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Two Novel Annexins from *Drosophila melanogaster*

CLONING, CHARACTERIZATION, AND DIFFERENTIAL EXPRESSION IN DEVELOPMENT*

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Paul A. Johnston‡, Mark S. Perin§, Gary A. Reynolds¶, Steven A. Wasserman‖, and Thomas C. Südhof‡§‡‡**

From the ‡Department of Molecular Genetics, §Howard Hughes Medical Institute, ¶Department of Internal Medicine, and the ‖Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235

The annexins are a family of homologous Ca^{2+}- and phospholipid-binding proteins that until now have only been found in vertebrates. cDNA clones encoding two novel annexins from *Drosophila melanogaster* were isolated and characterized. RNA blots indicate that the messages for the two *Drosophila* proteins are differentially expressed in development, with one message being expressed throughout development, while the other is only found in early embryos and adult flies. *In situ* hybridizations localize the two *Drosophila* genes to 93B and 19A-4,7. A similarly high degree of homology relates *Drosophila* annexins to different vertebrate annexins, indicating that the *Drosophila* annexins are not the invertebrate homologues of particular mammalian annexins but that they constitute novel members of the annexin gene family. In continuation with a recently established terminology, the *Drosophila* annexins will be named annexins IX and X. The biochemical properties of *Drosophila* annexin X were investigated using recombinant protein. Similar to vertebrate annexins, annexin X bound to liver membranes and liposomes containing phosphatidylserine in a calcium-dependent manner but not to liposomes containing phosphatidylcholine. In addition, annexin X partitioned into the detergent phase of Triton X-114 as a function of calcium. The conservation of the annexin family of Ca^{2+}-binding proteins in invertebrates suggests that they have a basic function in cells which is not peculiar to vertebrate biology, and the availability of the *Drosophila* sequences will open avenues for mutational studies of these functions.

The calelectrins were originally described as a family of Ca^{2+}-dependent membrane-binding proteins that were postulated to be related to each other because they exhibited similar biochemical properties and shared immunologic epitopes (1, 2). Sequencing (3, 4) revealed these proteins to be homologous to a number of other proteins that were independently characterized in recent years by several investigators. Members of this protein family have been described under the following names: lipocortins (5), p35 and p36, also named calpactin (6); synexin (7); endonexin I and II, which were the 32.5 and 35 kDA calelectrins (1, 8) renamed by Geisow *et al.* (3); proteins I, II, and III (9); lymphocyte-specific p68 (10); chromobindins (11), and human placental anticoagulant protein (12). All proteins of this gene family currently known were isolated from vertebrates. Although there is no established function for these proteins that is accepted by all investigators, a consensus has recently been reached to name all proteins of this gene family annexins (13).

The annexins are Ca^{2+}-binding proteins that bind to negatively charged phospholipids in a Ca^{2+}-dependent manner (1, 14–18). Their amino acid sequences predict the presence of four internal repeats for all annexins except for annexin VI (67-kDa calelectrin), which has eight such repeats (4). When the sequences of different members of the protein family are compared with each other, the repeats are found to be conserved better by position between distinct members of the family than within a given protein (4). The four repeats have differences in length that are conserved in all proteins with the third repeat being the longest (generally 85 residues) and the second the shortest (generally 72 residues). No sequences homologous to the EF-hand sequence of Ca^{2+}-binding proteins (19) can be found in the primary structure of the annexins, suggesting that they contain a different Ca^{2+}-binding structure.

In addition to binding to negatively charged phospholipids in a Ca^{2+}-dependent manner, members of the annexin family have several interesting characteristics. Annexins I and II (lipocortins 1 and 2, also referred to as p35 and p36 and as calpactin 1 and 2 (20–22)) are tyrosine phosphorylated by growth factor receptors in a stimulation-dependent manner (21–23). The functional significance of the tyrosine phosphorylation is unknown, but the fact that annexins I and II are major substrates for the growth factor receptor tyrosine kinases indicates that they are intracellular proteins (6). The different members of the annexin protein family are differentially distributed in vertebrate tissues, with some proteins showing very restricted localizations. For example, annexin IV (32.5-kDa calelectrin) is almost exclusively localized to ductal epithelia, such as those of biliary and pancreatic ducts, while annexin II (lipocortin 2/p36) is preferentially found in microvilli (24, 25). Other proteins, particularly annexin VI (67-kDa calelectrin), appear to be ubiquitously found in all cells examined but particularly high in endocrine cells (24).

Several sometimes conflicting hypotheses have been advanced regarding the functions of the annexins. As lipocortins, they are thought to represent humoral mediators of the glucocorticoid-dependent regulation of inflammation (26, 27), as calpactins, to represent Ca^{2+}-dependent actin-binding proteins (22), and as anticoagulants, to consist of circulating components of the coagulation system (12). Each of these functions is not universally accepted since it is controversial

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‡‡ To whom correspondence should be addressed.

whether the annexins are secreted or intracellular proteins, and since not all members of the protein family appear to bind actin (1). We originally hypothesized that these proteins might be involved in regulating membrane traffic (1), and recent evidence has implicated annexin I (lipocortin I/calpactin) in regulating exocytosis in chromaffin cells (28). However, a distinct cellular function for this protein family remains to be established.

Previously, the annexins have only been studied in vertebrates. To investigate if the annexins are phylogenetically widely distributed, and to explore the possibility of genetic approaches to this protein family, we have now studied their presence in *Drosophila melanogaster*. Two different novel members of the annexin protein family (annexins IX and X) were found in *Drosophila*, and one was biochemically characterized, demonstrating that it had calcium-dependent binding characteristics similar to vertebrate annexins. These results indicate a basic cellular function of these proteins that may be conserved between multicellular organisms.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (6000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from Du Pont-New England Nuclear and ^{35}S-labeled methionine and cysteine (Tran^{35}slabel) from ICN. *In vitro* transcription and translation reagents were from Stratagene and Du Pont-New England Nuclear, respectively. DNA modifying enzymes were from New England Biolabs (restriction enzymes, T_4 DNA ligase and kinase), U. S. Biochemicals (Sequenase) or Boehringer Mannheim (restriction enzymes, ligases). Phospholipids were obtained from Avanti Polar Lipids, and Triton X-114 from Calbiochem. All other chemicals and proteins were of reagent grade and used without further purification.

Complementary DNA Cloning and Sequencing—A cDNA library from adult *Drosophila* heads (kind gift of Dr. G. M. Rubin, University of California, Berkley) was screened with an oligonucleotide derived from the calelectrin/lipocortin consensus sequence as described (4). Of the five hybridization positive clones isolated, three were shown by sequencing not to be homologous to the calelectrins/lipocortins, one was λD3–6, and the fifth was identical with λD3–6 but was not studied further because it had a much smaller insert. The library was rescreened with a new consensus probe made to the first repeat of λD3–6 (sequence: GCGAAGCTTCTCGTCGGTGCCGAAGCCCTTTCAT) and λD3–16 was isolated. The *Eco*RI inserts of the two clones were subcloned into pBluescript (subclones are referred to as pD3–6 and pD3–16) and M13 phage vectors and sequenced by the dideoxy nucleotide chain-termination method (29). Sequence data were analyzed on an IBM-AT computer using Microgenie software (Beckman, Inc.).

RNA Blotting—10 μg of total RNA isolated from 0 to 2 h embryos, 2–4 h embryos, third instar larvae, and adult Oregon R *D. melanogaster* were electrophoresed and blotted onto nylon membranes as described (31), using Bethesda Research Laboratory RNA standards as molecular weight markers. Filters were hybridized with uniformly ^{32}P-labeled single-stranded DNA probes and washed at high stringency as described (30, 31).

In Situ Hybridizations—These were performed on squashed salivary gland chromosomes as described using the biotin-labeled total inserts of the cDNA clones as probes (32).

In Vitro Transcription and Translation of pD3–16—T3 and T7 transcription products of pD3–16 were obtained using an *in vitro* transcription kit and translated in the presence of [^{35}S]cysteine and -methionine using a rabbit reticulocyte *in vitro* translation system. Translation was terminated after 60 min by dilution in buffer A (10 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 5 mM EGTA[1] and 1 mM PMSF).

Bacterial Expression—In order to clone D3–16 into the bacterial expression vector pET3a (33), the coding region of D3–16 was amplified by the polymerase chain reaction (34, 35) using the following oligonucleotides CTGCATATGGAATACAAACCCGTGCC and CTCAGATCTAGGCGGATCCAAGTAGGGC. The oligonucleotides were designed to contain *Nde*I and *Bam*HI restriction sites for cloning into pET3a in such a manner that the recombinant protein produced would not contain any amino acid residues that are not predicted by the cDNA sequence. The amplified DNA was purified by PAGE and cloned into pET3a after restriction enzyme digestion. *Escherichia* coli BL21(DE3) were transformed with the expression construct pETd3–16 or with the parent vector pET3a as a control. Recombinant protein expression was induced by incubating a growing culture with 0.4 mM IPTG for 3 hours. Radiolabeled products were obtained from IPTG-induced cultures that were treated with rifampicin (200 μg/ml) 30 min after the addition of IPTG and pulse-labeled with [^{35}S]cysteine and -methionine in M9 minimal medium during the third hour of incubation. Bacterial cultures were pelleted by centrifugation, treated with 10 g/liter lysozyme in buffer A containing protease inhibitors (5 mg/liter each of leupeptin, pepstatin, and antipapain) on ice for 5 min, sonicated, and incubated with DNase (25 mg/liter) for 15 min at room temperature. Soluble proteins were separated from insoluble proteins and membranes by centrifugation (2200 × g_{av} for 10 min) and the resulting fractions were analyzed by SDS-PAGE and autoradiography.

Ca^{2+}-dependent Binding to Bovine Liver Membranes—Membranes were prepared from 30 g of bovine liver by first homogenizing the tissue in 100 ml of buffer A using a Polytron tissue homogenizer followed by 20 strokes in a Dounce homogenizer. The supernatant from a low speed centrifugation of the homogenate (800 × g_{av} for 5 min) was centrifuged at 100,000 × g_{av} for 60 min. The resulting membrane pellet was resuspended in buffer A (163 ml final volume) and the membranes (450 μg of protein) were incubated for 10 min at room temperature with radiolabeled annexin X (108 μg of protein) produced as a recombinant protein in bacteria. Incubations were carried out in buffer A containing 5 mM EGTA and 6 or 10 mM calcium or magnesium. Annexin X bound to the membranes was separated from free protein by centrifugation (30,000 × g_{av} for 10 min) and analyzed by SDS-PAGE and autoradiography.

Ca^{2+}-dependent Binding to Phospholipid Liposomes—For the preparation of liposomes composed of phosphatidylcholine/phosphatidylethanolamine or phosphatidylserine/phosphatidylethanolamine in a 1:1 ratio (w/w), chloroform solutions of these phospholipids were dried down under liquid nitrogen and dried further under vacuum for 1 h. The lipids were rehydrated in buffer A for 30 min, vortexed, and sonicated in a bath sonicator for 3 × 20 s. For binding studies, liposomes (300 μg of lipids) were incubated with radiolabeled recombinant annexin X (90 μg of protein) for 10 min at room temperature in buffer A containing 5 mM EGTA or 5 mM EGTA 5.5 mM $CaCl_2$. Bound protein was separated from free by centrifugation (150,000 × g_{av} for 30 min) and analyzed by SDS-PAGE and autoradiography.

Ca^{2+}-dependent Partitioning into the Detergent Phase of Triton X-114—Recombinant radiolabeled annexin X (97 μg of protein) in buffer A was adjusted to 1% Triton X-114 and 5 mM EGTA, 5 mM EGTA, 10 mM $CaCl_2$, or 5 mM EGTA, 10 mM $MgCl_2$ and incubated on ice for 10 min. Samples were incubated at 37 °C for 20 min to induce phase partitioning (36). Phases were separated by centrifugation on a 6% sucrose cushion and analyzed by SDS-PAGE and autoradiography.

RESULTS

Two different cDNA clones with homology to mammalian annexins were isolated from a *Drosophila* head cDNA library using oligonucleotide probes complementary to the annexin consensus sequence. The nucleotide and predicted amino acid sequences of the two clones are shown in Fig. 1. Clone D3–16 is full-length as indicated by the presence of in-frame stop codons in the 5′-untranslated region (*underlined* in Fig. 1) and by the size of its message (see below, Fig. 2), while D3–6 is partial although it probably contains most of the coding sequence as judged by the analysis of its repeat structure and message size (see below, Figs. 2 and 3). Both cDNA clones did not contain poly(A) tails. However, D3–16 has two overlapping polyadenylation consensus sequences at its 3′ end, while D3–6 has a sequence very similar to the polyadenylation sequence at the same position (*underlined* in the 3′-untranslated regions in Fig. 1). The poly(A) tails may have been lost during cDNA construction and amplification.

[1] The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; IPTG, isopropyl-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

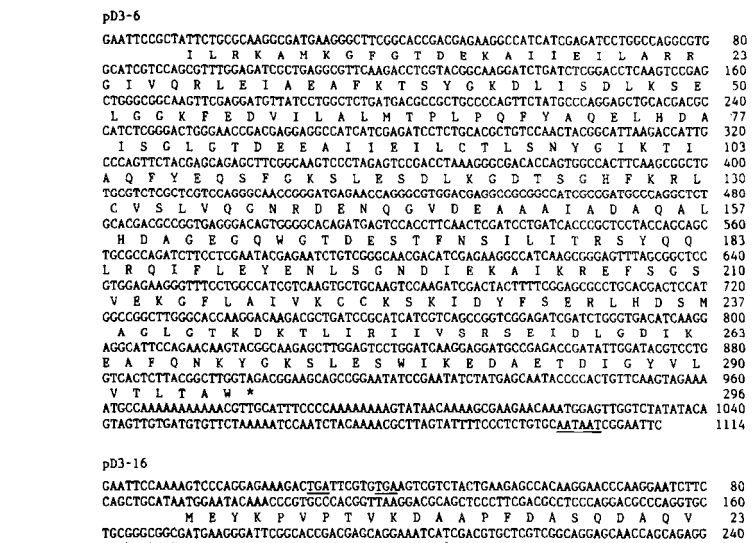

FIG. 1. **Nucleotide and translated amino acid sequences of two cDNA clones encoding annexins IX and X from *D. melanogaster*.** The sequence of clone pD3-6 (annexin IX) is shown on top and of clone pD3-16 (annexin X) on the bottom. Sequences are *numbered* on the right and flanked by *Eco*RI linkers derived from the cloning procedure (38). The translated amino acid sequence is given in *single-letter code* below the nucleotide sequence, with the *asterisk* denoting the stop codon. Although both clones lacked poly(A) tails, polyadenylation signals can be found in pD3-16 and a similar sequence in pD3-6 (*underlined* in the figure). The in-frame termination codons in the 5′-untranslated region of D3-16 are also *underlined*.

RNA blotting experiments were performed to determine the sizes of the messages for D3-6 and D3-16 and to study their expression during development (Fig. 2). D3-16 was encoded by a single message of 1.4 kb that was very abundant in adult flies and weakly expressed in early embryos (*lanes A* and *D*, respectively, *right panel* of Fig. 2) but absent from late embryos and late instar larvae (*lanes B* and *C*). This suggests that D3-16 is primarily expressed in adult flies and that the message observed in early embryos may be maternal. If the poly(A) tail is taken into account, the cDNA insert of the clone we isolated appears to be full-length. Probes derived from D3-6 hybridized to two messages of approximately 1.35 and 1.5 kb that were expressed at all stages in development, although most abundantly in adult flies (*left panel* of Fig. 2). The message sizes, particularly that of the larger species, appeared to vary slightly during development, with the late embryonal message being slightly smaller than the adult message (*cf. lanes B* and *D, left panel* of Fig. 2). The larger message of D3-6 was less abundant than the smaller message. As judged by the size of the smaller message for D3-6, the cDNA clone that we isolated appears to lack 100–250 bp at its 5′ end.

In Fig. 3, the amino acid sequences of the two *Drosophila* annexins are aligned with each other and with a consensus sequence obtained from a comparison of mammalian sequences (4–7), revealing a high degree of homology. The *Drosophila* proteins contain four internal repeats as is typical for the annexins (5, 6). While the homology between the four repeats within a given protein is rather weak, although significant, the degree of identity between the repeats found in the same position in different proteins is very strong. This allows the formulation of a separate consensus sequence for each repeat which is stronger than that for all four repeats combined (4). The *Drosophila* sequences clearly align well with the mammalian consensus sequence (Fig. 3) or with individual mammalian sequences (data not shown), although at some positions a highly conserved residue in the mammalian annexins is different in the *Drosophila* proteins (for example, the Leu and Lys at positions 96 and 118 of D3-6 and D3-16, respectively, is an Arg in all mammalian proteins). The alignment demonstrates that the clone encoding D3-6, although incomplete, contains all four repeats and is only missing its aminoterminal extension.

In individual comparisons between the *Drosophila* and the mammalian sequences, overall sequence identities observed ranged from 40 to 50%, with annexins I and II (lipocortins I and II) being least related to both *Drosophila* proteins, and annexin V (35-kDa calelectrin) and annexin VI (67-kDa calelectrin) being most related. The two *Drosophila* sequences, when compared with each other, were found to be 46% identical. These comparisons demonstrate that although there are differences in the relatedness between the *Drosophila* proteins and the mammalian sequences currently available, the differences are rather small and within the range generally observed in comparisons between members of the protein family. Accordingly, the *Drosophila* proteins do not appear to be the direct invertebrate equivalents of particular mammalian annexins, but rather independent members of the protein family and will therefore be referred to as annexins IX and X.

To investigate the biochemical properties of *Drosophila* annexins, a bacterial expression vector directing the inducible synthesis of full-length annexin X under the control of the T7 promoter was constructed (see "Experimental Procedures"). *E. coli* harboring the expression construct but not the parental vector exhibited the abundant inducible expression of a 35-kDa protein which by its molecular mass and expression characteristics was identified as recombinant annexin X (Fig. 4). Annexin X encoded by the expression vector can be selectively radiolabeled by growing the bacteria in the presence of [^{35}S]methionine and -cysteine under conditions under which the endogenous bacterial protein synthesis is inhibited (Fig. 4). In bacteria transformed with the expression construct, only annexin X was radiolabeled under those conditions while bacteria containing the parental vector exhibited a different single radiolabeled band that appears to be specified by a reading frame in the vector and is absent if the vector has an insert.

Most of the recombinant annexin was insoluble in *E. coli* and was segregated into inclusion bodies, but a small percentage (approximately 5%) was soluble (Fig. 4) and could be directly used for biochemical studies. Radiolabeled recombinant annexin X was used to assess the ability of the protein to bind to bovine liver membranes as a function of calcium. As demonstrated in Fig. 5, the recombinant protein only was copelleted with liver membranes in the presence of calcium but not in the presence of EGTA or magnesium. Identical results were also obtained with radiolabeled protein produced by *in vitro* transcription and translation of pD3-16. Only the sense but not the antisense strand of pD3-16 produced a protein product of 35 kDa after *in vitro* translation. This

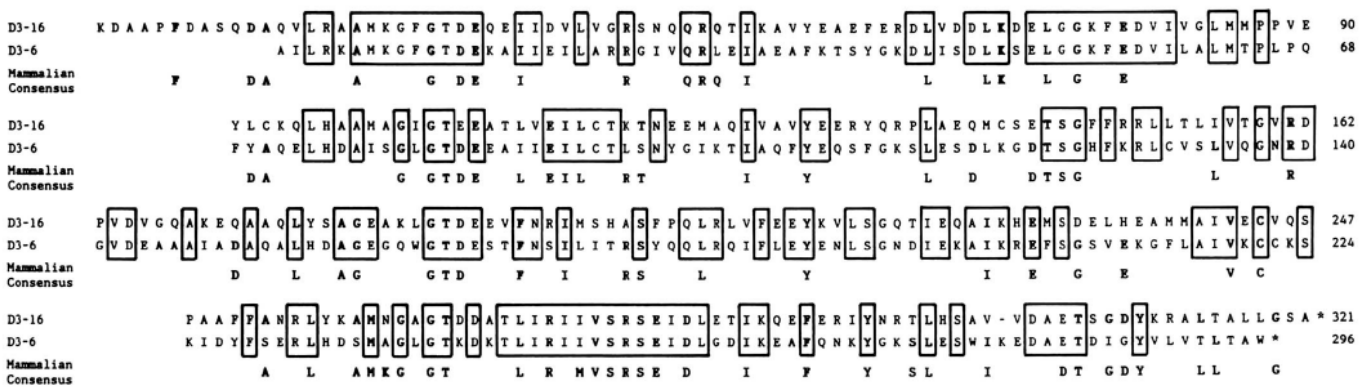

FIG. 2. **Alignment of the amino acid sequences of the *Drosophila* annexins with each other and with a consensus sequence derived from mammalian annexins.** Residues that are identical between the two *Drosophila* sequences are *boxed,* and residues that are shared between the mammalian consensus sequence and the *Drosophila* sequences are shown in *bold type*. Sequences are *numbered* to the right and identified on the left. Part of the first and third repeats are not shown because no shared residues were found in those portions. The mammalian annexin consensus sequence includes all residues that are present in more than 80% of the sequences (4–7).

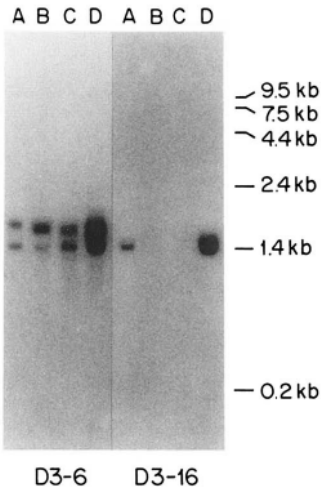

FIG. 3. **Differential expression of the messages for annexins IX and X during *Drosophila* development.** Total RNA (10 μg) from 0 to 2 h embryos (*lanes A*), 2–4 h embryos (*lanes B*), third instar larvae (*lanes C*), and adult flies (*lanes D*) was electrophoresed and blotted. The same blot was consecutively hybridized with probes derived from pD3–6 (annexin IX, *left panel*) and pD3–16 (annexin X, *right panel*). Position of molecular weight size markers are shown on the right. Both autoradiograms were exposed for 24 h at −70 °C with intensifying screens.

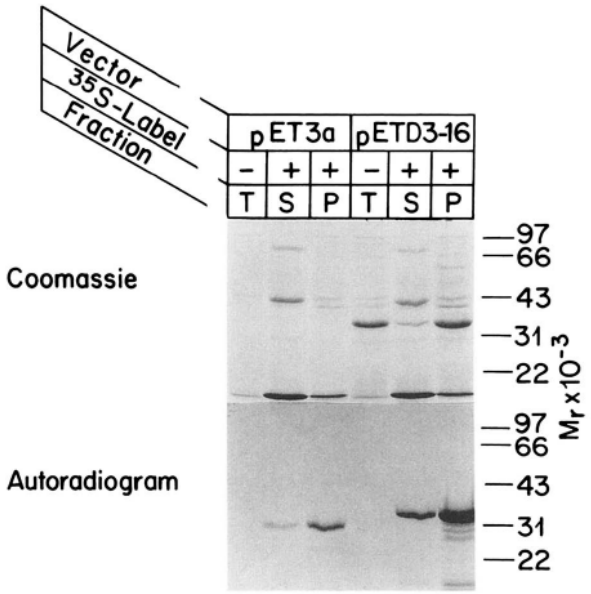

FIG. 4. **Production of recombinant annexin X in *E. coli*.** BL21(DE3) cells transformed with the parent vector pET3a (*left three lanes*) or with the annexin X expression vector pETD3–16 (*right three lanes*) were induced for 3 h with 0.4 mM IPTG. Half of the cells were radiolabeled and fractionated into soluble (*S*) and insoluble proteins (*P*) (see "Experimental Procedures"), and compared with total proteins in unlabeled bacteria (*T*). All fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining (*top*) and autoradiography (*bottom,* 48-h exposure). Bacteria transformed with the annexin X construct synthesize large amounts of a 35-kDa protein that is selectively radiolabeled in the presence of rifampicin which blocks endogenous bacterial transscription. The 35-kDa protein constitutes more than 30% of the total protein in the cells transformed with pETD3–16 but is absent from bacteria transformed with the vector only. These, however, exhibit a specific radiolabeled protein of 30 kDa upon rifampicin treatment that is specified by the insertless vector. Migration of molecular weight markers is shown on the right.

product again bound to liver membranes only in the presence of calcium but not of EGTA (data not shown).

To investigate the mechanism of binding of annexin X to liver membranes, the ability of recombinant *Drosophila* annexin X to bind to liposomes composed of either phosphatidylserine-phosphatidylethanolamine or phosphatidylcholine-phosphatidylethanolamine as a function of calcium was investigated (Fig. 6). Annexin X only bound to phosphatidylserine-containing liposomes but not to phosphatidylcholine liposomes and only in the presence of calcium, suggesting that it has binding characteristics similar to those of the mammalian annexins.

Some of the mammalian annexins (annexins IV, V, and VI, the original calelectrins (1)) are efficiently purified by calcium-dependent hydrophobic affinity chromatography, suggesting that they expose a hydrophobic site as a function of calcium (1, 18). In order to test if *Drosophila* annexin X would also bind to membranes by a mechanism that involves the calcium-dependent exposure of hydrophobic sites, the distribution of annexin X into the aqueous and detergent phases of Triton X-114 as a function of calcium was investigated. Proteins containing hydrophobic sites such as transmembrane proteins are selectively retained in the detergent phase of Triton X-114, while almost all soluble proteins partition into the aqueous phase. Annexin X, although fully soluble even at

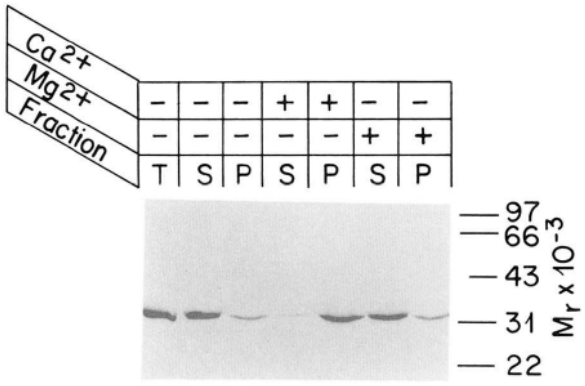

FIG. 5. **Binding of recombinant *Drosophila* annexin X to bovine liver membranes as a function of Ca^{2+}.** Bovine liver membranes were incubated with recombinant radiolabeled annexin X in buffers containing EGTA, EGTA-Ca^{2+}, or EGTA-Mg^{2+} and separated into bound and free annexin X by centrifugation (see "Experimental Procedures"). Pellets (*P*) and supernatants (*S*) were analyzed by SDS-PAGE and autoradiography (18-h exposure) with the migration positions of molecular weight standards being shown on the right. The left lane (*T*) contains the starting material used for the experiment. Migration of molecular weight markers is shown on the right.

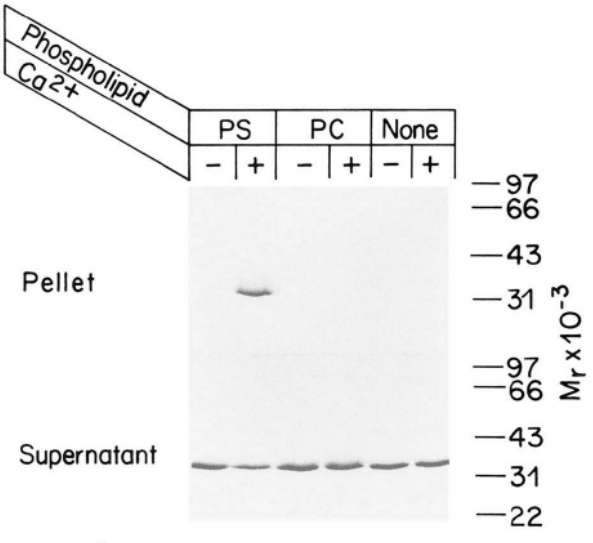

FIG. 6. **Ca^{2+}-dependent binding of recombinant *Drosophila* annexin X to phospholipids.** Radiolabeled annexin X was incubated with or without liposomes composed of phosphatidylserine-phosphatidylethanolamine (*PS*) or phosphatidylcholine-phosphatidylethanolamine (*PC*) in the presence or absence of Ca^{2+} (see "Experimental Procedures"). Bound and free annexins were separated by centrifugation and the liposome pellets (*top*) and supernatants (*bottom*) were analyzed by SDS-PAGE followed by autoradiography (18-h exposure). Annexin X is only pelleted if both calcium and phosphatidylserine are present. Molecular weight markers are shown on the right.

high calcium concentrations, showed a selective shift into the detergent phase of Triton X-114 that was not observed in the presence of EGTA only or of magnesium (Fig. 7).

In situ hybridization experiments were performed to localize the chromosomal positions of the genes for annexius IX and X. As shown in Fig. 8, annexin IX (D3–6) lies on chromosome 3 at position 93B, while annexin X (D3–16) is located on the *X* chromosome at position 19A–4,7. Several deletions have been described that encompass the regions to which D3–6 and D3–16 were localized (37). Future experiments will have to investigate if these deletions can be used as a first approach to genetically study the function of D3–6 and D3–16.

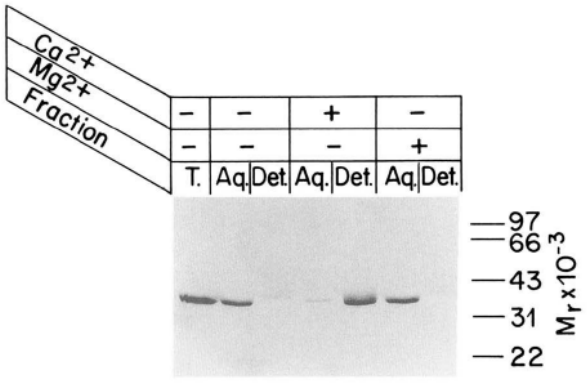

FIG. 7. **Ca^{2+}-dependent partitioning of *Drosophila* annexin X into the detergent phase of Triton X-114.** Radiolabeled recombinant annexin X was incubated on ice in 1% Triton X-114 with or without Ca^{2+} or Mg^{2+} and phase separation was induced at 37 °C (36). Fractions containing the detergent (*det.*) and aqueous (*aq.*) phases were analyzed by SDS-PAGE and autoradiography (18-h exposure). The *left lane* (*T*) contains the starting material, and the migration of molecular weight markers is shown on the right.

DISCUSSION

The annexins are a well-characterized family of Ca^{2+}-binding proteins that bind to phospholipids as a function of Ca^{2+} (1–7). Until now they have only been identified in vertebrates where at least eight different members named annexins I to VIII have been reported (1, 4–13). All vertebrate annexins are composed of four internal repeats (or a duplication of the four repeats to eight repeats in annexin VI) and exhibit sequence identities between 35 to 50% in pairwise comparisons. In addition to their common architecture, all vertebrate annexins appear to have similar phospholipid binding specificities, but there is currently no consensus on what their cellular functions might be.

We have now characterized two novel annexins from the fruitfly *D. melanogaster*. The sequences of these clones demonstrate that the structural characteristics of the annexin protein family are highly conserved between vertebrates and invertebrates. The consensus sequence elements established with the mammalian proteins are generally retained in the *Drosophila* sequences, including the observation that repeats are individually more conserved between proteins than within a protein. By and large, the *Drosophila* proteins are as homologous to the mammalian proteins as they are to each other or as the mammalian proteins are to each other (40–50% identities in pairwise comparisons). This indicates that the *Drosophila* homologues of the annexins described here are not the invertebrate equivalent of specific mammalian proteins but rather additional members of an already large gene family. As such, the *Drosophila* proteins are referred to as annexins IX and X.

The two *Drosophila* annexins described here are differentially expressed in development. Since the cDNAs encoding them were isolated from a library constructed with RNA derived from adult heads, it is not surprising that both proteins are most abundantly expressed in adult flies. However, they are differentially expressed in other stages of *Drosophila* development (Fig. 3). Eight different annexins have been described in mammals, raising the possibility that additional members of this protein family may be present in *Drosophila*.

Some of the biochemical characteristics of *Drosophila* annexin X were studied using radiolabeled recombinant protein produced in *E. coli* or synthesized by *in vitro* transcription and translation. Similar to mammalian annexins, *Drosophila* annexin X was found to bind to phospholipid membranes in

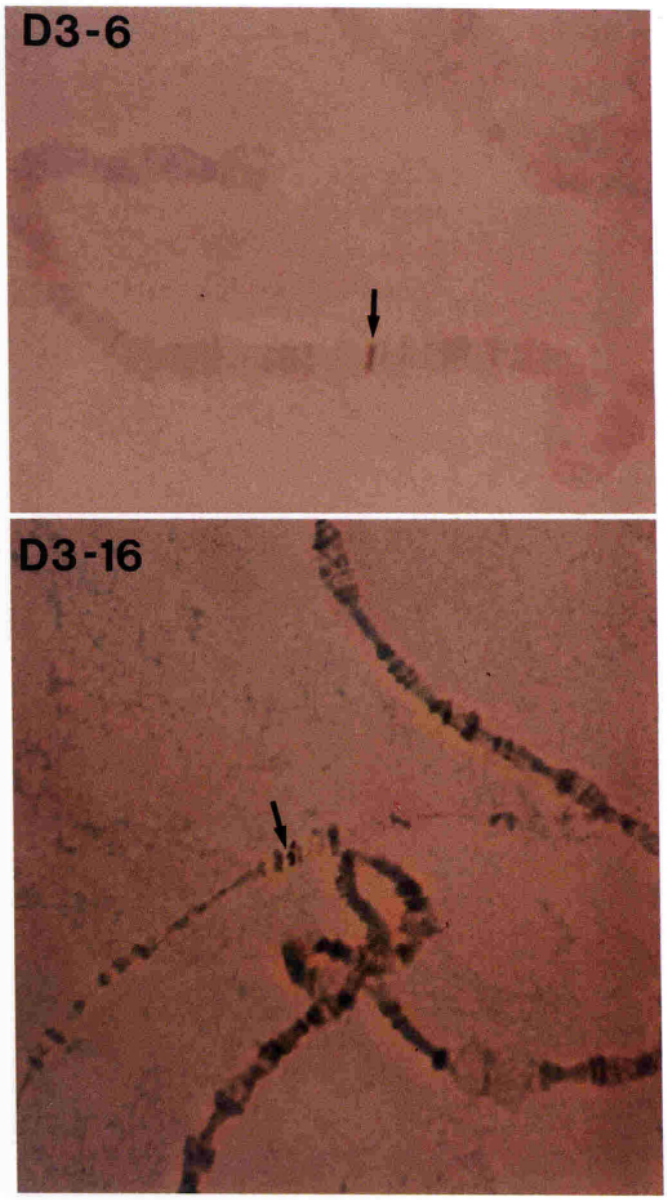

FIG. 8. **Localization of the genomic loci for annexin IX (pD3–6) and annexin X (pD3–16) by *in situ* hybridization using biotin-labeled probes on squashed salivary gland chromosomes (32).** Hybridization-positive bands are indicated by *arrows.*

a calcium-dependent manner. This binding was specific for calcium when compared with magnesium, and specific for phosphatidylserine as compared with phosphatidylcholine and phosphatidylethanolamine. In addition, it was found that annexin X could be selectively induced by calcium but not by magnesium to partition into the detergent phase of Triton X-114. Partitioning between the detergent and aqueous phases of Triton X-114 is a widely used method to assess the presence of hydrophobic sites such as transmembrane regions in a protein (36). The calcium-dependent movement of annexin X into the detergent phase of Triton X-114 suggests that calcium initiates binding of the hydrophobic Triton X-114 to annexin X, possibly by inducing a conformational change in annexin X that exposes hydrophobic sites. A similar mechanism has been postulated to explain the calcium-dependent purification of annexins IV, V, and VI (originally named calelectrins (1, 8)) on phenyl-Sepharose

The results of this study have several implications for our understanding of the annexin super-gene family. First, with the unequivocal demonstration of the presence of annexins in invertebrates a function such as anticoagulation or glucocorticoid-modulated inflammation that is only fully developed in vertebrates seems rather unlikely. Our data clearly support the notion that the annexins have a general role in cellular functions that is shared by all cell types and multicellular organisms.

Second, the similarity in the calcium-dependent phospholipid binding characteristics between *Drosophila* and vertebrate annexins suggests that these properties are encoded in structural elements shared by all annexins and that these properties are central to the unknown cellular functions of the annexins. However, no two annexins are more than 50% identical, and less than 10% of the amino acid residues are invariant in all annexins. Sequence characteristics such as hydrophobicity are much more strongly conserved. These results are compatible with the notion that the tertiary structures of all annexins are similar and that the actual calcium- and phospholipid-binding sites are only formed by a few key residues, while the variable residues may have a functional roles that are specific to individual annexins. The differential expression of the two annexins from *Drosophila* as well as the previously characterized tissue-specific expression of mammalian annexins clearly points toward tissue and stage-specific roles of individual annexins.

Third, the conservation of the four-repeat structure of the annexins in *Drosophila* supports an evolutionary model whereby all annexins were derived from the same four-repeat precursor which in turn may have evolved by gene duplications from precursors containing one and two repeats (4). It will be interesting to determine if the annexins are specific to multicellular organisms or are also present in unicellular eukaryotes such as yeast, and if a protein can be found that has less than four repeats. A function of the annexins in processes that are specific for vertebrates seem unlikely now in view of their presence in insects. However, the question now arises if the annexins have a role in functions associated with multicellularity, that is with the evolution of tissues, or if they participate in cellular processes shared by all eukaryotes.

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