyl β-d-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at
206 37ºC for 3h. The induced cells were harvested and lysed by sonication in a lysis buffer
207 containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.9. After a 15 min
208 centrifugation step at 40,000g, the lysate pellet was solubilised in binding buffer
209 containing 100 mM NaH_2PO_4, 10 mM Tris-Cl, and 8M urea, and re-centrifuged as
210 above. The new supernatant was passed through His-Bind resin (Qiagen) according to
211 the manufacturer's instructions. Before elution of the recombinant, urea was eliminated
212 by washing the column with wash buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 6.3)
213 containing decreasing concentrations of urea (6M, 4M, 2M). Then, the recombinant
214 protein was eluted with elution buffer (250 mM imidazole, 0.5 M NaCl, 50 mM
215 NaH_2PO_4, pH 7.9). The eluted rSbANX was dialysed against PBS for 24 h at 4ºC and
216 stored at -80ºC until use. The concentration of the recombinant protein was measured
217 using the DC Protein assay kit (Bio-Rad) and its purity was checked by SDS-PAGE.

Once the recombinant protein had been obtained, and in order to have a specific probe for immunofluorescence studies, a polyclonal rabbit serum against *S. bovis* annexin (rSbANX) was obtained. To accomplish this, two rabbits (New Zealand) were immunized subcutaneously with three doses of 100 μg of rSbANX, together with Freund's adjuvants. The first dose was administered with the complete adjuvant, the second one with incomplete adjuvant and the third one only in PBS. The rabbits were bled at 15 days after the third dose and the antibody titre was determined by ELISA, using a standard protocol (Oleaga-Pérez et al., 1994).

The reactivity and specificity of the polyclonal serum was tested by Western blotting against rSbANX and a tegument extract (TG) from adult worms following the protocol described by De la Torre-Escudero et al (2010). In this case the anti-rSbANX rabbit serum was used at a dilution of 1/1,600 and the peroxidase-conjugated anti-rabbit antibody (Sigma) was diluted at 1/1,000.

2.5. Bioassays with rSbANX

2.5.1. Plasminogen binding assays.

The binding of rSbANX to plasminogen was assessed by ELISA in a similar way to that described for the *S. bovis* enolase (De la Torre-Escudero et al., 2010). Briefly, plate wells were coated with 0.5 μg of rSbANX and non-specific binding sites were blocked with 1% BSA in PBS (PBS-BSA). After washing, the different wells were incubated with increasing amounts (from 0 to 3 μg) of human plasminogen (Acris Antibodies) diluted in PBS-BSA. After a new wash, the wells were incubated with peroxidase-conjugated goat anti-human plasminogen IgG (Cedarlane Laboratories) diluted 1/2,000 in PBS-BSA and finally ortho-phenylenediamine (OPD) was used as a

chromogenic substrate for peroxidase. The plate included negative control wells, coated only with BSA, and positive control wells, coated with 1 μg of TG extract.

To assess whether plasminogen binding to rSbANX occurs through lysine residues, a similar assay was performed in which the plate wells were coated with 0.5 μg of rSbANX and incubated with 0.5 μg of plasminogen and increasing concentrations (from 0 to 60 mM) of ε-aminocaproic acid (εACA, Sigma). εACA is a lysine analogue that competitively inhibits plasminogen from binding to its receptor. Then, bound plasminogen was developed by incubating with peroxidase-conjugated goat anti-human plasminogen IgG and OPD. Similarly, BSA-coated wells were used as negative controls.

2.5.2. Plasminogen activation assays.

The effect of rSbANX on plasminogen activation and plasmin generation was evaluated by measuring the amidolytic activity of newly generated plasmin on the chromogenic substrate D-Valyl-L-leucyl-L-lysine 4-nitroanilide dihydrochloride (Sigma). This assay was performed on ELISA plates according to the protocol described previously (Mundodi et al., 2008).

In each plate well, 2 μg of plasminogen, 3 μg of chromogenic substrate, 1 μg of rSbANX, and 15 ng of tissue-type plaminogen activator (t-PA) (Acris antibodies) were mixed in a total volume of 150 μl of PBS and incubated at room temperature. In a parallel assay, plasmin generation was measured in the absence of t-PA, in the absence of rSbANX, and substituting annexin by BSA or by the buffer in which the rSbANX had been dissolved. Plasmin generation was monitored by quantifying the hydrolysis of the substrate through absorbance measurements at 405 nm every 30 min over two hours

as from the time the reaction began. Each reaction was analysed in quadruplicate and the whole assay was repeated three times.

2.5.3. Prothrombin time and activated partial thromboplastin time of rSbANX.

The anticoagulant activity of rSbANX was analysed in two coagulation tests for general screening such as the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) tests. The PT and aPTT tests respectively detect disturbances in the extrinsic and intrinsic coagulation pathways. These tests measure the time elapsed between the addition of the corresponding coagulation initiation reagent (STA neoplastin plus or STA aPTT; Roche Diagnostics) to the plasma sample and clot formation. The PT results can also be expressed as a percentage of the normal activity.

To carry out these tests, plasma samples were obtained from lamb blood extracted in polypropylene tubes containing sodium citrate. The samples analysed consisted of 500 µl of plasma with increasing amounts of rSbANX (from 0 to 80 µg/ml). Two series of control samples were included in the analysis: one series contained BSA instead of rSbANX, and the other one contained no protein but equivalent volumes of the buffer in which the rSbANX had been dissolved.

Each sample was analysed in duplicate and the assays were repeated twice. Samples were analysed with the STA-R Evolution® analyser (Diagnóstica Stago, Inc.).

2.6. Immunolocalization of annexin in adult worms and lung-stage schistosomula

Tissue expression of annexin was determined in adult worms and schistosomula by indirect immunofluorescence and later analysis by confocal microscopy. The study was carried out on sections of adult worms, on whole adult worms, and on 6-day old

lung schistosomula. The worms were fixed in formaldehyde buffered at 4% for either 5 h (schistosomula) or 24 h (adults).

For assays on adult sections, the worms were dehydrated and embedded in paraffin following standard protocols. Microtome-cut 5-μm sections were placed on microscope slides, deparaffinised in xylene, and rehydrated. The sections were then blocked with 1% BSA in PBS containing 0.05% Tween 20 (PBST) for 1 h at 37ºC, after which they were incubated with the anti-rSbANX rabbit serum diluted 1/50 in blocking buffer for 1 h at 37ºC. Samples were washed three times with PBST and incubated at 4ºC overnight with an anti-rabbit IgG antibody conjugated to Alexa Fluor 594 (Invitrogen) diluted 1/400 in blocking buffer containing phalloidin-Alexa Fluor 488 (Invitrogen) diluted 1/200, which binds to actin microfilaments. The samples were then washed four times and mounted in antifade reagent (Prolong Gold, Invitrogen). All incubations were performed in a humid chamber.

A similar protocol was followed for whole-mount assays. The reactions were performed in 1.5-ml test tubes containing 100 schistosomula or 5 adult pairs each. Fixed parasites were blocked for 2 h at room temperature, incubated with the anti- rSbANX serum overnight at 4ºC, and then with the above-mentioned Alexa Fluor reagents, both at a 1/300 dilution, for 4 h at room temperature. Whole parasites were washed five times and then mounted in antifade reagent (schistosomula) or in PBS (adult worms). In each assay, serum from a non-immunized rabbit was used as a negative control. Samples were analysed with a Leica TCS-NT confocal microscope.

2.7. Statistics

The results of the PT and aPTT coagulation tests were first analysed with the one-way ANOVA test, and when significant differences were found a *post hoc* analysis

316 with the HDS Turkey test was conducted to determine the causes of the significance. A
317 similar procedure was applied to check whether there were any significant differences
318 as a function of the amount of rSbANX added to the plasma samples and, if so, to
319 establish which rSbANX concentration provided significantly different results.
320
321 **3. Results**
322
323 *3.1. Cloning and in silico characterization*
324 The ANXFw/ANXRev2 primer pair amplified a 1080 bp cDNA fragment
325 compatible with the expected size for a schistosome annexin and an open reading frame
326 coding for a protein of 359 amino acids, with a predicted MW and pI of 40,663 Da and
327 5.85, respectively (GenBank accession number EU595758).
328 BLAST searching of the SWISS-Prot database with the deduced amino acid
329 sequence of SbANX retrieved numerous annexins from different species. *S. bovis*
330 annexin showed the highest identity percentages with the *S. mansoni* (Q9XY89) and *S.*
331 *japonicum* (AAW25344) annexins (91% and 86%, respectively). Similarly, BLAST
332 searching of the SchistoDB database retrieved several annexin sequences including the
333 14 annexins sequences of *S. mansoni* reported by Hofmann et al. (2010). Among them,
334 the sequence showing the highest identity (91%) was Sm_074150, which corresponds to
335 sequence Q9XY89 from the SWISS-Prot database; the remaining 13 sequences
336 retrieved showed lower identities (from 37% to 23%).
337 Regarding non-schistosome helminths, SbANX showed the greatest identity to
338 *Taenia solium* ANX B1 and B2 (42% and 36%, respectively). Finally, SbANX showed
339 between 37% and 23% sequence identity to human annexins A1-A13, in particular 37%
340 to ANX A5 and 32% to ANX A2.

Fig. 1A shows the alignment of SbANX with *T. solium* B1 and B2 and human A2 y A5 annexins. The SbANX contained a 309-amino acid core domain and a 50-amino acid tail at its N-terminus. The core domain contained the four typical annexin repeats and each repeat harboured type II and III Ca^{2+}-binding sites (Fig. 1A, yellow-shaded and underlined residues, respectively). Type II calcium-binding sites were characterized by the M-K/R-G/R-X-G-T-(38 residues)-D/E sequence motif, and type III sites by the G-X-G-T-D/E sequence motif. In SbANX, each annexin repeat contained between 52 and 60 amino acid residues, mapping in the following sequence segments: 51-103, 123-183 229-281 and 304-356 amino acid. The *E*-values for the four annexin repeats ranged from $2.70e^{-20}$ to $113e^{-04.}$ (Fig. 1A).

The SbANX contains the KGLGT sequence motif within repeat II of the core domain as *T. solium* ANX B1 and human A2 annexins. In human A2 annexin, this motif and the aspartic acid located at position162 (D^{162}) appear to be required for the interaction of annexin with cell surface phospholipids (Fig. 1A) (Hajjar and Krishnan, 1999).

The SbANX also had the $^{100}LCQL^{103}/^{114}SL^{115}$ sequence motif, which showed 50% identity with the $^{8}LCKLSL^{13}$ sequence motif of human ANX A2. This motif is responsible for the interaction between human ANX A2 and t-PA (Hajjar et al., 1998). A similar motif ($^{38}LCK^{40}/^{69}SL^{70}$) is also displayed by the *T. solium* ANX B2. In Fig. 1B, the above-mentioned motif is highlighted only in the 3D model of SbANX, but not in that of ANX A2 because this molecule was modelled from the 32^{st} amino acid onwards not including the N-terminus fragment in with the motif is located.

SbANX and *T. solium* ANX B1 and B2 have an insert fragment in the linker region between repeats II and III, which is absent from human annexins (Figs. 1A and 1B).

In silico secondary structure prediction revealed that SbANX was mainly made up of α-helices although four beta sheets are also included. The model does not predict secretory signal, transmembrane helices or glycosyl-phosphatidyl inositol anchors. The secondary and tertiary structures of these annexins are highly conserved even though their amino acid sequence identity only ranges between 32% and 42% (Fig. 1). The alignment in Fig. 1A shows that the four typical annexin repeats and the predicted α-helices match in the five sequences. In Fig. 1B it can be observed that the four repeats are packed in a structure that resembles a flattened disc, with a slightly convex surface on which the Ca^{2+}-binding loops are located, together with a concave surface where the amino and carboxyl termini come into close opposition.

3.2. Expression and purification of recombinant S. bovis annexin

The full length SbANX cDNA was sub-cloned into the expression vector pQE-30 and transformed into M15 *E. coli* host cells. The his-tagged recombinant protein (rSbANX) was expressed abundantly, although it was 100% insoluble. After solubilisation and purification under denaturing conditions, 5.7 mg of protein per litre of cell culture was obtained. The purified rSbANX migrated as a single band of 39 kDa by SDS-PAGE.

3.3. Plasminogen binding and activation assays

The plasminogen-binding assays showed that plasminogen bound to rSbANX and that the amount of plasminogen bound increased with the rising amount of plasminogen added to the reaction medium. Likewise, in the plate wells in which tegument extract (TG) was included as a positive control in the assay the binding of the plasminogen to such proteins was observed. In the negative control wells, coated only

391 with BSA, unspecific binding of plasminogen did not occur (Fig. 2A). Competition
392 experiments with εACA revealed that plasminogen binding occurred through lysine
393 residues, and that 10 mM of εACA completely inhibited the binding of plasminogen to
394 rSbANX (Fig. 2B).

395 Fig. 3 shows the results of the assays on plasminogen activation, in which it may
396 be seen that rSbANX enhanced plasminogen activation in the presence of t-PA. It can
397 also be observed that rSbANX was unable to activate plasminogen and generate
398 plasmin in the absence of t-PA. In the controls, in which rSbANX was replaced by BSA
399 or by buffer, no reactivity was observed (not shown).

400

401 *3.4. Anticoagulant activity*

402 To investigate the anticoagulant effect of rSbANX, we evaluated the inhibition
403 of blood clotting in PT and aPTT assays (Figs. 4A and 4B).

404 Both assays revealed significant differences ($p < 0.01$) between the samples with
405 rSbANX and the controls with BSA or buffer. Additionally, there were no significant
406 differences between either type of control sample (those containing BSA or buffer).

407 The coagulant activity in the PT assay was lower in the plasma samples with
408 rSbANX than in the controls and clearly decreased as the concentration of annexin
409 increased. This difference was statistically significant ($p < 0.01$) in the samples
410 containing a concentration of 80 µg/ml of annexin with respect to the controls without
411 it. Likewise, coagulation time increased in parallel with the concentration of rSbANX.
412 This increase in coagulation time was significant at rSbANX concentrations equal to or
413 higher than 60 µg/ml (Fig. 4A).

414 The anticoagulant effect of rSbANX was considerably stronger in the aPTT
415 assay. In this assay, the coagulation time was significantly longer ($p < 0.01$) as from an
416 rSbANX concentration of 10 µg/ml in plasma (Fig. 4B).
417
418 *3.5. Reactivity and specificity of the anti-rSbANX rabbit hyperimmune sera*
419 The reactivity of the anti-rSbANX hyperimmune serum was tested in ELISA
420 against the recombinant protein prior to its use in the immunolocalization assays. The
421 antibody titre was 1/1,600. The specificity of the serum was assessed by Western
422 blotting against the rSbANX and the native TG extracts from *S. bovis* adult worms. As
423 shown in Fig. 5, the anti-rSbANX serum reacted strongly with the recombinant protein
424 and specifically recognized the native annexin in the TG extract. The negative serum
425 showed no reactivity with any of the proteins tested.
426
427 *3.6. Immunolocalization of S. bovis annexin*
428 Annexin localization in the parasite was analyzed in paraffin sections from adult
429 worms, as well as in intact adult worms and 6-day-old lung schistosomula.
430 The schistosomula incubated with the negative rabbit serum plus phalloidin (an
431 actin ligand) only resulted in green fluorescence, indicating a lack of non-specific
432 reactivity. The schistosomula incubated with the specific anti-rSbANX serum plus
433 phalloidin showed actin reactivity in green, as well as the specific annexin reactivity
434 (red) distributed on the tegument surface (Fig. 6). This figure shows the images
435 obtained at each of the laser wavelengths -594 (red) and 488 (green)- since the merged
436 image was difficult to interpret due to the co-localization of the specific reaction and the
437 actin fluorescence.

Paraffin-embedded sections from adult worms incubated with the negative serum plus phalloidin resulted in green fluorescence on the outermost part of the tegument. Sections of male and female worms incubated with the specific anti-rSbANX serum plus phalloidin showed besides the actin reactivity in green the specific annexin reactivity, in red, abundantly distributed in internal tissues and also in the tegument surface. In male tegument, the red signal appears as discontinuous patches (arrows) and in female tegument, apparently, as a more continuous signal (Fig. 7A).

Whole adult worms incubated with negative serum only showed the green florescence from actin. By contrast, adult worms incubated with the anti-rSbANX serum showed a red fluorescence pattern on the outermost part of the tegument. In males this pattern consisted of low abundant scattered tiny patches, and in females these patches were similarly distributed but more abundant (Fig. 7B).

4. Discussion

It is well known that schistosomes have adapted to the intravascular habitat of their hosts by developing mechanisms to modulate the haemostatic response. However, despite the relevance of the molecules involved in the biology of the parasite and their potential interest as vaccine antigens, these haemostatic molecules have aroused little attention.

This prompted us to undertake studies of the interaction of *S. bovis* with the host's haemostatic system. In previous work, we observed that adult worms activate the fibrinolytic system, presumably as a strategy to prevent the formation of clots around them, and that they did this through protein receptors for plasminogen expressed on the surface of their tegument, one of which has been determined to be enolase (Ramajo-

Hernandez et al., 2007; De la Torre-Escudero et al., 2010). Here we wished to determine whether the annexin of *S. bovis* possesses any anti-haemostatic activity. In this sense, for some time it has been known that other annexins, such as the human A2 and A5 and the B1 and B2 annexins of *T. solium* show anticoagulant and fibrinolytic activities (Hajjar et al., 1999; Gerke and Moss, 2002; Wang et al., 2006; Winter et al., 2006). So we cloned and sequenced the *S. bovis* annexin cDNA and obtained a peptide sequence of 359 amino acids that displayed structural characteristics typical of the annexin family. That is, a core domain containing four typical annexin repeats characterized by alpha-helices and a variable N-terminal region (Gerke and Moss, 2002). In this way, we confirmed the fact, already observed in vertebrate annexins, that the secondary and tertiary structures of annexins are highly conserved despite not showing high amino acid sequence identity among themselves (Moss and Morgan, 2004).

One particularity of the SbANX sequence, that human annexins do not have, is the long linker region between repeats II and III. This characteristic is also seen in other schistosome annexins and in those of *T. solium* (Wang et al., 2006; Hofmann et al., 2010; Tararam et al., 2010). As reported by Hofmann et al. (2010), these unique structural features combined with the immunogenic properties of several parasite annexins and unique epitopes not present in mammalian annexins may provide an opportunity to pursue these antigens as vaccine targets while preventing cross-reactivity with host annexins.

Sequence analysis also revealed that SbANX lacks motifs for its transport or expression on the cell surface (signal peptide, transmembrane motif or GPI anchors), even though it is present on the surface of schistosomula and adult worms. This is not an isolated phenomenon since the same occurs with other annexins, and in particular

488 with human A2 annexin, which is constitutively expressed on the endothelial cell
489 surface and the mechanism of its export from the cell is unknown. It has been described
490 that human annexin A2 interacts with cell surface phospholipids via a calcium-
491 dependent binding site that includes ^{119}KGLGT^{123} residues and the coordinating D^{162} of
492 core repeat 2 (Hajjar and Krishnan, 1999). In the *S. bovis* annexin we found the same
493 motif in residues 120-124 (^{120}KGLGT^{124}) as well as a D^{167}. It is therefore possible that
494 this motif could be responsible for the interaction between the *S. bovis* annexin and the
495 phospholipids of the plasma membrane at the surface of the tegument of the worms,
496 although confirmation of this point requires additional studies.

497 The plasminogen binding and activating assays indicated that the *S. bovis*
498 annexin, like the human A2 annexin (Hajjar et al., 1998), had fibrinolytic activity. It
499 was observed that rSbANX binds plasminogen through lysine residues and that it
500 enhanced t-PA mediated plasminogen activation. This is the first time that
501 profibrinolytic activity has been demonstrated in an annexin of parasite origin, and
502 particularly in a schistosome species.

503 Human A2 annexin interacts with t-PA through the ^{8}LCKLSL^{13} motif present at
504 the N-terminal end (Hajjar et al., 1998). In the rSbANX sequence, a similar motif has
505 been identified with an identity of 50% (^{100}LCQL^{103}/^{114}SL^{115}). However, despite this
506 similarity it does not seem likely that this motif would be responsible for the interaction
507 with t-PA since, as seen from the three-dimensional model of rSbANX, it is localized in
508 a zone of the molecule that is not very accessible (Fig. 1B).

509 The A5 extracellular annexin shows anticoagulant properties that depend on its
510 Ca^{2+}-regulated binding to anionic phospholipids; those exposed on the surface of
511 activated platelets or endothelial cells. In the presence of Ca^{2+} annexin competitively
512 binds to phospholipids with very high affinity and disturbs the form of some activated

coagulation complexes (Gerke and Moss, 2002). Regarding this type of activity, in known parasite annexins anticoagulant properties have only been demonstrated for the annexins B1 and B2 of *T. solium* (Wang et al., 2006). In *S. bovis*, the results of the PT and aPTT assays clearly demonstrate that rSbANX inhibits the extrinsic coagulation pathway and, more strongly, the intrinsic pathway. Inhibition of the extrinsic coagulation pathway was achieved with high concentrations of rSbANX (60-80 µg/ml), which were higher than those of *T. solium* annexin B2 producing the same effect (40 µg/ml); by contrast, inhibition of the intrinsic pathway was achieved with quite lower rSbANX concentrations (10 µg/ml) as compared to *T. solium* annexin B1 (60 µg/ml) (Wang et al., 2006).

Thus, this would be the first demonstration of anticoagulant activity by an annexin from a Schistosoma species. Additionally, bearing in mind the different functions of vertebrate annexins, it cannot be ruled out that *S. bovis* annexin could have additional activities, in particular some type of immunomodulatory activity, as has been demonstrated for the B1 *T. solium* annexin (Gao et al., 2007; Yan et al., 2008).

It is evident that for these anticoagulant and fibrinolytic activities of SbANX to have relevant physiological roles, the SbANX should be exposed on the surface of the worm into contact with the host blood. As reported in different studies addressing the tegument of schistosomes, annexin has been identified on the tegument surface of adult worms and schistosomula of *S. mansoni* and S. *japonicum* (Braschi and Wilson, 2006; Tararam et al., 2010; Castro-Borges et al., 2011). The SbANX immunolocalization studies performed in adults and schistosomula of *S. bovis* indicate that both developmental stages express this protein on the surface of their tegument. The schistosomula of *S. bovis* shows an annexin expression pattern similar to that of *S. japonicum* schistosomula, whereas on the surface of *S. bovis* adults the annexin is

538 expressed less abundantly than in *S. japonicum* adult worms (Tararam et al., 2010).
539 Additionally, the SbANX described in this work is homologous to Smp_074150. This *S.*
540 *mansoni* annexin was detected by mass spectrometry of material released by trypsin
541 shaving of live *S. mansoni* (Castro-Borges et al., 2011), providing additional support to
542 the notion that SbANX, as Smp_074150, might also be surface-located.

543 Bearing in mind these results, it is somewhat surprising that annexin, despite
544 being expressed on the surface of the tegument and also found in the secretion-excretion
545 products, should not be immunogenic in natural infections by *Schistosoma* (Mutapi et
546 al., 2005; Pérez-Sánchez et al., 2006). It would be interesting to test if vaccination with
547 this protein will lead to its recognition by the host immune system allowing the
548 evaluation of its protective potential against infections in ruminant schistosomiasis.

549 In sum, here we have cloned, sequenced and characterized a *S. bovis* annexin.
550 We show that, *in vitro*, the corresponding recombinant protein displays fibrinolytic and
551 anticoagulant activities; that the native protein is expressed on the tegument surface of
552 adult worms and schistosomula, and that it is therefore in direct contact with the host's
553 blood. All this suggests that this annexin could be used by *S. bovis*, together with other
554 tegument proteins of proven fibrinolytic activity, such as enolase, to prevent thrombus
555 formation and other haemostatic disturbances that could be lethal for the survival of the
556 parasite in the bloodstream. The anti-haemostatic activities observed, and other potential
557 immune modulatory functions make the *S. bovis* annexin and its homologues promising
558 antigenic targets for development of new anti-schistosome vaccines.
559

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677

678 **Figure legends.**

679

680 Figure 1. (A) Alignment of the SbANX (ACC78610) with annexins B1 (AAD34598)
681 and B2 (AAY17503) from *Taenia solium* and human A2 (AAH09564) and A5
682 (NP_001145). The conserved amino acids are labelled with asterisks; the conservative
683 and semi-conservative substitutions are labelled with two and one points, respectively.
684 Predicted α-helices are indicated in grey and β-sheets in green. The four-repeat domains
685 of the annexin sequence are indicated with a horizontal line over the conserved zone.
686 The type II Ca^{2+}- binding sites are shaded in yellow, and the type III Ca^{2+}- binding sites
687 are underlined. The t-PA-binding motif of human A2 annexin and other similar motifs
688 identified in the SbANX and in the *T. solium* B2 annexin are in red. The phospholipid-
689 binding motif of the cell surface identified in the human A2 annexin is boxed in. (B),
690 Molecular modelling of *S. bovis* annexin and human A2 annexin. The variable N-
691 terminal end is in black; and the annexin repeat domains I, II, III and IV are in red,
692 yellow, orange and blue respectively. The insert fragment in the linker region between
693 repeats II and III is in green.

694

695 Figure 2. (A) Plasminogen-binding assay to 0.5 μg of rSbANX and incubation with
696 increasing amounts of plasminogen, 0-3 μg (♦). Positive control using 1 μg of tegument
697 extract (TG) instead of rSbANX (**X**). Negative control coated with BSA (■). (B)
698 Competition assay. Wells coated with 0.5 μg of rSbANX (♦) or BSA (■) were
699 incubated with 0.5 μg of plasminogen and increasing amounts of εACA. Each point is
700 the mean of three replicates ± standard deviation.

Figure 3. Plasminogen activation assay with rSbANX or t-PA alone, rSbANX together with t-PA, and the negative control with the reaction substrate alone. Each point is the mean of four replicates ± standard deviation. The experiments were performed three times.

Figure 4. Coagulation assays in plasma samples with increasing concentrations of rSbANX (0-80 µg/ml)**.** (A) Prothrombin time assay (PT) expressed in time and percentage of activity. (B) Activated partial thromboplastin time (aPTT). (■) rSbANX, (❑)BSA and (□) buffer. Each point is the mean of two replicates ± standard deviation. The experiments were performed two times. * $p < 0.001$.

Figure 5. Western blot for study of the specificity of an anti-rSbANX serum on the rSbANX and a tegument extract (TG) from *S. bovis* adult worms. 1, negative control sera; 2, anti-rSbANX rabbit serum at 1:1,600 dilution.

Figure 6. Immunolocalization of *S. bovis* annexin in lung-schistosomula. Confocal microscope images of schistosomula after incubation with phalloidin-Alexa Fluor 488 (green) plus the negative or the anti-rSbANX rabbit sera and an anti rabitt IgG-Alexa Fluor 594 (red). Magnification 600x.

Figure 7. Immunolocalization of *S. bovis* annexin in adult worms. (A) Images of parasite sections, (B) magnification of the boxes highlighted in (A) showing the male and female tegument, and (C) images of whole parasites. Both, the sections and whole parasites were incubated with phalloidin-Alexa Fluor 488 (green) plus the negative or

725 the anti-rSbANX rabbit sera and an anti-rabbit IgG-Alexa Fluor 594 (red). The upper
726 row of panels (A) and (C) shows the merged projected images derived from sections
727 generated by the confocal microscope and the lower row shows the corresponding
728 transmitted light images. Magnification 600x.
729
730
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732

Figure 1

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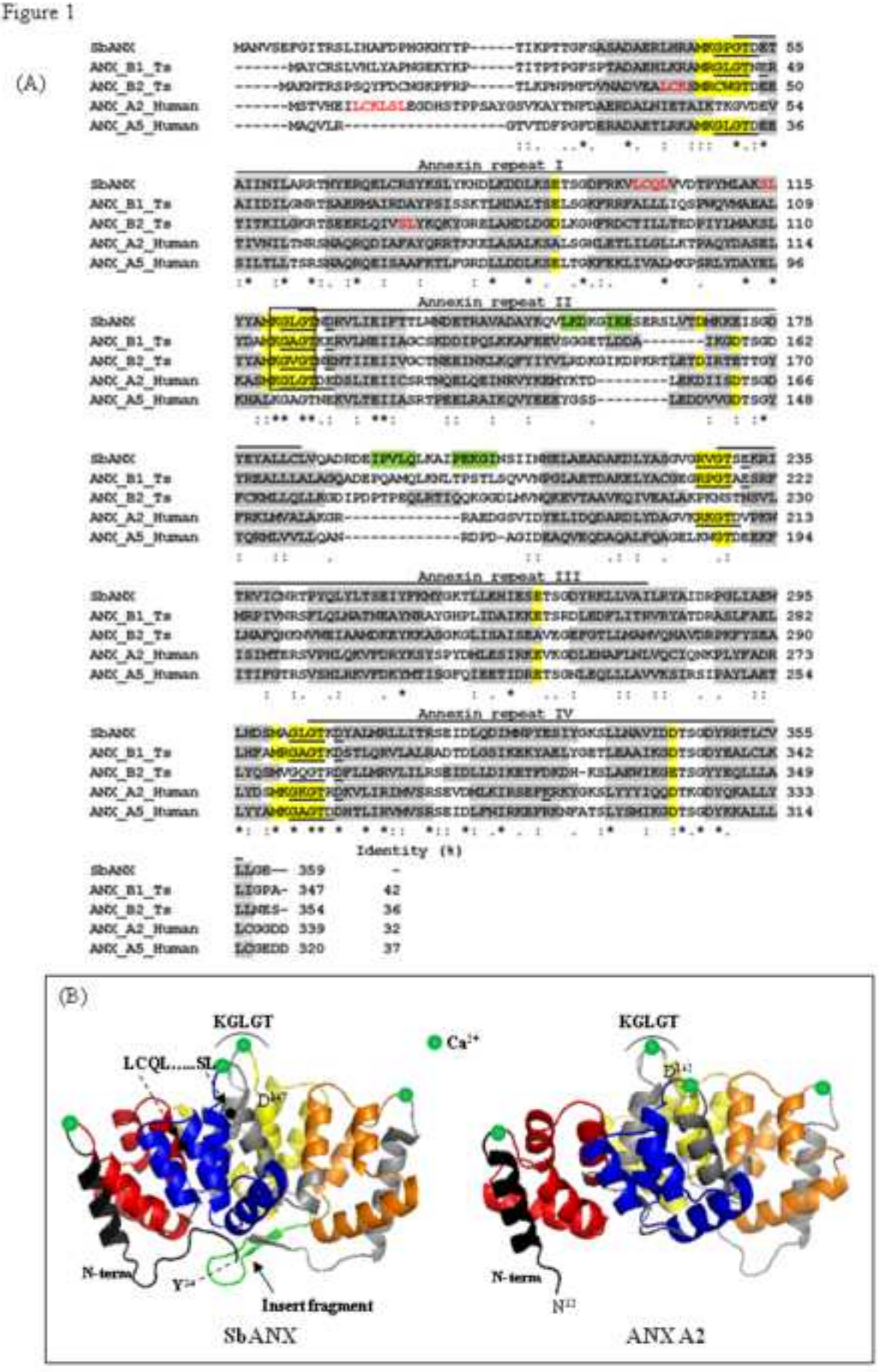

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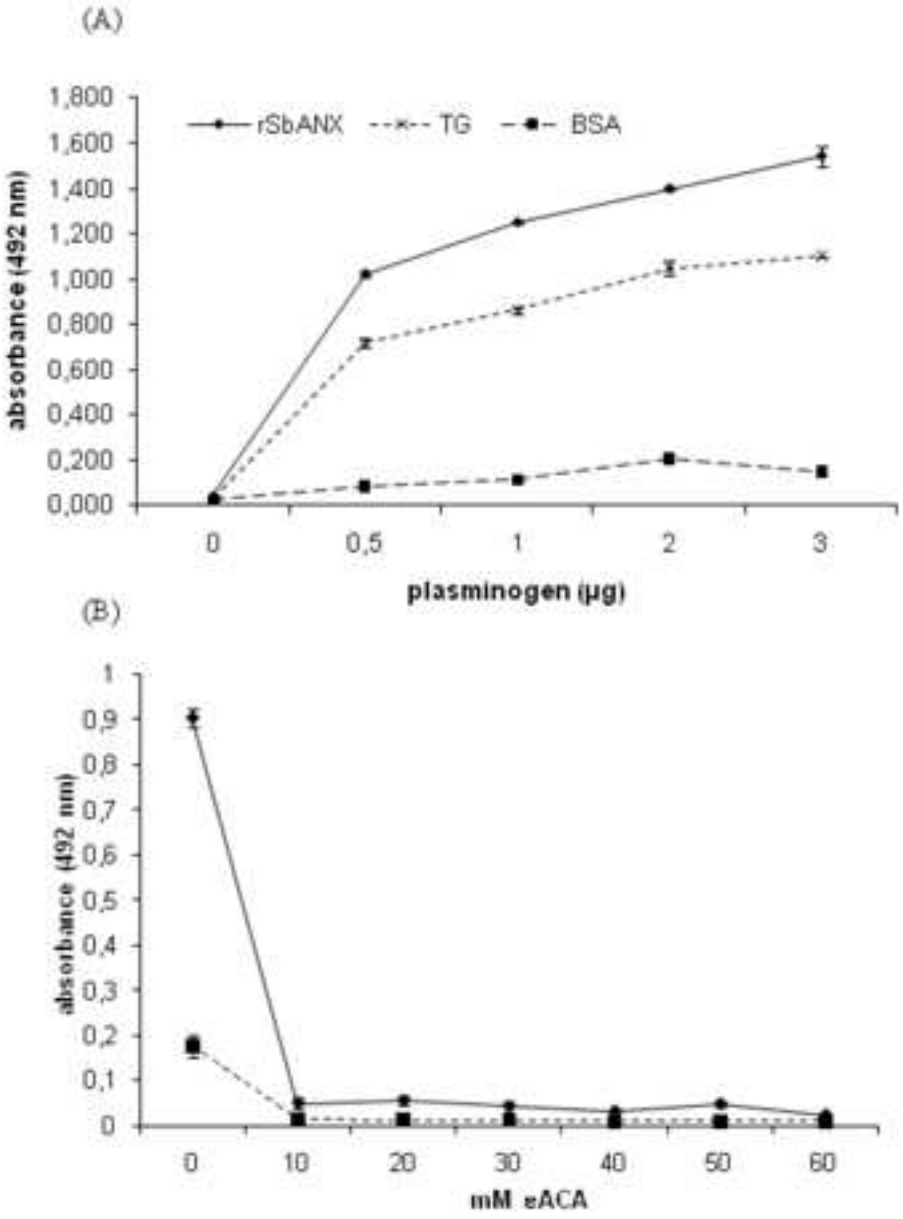

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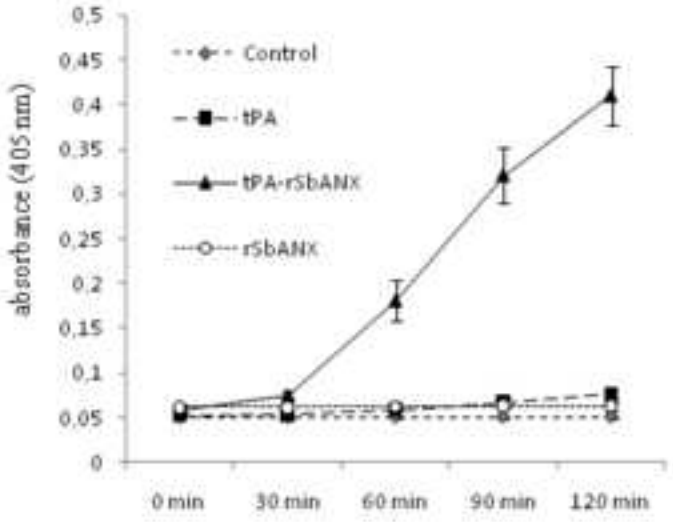

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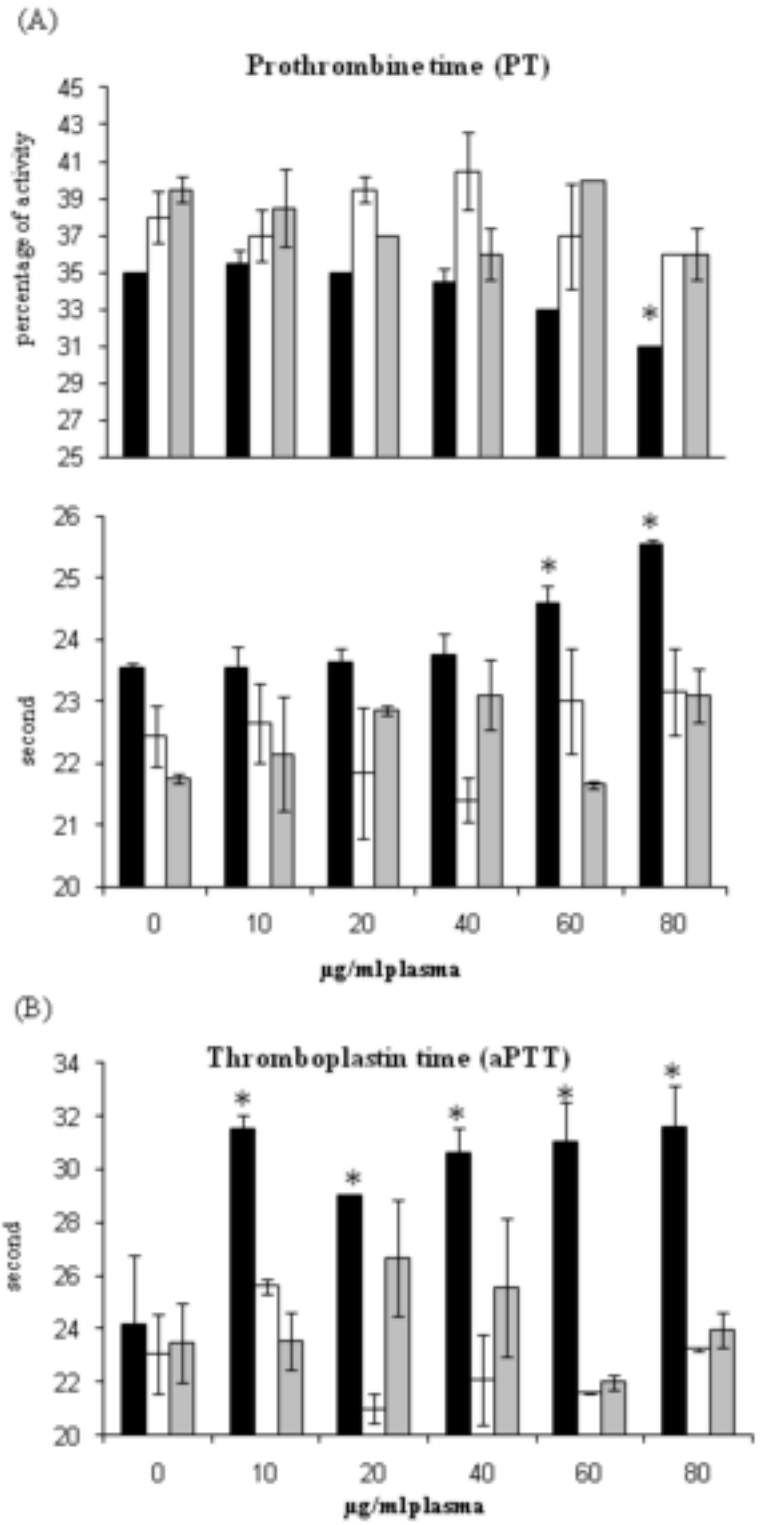

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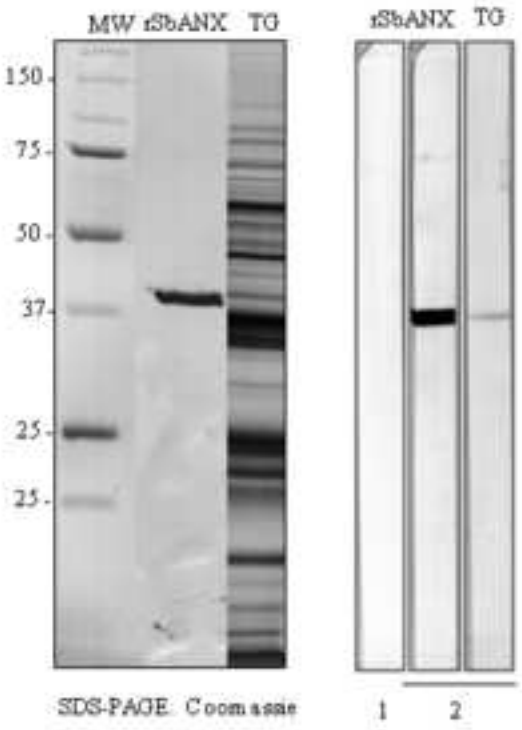

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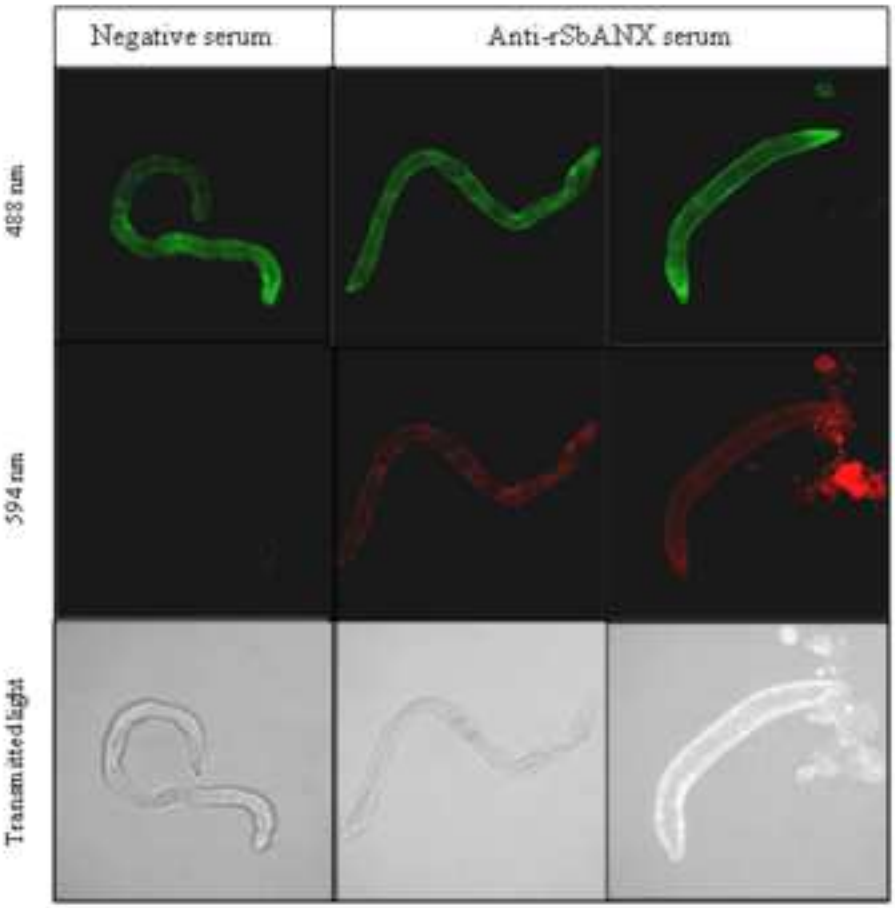

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