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Ligand-Binding Properties of Annexin from *Caenorhabditis elegans* (Annexin XVI, Nex-1)

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Annexins are structurally related proteins that bind phospholipids in a calcium-dependent manner. Recently, we showed that annexins IV, V, and VI also bind glycosaminoglycans in a calcium-dependent manner. Annexins are widely distributed from lower to higher eukaryotes, and the nematode *Caenorhabditis elegans* has been found to contain Nex-1, an annexin homologue. Here, we characterize the ligand-binding properties of Nex-1 using recombinant Nex-1. Nex-1 binds to liposomes containing phosphatidylserine. The apparent K_d was calculated by Biacore to be 4.4 nM. Compared to mammalian annexins, the Nex-1 phospholipid-binding specificities were similar whereas the K_d values were one order of magnitude larger. The Nex-1 glycosaminoglycan-binding specificities were investigated by affinity chromatography and solid-phase assays. Nex-1 binds to heparin, heparan sulfate, and chondroitin sulfate but not to chondroitin and chemically *N*- or *O*-desulfated heparin. Besides phospholipids, heparan sulfate and/or chondroitin (sulfate), probably on perlecan, could be endogenous ligands of Nex-1.

Key words: annexin, glycosaminoglycan, phospholipid.

Annexins are a family of related proteins that bind to phospholipids in a calcium-dependent manner. Many organisms, from plants and molds to mammals, have been shown to contain annexins. Several different annexin gene products are expressed in various human cells, excluding red blood cells. Recently, annexin 31 has been found in the database of human expressed sequence tags based on the ten known human annexin genes (*1*). Each annexin is composed of 4 or 8 repeating domains of around 70 amino acids and an N-terminal domain that is specific for each type. Crystallographic analyses have shown that the core structure, *i.e.*, 4 or 8 repeating domains, are highly conserved (*2*). The exact biological functions of annexins are not yet known, although a number of *in vitro* experiments indicate that annexins have membrane channel activity, inhibit phospholipase A_2 and blood coagulation, transduce mitogenic signals, and are involved in membrane-cytoskeleton interactions [reviewed by P. Raynal and H.B. Pollard (*3*)].

The nematode *Caenorhabditis elegans* is a very useful animal model, not only for genomic studies, but also because it contains only about 1,000 cells. It has a relatively small genome (1×10^8 bp), the sequence of which has been fully established. Annexin homologues (*nex-1*, *nex-2*, and *nex-3* genes) have recently been isolated from *C. elegans* by Creutz *et al.* (*4*). The inferred products of the *nex-2* and *nex-3* genes were not identified. These authors succeeded in isolating an annexin homologue, Nex-1 (Annexin XVI), from *C. elegans* extracts and have studied its phospholipid-binding properties and aggregating activities using bovine chromaffin granules (*5*).

By another method, we previously isolated annexin IV from bovine kidney extracts by affinity chromatography using sialoglycoprotein and heparin as ligands (*6*). Other annexins, namely bovine and human annexins I, II, V, and VI, have also been found to bind glycosaminoglycans (GAGs), including heparin (*7–9*, and our unpublished data).

To determine if this unique property is in fact common among annexin family members, we investigated the GAGs binding properties of *C. elegans* annexin Nex-1 (annexin XVI) using the recombinant protein.

MATERIALS AND METHODS

Production of Recombinant Nex-1—The open-reading frame (ORF) of *nex-1* was cloned by RT-PCR and sequenced. We used total RNA from *C. elegans* as a mRNA source, and PCR primers based on *EMBL/GenBank/DDBJ* CEU40483 with *Sma*I or *Eco*RI sites. The primers for PCR were the following sequences: 5′-TCCCCCGGGG-GAATGACTTCCCCATACGCTAC-3′,5′-gCCCATGGGAAG-TAAAGCTTGGGTTTGTCA-3′.

Recombinant Nex-1 proteins were expressed as glutathione *S*-transferase (GST)–Nex-1 fusion proteins in *Escherichia coli.* The expression vector, pGEX-3X, was obtained from Pharmacia (Pharmacia LKB Biotechnology, Piscataway, NJ). To express Nex-1, the cloned cDNA for *nex-1* was digested with *Sma*I and ligated to *Sma*I-digested

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Abbreviations: GAG, glycosaminoglycan; ORF, open-reading frame; GST, glutathione *S*-transferase; TBS, 10 mM Tris-buffered saline (pH 7.5); GSH, glutathione; CBB, Coomassie Brilliant Blue; HRP, horseradish peroxidase; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; HBS, 10 mM Hepes-buffered saline (pH 7.4); RU, resonance unit; RelResp, relative response.

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pGEX-3X in frame. The construct was used to transform *E. coli* strain DH5 cells. Overnight cultures of DH5 carrying the recombinant plasmid were grown at 37°C in LB medium containing 100 μg/ml ampicilin. A fresh overnight culture was diluted 10-times with LB medium and grown for 1 h. Isopropyl-1-thio-β-D-galactoside was then added to 0.1 mM, and the cultures were incubated at 37°C for an additional 4 h. Cell pellets were collected by centrifugation and frozen at –80°C. Frozen pellets from 250 ml of culture were resuspended in 20 ml of 10 mM Tris-buffered saline (pH 7.5) (TBS) containing 2 mM EDTA. The cells were sonicated on ice at maximum intensity for 1 min using the small probe of a sonicator. Lysates were clarified by centrifugation and the supernatants were applied to a glutathione (GSH)-Sepharose 4B (Pharmacia) column (0.8 × 1 cm). The column was then washed with 10 ml of 10 mM TBS containing 2 mM EDTA. Samples were eluted with 2.5 ml of 50 mM Tris buffer (pH 8.0) containing 5 mM GSH. To remove GST from fusion protein, we used the restriction enzyme factor Xa (Boehringer Mannheim Biochemicals, Indianapolis, IN).

SDS-PAGE and Immunoblot Analysis—SDS-PAGE analysis of purified Nex-1 was performed according to the procedure of Laemmli (10% acrylamide gel) (*10*). After electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB) or electroblotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was first blocked with 2% skim milk and then was incubated with the primary antibody at room temperature for 1 h. The blot was washed three times and subsequently incubated with horseradish peroxidase (HRP)–conjugated affinity purified goat anti–rabbit IgG (Kirkegaard & Perry Labs, Gaithersburg, MD) at a final dilution of 1:1,000 at room temperature for 30 min. The membrane was washed three times and then developed with 0.05% 4-chloro-1-naphthol and 0.01% H_2O_2. As the primary antibodies, we used polyclonal rabbit anti-GST antibodies prepared and affinity-purified in our laboratory or polyclonal anti–Nex-1 antibodies kindly provided by Dr. C.E. Creutz (University of Virginia).

Phospholipid-Binding Assay—Dimyristoylphosphatidylcholine (PC), phosphatidylserine (PS, bovine brain), and phosphatidylethanolamine (PE, egg york) were obtained from Sigma (St. Louis, MO). One milligram of the same amount of combinations of PC and PS or PE were dissolved in chloroform/methanol, and the solvents were removed with nitrogen gas. The phospholipids were suspended in 1 ml of TBS containing 1 or 5 mM $CaCl_2$, then vortexed and sonicated. The liposome (multi-lamellar vesicle) solutions containing 100 ng of phospholipids were incubated with 13 μg of GST-Nex-1 or GST in a final volume of 50 μl at room temperature for 1 h. Unbound proteins were collected by centrifugation for 5 min at 15,000 ×*g*. The pellets were washed twice and resuspended in 50 μl of TBS containing 2 mM EDTA for extraction. After 1 h, the liposomes were centrifuged and washed, and then the pellets were extracted with TBS containing 0.1% Triton X-100. The bound or unbound fractions were analyzed by SDS-PAGE, and the gels were stained with CBB.

Kinetic Analyses of the Interaction between Phospholipid and Nex-1 by the Biacore System—Phospholipid liposomes composed of only PC, PS:PC, and PE:PC in a ratios of 1:1, and PS:PC in a ratio of 9:1 were prepared by sonication and filtration (pore size: 0.1 μm polycarbonate filter). A hydrophobic chip HPA suitable for the study of hydrophobic interactions (Pharmacia) was used. The sensor chip was initially washed with 0.1 mM β-octyl glycoside for 5 min at a flow rate of 5 μl/min. The liposomes in 10 mM Hepes-buffered saline (pH 7.4) (HBS) were then injected for 30 min at a flow rate of 1 μl/min. An increase in the flow rate to 5 μl/min, as well as a brief injection of 10 mM NaOH, were sufficient to wash off the unbound phospholipids and to plane the phospholipid surfaces. The chip had the following phospholipid mixtures immobilized on it: PC (1,900 RU), 1:1 ratios of PS:PC (1,800 RU), PE:PC (2,200 RU), and 9:1 ratio of PS:PC (1,800 RU) respectively. Forty microliters of Nex-1 solution was then injected in the presence of 1 mM calcium ion at a flow rate of 20 μl/min at 25°C. The sensor chip was regenerated after each cycle of measurement by injecting 10 μl of 100 mM EDTA. For assays at pH 4.0, 30 mM acetate-buffered saline (pH 4.0) as the flow buffer and 100 mM EDTA containing 10 μM NaOH as the regeneration buffer were used.

GAG Affinity Chromatography—GAG-immobilized affinity columns as previously described were used (7). Five micrograms of GST-Nex-1 or GST in 10 mM TBS were applied to the columns in the presence of 5 mM calcium ion. The columns were washed with the same buffer and the adsorbed proteins were eluted with 2 mM EDTA. The pass-through, wash, and eluted fractions were collected, and then the fraction containing Nex-1 was identified by ELISA using anti-GST antibodies. The bound antibodies were detected with HRP-conjugated anti–rabbit IgG by developing with 0.04% *o*-phenylenediamine and 0.01% H_2O_2 in 100 mM citrate buffer (pH 5.0). Then, the absorbance of each well was read at 490 nm using a Model 3550 microplate reader (Bio-Rad).

BSA-Conjugated GAG-Binding Assay—Two hundred microliters of bovine serum albumin (BSA)–conjugated GAG (7) was placed in the wells of a 96-well microtiter plate and incubated overnight at 4°C. The wells were then washed and blocked with 3% bovine serum albumin (BSA), 50 μl of 5 μg/ml recombinant GST-Nex-1 or GST containing 5 mM $CaCl_2$ or 2 mM EDTA. After incubation for 1 h, the wells were washed and the bound proteins were detected with polyclonal anti-GST antibodies.

Kinetic Analyses of Annexin IV–GAG Interactions by the Biacore System—Immobilization of GAGs to the Biacore sensor chip was performed according to the procedure of Satoh and Matsumoto (*11*). Briefly, to introduce hydrazino groups, carboxylated dextran matrix, CM5 sensor chip (Pharmacia) was activated with EDC [*N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide]/NHS (*N*-hydroxysuccinimide). Heparin, and chondroitin sulfate A and C were from Wako Pure Chem. (Osaka), and Seikagaku (Tokyo), respectively. To expose reducing end, the GAGs were treated by NaOH. The surface was initially activated with the NHS/EDC mixture. Then, adipic acid dihydrazide solution was injected. GAGs (50 mg/ml) or 0.1 M of lactose in 10 mM Hepes-buffered saline (pH 7.4) (HBS) were then injected, followed by injection of $NaBH_3CN$. After that ethanolamine was injected and NaCl was injected to wash off the nonspecific bound. Ten microliters of Nex-1 solution in HBS was then injected in the presence of 1 mM calcium ion at a flow rate of 5 μl/min. The surface was regenerated after each cycle of measurement by injecting 10 μl of 0.5 M EDTA.

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The kinetic parameters were calculated by the evaluation program BIAevaluation 3.0.

RESULTS

Expression of Nex-1 in E. coli—Nex-1 was expressed in *E. coli* with pGEX, *E. coli* expression vector, pGEX. The vector expressed a recombinant protein in the form of a GST-fusion protein. The fusion protein obtained exhibited sizes of 57 and 55 kDa on SDS-PAGE (Fig. 1a). The expressed GST-Nex-1 reacted with the polyclonal anti–Nex-1 antibody (Fig. 1b). The protein digested with factor Xa to remove GST exhibited sizes of 37 and 33 kDa. Nex-1 amino acid sequencing was performed after endopeptidase digestion and fractionation of the peptides obtained. Amino acids from the 316th residue until the end could not be detected. The smaller protein size may allow digestion at residue 316 by endogenous *E. coli* proteases. Protease inhibitors such as aprotinin and phenylmethanesulphonyl fluoride were not able to inhibit this degradation.

Phospholipid-Binding Activities of Nex-1—We previously reported that bovine annexin IV binds to PS-, PE-, and PI-containing liposomes in the presence of calcium ions (*12*). We found that bovine annexins II, V, VI, and I have similar properties (our unpublished data). Nex-1 has been isolated from *C. elegans* extracts using its binding properties to brain lipid vesicles (*4*). We performed liposome-binding assays and Biacore analysis to clarify Nex-1 phospholipid-binding properties. In the presence of 1 and 5 mM calcium ions, Nex-1 bound PS- and PI-containing liposomes (Fig. 2 and data not shown), and was released by 2 mM EDTA but not by 0.1% Triton X-100. GST did not bind to phospholipids under the same conditions (data not shown).

We analyzed and calculated the kinetic parameters of the interaction between Nex-1 and phospholipids by the Biacore 2000 system and BIAevaluation 3.0 program from the concentration of Nex-1 *versus* the Req (Equilibrium binding plot analysis). Nex-1 bound to the PS-immobilized surface in the presence of 1 mM $CaCl_2$ (Fig. 3). Unexpectedly, Nex-1 bound to the PE-immobilized surface, but did not bind to PE-containing liposomes at pH 7.4. The apparent K_d of the interaction between PS and Nex-1 was 4.4 nM. This value is one order of magnitude larger than that of annexins IV, V, and VI (our unpublished data and Refs. *13* and *14*). To bind to phospholipids, at least 0.2 mM calcium ion was necessary for Nex-1, instead of the 0.05 mM for annexins IV and V (data not shown). Under the same experimental conditions, GST did not bind to phospholipid surfaces (data not shown).

GAG-Binding Properties of Nex-1—We examined whether Nex-1 has GAG-binding properties similar to

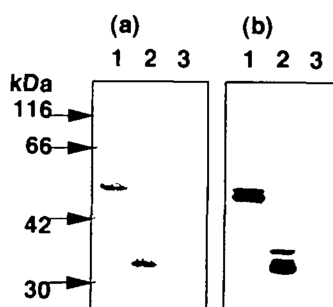

Fig. 1. **Expression of recombinant Nex-1.** Arrows, molecular mass standards; lane 1, GST-Nex-1; lane 2, factor Xa–digested GST-Nex-1; lane 3, GST. The proteins were separated by SDS-PAGE under reducing conditions, blotted, and then stained with CBB (a) or polyclonal anti–Nex-1 antibody (b).

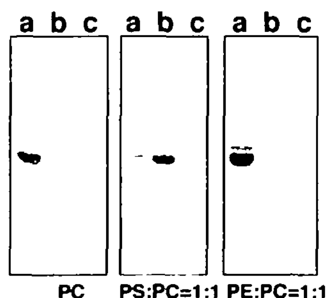

Fig. 2. **Liposome-binding assay.** CBB-stained 10% SDS-PAGE of equal proportions of unbound (lane a), EDTA-eluted (lane b), and Triton-extracted (lane c) fractions following incubation of the proteins with liposomes, as described in "MATERIALS AND METHODS."

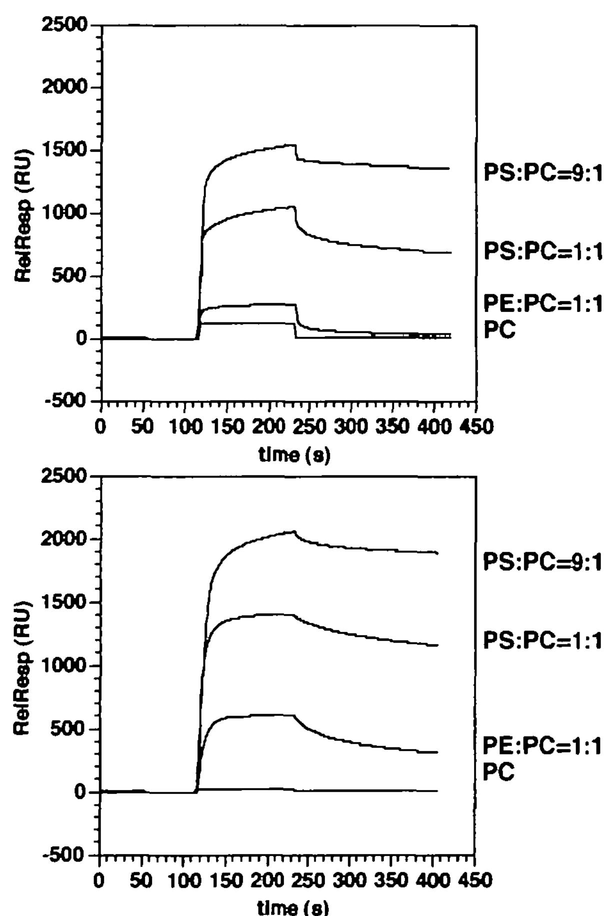

Fig. 3. **Phospholipid-binding analysis by the Biacore system.** Two hundred micrograms of Nex-1 was passed over various immobilized phospholipids (PC, PS:PC=1:1, PS:PC=9:1, and PE:PC=1:1) on HPA sensor chips at a flow rate of 20 µl/min in the presence of 1 mM calcium ion at pH 4.0 (upper panel) or pH 7.4 (lower panel). The amounts of interacting Nex-1 as a relative response [resonance units (RU)] are shown on the *y*-axis; the *x*-axis is the flow time [seconds (s)].

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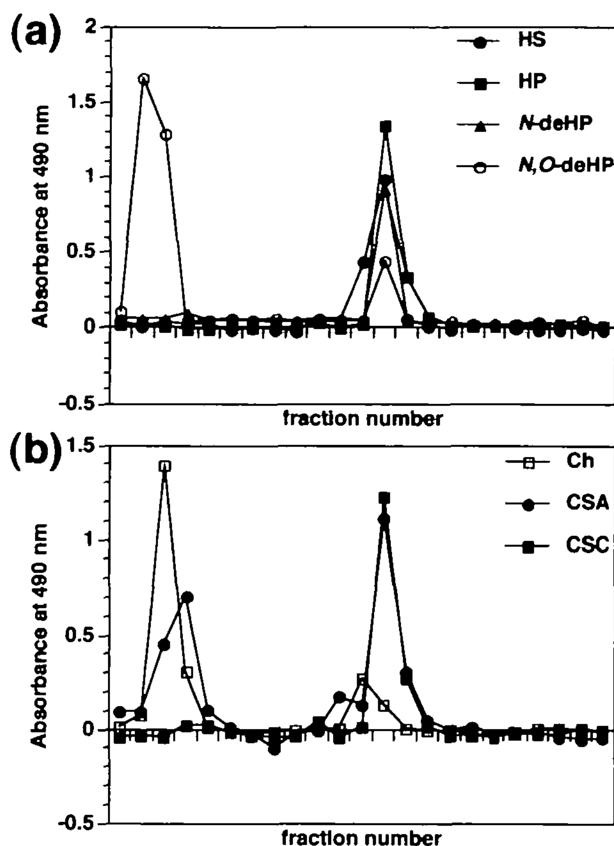

Fig. 4. **Affinity chromatography of Nex-1 on a GAG column.** GST-Nex-1 was applied to heparin-related GAG columns of heparan sulfate (●), heparin (■), *N*-desulfated heparin (▲), and *N,O*-desulfated heparin (○) (a), and to chondroitin sulfate-related GAG columns of chondroitin (□), chondroitin sulfate A (●), and C (B) (■) in the presence of 5 mM $CaCl_2$. Elution was with 2 mM EDTA. The bound Nex-1 was detected by ELISA with anti-GST antibodies.

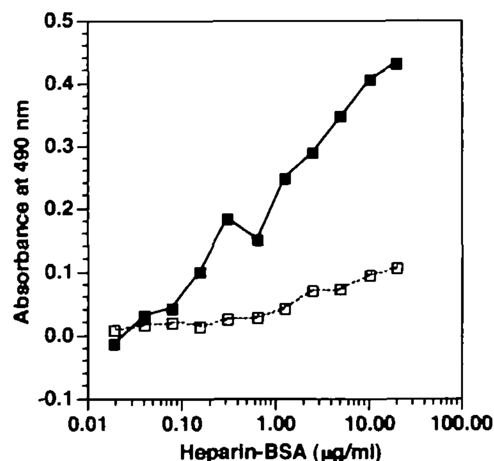

Fig. 5. **BSA-conjugated GAG binding assay of Nex-1.** GST-Nex-1 was incubated with immobilized BSA-heparin on an ELISA plate in the presence of 5 mM $CaCl_2$ (■) or 2 mM EDTA (□). The bound Nex-1 was detected by ELISA with anti-GST antibodies.

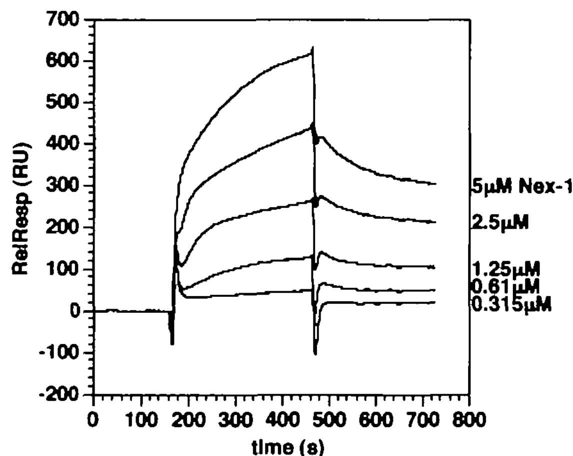

Fig. 6. **GAG binding analysis by the Biacore system.** Ten microliters of Nex-1 was passed over immobilized heparin on CM5 sensor chips at a flow rate of 5 μl/min in the presence of 1 mM calcium ion at pH 7.4. The amounts of interacting Nex-1 as a relative response [resonance units (RU)] are shown on the *y*-axis; the *x*-axis is the flow time [seconds (s)].

those of mammalian annexins. Nex-1 was applied to a chromatography column containing GAG-immobilized beads in the presence of 5 mM $CaCl_2$, and eluted with 2 mM EDTA. Nex-1 bound to heparin, heparan sulfate, and chemically *N*-desulfated heparin, but not to *N*-, *O*-desulfated heparin. Nex-1 also bound to chondroitin sulfates A and C but not to chondroitin (Fig. 4, a and b). Under the same experimental conditions, GST did not bind to GAGs (data not shown).

Semi-quantitative Nex-1 binding assays were also performed with heparin-conjugated BSA. Nex-1 bound to heparin immobilized on ELISA plates in the presence of 5 mM $CaCl_2$ in a dose-dependent manner (Fig. 5). No binding was observed in the presence of 2 mM EDTA. Under the same experimental conditions, GST did not bind to BSA-heparin (data not shown).

Then we determined the kinetics parameter of the binding of Nex-1 to heparin by Biacore (Fig. 6). The apparent K_d of the interaction between heparin and Nex-1 was 4.9 nM. Unfortunately, we could not observe the binding of Nex-1 to chondroitin sulfate by Biacore. This may be because the concentration of chondroitin sulfate immobilized on the sensor chip was not high enough to detect the specific binding of Nex-1 from its nonspecific binding to the chip. The apparent K_d of the interaction between monoclonal anti-chondroitin sulfate antibody (CS-56) and chondroitin sulfate was estimated to be 45 nM by the same system (*11*).

DISCUSSION

Similar to mammalian annexins, the *C. elegans* annexin Nex-1 was found to bind phospholipids and GAGs. Phospholipids such as PS and PE are ubiquitous because they are the main components of the inner leaflet of biological membranes. In contrast, the expression of carbohydrates, including those on glycoconjugates, varies dramatically from one tissue to another, from one cell type to another, and with developmental stage. GAGs are unbranched polysaccharide chains composed of repeating disaccharide units containing an amino-sugar and a uronic acid. Since sugar residues are replaced by sulfate or carboxyl groups, GAGs are highly negatively charged. All GAGs, with the exception of hyaluronan, are covalently attached to a protein

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backbone *via* a specific tetrasaccharide link synthesized by most animal cells. GAGs comprise some of the most complex and multifunctional molecules in the animal kingdom and play very important biological roles. GAGs provide structural constraints, function as growth-supportive or suppressive molecules, possess adhesive and anti-adhesive properties, and act as major biological filters; they also promote angiogenesis, and induce neurite outgrowth. In addition, they bind, store, and deliver growth factors to target cells during normal development as well as during pathological states [reviewed by Iozzo and Murdoch (*15*)].

Some authors have reported sugar chains in *C. elegans*. Specifically, glycolipids have been isolated and their sugar chains have been characterized (*16*). Various lectins and periodic acid thiosemicarbazide-silver proteinate stainings were successfully used for the characterization of cytochemical sugars (*17*, *18*). Taken together with the results of the inhibition of lectin binding by periodate oxidation and haptenic sugars, these data strongly suggest that *C. elegans* has glycoconjugates, especially *N*- and *O*-linked glycoproteins, as found in mammals. The existence of glycoproteins in *C. elegans* is consistent with the detection of $\alpha 1 \rightarrow 3$ fucosyltransferase activity specifically transferring Fuc in $\alpha 1 \rightarrow 2$ linkages to Gal on type-1 chains, and the cloning of the putative cDNA for the *C. elegans* $\alpha 1 \rightarrow 3$ fucosyltransferase (*19*). Staining with cationized ferritin particles showed that the surface (epicuticle) of *C. elegans* is highly negatively charged. The presence of chondroitin sulfate chains was strongly suggested by the reduction of staining by chondroitinase ABC digestion but not by neuraminidase digestion (*17*). In the *unc*-52 gene in *C. elegans*, which is homologous to the core protein of a mammalian basement membrane, heparan sulfate proteoglycan perlecan, extracellular component of all contractile tissues, was identified (*20*). Recently, chondroitin and heparan sulfate were found in *C. elegans* (*21*). These lines of evidence strongly suggest the existence of GAGs in *C. elegans* and that the GAGs are involved in fundamental biological processes.

Nex-1 has been detected in *C. elegans* by immunofluorescence and by electron microscopy using immunogold labeling (*4*). Nex-1 was detected in the membrane systems of the secretory gland cells of the pharynx, the sites of cuticle formation in the grinder in the pharynx, the yolk granules in oocytes, and the uterine wall and vulva, as well as membrane systems in the spermathecal valve.

In this study we have shown that recombinant Nex-1 binds not only to phospholipids but also to GAGs. We also showed that the recombinant Nex-1 protein has almost the same binding activities as the mammalian species. The recombinant protein used in this study could be an active form as well as an intact protein in *C. elegans*, since recombinant annexins are generally used in many crystal structure and biochemical studies, and their structures include many stable α-helices. Further investigation of the *C. elegans* sugar chains is necessary to identify the glycoconjugate ligands and to understand the functions of Nex-1.

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