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Ligand-binding Activity and Expression Profile of Annexins in *Caenorhabditis elegans*

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Mammalian annexins are implicated in several physiological mechanisms based on their calcium-dependent phospholipid/membrane binding and carbohydrate-binding activities. In this study, we investigated gene expression profiles of all four *Caenorhabditis elegans* annexins, *nex-1*, *-2*, *-3* and *-4*, throughout the development, and compared phospholipid- and carbohydrate-binding properties of their protein products, NEX-1, -2, -3 and -4. We found that *nex-1* and *-3* are transcribed continuously during the developmental stages, while expression of *nex-2* and *-4* appeared to be temporal, peaking at the L1 stage followed by a gradual decrease toward the adult stage. NEX-1 and -3 were detected as single protein band in total worm extracts by immunoblotting, but NEX-2 was heterogenic in size. NEX-1, -2, and -3 showed the binding activities to phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine, but not to phosphatidylcholine. In contrast to their uniform phospholipids-binding properties, their glycosaminoglycan-binding activities were distinctive. NEX-2 bound to heparan sulfate and chondroitin, NEX-3 bound only to heparan sulfate, and NEX-1 showed no lectin activities under tested conditions. NEX-4 had neither phospholipids- nor carbohydrate-binding properties. Differentiated expression profiles and ligand-binding properties of NEX-1, -2, -3 and -4, shown in our study, may represent distinctive roles for each *C. elegans* annexins.

Key words: annexin, lectin, *C. elegans*, glycosaminoglycan, phospholipid.

Abbreviations: GST, glutathione S-transferase; GSH, glutathione; CBB, Coomassie Brilliant Blue; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; FPLC, fast protein liquid chromatography; TBS, Tris-bufferd saline; GAG, glycosaminolycan; PC, phosphatidylchorine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; POPC, palmitoyl-oleoyl-phosphatidylcholine; POPE, palmitoyl-oleoyl-phosphatidylethanolamine; POPS, palmitoyl-oleoyl-phosphatidylserine.

INTRODUCTION

Annexins are a family of proteins that gain their phospholipid-binding activities upon their binding to calcium. They are ubiquitously distributed in a wide range of eukaryotes, from fungi to mammals, and 12 annexin genes were identified in the human and mouse genomes (*1*). Annexins comprise a bipartite characteristic structure: the diverse N-terminal domain and the evolutionally well-conserved C-terminal domain with endonexin fold motifs. Several roles have been proposed for annexins, such as anti-inflammation (*2, 3*), regulation of blood coagulation (*4*), vesicle trafficking, intracellular calcium signaling, and regulation and formation of ion channels (*5, 6*). Some of those roles were confirmed by the recent studies using the annexin knockout mice. Phenotypes observed in the mice deficient of annexins are diverse, such as changes in the inflammatory response (*7*), defects in neovascularization and fibrin homeostasis (*8*), embryonic lethal (*9*) and changes in calcium homeostasis (*10*), indicating different functions for individual annexins.

A classic ligand, that may contribute to above annexins' functions, is phospholipid. In addition, we have first identified a lectin activity in annexin A4, where sialylated N-glycans and heparin serves as ligands (*11, 12*). Another ligand for annexin A4, sialoglycoprotein GP-2, was then identified, and a possible involvement in the formation of pancreatic zymogen granules was suggested (*13*). Evidences for physiological importance of the annexin–carbohydrate interaction are on the increase. Binding activities to sulfated glycoconjugates (*14, 15*) and chondroitin sulfate chains (*16*) were found for some mammalian annexins. Furthermore, the lectin activity of annexin A1 is implicated in leukocyte trafficking (*17*), and that of annexin A5 in anticoagulation (*18*) and Hsp47 binding (*19, 20*).

The complete sequencing of *C. elegans* genome revealed the significant similarities to a large number of mammalian genes involved in glycosylation. This means that worms can synthesize various glycoconjugates; glycoproteins, glycosaminoglycans and glycolipids comparative

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to those found in mammals. Indeed, a large amount of chondroitin with a rare amount of heparan sulfate were found in *C. elegans* (*21, 22*), and presences of *N*- and *O*-glycans have shown by primarily mass spectrometry (*23–26*). Furthermore, genetic removal of the enzymes or nucleotide-sugar transporters involved in the synthesis of proteoglycans, such as *sqv-1* ~ *sqv-8*, *rib-2*, *hse-5*, *hst-2* and *hst-6*, caused severe defects such as vulval morphogenesis (*27*), zygotic cytokinesis (*27, 28*), axonal guidance (*29*) and cell migration (*30*).

Identifying individual functions of 12 annexins in higher eukaryotes is difficult, since more than two annexins are thought to be readily expressed in each cell. On the other hand, according to the genome database, only four annexin genes (*nex-1*, *nex-2*, *nex-3* and *nex-4*) exist in *C. elegans*, and among them, transcription of *nex-1, -2* and *-3* was actually confirmed (*31*). In spite of that *C. elegans* is a simple eukaryote, it has well-organized tissues and systems similar to mammals, such as neurons, muscles and organs for reproduction and digestion. Therefore, studying properties of simple constituents of the annexin family in *C. elegans* would provide important insights into understanding of the annexins' functions in the more complex mammalian system.

In this study we investigated the gene expression profile and ligand-binding properties of all four members of annexin in *C. elegans*, NEX-1, -2, -3 and -4. Our findings suggested developmental stage specific roles of selective NEXs and diversity in physiological ligands among NEXs. We propose that *C. elegans* will serve as a powerful tool to study the significance of the interaction between annexin and carbohydrate *in vivo*, transferable to the mammalian system.

MATERIALS AND METHODS

Materials—DNase (DNase I, amplification grade) was purchased from Invitrogen corporation (Carlsbad, CA). Reverse transcriptase (SuperScript II Rnase H- Reverse Transcriptase) was obtained from Gibco Industries, Inc. (Los Angels, CA). Taq DNA polymerase (AmpliTaq Gold™) was purchased from Applied biosystems (Foster city, CA). pGEM-T vector and a HRP-conjugated goat anti-rabbit IgG antibody were purchased from Promega corporation (Madison, WI). Origami B cells were obtained from Novagen EMD Biosciences Inc. (Darmstadt, Germany). pG-KJE8 (Chaperone set) and *Escherichia coli* JM109 cells were purchased from Takara Bio Inc. (Shiga, Japan). pGEX-6P-3 Vector, Glutathione (GSH)-Sepharose 4B, PreScission protease, Hitrap™ Heparin and chemiluminescent substrate system (ECL blotting detection kit) were purchased from Amersham Bioscience corporation (Piscataway, NJ). Factor Xa was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Phosphatidylcoline (PC) from egg yolk, and chemically synthesized phospholipids of palmitoyl-oleoyl-phosphatidylcholine (POPC), palmitoyl-oleoyl-phosphatidylethanolamine (POPE) and palmitoyl-oleoyl-phosphatidylserine (POPS) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Phosphatidylserine (PS) from bovine brain, phosphatidylinositol (PI) from bovine liver and Phosphatidylethanolamine (PE) from egg yolk were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Heparan sulfate (bovine kidney, average molecular weight: 11,000) and chondroitin (shark cartilage, average molecular weight: 4700) were obtained from Seikagaku corporation (Shiga, Japan). Ultrafree-4 centrifugal filter unit (cut-off size: MW 100,000) was purchased from Millipore Corporation (Bedford, MA).

C. elegans Strains and Culture Conditions—Wild-type Bristol N2 and VC205 *cav-1* (*ok270*) IV were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota (St. Paul, MN). Worm breeding and handling were conducted as described (*32*).

Reverse Transcriptase Polymerase Chain Reaction—Worms were grown at 20°C (embryo) or 16°C (all other stages). Total RNA was prepared from synchronized stages of nematodes as described (*32*). First-strand cDNA for each developmental stage was prepared from 1 μg of total RNA. The reverse transcription was primed with oligo (dT) primers according to the manufacture's protocol. Primers for *ama-1*, the large subunit of RNA polymerase II were used as an internal control (*33*). The sequences of forward and reverse primers were as follows: 5′-CTTTTGGTCAGCTTGGTCAC-3′ and 5′-GCAG GTAACATTTTAGGGGTTG-3′ for *nex-1*, 5′-GAATCCAGC CAAACCGACGGTC-3′ and 5′-GACACCACGTACAAGTT GAGATTGC-3′ for *nex-2*, 5′-GATTCGCTGATACATTCA AAG-3′ and 5′-CAATGTAAAGCAATTTCAAACAG-3′ for *nex-3*, 5′-CTTCTGTGCAACAACCGCGAG-3′ and 5′- GGG CATGAGGGTATGTGTTG-3′ for *nex-4*, and 5′-TTCCAAG CGCCGCTGCGCATTGTCTC-3′ and 5′-CAGAATTTCC AGCACTCGAGGAGCGGA-3′ for *ama-1*. The number of PCR cycles was determined empirically after testing each pair of primers separately; 26 cycles for *nex-1*, 29 cycles for *nex-2*, and 32 cycles for *nex-3*, *nex-4* and *ama-1*. PCR was performed with the 2 U Taq DNA polymerase. After denaturing at 95°C for 10 min the cycle reactions were carried out by following conditions; 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and then incubated at 72°C for 10 min. PCR products were detected by electrophoresis on 1% agarose gels and staining with ethidium bromide. Nucleotide sequences were analysed by DNA sequencer (ABI PRISM 377™ DNA sequencer).

Preparation of Recombinant Proteins—Construction of pGEX-3X-NEX-1 had been described previously (*34*). cDNAs of *nex-2*, *-3* and *-4* were prepared by reverse transcription using the total RNA from a mixed culture of wild-type worms (provided by Dr J. Hirabayashi, Research Center for Glycoscience, AIST, Japan). First-strand cDNA was then utilized to amplify cDNA for coding region of each annexin by PCR. Primers used would be informed upon request. Each cDNA of *nex-2*, *nex-3* and *nex-4* obtained by RT-PCR was subcloned into pGEM-T vector and used for PCR to obtain the glutathione *S*-transferase (GST) fusion constructs with annexins. The sequences of forward and reverse primers were as follows: 5′-AGCCCGGGTGTCGCTTCGCAAATT CGTCATG-3′ and 5′-CTGCGGCCGCTTTATCCACGATT TCCATTAAC-3′ for *nex-2*, 5′-AGCCCGGGTGGCAACTGC AAAGATCCATTTC-3′ and 5′-CTGCGGCCGCTCTAAGG TCTTGGATTTCCCTTG-3′ for *nex-3*, 5′-AGCCCGGGTGA TTTTCCAAATGTACGAATC-3′ and 5′-CTGCGGCCGCT

TTATTCTTCAATTGATTCTG-3′ for *nex-4*. PCR products were digested with *Sma* I and *Not* I and the resulting fragments were then inserted into the pGEX-6P-3 vector. To obtain recombinant proteins, pGEX-3X-NEX-1, pGEX-6P-NEX-2 and pGEX-6P-NEX-3 were introduced into *E. coli* Origami B cells, while pGEX-6P-NEX-4 was transformed with pG-KJE8 into *E. coli* BL21 cells. Expressions of GST-fused proteins were induced using 0.1 mM IPTG. *E. coli* lysates were clarified by centrifugation and the supernatants were applied to a glutathione (GSH)-Sepharose 4B column as described previously (*34*). Fusion proteins were eluted with 50 mM Tris-HCl (pH 8.0) containing 10 mM GSH.

Generation of Rabbit Polyclonal Antibodies—A rabbit anti-GST polyclonal antibody was prepared and affinity-purified in our laboratory (*34*). The GST-fused annexins were cleaved in the linker region by digestion with factor Xa (for GST-NEX-1) or PreScission protease (for GST-NEX-2 and GST-NEX-3) according to the manufacture's protocol. After purification by FPLC system on a heparin column (Hitrap™ Heparin, 1 ml), NEX-1, -2 and -3 were utilized for immunization to rabbit. Specificities of each antibody were confirmed using recombinant GST-NEXs (data not shown).

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western blot analysis—About 50 worms were mixed in Laemmli's PAGE sample buffer with dithiothreitol (*35*), denatured at 100°C for 5 min, and applied to 10% SDS-polyacrylamide gels. After electrophoresis the proteins were transferred electrically to a polyvinylidene difluoride (PVDF) membrane and allowed to react with rabbit polyclonal anti-annexin antibodies, followed by incubation with HRP-conjugated goat anti-rabbit IgG antibodies. Detection of immunoreactive proteins was performed by a chemiluminescent substrate system.

Liposome-Binding Assay—Lipids were suspended in TBS (10 mM Tris-HCl (pH 7.6), 150 mM NaCl), and then sonicated by a bath-type sonicator to generate liposomes. Liposomes containing PC, POPC, or a mixture of (*1*) PE and PC, (*2*) PS and PC, (*3*) PI and PC, (*4*) POPE and POPC, or (*5*) POPS and POPC in the equal weight ratio was prepared to perform binding experiments. Reaction mixture was composed of 50 μg of phospholipids in liposome-form and 15 μg or 5 μg pf each annexin for natural (PC, PE, PS and PI) or synthesized (POPC, POPE and POPS) compounds, respectively. Reaction was then performed for 30 min on ice in a final volume of 100 μl, in the presence of 5 mM $CaCl_2$ or 2 mM EDTA, followed by centrifugation at 18,000*g* for 10 min at 4°C. The liposomes in the fraction of a sediment were washed with 200 μl of TBS containing 5 mM $CaCl_2$ or 2 mM EDTA, followed by centrifugation at 18,000*g* for 5 min at 4°C. Finally the liposomes were resuspended in 10 μl of TBS. Annexins coprecipitated with liposomes were analysed by SDS-PAGE and CBB staining. All reactions were performed in duplicate. The intensities of each band were quantitated by L process and Image Gauge software (Fujifilm, Tokyo, Japan).

Binding Assay to Phospholipid Immobilized on Plastic Microtiter Plate—Fifty microliters of methanol solution of phospholipids at various concentrations was added to each well in a microtiter plate (Immulon 1B, ThermoLabsystems, Vantaa, Finland) and evaporate methanol by warming at 37°C. All other procedures were performed at room temperature. After blocking with 5% BSA in TBS for 1 h, 50 μl of GST-annexins (0.38 μg/ml) were applied to each well and incubated for 2 h in the presence of 5 mM $CaCl_2$. The amounts of GST-annexins bound to phospholipids were monitored by enzyme-linked immunosorbent assay by use of anti-GST polyclonal antibodies, HRP-conjugated anti-rabbit IgG antibodies, and *o*-phenylene diamine. All reactions were performed in duplicate.

Binding Assay to BSA-conjugated GAG—Bovine serum albumin (BSA)-conjugated glycosaminoglycans were prepared by following methods. Five milligrams of heparan sulfate in 400 μl of H_2O was added to 5 mg of *N*-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline (EEDQ) in 500 μl of EtOH and incubated at room temperature for 1 h. Then, 10 mg of BSA in 500 μl of H_2O was added and incubated at 4°C for 18–24 h. Two mg of chondroitin and 4 mg of BSA in 900 μl of H_2O were added to 3 mg of EEDQ in 600 μl of EtOH and incubated at 4°C for 18–24 h. Uncoupled glycosaminoglycans were removed by centrifugation using a filter unit (cut off size: <MW 100,000). Various amounts of BSA-conjugated heparan sulfate or chondroitin in 50 μl were added to wells in a microtiter plate (Iwaki ELISA plate, Asahi Technoglass, Tokyo, Japan) and incubated at 4°C for 18–24 h. The amounts of glycosaminoglycan added on each well were indicated in horizontal axis in Fig. 7. After washing with TBS the wells were blocked with 3% BSA in TBS for 1 h. Fifty microliters of GST-annexins (0.38 μg/ml) were added and incubated for 2 h in the presence of 5 mM $CaCl_2$. The amounts of GST-annexins bound to BSA-GAGs were measured by enzyme linked immunosorbent assay as described earlier.

RESULTS AND DISCUSSION

Structural Characteristics of Four Annexins Expressed in C. elegans—The completion of the *C. elegans* genome sequencing project revealed that there are four genes (*nex-1*, *-2*, *-3* and *-4*) encoding polypeptides similar to mammalian annexins (http://www.wormbase.org/). In this work, we successfully cloned *nex-2, -3* and *-4* cDNAs, in addition to the previously cloned cDNA *for nex-1* (*34*). The predicted amino acid sequences of all four *C. elegans* annexins are compared in Fig. 1. Domain structures and repeating units are represented according to the crystal structure of human annexin A5 (*36*). The amino acid sequence identity in the C-terminal domain of NEX-2, -3 and -4 to NEX-1 is 44.0, 35.7 and 22.7%, respectively. They also exhibit significant structural similarities to mammalian annexins. When compared to human annexin A5, the amino acid sequence identity of the C-terminus of NEX-1, -2, -3 and -4 is 39.9, 46.5, 38.6 and 31.7%, respectively. Overall domain structures of NEX-1 and NEX-3 are similar to most annexins found in mammals, composed of a short N-terminal domain (<50 a.a.) and the C-terminal repeating units including 2∼4 endonexin fold motif.

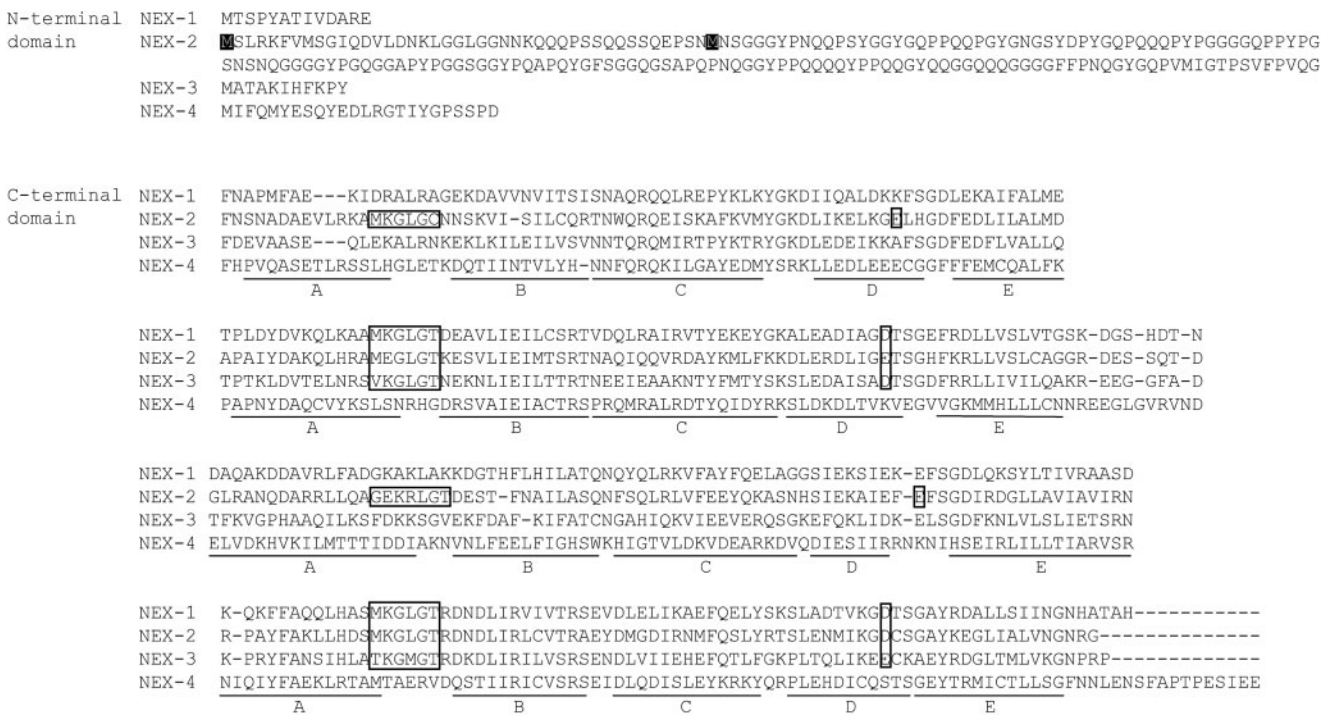

Fig. 1. **Sequence comparison of *C. elegans* annexins.** Putative sequences corresponding to five α-helices based on the X-ray crystal structure of human annexin A5 (*36*) are undefined and designated as 'A to E'. The translational initiation codons of NEX-2 are indicated by reverse display. Endonexin fold motif and acidic residues included in type II calcium-binding sites are surrounded by boxes.

NEX-2 has a particular long N-terminal domain, which is rich in glycine, tyrosine and proline residues. Similar sequences are situated in the N-terminus of human annexin A7 and A11, that contribute to the association with their partner proteins, such as Sorcin and apoptosis linked protein ALG-2. Interaction between annexin A7 and Sorcin is associated with the induction of calcium signaling and proliferation of astrocytes (*37*) and in the regulation of granule aggregation in chromaffin cells (*38*). Also, the binding of annexin A7 and A11 to ALG-2 is implicated in enhancement of the apoptosis pathway (*39, 40*). Thus, NEX-2 may play similar modulatory roles by interacting with some proteins through the long N-terminal domain.

Striking structural feature of NEX-4, characterized for the first time in this article, is a lack of the endonexin fold motif in the type II calcium-binding site. This suggests a reduced calcium-binding activity of NEX-4. Annexin A9 is known as only mammalian annexin lacking the endonexin fold motif. The overall C-terminus domain of NEX-4 shares a 22.1% amino acid sequence similarity to that of annexin A9.

Expressions of mRNA of Annexins During C. elegans Development—We next examined expression profiles of all four *C. elegans* annexin genes through the developmental stages. The expression was analysed by RT-PCR using total RNA prepared from synchronized cultures. One side of primers for each *nex* was designed corresponding to nucleotide sequences localized in 3′ untranslated region to avoid non-specific amplification by misannealing to other *nex* genes. The amplified region of *nex-2* was common to two isoforms, which are possibly produced by different initial methionines. The amount of cDNA in each reaction was adjusted by using *ama-1*, RNA Polymerase II large subunit, as a housekeeping gene (*33*). All annexins were expressed at low level at the embryonic stage and total amounts of annexins reached to a peak at the L1 stage. The expressions of *nex-1* and *-3* were continuous in all larval stages after hatching to the adulthood (Fig. 2). The expression level of *nex-1* was higher than that of *nex-3*, as more PCR cycles were required for detection of nex-3. In contrast, the mRNA levels of *nex-2* and *-4* decreased gradually after the L1 stage towards the adult stage. Considering that annexins play some roles in membrane fusion in many mammalian cell types, it is not surprising that in *C. elegans*, annexins are expressed at high levels during embryonic and post-embryonic developments when exoplasmic cell fusion occurs frequently (*41*). It is noteworthy that expression of *nex-2* and *-4* is limited to the period of exoplasmic cell fusion. This suggests that temporal changes in expression of *nex-2* and *-4* might be important for the development of *C. elegans*.

Preparation of Recombinant Annexins in E. coli—In previous work (*34*) we successfully obtained recombinant NEX-1 as a fusion protein with GST in *E. coli*. We further planned to express recombinant NEX-2, -3 and -4 in a similar manner. NEX-2 and -3 were obtained in a good yield similar to NEX-1. Due to its insolubility, NEX-4 required an aid of the coexpression of chaperons (dnaK, dnaJ, grpE, groE and groEL) to obtain enough amounts for further studies. As shown in Fig. 3,

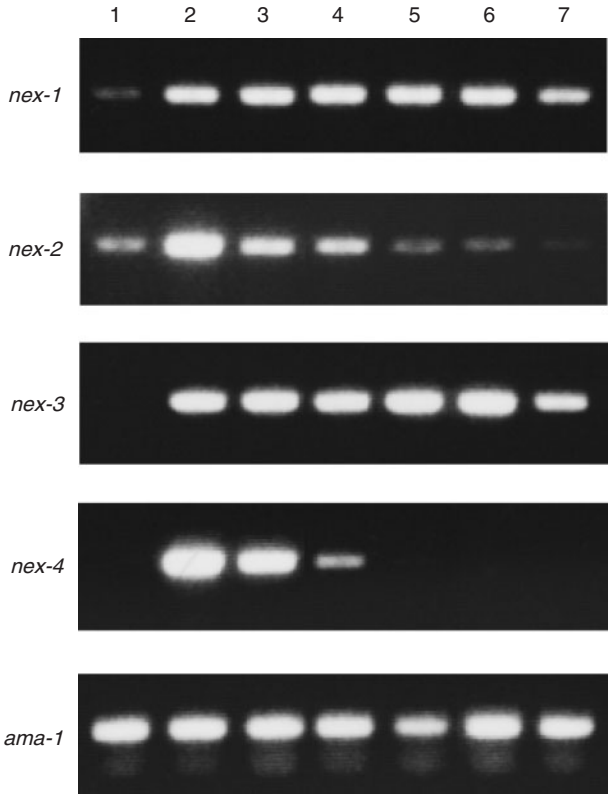

Fig. 2. **RT-PCR detection of transcripts for each annexin mRNA in *C. elegans*** during development. Specific primers for each annexin were used for the reaction. *ama-1* was used as an internal control. First strand cDNAs from embryo (lane 1), L1 (lane 2), L2 (lane 3), mix of L3 and L4 (lane 4), L4 (lane 5), young adult (lane 6) and adult (lane 7) were used as templates.

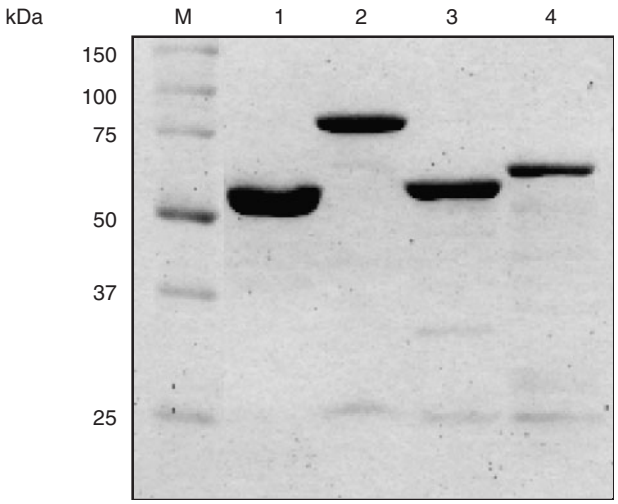

Fig. 3. **Preparation and purification of recombinant *C. elegans* annexins. GST-fused *C.*** *elegans* annexins were expressed in *E. coli*. Gels were stained with CBB. Lane 1, GST-NEX-1; lane 2, GST-NEX-2; lane 3, GST-NEX-3; lane 4, GST-NEX-4. The positions of molecular mass markers (lane M) are indicated on the left.

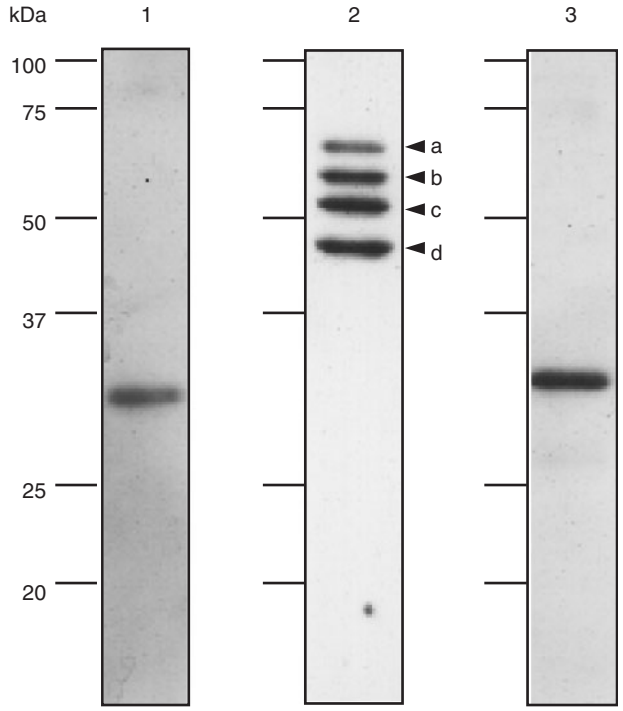

Fig. 4. **Detection of *C. elegans* annexins in worm extracts by antibodies prepared against each annexin.** Total worm extracts were prepared from mixed stage population and subjected to SDS-PAGE and western blotting. Four species of about 65, 60, 51 and 47 kDa detected by anti-NEX-2 are indicated as a to d, respectively. Wild-type worm extract was probed with anti-NEX-1 (lane 1), anti-NEX-2 (lane 2) and anti-NEX-3 (lane 3). The positions of molecular mass markers are indicated on the left.

homogeneous protein bands of 55, 78, 57 and 60 kDa were detected by SDS-PAGE in GST-NEX-1, -2, -3, and -4 preparations, respectively. The size of each protein band was in a good agreement with estimated molecular mass for the corresponding GST-annexins. The purified proteins were subjected to ligand binding assays.

Immunoblot Analysis of Annexins in Total Worm Extracts—We prepared rabbit polyclonal antibodies specific to NEX-1, -2 or -3 to detect endogenous proteins. The antibodies against NEX-1 and -3 detected 32 kDa and 34 kDa proteins in total worm extracts (Fig. 4, lanes 1 and 3), which was approximate to the estimated molecular mass from the predicted amino acid sequence (Fig. 1), 35,685 and 36,090 Da, respectively.

As to NEX-2, the antibody recognized four molecular species of 65, 60, 51 and 47 kDa (Fig. 4, lane 2, a–d). The amounts of those proteins were decreased by RNA interference (RNAi), suggesting that all four species derived from products of *nex-2* gene (S. Nishioka *et al*. manuscript in preparation). Mobility and intensity of each band were not changed after the extended treatments of denaturing by heating, SDS and other reducing reagents. Two translational start-sites were predicted for NEX-2 (Fig. 1), yielding polypeptides with calculated

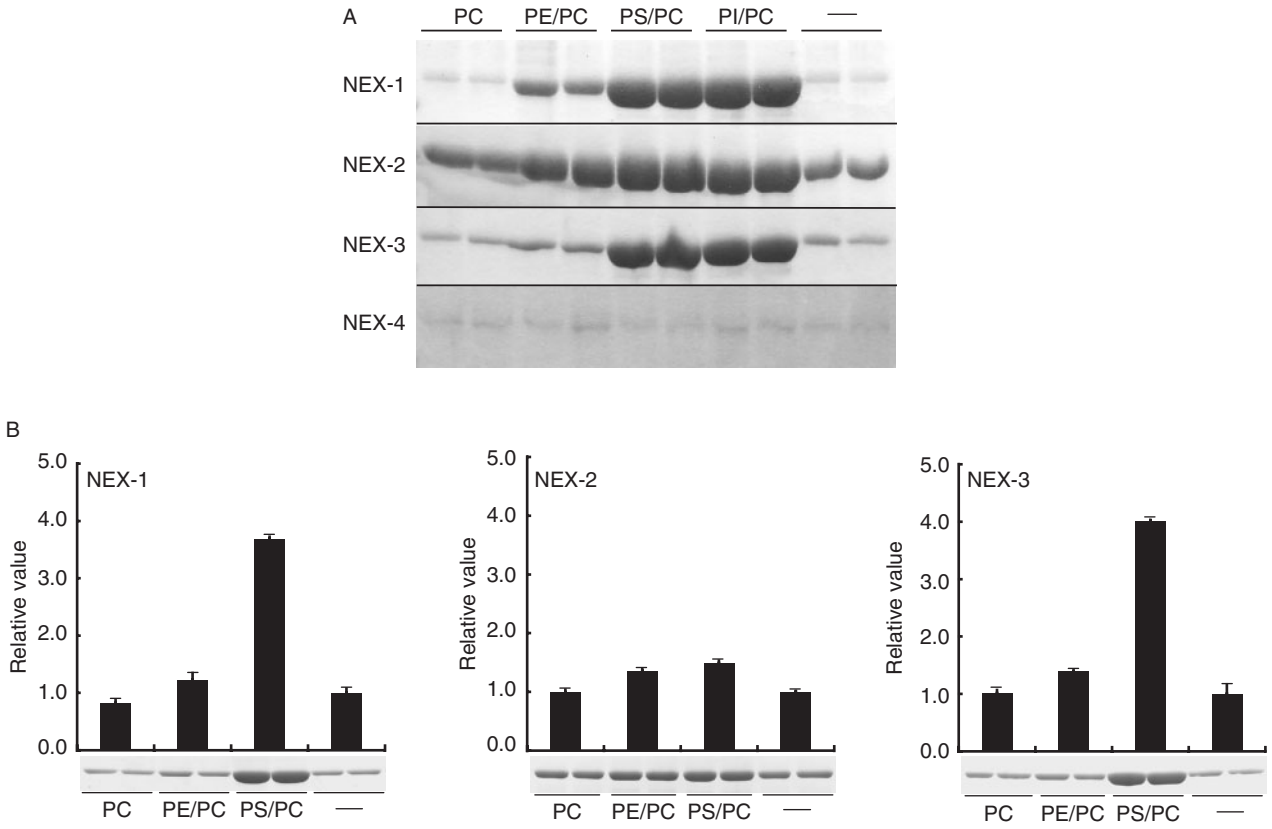

Fig. 5. **Binding properties of *C. elegans* annexins to phospholipid-containing liposomes.** GST-annexins were incubated with liposomes in the presence of 5 mM $CaCl_2$. Proteins coprecipitated with liposomes were subjected to SDS-PAGE and detected by CBB staining. (A) Liposomes were composed of PC only (PC), a mixture of PE and PC (PE/PC), PS and PC (PS/PC), PI and PC (PI/PC), or no phospholipids (–). The lipid mixtures contain equal amounts of the each lipid. Duplicate reactions were indicated in two lanes. (B) Liposomes were composed of POPC only (PC), a mixture of POPE and POPC (PE/PC), POPS and POPC (PS/PC), or no phospholipid (–). The bands intensities were quantitated as described in 'Materials and Methods' and indicated as the relative values to the intensity of the control reaction with no phospholipid. The gel images were shown in inlets. Error bar indicates the values in duplicate experiments.

molecular mass of 53,960 and 49,445 Da. These may correspond to the two bands with lower molecular species, a 51 and a 47 kDa protein. One possibility for the bands appearing around 65 and 60 kDa is the formation of detergent-resistant protein complexes with the caveolin family, as reported for annexin A2 in enterocytes (*42*). However, this was not the case for NEX-2, because those bands were present in the worm extracts from CAV-1-null mutant, *cav-1* (*ok270*) (data not shown). Other possibilities, such as post-translational modification or formation of stable heterocomplexes with unidentified molecules, still remain to be elucidated to see whether structural diversities of NEX-2 represent their specific and temporal functions in living nematode. We also aimed to detect NEX-4 in worm lysate using an antibody prepared by immunization of the synthetic peptide composed of 14 amino acid residues in NEX-4. However, the titer of this antibody was not sufficient to perform specific and sensitive detection of NEX-4 in crude extracts.

Phospholipid Binding Properties of C. elegans Annexins—To examine ligand specificity of NEXs, we first tried cosedimentation of GST-annexins with liposome composed of PE:PC, PS:PC, PI:PC, and PC only. As shown in Fig. 5A, GST-NEX-1, -2 and -3 bound to liposomes of PE:PC, PS:PC, and PI:PC, but not to the PC only liposome in the presence of 5 mM $CaCl_2$. NEX-4 failed to bind to any phospholipids. Binding of GST-NEX-1, -2 and -3 to PE:PC liposomes was weaker than to PS:PC and PI:PC liposomes. This may reflect heterogeneities in the fatty acid moieties of phospholipids from natural source, therefore, we examined binding assay using liposomes prepared from chemically synthesized phospholipids; POPC, POPE and POPS. In good agreement with the results using phospholipids from natural sources (Fig. 5A), GST-NEX-1, -2 and -3 bound to liposomes of POPS:POPC strongly and to that of POPE:POPC weakly, but not to the POPC only liposome in the presence of 5 mM $CaCl_2$ (Fig. 5B). Those results suggest that the differences in binding affinity between annexins and phospholipids derived from the nature of phospholipids polar groups, but not fatty acids. An appreciable amount of NEX-2 was detected in the control reaction without lipids, perhaps due to its self-aggregation in the presence of 5 mM $CaCl_2$. This prevented quantitative comparisons among types

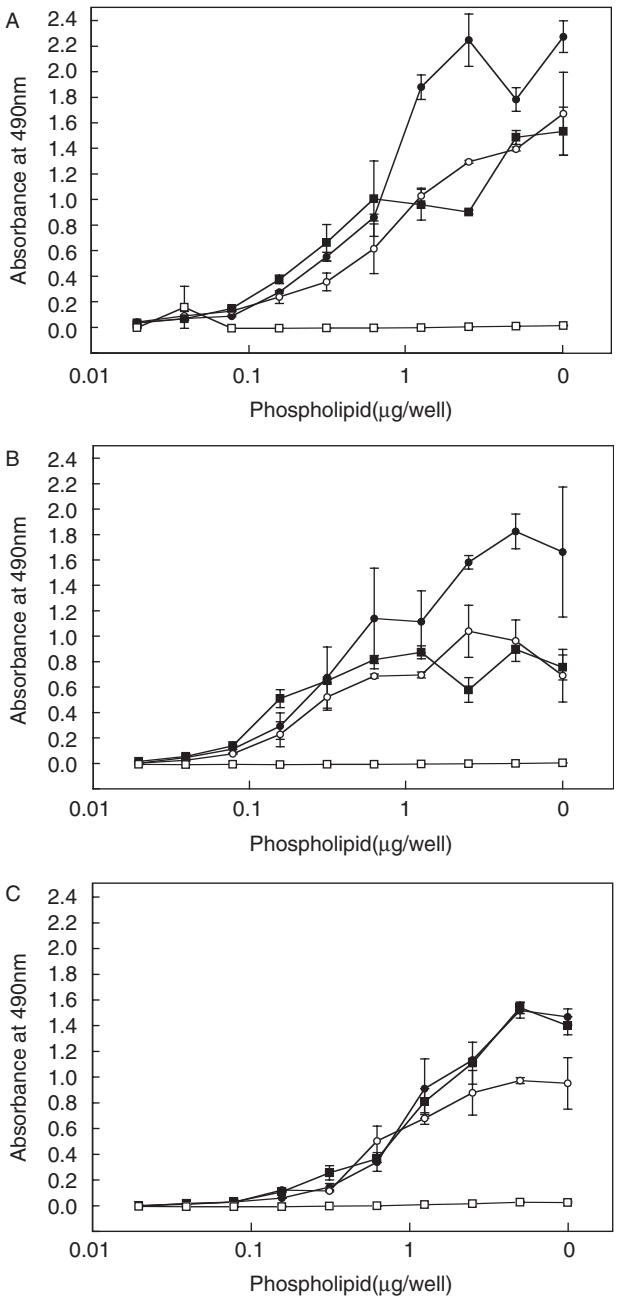

Fig. 6. **Binding properties of *C. elegans* annexins to phospholipid immobilized on a microtiter plate.** NEX-1 (closed circle), NEX-2 (open circle), NEX-3 (closed square) and NEX-4 (open square) were incubated with PS (A), PI (B) and PE (C) immobilized on plates in the presence of 5 mM $CaCl_2$. Annexins bound to phospholipids were detected by using anti-GST antibodies. Average values were calculated from duplicate experiments. Error bar indicates values of two experiments.

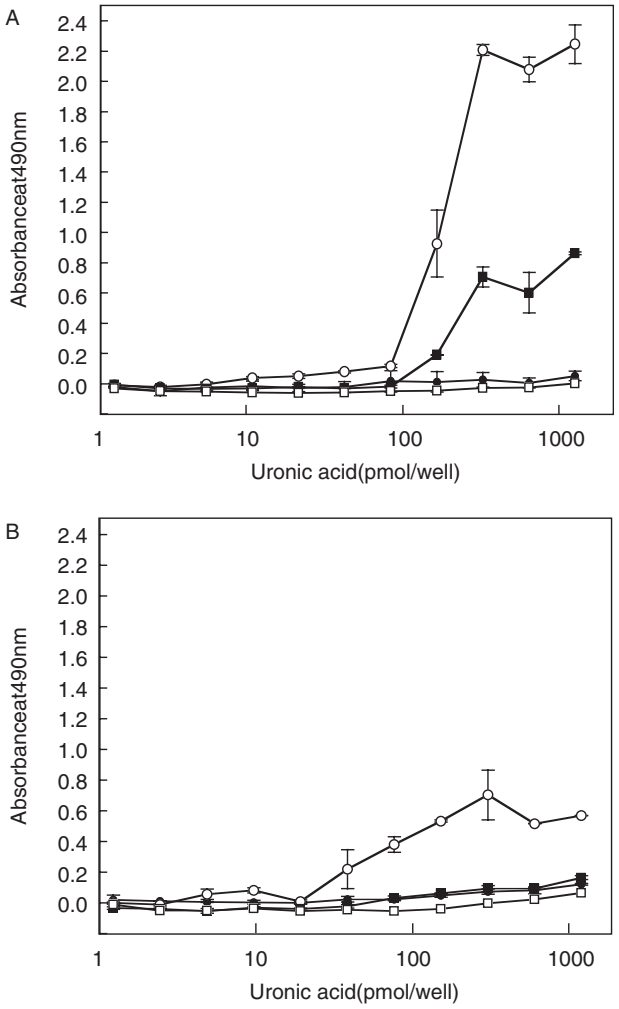

Fig. 7. **Binding properties of *C. elegans* annexins to BSA-conjugated glycosaminoglycan immobilized on a microtiter plate.** NEX-1 (closed circle), NEX-2 (open circle), NEX-3 (closed square) and NEX-4 (open square) were incubated with BSA-heparan sulfate (A) or BSA-chondroitin (B) immobilized on plates in the presence of 5 mM $CaCl_2$. Annexins bound to BSA-glycosaminoglycan were detected by using a anti-GST antibodies. Average values were calculated from duplicate experiments. Error bar indicates values of two experiments.

of liposomes, and also among four types of NEXs. Therefore, we next examined the binding activity of GST-NEXs to lipids adsorbed to the surfaces of plastic plates. As shown in Fig. 6, GST-NEX-1, -2 and -3 reacted with PS, PE and PI in a dose dependent manner in a range from 0.1 to 10 μg of the lipids applied to each well. In good agreement with the cosedimentation results, GST-NEX-4 bound to none of lipids tested on the solid phase assay.

GAG-binding Properties of C. elegans Annexins—Presences of Chondroitin and heparan sulfate have been identified in *C. elegans* by chemical analysis (*22, 23*) and genes for enzymes involved in the biosynthesis of chondroitin and heparan sulfate were identified (*43*). Therefore, we studied whether *C. elegans* annexins binds to chondroitin and heparan sulfate. We first examined the glycosaminoglycan binding activities of four NEXs by affinity chromatography and revealed that GST-NEX-1, -2 and -3, but not GST-NEX-4, were adsorbed to the heparin column (data not shown). To compare relative binding activities of GST-NEX-1, -2, -3 and -4 solid phase binding assay was performed using BSA-conjugated

heparan sulfate and chondroitin. As shown in Fig. 7, GST-NEX-2 bound to both heparan sulfate and chondroitin in a dose dependent manner, while GST-NEX-3 bound only to heparan sulfate. The binding of GST-NEX-1 was not observed at the protein concentration tested in this experiments, suggesting that relative binding activity of NEX-1 is remarkably lower than that of NEX-2 and -3. The solid-phase assay also confirmed a lack of GAG-binding activities of GST-NEX-4. Since NEX-4 appears not to possess binding activities to neither of ligands (Figs. 5A, 6, 7), one would argue whether the recombinant NEX-4 represents the correctly active form in our preparation. Coexpression of chaperons is, however, unlikely to cause incorrect folding of proteins (*44, 45*). Rather, considering that the ligand-binding activities tested here are Ca^{2+}-dependent, a lack of the endonexin fold motif, the most outstanding structural difference between NEX-4 and others, may be the reason for the unique binding characteristics of NEX-4. Further works are required to elucidate exact ligands for NEX-4.

It is known that heparan sulfate in *C. elegans* is modified by *N*-, *2-O*-, and *6-O*-sulfations similar to mammalian cells, but exhibits less variety in disaccharide units of heparan sulfate than that in bovine kidney (*23*). Hearan sulfate/heparin requires minimally five sugar units to interact with heparin-binding proteins and modification of oligosaccharides is important for their interaction (*46*). Our results using heparan sulfate from bovine kidney, therefore, may not be a precise representation of NEXs' activity to GAG ligands. Hence, further investigation using heparan sulfate with a similar structure to that produced in *C. elegans* will be required to confirm their exact interaction partner *in vivo*.

In this study we investigated the binding activity of *C. elegans* annexins toward two types of ligands, phospholipids and glycosaminoglycans. NEX-1, -2 and -3, but not NEX-4, possess binding activity to both types of ligands. While their phospholipids-binding properties appear relatively uniform, their binding activities to glycosaminoglycans varied significantly. This may suggest that the phospholipid binding properties are required for a general function of those annexins, but the glycosaminoglycan binding properties contribute to their specific roles. Functional analysis focused on glycosaminoglycan-binding properties by use of a genetic approach will clarify functional differences in each annexin in *C. elegans*.

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