Characterization of a Secretory Annexin in *Echinococcus granulosus*

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Abstract. Cystic echinococcosis, caused by *Echinococcus granulosus*, is a widespread parasitic zoonosis causing economic loss and public health problems. Annexins are important proteins usually present in the plasma membrane, but previous studies have shown that an annexin B33 protein of *E. granulosus* (Eg-ANX) could be detected in the excretory/secretory products and cyst fluid. In this study, we cloned and characterized Eg-ANX. In silico analysis showed that the amino acid sequence of Eg-ANX was conserved and lacked any signal peptides. The phospholipid-binding activity of recombinant Eg-ANX (rEg-ANX) was tested; liposomes could bind to rEg-ANX only in the presence of Ca^{2+} . In addition, we performed western blotting and immunohistochemical analyses to further validate the secretory properties of Eg-ANX. The protein could be detected in the cyst fluid of *E. granulosus* and was also present in the intermediate host tissues, which suggested that Eg-ANX might play an important role in parasite–host interaction.

INTRODUCTION

Echinococcus granulosus is a tapeworm with a two-host life cycle. Dogs and other canids are the definitive hosts and humans and domestic animals are the intermediate hosts for the metacestode (larval) stage. *Echinococcus granulosus* is the causative agent of cystic echinococcosis (CE), which is a substantial cause of morbidity and mortality in many parts of the world.^{1–3} This zoonosis is characterized by long-term growth of CE cysts in humans and domestic animals; more than 90% occur in the lung, liver, or both organs.⁴ The global burden of human CE exceeds 1 million disability-adjusted life years and losses are estimated at US\$760 million a year. The livestock-associated losses range from US\$1.2 billion to US\$2.1 billion.⁵ The mortality rate of CE is about 2–4% in humans, and it may increase notably with poor treatment and care.^{6,7} Hence, CE remains an important zoonosis and public health threat.

Annexins belong to a multigene family, are widespread in all eukaryotes and are characterized by their ability to bind reversibly to phospholipids in a Ca^{2+} -dependent manner. Structurally, annexins contain a variable N-terminal domain and a conserved core C-terminal domain.⁸ The C-terminal domain contains four repeat domains, and each repeat generally has type II and/or type III Ca^{2+} -binding sites. Annexins are often associated with the plasma membrane, inner membrane, and cytoskeletal proteins, playing key roles in a broad range of important biological processes such as inflammatory response, apoptosis, signal transmission, membrane fusion and exocytosis, anticoagulation, ion channel regulation, and cell migration.^{8–11} In parasites, some annexins were considered to protect the parasite from structural breakdown during parasitism and to regulate the immune responses of the hosts, which is linked to parasite survival.^{12,13} For *Taenia solium* and *Heterodera glycines*, annexin was demonstrated to downregulate the immune response of the host.^{14,15} Annexin from *Schistosoma mansoni* was closely associated with tegument development, which is the most important host–parasite interface; therefore, annexin might be a potential vaccine candidate for *S. mansoni*.¹⁶ Annexin B30 of *Clonorchis sinensis* was shown

to be involved in host–parasite interaction and affected the immune response of the host during *C. sinensis* infection.¹⁷

Interestingly, some annexins without any secretory signals have been found in the extracellular space (e.g., annexin A1, A2, and A5 of human and annexin B1 of *T. solium*).^{15,18–20} The mechanism by which this occurs remains unclear and is worthy of investigation.^{15,21} Recently, proteomic research on *E. granulosus* has shown that annexin B33 protein (Eg-ANX) could be detected in the excreted/secreted (ES) product and hydatid cyst fluid, which might potentially play important roles in parasite survival mechanisms during infection.^{22,23} However, the secretory properties of Eg-ANX in the proteomic research on *E. granulosus* was not very specific. In this study, we validated the existence of Eg-ANX in hydatid cyst fluid and found that Eg-ANX could be secreted in the host-derived layer (granulation tissue) of host liver. This could be an important finding of Eg-ANX in parasite–host interaction. In addition, to understand other characteristics of Eg-ANX gene, we identified and located Eg-ANX, and the classical Ca^{2+} -dependent phospholipid-binding properties of Eg-ANX was also demonstrated.

MATERIALS AND METHODS

Parasites and animals. Cysts of *E. granulosus* were isolated from naturally infected sheep livers at a slaughterhouse in Qinghai Province, China. The liver of healthy sheep was obtained from Sichuan Province, China. The hydatid fluid and protoscoleces (PSCs) were separated aseptically by centrifugation at $600 \times g$ for 5 minutes. Then the hydatid fluid was filtered through a 0.22- μm membrane and stored at -80°C for western blotting. Adult worms were collected from a 2-month-old dog 35 days postinfection with 20,000 PSCs. From the Laboratory Animal Center of Sichuan University (Chengdu, China), 6- to 8-week-old female-specific pathogen-free Institute for Cancer Research mice were purchased. Nine-week-old female New Zealand white rabbits and dogs were obtained from the Laboratory Animal Center of Sichuan Agricultural University. All animals were handled in strict accordance with the animal protection law of the People's Republic of China (a draft animal protection law was released on September 18, 2009). All procedures were approved by the Institute of Health Animal Care and Use Committee of Sichuan Agricultural University of China.

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Bioinformatic analysis. The complementary DNA (cDNA) sequence of Eg-ANX (EgrG_000041300) was obtained from GeneDB (<http://www.genedb.org/Homepage>). DNASTAR software (Madison, WI) was used to deduce the amino acid sequence of Eg-ANX. Multiple sequence alignment was performed using Clustal X software version 1.83 (EBI, Cambridge, UK). The phylogenetic tree was constructed using the neighbor-joining method with MEGA software (version 5.05, The Biodesign Institute, Tempe, AZ). Conserved domains were searched using SMART (<http://smart.embl-heidelberg.de/>). The physicochemical properties including molecular weight (MW) and isoelectric point (pI) were predicted by ProtParam (<http://www.expasy.ch/tools/protparam.html>). The signal sequence was predicted with the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>).

Cloning, expression, and purification of recombinant Eg-ANX. Total RNA from PSCs was isolated using the RNAPrep Pure Tissue Kit (Tiangen, Beijing, China) and reverse transcribed into cDNA using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). The expression sequence of Eg-ANX was amplified by polymerase chain reaction (PCR) using primers 5'-CGCGGATCCATGAATTACGACTATAATAATGA-3' and 5'-CCGGAATTCCTTCTCTCCCAAAGGGT-3' with *Bam*HI and *Eco*RI restriction enzyme sites (underlined), respectively. The PCR products of Eg-ANX were ligated into the expression vector pET32a(+) (Novagen, Madison, WI). The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) cells (Invitrogen, Carlsbad, CA). Subsequently, the transformants were induced with 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG; Sigma, St Louis, MO) at 37°C for 6 hours. The bacterial cells were collected, resuspended in lysis buffer (50 mM NaH₂PO₄ [pH 8.0], 10 mM Tris-HCl [pH 8.0], and 100 mM NaCl), ultrasonicated on ice, and centrifuged at 4°C. The recombinant protein was purified using a Ni²⁺ affinity column (Bio-Rad, Hercules, CA) from the bacterial lysate following the manufacturer's instructions. The expression and purification of proteins were examined by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined with micro-bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

Sera. Mouse anti-PSC sera was obtained as previously described.²⁴ Every mouse was injected with 2,000 PSCs, and serum samples were collected after 9 months for subsequent western blot analysis. Polyclonal antibody against Eg-ANX was produced after a previous study.²⁵ In brief, each rabbit was immunized with 200 μ g recombinant Eg-ANX (rEg-ANX) emulsified in Freund's complete adjuvant (FCA; Sigma) subcutaneously, followed by two boosters (2 weeks apart) using the same route and dose in FCA. Two weeks after the final vaccination, rabbit anti-rEg-ANX serum was collected and the antibody titer was detected by enzyme-linked immunosorbent assay. Anti-rEg-ANX serum with high antibody titer was purified by HiTrap Protein A affinity chromatography (Bio-Rad) for subsequent western blotting and immunohistochemistry.

Quantitative real-time PCR. To quantify the transcript level of Eg-ANX in PSCs, germinal layer, and laminated layer, real-time PCR was performed as described previously with primer pairs 5'-GGAGAACTGGCAGCAATC-3'/5'-TGGACTGAGGCAGCAAATAA-3' (for Eg-ANX) and 5'-TTTGTAGAAAGAGGCGGTGAGATG-3'/5'-TAATAAAGTACAGTAGACCGGGCG-3' (for elongation factor 1 alpha).²⁶ The data were calculated using the 2^{- $\Delta\Delta$ CT} method.²⁷

Western blot analysis. The total proteins of PSCs and healthy sheep liver were obtained with a mammalian protein extraction kit (CWBIO, Beijing, China). The cyst fluid, total PSCs extracts, total liver extracts, and recombinant proteins were run on 12% SDS-PAGE and transferred onto nitrocellulose membranes, respectively. The membranes were washed with Tris-buffered saline Tween-20 buffer five times and subsequently blocked with 5% (w/v) skimmed milk at 37°C for 2 hours. After five washes, the membranes with cyst fluid, total PSCs extracts, and total liver extracts were incubated with rabbit anti-rEg-ANX serum (1:200 v/v dilution), while the membrane containing recombinant proteins was incubated with serum of anti-rEg-ANX rabbit or serum of infected mice (1:200 v/v dilutions) overnight at 4°C. After washing procedures, the membranes were incubated for 2 hours with 1:2,000 diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or anti-mouse IgG (Bio-Rad), respectively. The signals were detected by Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen).

Immunolocalization of Eg-ANX protein. Immunolocalization of Eg-ANX in the different life cycle stages of *E. granulosus* and the host-derived layer was determined by immunofluorescence. Fresh adult worms, PSCs, the germinal layer, the healthy sheep liver, and the host-derived layer were washed in phosphate-buffered saline (PBS, pH 7.4) three times and then fixed in 4% (w/v) paraformaldehyde for 36 hours. These samples were paraffin embedded and sliced into 5 μ m-thick sections. After dewaxing in xylene and dehydration in an ethanol series, the slices were treated with 0.01 M citrate buffer (pH 6.0) at 95°C for 15 minutes. Then 4% (w/v) bovine serum albumin in 0.01 M PBS was used to block the slices at 37°C for 2 hours, followed by washing three times with PBS for 5 minutes. The slices were incubated with anti-rEg-ANX antibodies or normal rabbit serum (1:200 v/v dilutions) overnight at 4°C. After three washes, some of the slices were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (H+L) (Bethyl Laboratories, Montgomery, TX), diluted 1:200 in 1% Evans Blue (Leagene Biotechnology, Beijing, China) at 37°C for 1 hour in darkness and imaged under a fluorescence microscope (Nikon, Tokyo, Japan). Others were incubated with HRP-conjugated goat anti-rabbit IgG (Boster, Wuhan, China) at 37°C for 1 hour. After washing with PBS, the slices were stained with DAB substrate (Boster) and the nuclei were counterstained with hematoxylin.

Phospholipid-binding bioactivity assay. Liposomes were produced based on the previous description with some modifications.²⁸ In brief, 8 mg phosphatidylcholine (Sigma) and 2 mg phosphatidylserine (Sigma) were mixed and dissolved into chloroform in a round bottomed flask, followed by rotary evaporation to remove the chloroform. Then the residue in the flask was dissolved in 50 mM Tris-HCl (pH 8.0) and ultrasonicated on ice for 5 minutes. The phospholipid-binding assay was performed as described by Choi and others.²⁹ The experiment was divided into three sample groups (A, B, and C). The reaction mixture for each group was composed of 1 mM CaCl₂ (except group C), 20 μ g liposomes, and 10 μ g Eg-ANX and supplemented with 50 mM Tris-HCl (pH 7.5) to a total volume of 200 μ L. After incubation at 37°C for 30 minutes, all groups were centrifuged at 10,000 $\times$ g for 10 minutes to separate the bound (pellets) and unbound (supernatant) proteins. The supernatant samples were collected and the pellets

were washed three times with 500 μ L of 50 mM Tris-HCl. Subsequently, 1 mM EGTA (a kind of calcium chelating agent) and 50 mM Tris-HCl (total 200 μ L) were added to the pellets of group B and incubated at 37°C for 30 minutes. The supernatant and pellets were separated by centrifuge at 12,000 $\times$ g for 10 minutes. All the supernatant and pellet samples were run on 12% SDS-PAGE and observed by Coomassie staining.

Statistical analysis. Statistical analysis was performed with Student's *t* test for comparison between two experimental groups using the software package GraphPad Prism (San Diego, CA).

RESULTS

Molecular characterization. The *Eg-ANX* gene contained an open reading frame of 996 bp, encoding a putative pro-

tein of 331 amino acids with a predicted MW of 37.3 kDa and a pI of 5.9. Sequence analysis revealed that *Eg-ANX* did not have a signal peptide. *Eg-ANX* was aligned with other members of the annexin family (Figure 1), which showed that the core domain contained four annexin repeat domains, each repeat containing 52–62 amino acids. Alignment analysis revealed that *Eg-ANX* shared 95.8% identity with an annexin from *Echinococcus multilocularis* (Em-ANX) (GenBank: CDI96708.1), 79% identity with *Hymenolepis microstoma* annexin (Hm-ANX) (GenBank: CDS34213.1), and 34–45% identity with annexin from *Schistosoma japonicum* (Sj-ANX) (GenBank: CAX70812.1), *S. mansoni* (Sm-ANX) (GenBank: ACO90180.1), *Schistosoma haematobium* (Sh-ANX) (GenBank: KGB36040.1), and *C. sinensis* (Cs-ANX) (GenBank: GAA40121.2).

FIGURE 1. Sequence alignment analysis of annexin B33 protein of *Echinococcus granulosus* (*Eg-ANX*) with homologous annexins. Alignment of the deduced amino acid sequence of *E. granulosus* annexin (GeneDB: EgrG_000041300) with homologs from other species. The following sequences were retrieved from the GenBank protein sequence database and aligned using the Clustal X program: *Echinococcus multilocularis* annexin (Em-ANX) (GenBank: CDI96708.1), *Hymenolepis microstoma* annexin (Hm-ANX) (GenBank: CDS34213.1), *Clonorchis sinensis* annexin (Cs-ANX) (GenBank: GAA40121.2), *Schistosoma mansoni* annexin (Sm-ANX) (GenBank: ACO90180.1), *Schistosoma haematobium* annexin (Sh-ANX) (GenBank: KGB36040.1), *Mus musculus* annexin (Mm-ANX) (GenBank: NP_081487.1), *Homo sapiens* annexin (Hs-ANX) (GenBank: CAG46637.1), and *Schistosoma japonicum* annexin (Sj-ANX) (GenBank: CAX70812.1). According to the Clustal X algorithm, regions of high identity and similarity between annexin sequences are shown as black and gray columns, respectively. The four repeat domains of annexin sequences are marked with red boxes. The actin-binding motifs (in repeat IV) are indicated by green letters. The type II Ca^{2+} -binding sites are in white letters with blue backgrounds; the type III Ca^{2+} -binding sites are in white letters with red and blue backgrounds. The KGD motifs are in black letters with green backgrounds. The percentage homology of *Eg-ANX* with other species is shown at the end of the alignments.

Annexins share a core domain made up of four similar repeats, and each repeat usually harbors type II and/or type III Ca^{2+} -binding sites. Type II sites bind calcium ions using the sequence M-K/R-G/R-X-G-T-(38 residues)-D/E and type III using G-X-G-T-D/E.^{30,31} Some annexins contain both a type II and type III motif in all repeats (e.g., *Homo sapiens* (Hs)-ANX, *Mus musculus* (Mm)-ANX and *Sj-Annx*) (Figure 1). However, other annexins, such as Eg-ANX, Em-ANX, and Cs-ANX showed an absence of Ca^{2+} -binding sites in some repeats. A KGD motif was found in repeat III of Cs-ANX, Sm-ANX, and Sh-ANX, where the Ca^{2+} -binding site was lost. In repeat I or II of Sj-ANX, Sm-ANX, and Cs-ANX, the interval in the type II Ca^{2+} -binding site was 42 amino acid residues. In addition, we identified an actin-binding (IRI) motif in repeat IV of Sm-ANX, Sh-ANX, Mm-ANX, and Hs-ANX but not in the Eg-ANX sequence.

A phylogenetic tree was constructed based on multiple annexin sequences from various species (Figure 2), showing that Eg-ANX has near relationship with vertebrate annexin A13 family.

Cloning, expression, and purification of rEg-ANX. The *Eg-ANX* gene was amplified from PSCs and the fragment was expressed in *E. coli* with a ~20-kDa epitope tag fusion peptide from pET-32a. The molecular mass of the recombinant protein was approximately 57 kDa (Figure 3). The

rEg-ANX protein was partly expressed as soluble and was purified by Ni^{2+} affinity chromatography in non-denaturing conditions (Figure 3).

The mRNA expressions at different parts of metacystode (larval) stage. The transcripts of *Eg-ANX* were detected both at the PSCs and germinal layer, but no signal at laminated layer. And the transcriptional levels of PSCs and germinal layer showed no significant difference (Figure 4B).

Western blotting. The rEg-ANX protein was recognized by antibodies from immunized rabbits as well as the sera from mice infected with *E. granulosus*, but was not recognized by negative controls (Figures 3 and 4A). These results showed strong immunogenicity and immunoreactivity of rEg-ANX. The native *Eg-ANX* protein in cyst fluid and PSCs was identified using rabbit anti-rEg-ANX antibody. A single band of ~37.3 kDa was observed in each lane but not in the negative controls (Figure 4A). This result indicates that *Eg-ANX* is present in cystic fluid and PSCs.

Localization of *Eg-ANX* by immunohistochemistry. Localization of *Eg-ANX* in different life cycle stages of *E. granulosus* was performed by immunofluorescence using rabbit anti-rEg-ANX antibody. The results showed that the *Eg-ANX* was located on the tegument and eggs of adult worms. The weak fluorescence signals were detected in the whole germinal layer (Figure 5A). For PSCs, the fluorescence and brown signals were both detected on the tegument and

FIGURE 2. Phylogenetic analysis of annexin B33 protein of *Echinococcus granulosus* (Eg-ANX) with homologous annexins. The phylogenetic tree was constructed based on the neighbor-joining method. gb = GenBank ID; gd = GeneDB ID; up = UniProt ID.

FIGURE 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analysis of recombinant annexin B33 protein of *Echinococcus granulosus* (rEg-ANX). Expression, purification, and identification of rEg-ANX by serum of *E. granulosus*-infected mice. M = molecular mass marker in kDa; lane 1 = whole cell protein from isopropyl- β -D-1-thiogalactopyranoside-induced *Escherichia coli* cells containing pET-32a-Eg-ANX; lane 2 = sample after Ni column purification of recombinant protein; lane 3 = purified rEg-ANX was probed with the serum of *E. granulosus* infected mice; lane 4 = purified rEg-ANX was probed with native mouse serum.

calcareous corpuscle (Figure 5A). At the cellular level, Eg-ANX was located in the cytosol and cellular membrane of protoscolex (Figure 5C). In addition, weak fluorescent and brown signals were observed on the inflammatory cells and fibroblasts of host-derived layer. No fluorescence or brown signal was detected in controls (Figure 5B).

Phospholipid-binding bioactivity analysis. To examine the calcium-dependent phospholipid-binding properties of rEg-ANX, the recombinant protein was reacted with a liposome in the absence or presence of Ca^{2+} . Mixtures were then centrifuged, bound protein located to the pellet and unbound protein to the supernatant. Recombinant Eg-ANX was observed only in the supernatant without Ca^{2+} (group C) (Figure 6), while rEg-ANX was observed in the pellets, but not in the supernatant, in the presence of 1 mM Ca^{2+} (group A) (Figure 6). When Ca^{2+} was removed from the recombinant protein by EGTA, rEg-ANX lost the ability to bind to liposomes and returned into the supernatant (group B).

DISCUSSION

Annexins are a multigene family widely distributed in eukaryotes and are proposed to be involved in a wide range of important biological processes, such as membrane trafficking and fusion, anticoagulation, and anti-inflammatory action. Recent research has shown that annexins play important roles in host-parasite relationships. In *T. solium* metacystodes, annexin B1 was detected in cyst fluid and the sera of hosts as well as in the host-derived layer surrounding the cysts.³² Subsequently, other research demonstrated that *T. solium* annexin B1 was secreted by living cells and downregulated the host immune response, which suggested that secretion of annexin B1 might be a strategy for *T. solium*

FIGURE 4. Transcription and expression of endogenous annexin B33 protein of *Echinococcus granulosus* (Eg-ANX) in *E. granulosus*. (A) Identification of endogenous Eg-ANX from *E. granulosus*. M = molecular mass marker in kDa; lane 1 = purified rEg-ANX was probed with anti-rEg-ANX rabbit serum; lane 2 = purified rEg-ANX was probed with native rabbit serum; lane 3 = the anti-rEg-ANX rabbit serum detected a single band at a mass of 37 kDa in *E. granulosus* cystic fluid; lane 4 = the cystic fluid was probed with native rabbit serum; lane 5 = endogenous Eg-ANX from PSCs was probed with anti-rEg-ANX rabbit serum; lane 6 = the total protein of PSCs was probed with native rabbit serum; lane 7 = the total extracts of healthy sheep was probed with anti-rEg-ANX rabbit serum. (B) Endogenous Eg-ANX transcription of *E. granulosus* metacystode determined by quantitative real-time polymerase chain reaction. The transcripts of Eg-ANX in laminated layer were not detected. The elongation factor 1 alpha was used as the transcriptional control, and the data were presented as mean $\pm$ SD. GL = germinal layer; PSCs = protoscolexes.

FIGURE 5. Immunolocalization of annexin B33 protein of *Echinococcus granulosus* (Eg-ANX). (A) Immunofluorescence localization of Eg-ANX in different stages of *E. granulosus*. Eg-ANX in the protoscolex, germinal layer, and adult was immunofluorescently labeled using specific anti-rEg-ANX IgG (positive), or control pre-immune serum (negative), followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG. CC = calcareous corpuscle; Teg = tegument. (B) Immunohistochemical localization of Eg-ANX in the host-derived layer and host liver. Eg-ANX in the host liver and host-derived layer was labeled using specific anti-rEg-ANX IgG (positive) or control pre-immune serum (negative), followed by FITC-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated goat anti-rabbit IgG. The healthy liver was incubated by specific anti-rEg-ANX IgG and stained with hematoxylin-eosin. Fib = fibroblasts; GL = germinal layer; HDL = host-derived layer; IC = inflammatory cells; LL = laminated layer. (C) Eg-ANX localization in cytosol, cellular membrane of protoscolexes. CM = cellular membrane; Cyt = cytosol. Fluorescence-labeled or brown-labeled regions are marked with arrows. Scale bars: 50 μ m.

metacestodes to survive the host immune system.¹⁵ These results indicate the importance of parasite annexin and suggest a possible intervention target for the therapy and control of parasitic diseases. Two proteomic studies on the zoonotic pathogen *E. granulosus* found that an annexin was present in the ES product and hydatid cyst fluid.^{22,23} Therefore, we performed a primary study characterizing the *Eg-ANX* gene

and validated the secretory and calcium ion binding properties of Eg-ANX protein.

As shown in many reports, annexins generally have Ca^{2+} -binding sites in each annexin repeat domain, but some annexins have lost some of the Ca^{2+} -binding sites.^{16,17,33,34} Our bioinformatic analysis showed that Eg-ANX has lost Ca^{2+} -binding sites in repeats II and III, and this loss was

FIGURE 6. Phospholipid-binding properties of annexin B33 protein of *Echinococcus granulosus* (Eg-ANX). Lane M = molecular mass marker in kDa; Group A = rEg-ANX was incubated with liposomes in buffer containing 1 mM Ca^{2+} ; Group B = rEg-ANX was incubated with liposomes in buffer containing 1 mM Ca^{2+} and 1 mM EGTA was then added; Group C was the control group (no Ca^{2+} or EGTA). Ca^{2+} + EGTA (mM) = the concentrations of added Ca^{2+} and EGTA, respectively; P = pellet; S = supernatant.

not substituted by a “KGD” motif as in Em-ANX and Hm-ANX. However, we did identify a KGD motif in repeat III of Cs-ANX, Sm-ANX, and Sh-ANX, which may substitute for the loss of a Ca^{2+} -binding site.³³ Because of the Ca^{2+} -binding sites in repeats I and IV, Eg-ANX had Ca^{2+} -dependent phospholipid-binding properties, which might provide a link between Ca^{2+} signaling and membrane functions, such as the regulation of ion flux across membranes, organization of membranes, endocytosis, exocytosis, vesicle fusion, and so on.⁸ In the presence of EGTA, the Ca^{2+} -dependent binding properties of Eg-ANX protein were lost. All this was consistent with previous reports, showing the reversible Ca^{2+} -dependent phospholipid-binding properties of annexins, which are considered to be their biochemical hallmark.^{9,28,29} Further, the Eg-ANX sequence showed the absence of an IRI actin-binding motif, which is very common in other annexins (e.g., Sm-ANX, Sh-ANX, Mm-ANX, and Hs-ANX). This may suggest that Eg-ANX has lost the ability for membrane-actin interactions.³⁴

Annexins are usually localized either in the cytosol or associated with cellular membranes and cytoskeletal proteins.⁸ Intriguingly, many previous reports have documented that some annexin could be secreted and released extracellularly despite the absence of any secretory signal peptide.^{15,32,35,36} In this study, based on an Eg-ANX-specific antibody, our western blot and immunolocalization analyses revealed that Eg-ANX could be secreted to extracellular space, even to the outside of *E. granulosus*. Given the fact that Eg-ANX has no signal peptide, we can hypothesize that Eg-ANX may have a special secretion pathway. Regrettably, the secretion mechanism of annexins is still at the stage of conjecture and hypothesis. Researchers hold different opinions on the secretion mechanism. Christmas and others³⁷ reported that even though annexin A1 lacks hydrophobic

signal sequences, the human prostate gland could selectively secrete high concentrations of the protein. Thus, the secretion of annexin A1 was considered to involve a highly selective mechanism and to be independent of a hydrophobic signal sequence and the endoplasmic reticulum. Faure and others³⁸ believed that the secretion of annexin was caused by membrane disruption during exocytosis. Danielsen and others³⁹ found a majority of enterocyte annexin A2 was located on the luminal side of microvilli, implying an apical secretion by a “nonclassical” mechanism. More recent studies showed that annexin A2 was an important component of exosomes in dendritic cells⁴⁰ and could be transferred from one cell to another in exosomes in a raft-associated manner.^{19,41} More research is required to better understand the secretory annexins.

In conclusion, we characterized a secretable annexin from *E. granulosus*, which had typical Ca^{2+} -dependent phospholipid-binding properties. Our results revealed that the Eg-ANX was located on the inflammatory cells and fibroblasts of host-derived layer, which implied that this protein might be an important molecule in host-parasite interactions. The exact function of Eg-ANX, especially in the host-parasite interface, will be investigated further.

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