Identification, Localization, and Functional Implications of an Abundant Nematode Annexin

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Abstract. Cultures of the nematode *C. elegans* were examined for the presence of calcium-dependent, phospholipid-binding proteins of the annexin class. A single protein of apparent mass on SDS-polyacrylamide gels of 32 kD was isolated from soluble extracts of nematode cultures on the basis of its ability to bind to phospholipids in a calcium-dependent manner. After verification of the protein as an annexin by peptide sequencing, an antiserum to the protein was prepared and used to isolate a corresponding cDNA from an expression library in phage λ gt11. The encoded protein, herein referred to as the nex-1 annexin, has a mass of 35 kD and is 36–42% identical in sequence to 10 known mammalian annexins. Several unique modifications were found in the portions of the sequence corresponding to calcium-binding sites. Possible phosphorylation sites in the NH$_2$-terminal domain of the nematode annexin correspond to those of mammalian annexins. The gene for this annexin (*nex-1*) was physically mapped to chromosome III in the vicinity of the *dpy-17* genetic marker. Two other annexin genes (*nex-2* and *nex-3*) were also identified in chromosome III sequences reported by the nematode genomic sequencing project (Sulston, J., Z. Du, K. Thomas, R. Wilson, L. Hillier, R. Staden, N. Halloran, P. Green, J. Thierry-Mieg, L. Qiu, et al. 1992. *Nature (Lond.)*. 356:37–41). The nex-1 annexin was localized in the nematode by immunofluorescence and by electron microscopy using immunogold labeling. The protein is associated with membrane systems of the secretory gland cells of the pharynx, with sites of cuticle formation in the grinder in the pharynx, with yolk granules in oocytes, with the uterine wall and vulva, and with membrane systems in the spermathecal valve. The presence of the annexin in association with the membranes of the spermathecal valve suggests a novel function of the protein in the folding and unfolding of these membranes as eggs pass through the valve. The localizations also indicate roles for the annexin corresponding to those proposed in mammalian systems in membrane trafficking, collagen deposition, and extracellular matrix formation.

T HE annexins are a family of calcium-dependent, phospholipid-binding proteins that have been suggested to underlie a number of activities on the surfaces of biological membranes (8, 16, 25). They are abundant proteins, comprising up to 1% of mammalian cell proteins, and represent a diverse family of calcium-binding proteins that have characteristic sequence motifs distinct from those that define the EF-hand containing calcium-binding proteins such as calmodulin (15, 22). However, the hypothesized roles for these proteins are based on experimental descriptions of the in vitro properties of the proteins and not on genetic tests of function. Therefore, it remains important to develop genetic models in which to investigate the functions of these proteins. Annexin homologues have been identified in *Dictyostelium* (18), *Drosophila* (20), and *Hydra* (31), indicating their presence in several lower eukaryotes that may permit genetic analyses to be performed.

The present study was undertaken to determine if annexin homologues exist in the nematode, *C. elegans*, as this system also provides the opportunity for reverse-genetic analysis of gene function through the isolation of transposon insertion and excision mutants (29, 30, 39). Furthermore, this organism is moderately complex, possessing several cell and tissue types so it may be possible to define differential roles for annexins in the different cell types in which they are expressed. The active program to sequence the nematode genome also provides access to genomic sequence data that will assist in the genetic analysis (33).

The characterization and localization of a major nematode annexin is reported here, establishing this organism as a possible model for analysis of annexin function. In addition, several novel localizations of this annexin are reported which lead to additional hypotheses for the functions of the annexin family of proteins. The name "nex-1 annexin" is proposed for this annexin, and "*nex-1*" for the corresponding gene. In a recent analysis of the apparent

phylogeny of annexins (24), it was suggested that the nex-1 annexin be classified as annexin XVI in the reference nomenclature for the annexins (12).

Materials and Methods

Isolation of the Nematode Annexin

Cultures of wild-type *C. elegans* (strain N2) were maintained on plates of NGM medium (containing peptone, cholesterol, and salts as described [38]) with a lawn of *E. coli* strain OP50 as described (38). For biochemical extractions, nematodes were eluted from four 9-cm petri plates and seeded into 2 liters of a suspension of OP50 cells in S medium (a balanced salt solution containing trace metals as described [38]). Suspension cultures were grown with continuous agitation at 20–25°C for 6–8 d, until the bacteria were consumed. The nematodes were harvested by flotation on a sucrose shelf as described (38).

5–25 g of nematodes harvested from 2 to 6 liters of culture were homogenized in 50 ml of 150 M KCl, 20 Mm Hepes-NaOH (pH 7.4), 2 Mm $MgCl_2$, 2 mM EGTA, 0.5 mM phenylmethane sulfonate, 0.5% aprotinin, and 1% β-mercaptoethanol by grinding in a glass ball mill (Beadbeater, Biospec Products, Bartlesville, OK) for 7.5 min (five grinding cycles of 90 s with 60-s intervals). The homogenate was centrifuged at 3,000 *g* for 10 min and then at 100,000 *g* for 60 min to prepare a postmicrosomal supernatant. The annexin was isolated from this supernatant by binding to brain lipid vesicles, using a procedure previously used to isolate recombinant annexins from yeast extracts (11). Briefly, 250–500 mg of brain lipid vesicles (Sigma B-1502, 80% phosphatidylserine) were prepared by suspension in 10 ml of 150 mM NaCl, 50 mM Hepes-NaOH (pH 7.4) and sonication until a uniform suspension was obtained (~2 min with a probe sonicator at medium output level). The vesicles were added to the postmicrosomal supernatant along with 6 mM $CaCl_2$ to give a final free Ca^{2+} concentration of ~4 mM. After 5 min the vesicles with bound annexin were isolated by centrifugation at 38,000 *g* for 20 min. The vesicles were washed twice by resuspension in a Dounce homogenizer in 40 ml of calcium containing buffers (150 mM KCl, 20 mM Hepes-NaOH [pH 7.4], 2 mM $MgCl_2$, 2 mM $CaCl_2$ followed by the same buffer without KCl), and then the annexin was extracted by resuspending the vesicles in 10 ml of a buffer containing EGTA (25 mM Hepes-NaOH [pH 7.4], 10 mM EGTA) and sedimenting the vesicles at 100,000 *g* for 30 min. A second extraction was performed typically yielding another 10–50% of the amount of annexin obtained in the first extraction.

The annexin in the EGTA extracts from the lipid vesicles was further purified by application to an FPLC mono-Q column equilibrated with 20 mM Tris-HCl (pH 7.4). The annexin was not retained by this column and was obtained in the flow-through fractions.

Isolation and Sequencing of Peptides from the Annexin

The annexin purified as described above was subjected to digestion with trypsin (80 μg annexin digested with 1 μg trypsin in 0.1 M ammonium bicarbonate, 20 mM $CaCl_2$, at 37°C overnight) and the resulting peptides isolated by reversed-phase HPLC on a C18 column and subjected to Edman degradation on a gas-phase sequenator (Appl. Biosystems, Inc., Foster City, CA).

Preparation of Antiserum and Affinity-purified Antibodies

Approximately 250 μg of the nematode annexin was applied to a preparative 10% SDS polyacrylamide gel and the 32-kD band excised after electrophoresis and brief staining and destaining of the gel (10% methanol, 1% acetic acid, ±1% Coomassie blue). The band was homogenized in 2 ml of PBS by repeated extrusion through a stopcock between two syringes, and the protein then permitted to diffuse from the gel particles at 4°C overnight. The gel particles were then removed by centrifugation and the supernatant dialyzed against PBS. The protein sample was then combined 1:1 with Freund's complete adjuvant and used to immunize two rabbits through injections in the popliteal lymph nodes and intradermal sites on the back. Another 250 μg of annexin was similarly isolated and combined with Freund's incomplete adjuvant for booster injections at 24, 40, and 75 d. Serum was tested for a positive reaction by Western blotting.

Affinity-purified antibodies were prepared by applying the serum to a 15-ml column of 2 mg of purified nematode annexin coupled to CNBr-activated Sepharose (Pharmacia LKB Biotechnology, Piscataway, NJ). The column was washed in PBS, and specific antibodies eluted with a low pH buffer (2 M glycine-HCl, pH 2.4).

Isolation of a cDNA Encoding the Nematode Annexin

The affinity-purified antibodies, at a concentration of 10 μg/ml, were used to screen λ gt11 cDNA libraries prepared from a mixed stage culture or from embryos of *C. elegans* strain N2 (libraries kindly provided by Dr. A. Fire, Carnegie Institution of Washington, Baltimore, MD). In the initial screening, nitrocellulose filters lifted from 10 9-cm plates of phage with plaque density, 2,000 plaques per plate from the mixed stage library, were incubated with the antibody in TNT buffer (10 mM TRIS-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20, and 5% nonfat dry milk powder). Positive plaques were subsequently localized by incubation of the filters with a goat anti–rabbit antibody coupled to horseradish peroxidase followed by incubation with 4-chloro napthol for detection.

Phages were amplified from pure plaques and the inserts subcloned into Bluescript (Stratagene, La Jolla, CA) for sequencing by the Sanger technique. All molecular biological procedures were conducted according to standard protocols (2).

Physical Mapping of the Nex-1 Gene

An ordered grid of yeast artificial chromosomes containing fragments of the nematode genome applied to a nitrocellulose filter was kindly provided by Dr. Alan Coulson (Cambridge University, Cambridge, England). The coding sequence of the nex-1 protein was excised from the cDNA in Bluescript, labeled with ^{32}P by random prime labeling (2) and hybridized to the filter at 65°C. The filter was washed at 55°C and used to expose X-ray film (Kodak XAR). The physical map location was interpreted using the ACEDB database (33).

Expression and Purification of Recombinant Nematode Annexin

The coding sequence of the annexin was amplified by polymerase chain reaction from a full-length λ gt11 clone obtained from the embryonic cDNA library. Primers were designed to incorporate NCOI sites (underlined below) at the initiation codon and adjacent to the termination codon to facilitate subcloning:

Primer 1: 5′ GA<u>CCATGG</u>CTTCCCCATACGCTACAATCG 3′
Primer 2: 5′ CAA<u>CCATGG</u>CATCTAGTGGGCAGTAGCG 3′

Use of the NCOI site at the initiation codon introduced a change in the first codon following the initiation codon from ACT (THR) to GCT (ALA) so that the recombinant protein that is expressed is the T1A nex-1 protein (threonine at position 1 changed to alanine). The amplified coding sequence was subcloned into the NCOI site of the yeast expression vector $YEpC^21$ (11) which uses the *GAL10* promoter. Recombinants with the proper orientation of the coding sequence were selected by restriction analysis and used to transform yeast strain NY606 by the lithium acetate procedure (19). Production of the annexin was induced by growth on galactose medium and the annexin isolated as previously described in detail for the expression and isolation of mammalian annexins using $YEpC^21$ (11).

Immunofluorescence

The immunofluorescence procedure was adapted from that described by Nonet et al. (27). Nematodes were harvested from suspension cultures and fixed in freshly prepared 4% paraformaldehyde in PBS at 4°C overnight. After four washes in PBS, nematodes were then incubated overnight in 1% triton, 100 mM Tris-HCl (pH 7.0), 1% β-mercaptoethanol at 37°C. After four washes in PBS the nematodes were incubated for 6 h in 900 U/ml of collagenase type IV (Sigma Chem. Co., St. Louis, MO) in 100 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$ at 37°C. After four washes in PBS, the nematodes were stored at 4°C in buffer A (PBS with 0.1% Triton X-100, 1% bovine serum albumin, and .05% NaN_3).

Fixed and permeabilized nematodes were incubated on a rocking plat-

form with primary antiserum at a dilution of 1:200 to 1:100 in buffer A at room temperature for 1–2 d. After four washes in buffer A with 0.1% in place of 1% bovine serum albumin, the nematodes were incubated with rhodamine-conjugated anti–rabbit antibodies (Hyclone Labs., Logan, UT; model EA-1022-U, heavy and light chain specific) at a dilution of 1:100 to 1:50 at room temperature overnight. After four washes in buffer A, nematodes were then examined in a Nikon Optiphot microscope equipped for epifluorescence illumination.

The absorbed-serum control was prepared by incubating the diluted primary antiserum (1:100) with 40 μg/ml purified recombinant T1A nex-1 protein for 30 min before and during incubation with the nematodes.

Immunogold Staining and Electron Microscopy

Worms prepared for immunofluorescence as described above were further processed for electron microscopy. The fixed worms were washed in 0.1 M phosphate buffer (24°C, 1–2 h), dehydrated in graded ethanol at −20°C, embedded in Lowicryl resin, and polymerized for 48 h at −20°C with ultraviolet light. Sections 70–80 nm in thickness were collected on nickel grids, and processed at 20°C (unless otherwise indicated) through: (a) 0.01 M glycine in 0.1 M tris-buffered saline (TBS), pH 7.4, 10 min; (b) 5% bovine serum albumin (Sigma, Fraction V) in TBS, 10 min; (c) either preimmune rabbit antiserum or rabbit antiserum to annexin, diluted 1:1,000–1:3,000 in TBS, overnight, 4°C; (d) TBS wash, 5 × 5 min; (e) goat antiserum to rabbit IgG, coupled to 15-nm colloidal gold (E.Y. Laboratories) diluted 1:25 in TBS, for 1 h; (f) TBS wash, 5 × 5 min; (g) a solution containing equal parts protein A coupled to 15-nm gold and protein A coupled to 30-nm gold (E.Y. Laboratories) diluted 1:10 in TBS, for 1 h; (h) TBS wash, 5 × 5 min; distilled water wash, 3 × 10 min, and (j) air drying. The sections were contrast stained with uranyl acetate and lead citrate according to routine procedure, and examined in a Joel 100CX electron microscope.

Analytical Procedures

SDS-PAGE was conducted in 10% polyacrylamide gels according to Laemmli (23). Western blotting was performed according to Burnette (5), on nitrocellulose membranes using 1% nonfat dry milk in PBS as a blocking agent, and 4-chloronapthol for detection of peroxidase-conjugated secondary antibodies. Protein concentrations were determined according to Bradford (4) using bovine serum albumin as standard.

Figure 1. A single major annexin can be isolated from mass cultures of *C. elegans* by calcium-dependent binding to phospholipid vesicles. This Coomassie-stained, SDS-polyacrylamide gel illustrates the fractions obtained during purification of the annexin: *H*, homogenate; *S*, postmicrosomal supernatant; *SC*, Supernatant obtained after adding 2 mM Ca^{2+} and phosphatidylserine vesicles to the postmicrosomal supernatant and removing bound annexins by sedimentation of the vesicles. Note that this procedure removes a 32-kD protein from the postmicrosomal supernatant. *W1* and *W2*, wash fractions obtained during washing of the lipid vesicles in calcium-containing buffer. *E1* and *E2*, sequential extractions of the lipid vesicles in 10 mM EGTA. Note that primarily a single protein of mass 32 kD is obtained. *Q*, Annexin purified from the E1 extract by FPLC on Mono Q resin.

Results

Identification of a C. elegans Annexin

To determine if proteins that exhibit the biochemical properties of annexins could be extracted from *C. elegans*, a procedure for the isolation of annexins based on calcium-dependent affinity for lipid vesicles was applied to mixed cultures of worms. As detailed under Materials and Methods, ~10 g of nematodes were isolated from suspension cultures and homogenized in a ball mill. A postmicrosomal supernatant was prepared in EGTA-containing buffers to chelate Ca^{2+} and solubilize calcium-dependent, lipid-binding proteins. Phospholipid vesicles (80% phosphatidylserine) were added with additional calcium (4 mM free Ca^{2+}) and the vesicles were sedimented and washed in calcium-containing buffers to retain lipid-binding proteins. The vesicles were then extracted with excess EGTA to release calcium-dependent phospholipid-binding proteins.

The EGTA extracts from the vesicles contained a single major protein of 32 kD as visualized on SDS gels (Fig. 1). The typical yield of this protein was 60–80 μg per gram of nematodes (wet weight). It was apparent by examination
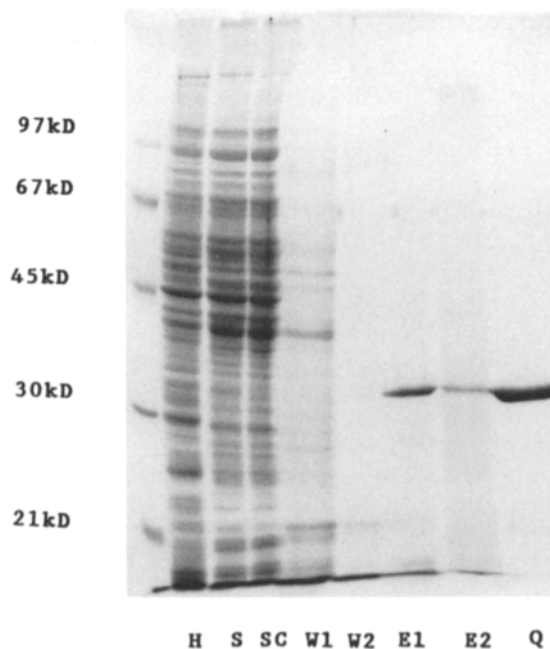
of the gels of the supernatant fractions that this protein corresponded to a significant band and that this protein was quantitatively removed from the supernatant by binding to the lipid vesicles (Fig. 1). The protein was then further purified by passing the extracts over an FPLC mono Q anion exchange column, to which the protein failed to bind at neutral pH.

The purified protein was subjected to digestion with trypsin and the resulting peptides isolated by HPLC. Several peptides of 6–15 residues were sequenced and the sequences were found to be 20–40% identical to sequences found in mammalian annexins (included in Fig. 3, below).

The putative nematode annexin (nex-1 annexin) was then excised from a preparative SDS gel and used to raise an antiserum in rabbits. The antiserum obtained proved to be of high titer and specificity. When tested on Western blots of total worm homogenates, the antiserum reacted only with the 32-kD antigen, and the preimmune serum showed no reaction with nematode proteins (Fig. 2). Specific antibodies were prepared from the antiserum by affinity chromatography on the isolated antigen coupled to Sepharose beads, and these showed similar specificity on Western blots (Fig. 2).
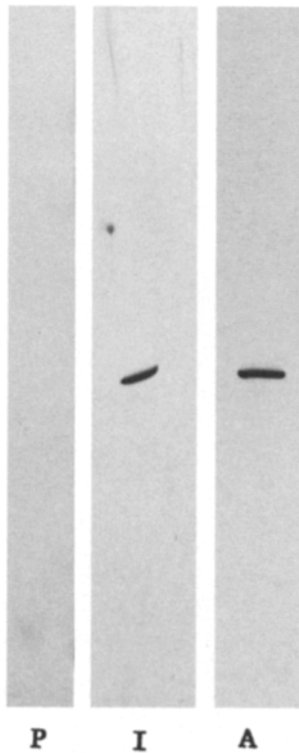
Figure 2. An antiserum prepared against the *C. elegans* nex-1 annexin reacts with a 32-kD antigen in Western blots of total worm homogenates. (Lane *P*) Reaction with preimmune serum. (Lane *I*) Reaction with immune serum. (Lane *A*) Reaction with antibodies affinity-purified immobilized antigen.

P   I   A

Cloning of the nex-1 Annexin cDNA

The affinity-purified antibodies were used to screen a *C. elegans* (mixed stage) cDNA library in the expression vector λ gt11. In an initial screening of 20,000 plaques, four clones were obtained, all of which contained a 1.2-kb insert. This insert contained an open reading frame encoding the nematode annexin as verified by the presence of all of the peptide sequences determined directly from the protein. The clones did not contain an apparent initiation codon, so an embryonic cDNA library in λ gt11 was screened with the antibody to obtain a clone with a larger insert. The 5' sequence of the insert from this clone revealed an additional eight nucleotides of the cDNA, including the probable initiation codon. The complete sequence is presented in Fig. 3.

The annexin cDNA encodes a protein of 321 amino acids with a calculated mass of 35,474 D. The sequence is 42% identical to that of bovine annexin IV, the closest homologue in current databases, and contains the fourfold 70–amino acid repeat characteristic of the annexins. The highly conserved endonexin fold consensus sequences are present in the nematode protein, although repeats 1 and 3 show significant deviations from other annexin family members. In the short amino-terminal domain of the worm annexin potential phosphorylation sites are present that can be aligned with the serine/threonine sites that are known to be phosphorylated by protein kinase C in mammalian annexins, and with the tyrosine that is a substrate for the *src* kinase in mammalian annexin II:

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      1    AGAATGACTTCCCCATACGCTACAATCGTCGACGCT            36
                M  T  S  P  Y  A  T  I  V  D  A

     37 AGAGAGTTCAATGCTCCGATGTTCGCTGAGAAGATCGACCGT        78
           R  E  F  N  A  P  M  F  A  E  K  I  D  R

     79 GCTCTTCGTGCCGGAGAGAAGGACGCTGTTGTCAACGTCATC       120
           A  L  R  A  G  E  K  Q  A  V  V  N  V  I

    121 ACCTCGATCTCCAACGCTCAGCGCCAGCAGCTCCGCGAGCCA       162
           T  S  I  S  N  A  Q  R  Q  Q  L  R  E  P

    163 TACAAGCTCAAGTACGGAAAGGACATCATCCAGGCTCTTGAC       204
           Y  K  L  K  Y  G  K  D  I  I  Q  A  L  D

    205 AAGAAGTTCTCTGGAGATCTCGAGAAGGCCATCTTCGCTCTC       246
           K  K  F  S  G  D  L  E  K  A  I  F  A  L

    247 ATGGAAACTCCATTGGATTATGATGTGAAGCAGCTTAAGGCT       288
           M  E  T  P  L  D  Y  D  V  K  Q  L  K  A

    289 GCCATGAAGGGATTGGGAACTGACGAGGCTGTGCTCATCGAG       330
           A  M  K  G  L  G  T  D  E  A  V  L  I  E

    331 ATTTTGTGCTCCAGAACTGTTGATCAACTCCGCGCCATTCGT       372
           I  L  C  S  R  T  V  D  Q  L  R  A  I  R

    373 GTGACCTACGAGAAGGAGTATGGAAAGGCTTTGGAAGCTGAC       414
           V  T  Y  E  K  E  Y  G  K  A  L  E  A  D

    415 ATCGCTGGAGATACTTCTGGAGAATTCCGTGATCTTTTGGTC       456
           I  A  G  D  T  S  G  E  F  R  D  L  L  V

    457 AGCTTGGTCACTGGATCCAAAGACGGATCTCATGATACCAAC       498
           S  L  V  T  G  S  K  D  G  S  H  D  T  N

    499 GACGCTCAGGCCAAGGATGATGCTGTTCGCCTGTTCGCCGAC       540
           D  A  Q  A  K  D  D  A  V  R  L  F  A  D

    541 GGAAAAGCCAAGCTTGCCAAGAAGGACGGAACACACTTCCTT       582
           G  K  A  K  L  A  K  K  D  G  T  H  F  L

    583 CACATCCTCGCCACTCAAAATCAATACCAGCTCCGCAAGGTC       624
           H  I  L  A  T  Q  N  Q  Y  Q  L  R  K  V

    625 TTCGCCTACTTCCAGGAACTCGCTGGTGGCTCTATCGAGAAA       666
           F  A  Y  F  Q  E  L  A  G  G  S  I  E  K

    667 AGCATCGAGAAGGAGTTTTCCGGAGATCTCCAGAAATCCTAC       708
           S  I  E  K  E  F  S  G  D  L  Q  K  S  Y

    709 CTCACCATTGTCCGTGCCGCTTCCGACAAGCAGAAGTTCTTC       750
           L  T  I  V  R  A  A  S  D  K  Q  K  F  F

    751 GCTCAGCAGCTCCACGCTTCCATGAAAGGACTCGGAACTCGC       792
           A  Q  Q  L  H  A  S  M  K  G  L  G  T  R

    793 GATAACGACTTGATCCGTGTCATCGTCACTCGTTCCGAAGTC       834
           D  N  D  L  I  R  V  I  V  T  R  S  E  V

    835 GACTTGGAGCTCATCAAGGCCGAATTCCAAGAGCTCTACTCT       876
           D  L  E  L  I  K  A  E  F  Q  E  L  Y  S

    877 AAGAGCTTGGCCGACACAGTGAAGGGAGACACTTCTGGAGCC       918
           K  S  L  A  D  T  V  K  G  D  T  S  G  A

    919 TACCGTGACGCTCTGCTCTCCATCATCAATGGAAACCACGCT       960
           Y  R  D  A  L  L  S  I  I  N  G  N  H  A

    961 ACTGCCCACTAGATGCTGAAATTGACAAACCCAAGCTTTACT      1002
           T  A  H  Z

   1003 TCATTAATTTCGTATTAAAATTCACTTTTCAACCCTAAAATG      1044
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Figure 3. Nucleotide sequence of the nex-1 annexin cDNA. The interpreted protein sequence is shown beneath the cDNA sequence. Underlined amino acids represent the sequences of peptides obtained from the isolated protein. These sequence data are available from GenBank under accession number U40483.

However, the threonine (residue 7) is not followed by a basic residue as is usually the case for protein kinase C phosphorylation sites. The COOH terminus of the protein does not contain the conserved CYS residue and the consensus sequence CGGDD seen in many mammalian annexins (3), but absent from annexins previously characterized from the other lower eukaryotes Hydra (31) and *Dictyostelium* (18).

The coding portion of the annexin cDNA was hybridized to an ordered grid of yeast artificial chromosomes (YACs)[1] containing genomic fragments from *C. elegans* to

1. *Abbreviation used in this paper:* YAC, yeast artificial chromosome.

```
            MASPYATIVDAREFN   .  .  .  .  C. elegans ANNEXIN
            MAAKGGTVKAASGFN   .  .  .  .  BOVINE ANNEXIN IV
 MSTVMEILCKLSLEGDHSTPPSAYGSVKPYTNFD  .  .  .  .  . HUMAN ANNEXIN II
```
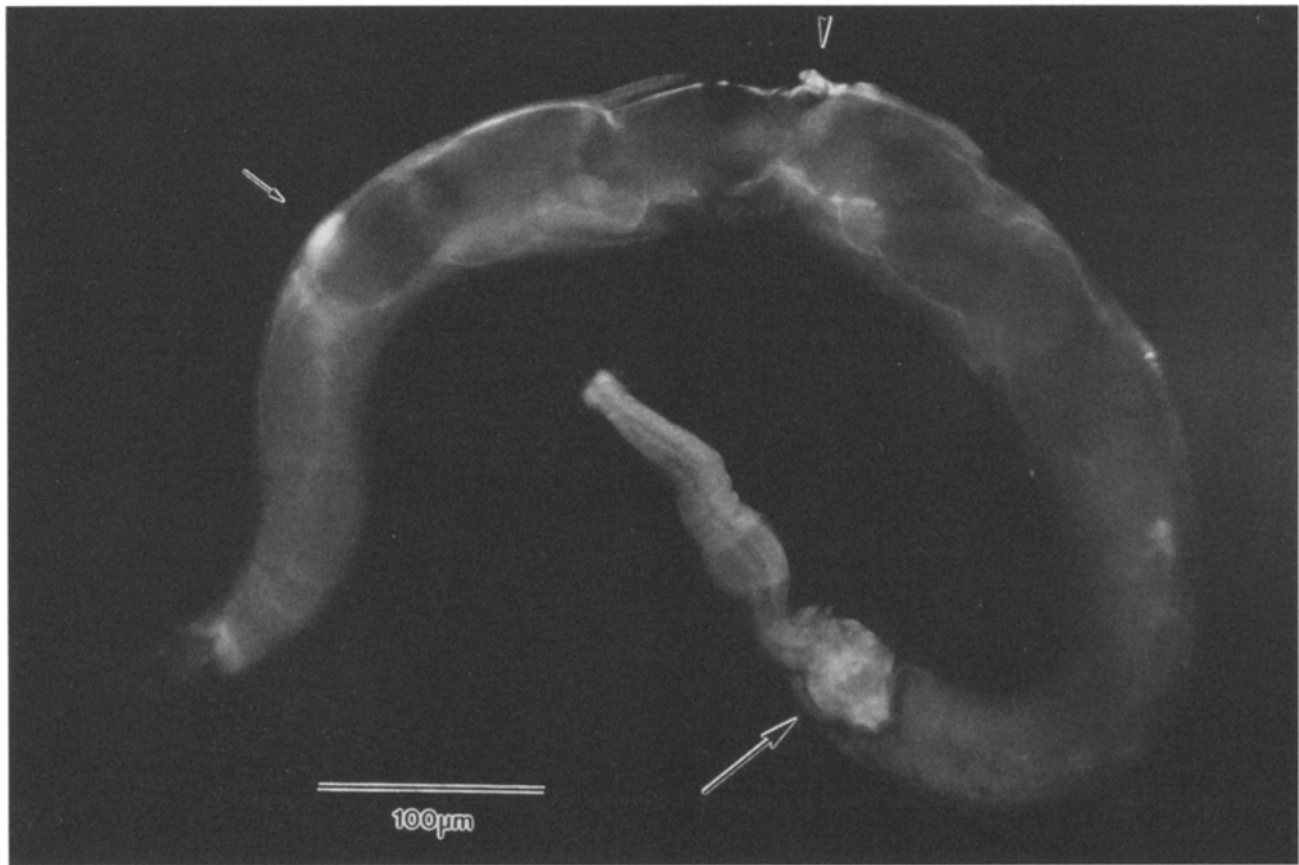
Figure 4. Localization of the annexin by immunofluorescence in the whole nematode. Particularly intense fluorescence is seen in the pharynx (*large arrow*), the vulva (*arrowhead*), and the spermathecal valve (*small arrow*).

determine the location of the annexin gene on the physical map of the nematode genome. The cDNA hybridized with three overlapping YACs (Y44H9, Y54H5, and Y74G2), indicating a single locus for the annexin gene left of center of linkage group III, near the *dpy-17* genetic marker. The region of overlap of the three YACs corresponds to cosmid ZC155 (GenBank reference CELZC155); Southern blotting of this cosmid with the cDNA confirmed the presence of the *nex-1* gene on this cosmid (see Discussion).

Localization of the nex-1 Annexin

The antiserum raised against the annexin was used in immunofluorescence and immunogold techniques to localize the annexin in the nematode. By immunofluorescence, significant annexin was found in the pharynx, the uterine wall, the vulva, and the spermathecal valves. Staining in these areas was not apparent when the preimmune serum from the same rabbit was used, or when the immune serum was preincubated with the antigen as described under Materials and Methods. In the figures that follow, the prints have been exposed so that nonspecific staining appears as a gray background, and specific staining stands out as white (e.g., compare Fig. 5 and Fig. 7, below). Figs. 4 and 5 *a* illustrate staining of the pharynx which was of greatest intensity within the terminal bulb in the region of the grinder and the gland cells at the back of the bulb. Additional strong staining was found along the length of the pharynx.

The intense staining of the uterine wall was seen either as an outline around the eggs in the gravid adults (Fig. 4), or as intense staining of the entire uterus and vulva in young adults that did not have eggs (Fig. 5 *b*). The staining of the spermathecal valves was most apparent in the case of uteri which had fallen out of adults during the staining procedure. The intensely staining valves were seen at the two ends of the uteri (Fig. 6).

Higher resolution localization of the annexin was obtained by electron microscopy and immunocytochemical staining with gold-conjugated secondary and tertiary reagents. In the figures that follow, localization of the annexin is indicated by the 15-nm gold particles attached to the secondary antibody, as well as the 30-nm particles attached to protein A which was used as a tertiary reagent. Therefore, gold particles of both sizes indicate the presence of the same antigen. Only a few randomly associated particles were seen when sections were stained using the preimmune serum from the same rabbit (e.g., Fig. 10 *a*, below).

In sections of the pharynx it was readily apparent that the staining did not occur in the contractile apparatus of the muscle cells, so the intense staining seen by immunofluorescence is likely associated with other cell types and structures in this highly muscular organ. Significant staining was found within the gland cells at the back of the terminal bulb (Fig. 8 *a*). The staining was distributed throughout the cytoplasm of these cells, with increased concentration in association with intracellular membrane systems. Intense staining was found underneath the folds of cuticle
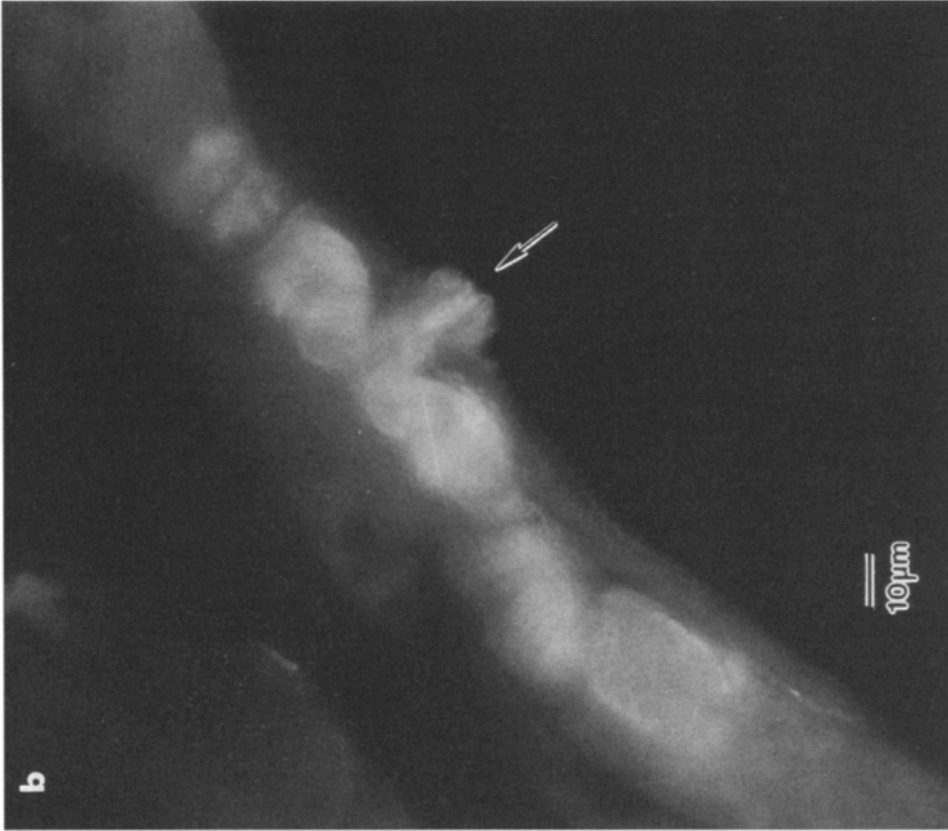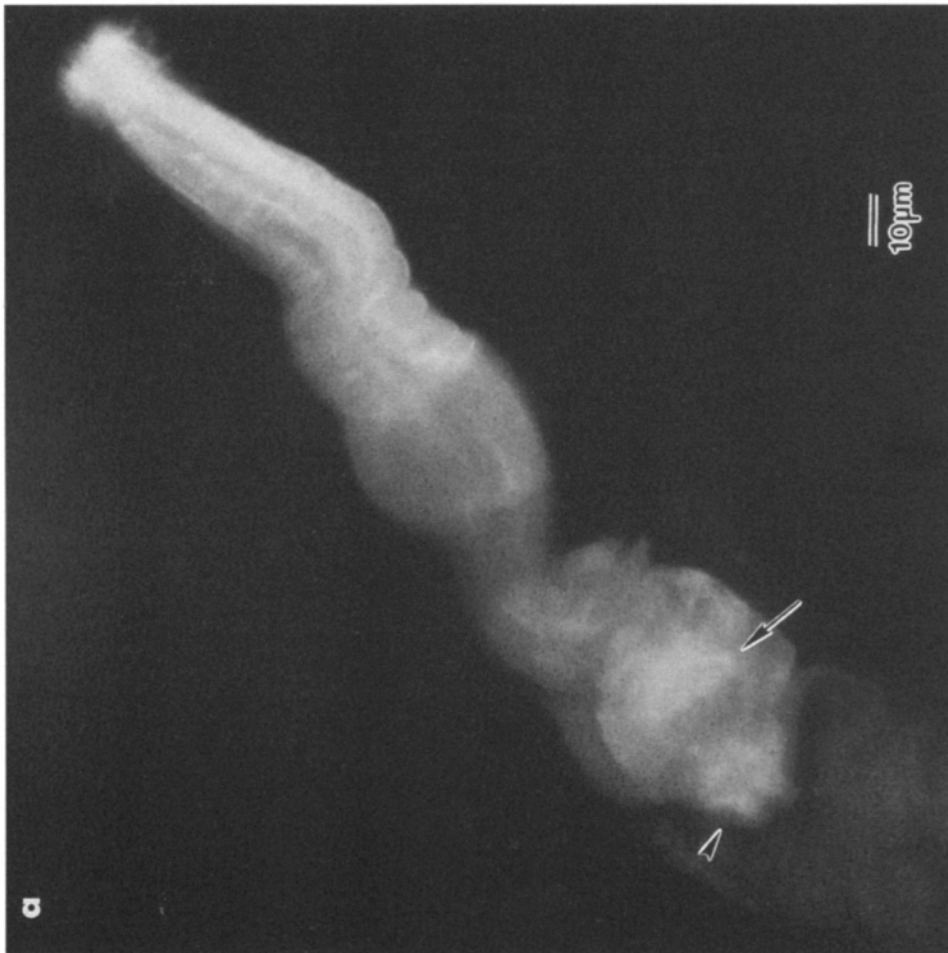
Figure 5. Localization of the annexin by immunofluorescence in the pharynx and uterus. (*a*) Specific staining is seen throughout the length of the pharynx, in the grinder (*arrow*), and in the gland cells (*arrowhead*) at the back of the terminal bulb. (*b*) Specific staining in the uterus and vulva (*arrow*) of a young adult nematode.
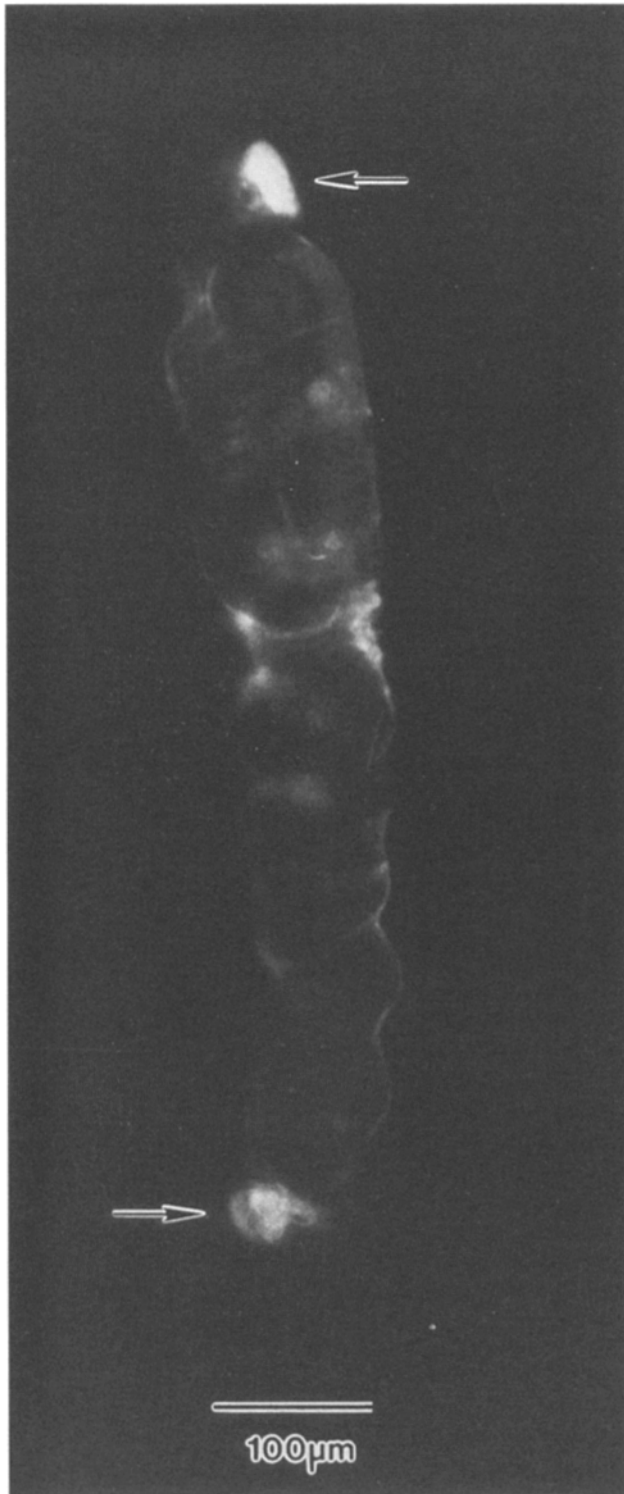
Figure 6. Localization of the annexin by immunofluorescence in an isolated pair of uteri containing embryos. Particularly intense staining is seen in the spermathecal valves at the ends of the uteri (arrows).

Figure 7. Immunofluorescent staining of the nematode seen with the preimmune serum. Staining is virtually absent from the pharynx (a, right side; compare with, for example, Fig. 4) and the spermathecal valve at the end of the uterus (b, area adjacent to the dark central image of an embryo; compare with, for example, Fig. 6).

that make up the "teeth" of the grinder (Fig. 8 b). These specialized areas of cuticle are used to grind bacteria and are thought to be a region of continuous deposition of cuticle throughout the lifetime of the animal (1). Additional immunogold staining of lower intensity was present under-

neath the cuticle of other parts of the oral cavity and distributed in nonmuscle cells, which may account for the general staining of the pharynx seen by immunofluorescence.

Several areas of the reproductive tract were found to stain significantly for the annexin by the immunogold procedure. In mature oocytes the annexin appears localized to the vicinity of the membranes of clear vesicles in the cytoplasm (Fig. 9 a). These are likely to be yolk granules as they appear in only the last few oocytes closest to the spermatheca (13). In sections stained with osmium, the yolk granules appear dark as they are highly osmiophilic (13). As osmium was avoided in the protocol used here for immunocytochemistry, the vesicles appear essentially empty. As fertilization occurs, the yolk granules break down as their contents are used. During this process the annexin is found concentrated in the membrane systems associated with the vesicle breakdown (Fig. 9 b).

The membranes of the spermathecal valve are seen clearly in a cross section of the valve stained with the preimmune serum (Fig. 10 a). This valve is several times smaller than the diameter of the eggs that must pass through it (37). Therefore, the membrane reticulum must unfold as the egg passes through, and then refold to form an opening of the original size after the egg passes through. When this structure is stained with the immune serum, the membranes in the valve are richly covered with gold particles (Fig. 10 b), suggesting a remarkably high concentration of annexin is associated with these membranes.

Fig. 11 also shows membranes associated with the spermathecal valve, including desmosomal junctions between the peripheral cells of this organ. These membranes are
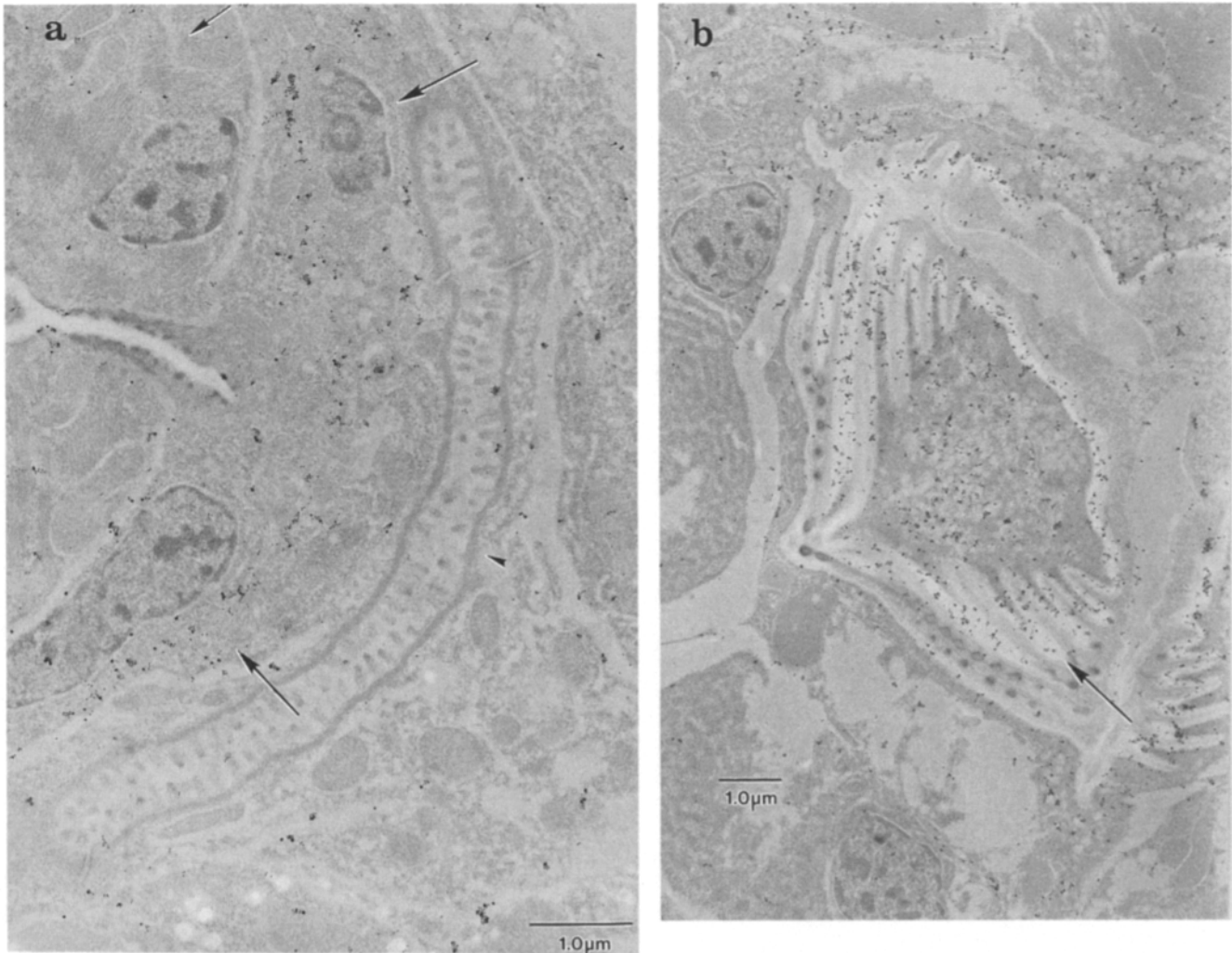
Figure 8. Localization of the annexin in the terminal bulb of the pharynx by immunogold staining. (*a*) The concentration of 15- and 30-nm gold particles indicates the presence of the annexin in the cytoplasm of the two gland cells (*large arrows*). Levels of staining seen in the muscle cells (*small arrow*) or gut cells (terminal disk; *small arrowhead*) are similar to those seen with the preimmune serum. (*b*) Localization of the annexin in the grinder portion of the pharynx. Gold particles are strongly associated with the teeth of the grinder, formed by folds of the cuticle (*arrow*).

also intensely stained with the annexin antibody. In general, the staining does not appear in the extracellular portion of the junctions between membranes, but is confined to the intracellular region or to the membranes themselves. In some cases, only one membrane of a pair in the junction is stained. In addition, some regions of some junctions are unstained although adjacent regions are intensely stained (Fig. 11).

Beyond the spermathecal valve, the fertilized eggs enter the uterus. The luminal wall of the uterus is seen to stain with the annexin antibody (Fig. 12). Stain appears present both within the cells comprising the uterine wall, as well as in association with extracellular matrix material facing the uterine cavity.

Discussion

At the time the sequencing of the nematode annexin cDNA was being completed, the nematode genomic se-

quencing project (33) was, coincidentally, sequencing the corresponding region of chromosome III (cosmid ZC155; GenBank reference CELZC155). The annexin gene was found to contain only one small intron of 50 bases near the center of the coding sequence. When most of the sequence of chromosome III was reported, it was evident that there are two other potential annexin genes on this chromosome, present in cosmids T07C4 and C28A5 (GenBank CET0744 and CEC28A5). We suggest the names *nex-1*, *nex-2*, and *nex-3* for these three annexin genes, in the order in which their sequences were released to GenBank. Hence, *nex-1* would be the gene corresponding to the protein characterized in this study. The *nex-2* and *nex-3* genes are complex genes possessing multiple introns. However, if the introns were properly removed, it appears these genes also encode intact proteins clearly homologous to the annexins. Our failure to detect the products of the *nex-2* and *nex-3* genes in this study suggests they are either not expressed, expressed to very low levels or only briefly during development, or may be trapped in compartments or
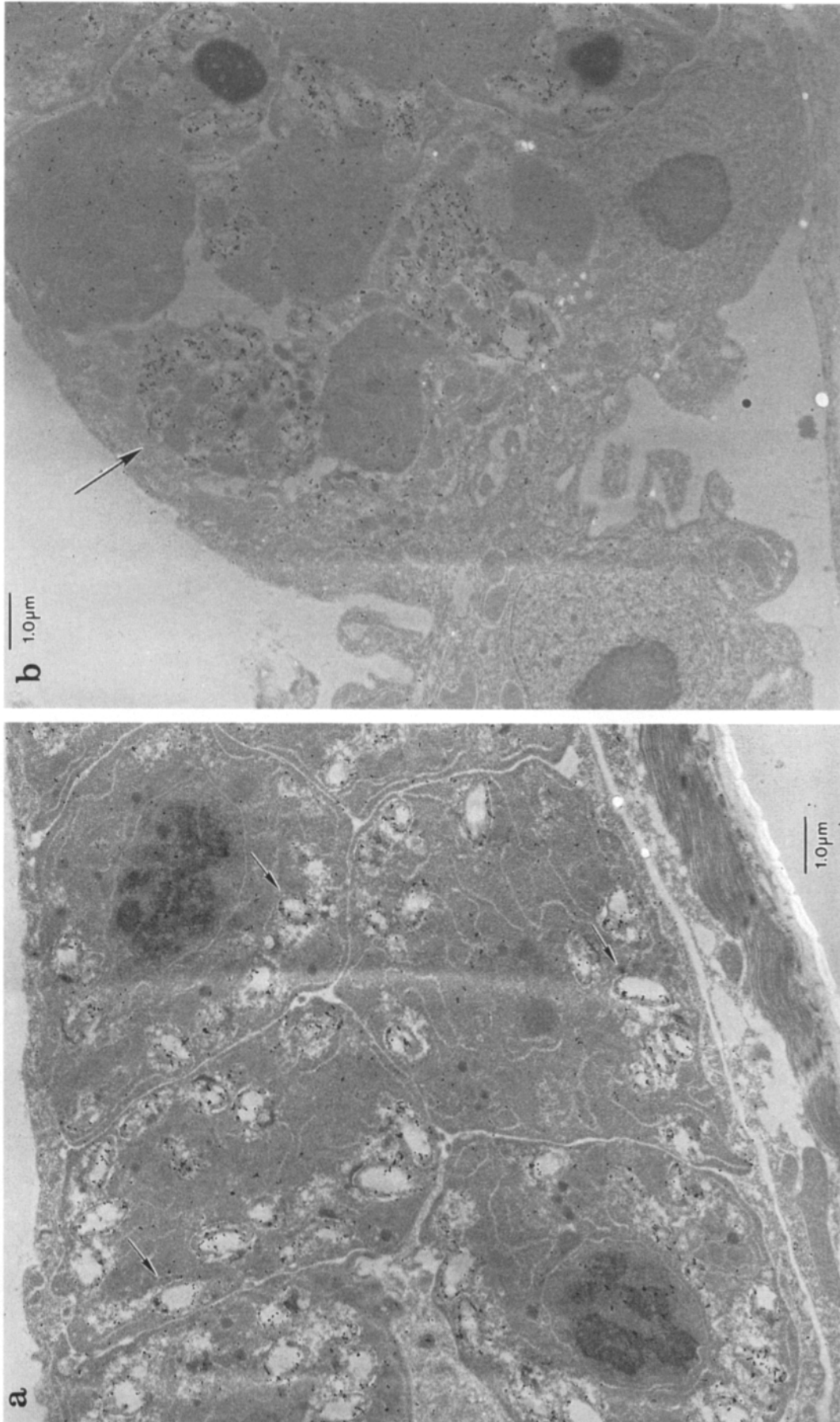
Figure 9. Localization of the annexin in oocytes in the proximal portion of the ovary of the nematode. (*a*) Gold particles are strongly associated with the periphery of the yolk granules in maturing oocytes (*arrows*). (*b*) In an oocyte undergoing fertilization gold particles are present on membranous complexes associated with breakdown of the yolk granules (*arrow*). Sperm cells show no specific staining for the annexin (left portion of *b* of the figure).
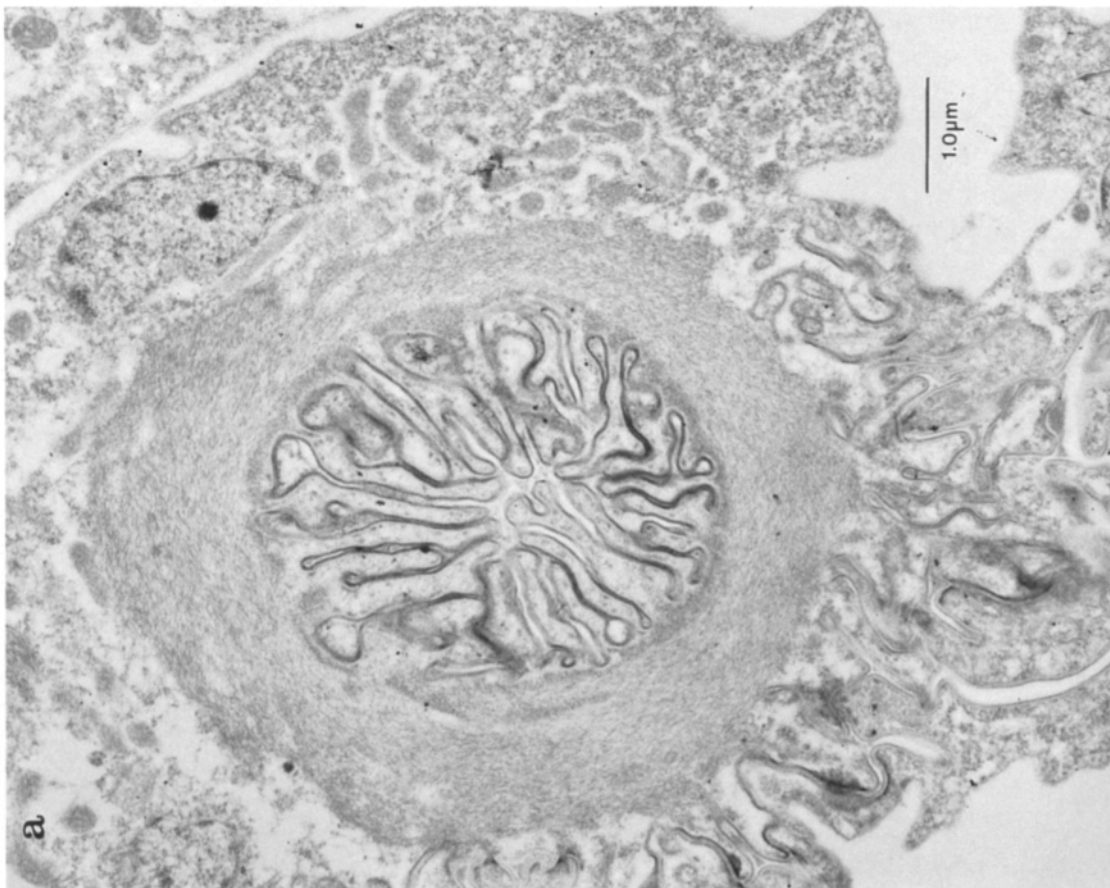
Figure 10. Immunogold staining of a cross section of the spermathecal valve. (*a*) Section stained with the preimmune serum. (*b*) Section stained with the immune serum. Gold particles are strongly associated with the folded membranes of the interior of the valve.
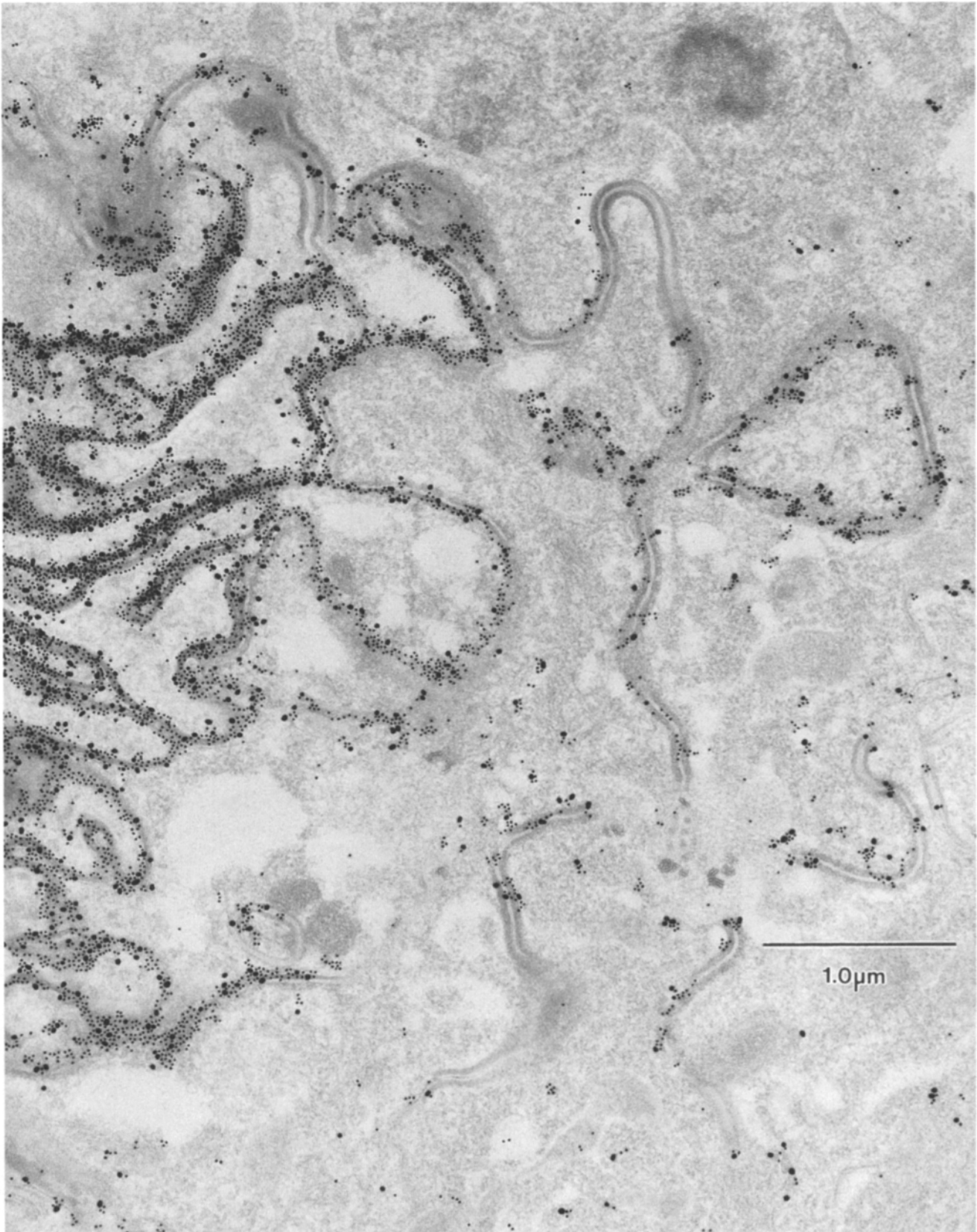
Figure 11. Localization of the annexin in association with the desmosomes near the spermathecal valve by immunogold staining.
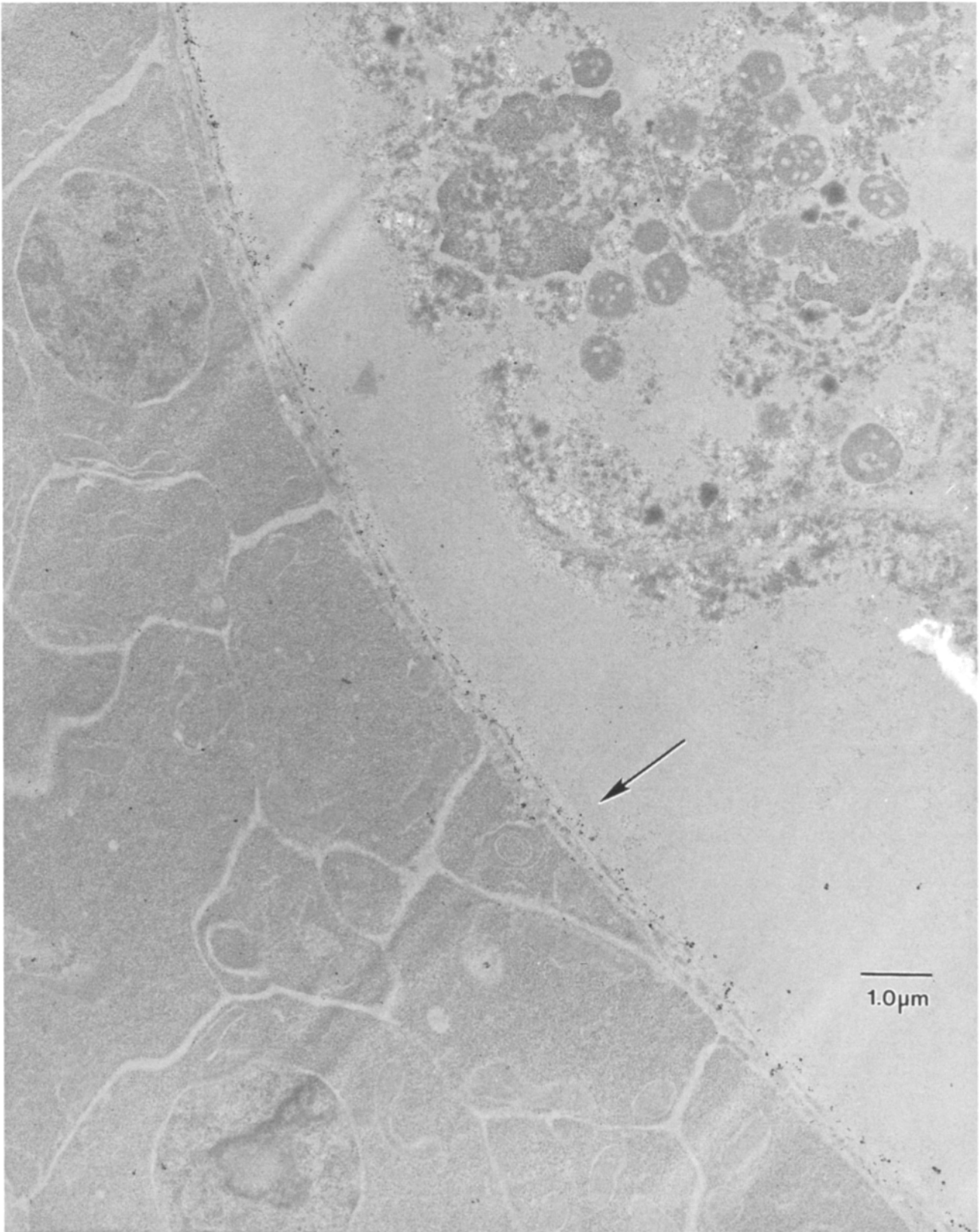
Figure 12. Localization of the annexin by immunogold staining in association with the uterine wall (*arrow*). The distal portion of the ovary is on the left, and an embryo within the uterus is on the right.

otherwise insoluble so that they were not obtained by the extraction procedures we applied.

A pattern of eight nucleotides (ATGCACTA) is found 212 bases upstream of the initiation codon in the *nex-1* gene sequence which is similar to a sequence motif present in the four cuticulin genes of *Drosophila* (32). This element may be important in controlling the expression of the *nex-1* gene during assembly of the cuticular structures the gene product is found associated with, such as the teeth of the grinder.

The amino acid sequence of the nex-1 annexin is ~36–42% identical to mammalian annexin sequences in the 300 residue conserved lipid- and calcium-binding core of the molecule. Therefore, it is not possible to identify this nematode annexin as a homolog of any specific one of the ten known mammalian annexins. The inferred products of the *nex-2* and *nex-3* genes show a similar degree of divergence in sequence. However, the genomic sequence at the 5' end of the sequence encoding the core of the nex-2 protein encodes a long motif rich in repeating tyrosines, glycines, and prolines similar to the NH_2-terminal domains of synexin (annexin VII; references 6, 10) and annexin XI (the calcyclin-binding annexin; references 34, 35). Thus, the family of nematode annexin genes shows some of the same types of diverse modifications present in mammalian annexins (3). Unless the nematode annexin genes are coincidentally grouped on chromosome III, it seems probable that additional annexin genes will be discovered in the nematode genome as sequencing of the entire genome proceeds.

The localization studies indicate a broad distribution for the nex-1 annexin, possibly underlying a wide range of activities. The involvement of the annexin in the formation and breakdown of the yolk vesicles and in elaboration of secretory product from the gland cells may be similar to the postulated roles of annexins in intracellular membrane trafficking and sorting (8, 9). Involvement in the expansion and contraction of the membranes of the spermathecal valve may reflect a more general role for annexins in maintaining membrane shape, particularly in those cells where annexins are found underlying the plasma membrane (for example see reference 26). Specialized functions related to maintenance of the extracellular matrix of the uterine wall, the cuticle layer of the vulva, and formation of the teeth of the grinder may be related to the presence of annexins in mammalian matrix vesicles where they are postulated to be involved in collagen deposition and bone mineralization (17). The association of the annexin with structures in the reproductive tract may indicate that the annexin plays a role in reproductive physiology related to that of the annexins that are abundant components of human seminal fluid (7). Both intracellular and extracellular localizations are indicated for the annexin, reflecting the reports of both intracellular and extracellular localizations of mammalian annexins (7, 14, 25, 28, 36). In all cases, the nematode annexin was found in association with membranous structures of some sort, emphasizing the probable importance in vivo of the ability of the protein to bind phospholipids in a calcium-dependent fashion.

The diversity of the localizations seen in the immunocytochemical experiments raises the question of whether multiple antigens were in fact being detected. In this regard, it is important that all staining seen by immunofluo-rescence was absent from controls in which the antiserum was preincubated with the annexin. Since the annexin used in the preincubation controls was recombinant protein produced in yeast, the specific staining seen is not due to the presence of other antigens that may have copurified with the annexin when it was prepared from nematodes for immunization. It is also important that there was an excellent correlation between observations made by immunofluorescence and by electron microscopy. All structures that were found to stain by immunofluorescence were also stained by the immunogold technique, with the possible exception of the vulva for which a proper section was not obtained in this study to examine. Significant staining was seen by electron microscopy in the oocytes, however, that was not detected by immunofluorescence. We think this is likely due to the inability of the antibody to penetrate these structures in the immunofluorescence procedures used. Indeed, even the background staining with the preimmune serum did not extend to the interior of the oocytes as they generally appeared dark in the fluorescence micrographs.

However, a remaining caveat when working with a family of homologous proteins such as the annexins is the possibility that another annexin is expressed that has some epitopes in common with the nex-1 protein. In general, we have found that antisera raised against annexins that show only 40% identity in sequence, similar to the divergence that has occurred with the *nex-1*, *nex-2*, and *nex-3* genes, are highly specific in the immunochemical and immunocytochemical procedures used here. However, there may be other closely related annexins in the nematode that have not been identified yet. This caveat may best be addressed by future examination of nematodes in which the expression of the *nex-1* gene has been altered.

It is intriguing that the physical location of the *nex-1* gene is close to the *dpy-17* genetic marker. In general, mutations in the *dpy* genes yield a shortened ("dumpy") worm. Several *dpy* genes have been found to encode collagen structural genes or genes for proteins that interact with collagen, such as proline hydroxylase (21). Furthermore, as described above, the genomic sequence in the vicinity of *nex-1* contains an eight base pattern that has been defined in the promoter region of the cuticulin genes of *Drosophila* (32). However, we did not detect any differences in the expression levels or size of nex-1 proteins extracted from three different *dpy-17* alleles (e164, e1295, and e1345; data not shown). In addition, recent fine structure analysis of this region of the genome suggests that *dpy-17* lies outside the cosmid, ZC155, that contains the annexin gene (Ewbanks, J., and B. Lakowski, personal communication).

The demonstration of high level expression of an annexin in *C. elegans*, plus its localization to a number of membranous structures, suggests this organism may prove to be a valuable system for genetic analysis of the diverse functions of this protein family in membrane trafficking, membrane shape changes, membrane–membrane junctions, and the expression and organization of extracellular matrices.

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