Identification and partial sequence analysis of novel annexins in *Lytechinus pictus* oocytes

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The annexins are a major class of calcium-binding proteins with unknown functions. In an attempt to define novel model systems in which to study members of the annexin family, we have investigated the expression of annexins in eggs from the sea urchin *Lytechinus pictus*. Western blot analysis of *L. pictus* eggs using antisera raised against human annexins I, V and VI revealed the presence of immunoreactive proteins of approximately 34 kDa, 35 kDa and 68 kDa respectively. The sea urchin annexins behaved similarly to their mammalian counterparts, both during purification and in their ability to bind calcium-dependently to anionic phospholipids. Of the three sea urchin

annexins, the 34 kDa form was most abundant, yielding sufficient quantities for peptide microsequencing. The amino acid sequences derived in this way showed the *L. pictus* annexin to be closely related both to mammalian annexin I and to annexins IX, X and XII from *Drosophila* and *Hydra*. However, N-terminal sequence from the *L. pictus* annexin showed it to be a novel member of the annexin super-gene family. The results are interesting in view of the complex evolution of the annexin gene family, and also point to the potential usefulness of echinoderm eggs as a model system in which to study annexin function.

INTRODUCTION

The annexins are a family of calcium-dependent phospholipid-binding proteins, of which there are known to be at least ten mammalian genes (Moss, 1992), with further unrelated annexins identified in *Hydra vulgaris* and *Drosophila melanogaster* (Schlaepfer et al., 1992a; Gerke, 1989). Despite a lack of persuasive functional data, the annexins have been proposed to have roles as phospholipase A₂ inhibitory proteins (Davidson and Dennis, 1989), anticoagulants (Tait et al., 1988), calcium channels (Huber et al., 1990), protein kinase C inhibitors (Schlaepfer et al., 1992b) and participants in both exocytotic (Creutz, 1992) and endocytic pathways (Futter et al., 1993; Emans et al., 1993). Members of the annexin family share a common biochemical property, namely the ability to bind calcium-dependently to phospholipids. However, they are distinct from other proteins that have this property (such as protein kinase C and members of the E-F hand family of calcium-binding proteins), in that the structural determinant that confers calcium-binding is a highly conserved 70 amino acid motif that occurs as a tetrad repeat in all annexins except annexin VI, which has eight repeats (Moss et al., 1988). Given that annexins are broadly similar to one another with respect to structure, it might be anticipated that they share related functions. Although this is a possibility, the N-terminal domains of annexins differ greatly between members of the family, and it seems likely that these important sequences determine functional individuality (Crompton et al., 1988).

Of the suggested functions for annexins, roles for annexins II and VI in both exocytosis and endocytosis (Drust and Creutz, 1988; Emans et al., 1993; Creutz et al., 1992; Lin et al., 1992) and for annexin I in endocytosis (Futter et al., 1993) have recently

received close attention. Annexin II is able to promote the aggregation of isolated chromaffin granules at physiological calcium concentrations (Drust and Creutz, 1988) and can prevent exocytotic run-down of permeabilized chromaffin cells (Ali et al., 1989). The observation that annexin II is also associated with endosomal vesicles (Emans et al., 1993) implicates this protein in both pathways. The role of annexin VI in exocytosis and endocytosis is less clear, it has been shown to inhibit annexin II-mediated granule aggregation (Creutz et al., 1992) and also to promote budding of clathrin-coated pits *in vitro* (Lin et al., 1992) but not *in vivo* (Smythe et al., 1994). Annexin I, which is a major substrate for phosphorylation on tyrosine by the epidermal growth factor receptor (EGFR), has been co-localized with the EGFR to multivesicular bodies, suggesting involvement in lysosomal targeting and/or receptor recycling (Futter et al., 1993). In addition to roles in membrane interactions, it has also been suggested that annexins may have cell-cycle regulated functions. For example, annexin II mRNA levels are known to be influenced by progression through the cell cycle (Keutzer and Hirschhorn, 1990; Chiang et al., 1993) and annexin VI is subject to a growth-dependent post-translational modification (Moss et al., 1992).

Given the confusion surrounding the proposed roles of annexins in these diverse cellular activities, clarification may be sought by investigating annexin function in simpler model systems. Sea urchin eggs have been extensively used for the study of both exocytosis and cell cycle regulation (Vacquier, 1975; Baker and Whitaker, 1978) and may thus be useful for studying the annexins. Indeed, Maekawa et al. (1991) described a protein with similar properties to annexin II, during an investigation of calcium-binding proteins in the mitotic apparatus of sea urchin eggs. In this paper we have examined *Lytechinus pictus* eggs and

Abbreviations used: EGFR, epidermal growth factor receptor; ASW, artificial sea water; IM, intracellular medium.

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by a variety of biochemical and immunological criteria have identified homologues of several mammalian annexins. On the basis of protein sequence analysis, we also report the presence of a novel annexin in *L. pictus* eggs. We conclude that sea urchin eggs represent a potentially useful system for investigating the proposed roles of annexins in vesicle trafficking and/or cell cycle, and also comment on the implications of our findings for the complex evolution of the annexin gene family.

EXPERIMENTAL

Materials

Sea urchins (*L. pictus*) were routinely maintained in artificial sea water (ASW: 435 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM EDTA, pH 8.0). Eggs were collected following intracoelomic injection of 0.5 M KCl, de-jellied in ASW and washed with Ca²⁺-free sea water before use (Whalley et al., 1991). All phospholipids were purchased from Sigma (U.K.).

Preparation of sea urchin egg cortices

De-jellied eggs were applied to Petri dishes precoated with poly(L-lysine) to produce a confluent layer of eggs. Sea water was removed and the dishes were washed three times with 10 ml of intracellular medium (IM: 220 mM potassium glutamate, 500 mM glycine, 10 mM NaCl, 5 mM MgCl₂, 2.5 mM ATP, 10 mM EGTA, pH 6.7). Eggs were lysed by pipetting vigorously in 5 ml of IM, cortices remained bound to the dish and the cytosol was removed by 3–5 further washes with 5 ml of IM. Any residual IM was drained from the dish and cortical proteins were dissolved in 1% (v/v) Triton X-100. Proteins were concentrated with chloroform/methanol (2:1) and washed with water before storage at –20 °C.

PAGE and Western blotting

SDS/PAGE was performed as described (Moss et al., 1992). After transfer to Immobilon P (Millipore) by Western blotting (Moss et al., 1992) proteins were detected using polyclonal antisera to annexins I, V and VI and CP2 (Gerke, 1989, a polyclonal antibody to an annexin consensus peptide, generously provided by Dr. Volker Gerke, Goettingen, Germany). The antisera to annexins I and VI have been described elsewhere (Moss et al., 1988; Futter et al., 1993), the antibody to annexin V was generated by immunization of rabbits with purified human placental annexin V. The anti-annexin V serum was found to exhibit specificity for annexin V when tested against a range of human annexins (results not shown). Immunoreactive bands were detected using either goat-(anti-rabbit) IgG coupled to alkaline phosphatase (Sigma, U.K.) followed by incubation in Western Blue (Promega, UK), or goat-(anti-rabbit) IgG coupled to horseradish peroxidase with detection by enhanced chemiluminescence (ECL) (Amersham, plc., U.K.) according to the manufacturer's instructions.

Purification of sea urchin annexins

Whole de-jellied sea urchin eggs were homogenized on ice for 20 min in buffer A [10 mM Hepes (pH 7.4), 75 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol] containing 5 mM EGTA and 2% (v/v) Triton X-100, and centrifuged at 10000 g for 10 min at 4 °C to pellet nuclei and cell debris. The supernatant was adjusted to 6 mM free Ca²⁺ from a stock solution of CaCl₂ and dialysed for 18 h at 4 °C against buffer A containing 2 mM CaCl₂ and 1% (v/v) Triton X-100. Insoluble material was collected by

centrifugation at 50000 g for 60 min and the pellet was washed successively with buffer A containing 2 mM CaCl₂ and 1% Triton X-100, 2 mM CaCl₂ and 0.1% Triton X-100 and finally 2 mM CaCl₂ with no detergent.

The pellet was resuspended in 5 ml of buffer A containing 2 mM EGTA and dialysed against the same buffer for 18 h at 4 °C. The dialysate was centrifuged for 60 min at 50000 g and the supernatant was examined for protein content by SDS/PAGE and Coomassie Blue staining (results not shown). Following dialysis against 20 mM Tris/HCl, pH 7.4, containing 2 mM EGTA, the sample was applied to a Mono Q FPLC column (Pharmacia). Bound proteins were eluted using a linear salt gradient from 0 M to 0.5 M NaCl. Fractions (1 ml) were collected and Western blotted using antisera against annexins I, V and VI.

Calcium-dependent phospholipid binding

Fractions enriched in annexins I, V and VI (see above) were tested for their ability to bind calcium-dependently to phospholipids. Mini-columns of phospholipids immobilized on phenyl-Sepharose (Sigma, U.K.) were prepared as described elsewhere (Edwards and Crumpton, 1991). The three sea urchin annexins shown to be immunologically recognized by antisera to annexins I, V and VI were applied to the phospholipid columns in the presence of 1 mM CaCl₂ in buffer A. Unbound protein was washed through with the same buffer (3 ml) and retained protein was eluted with 3 ml of buffer A containing 8 mM EGTA. Proteins in the eluates were precipitated with ice-cold acetone, dried and resuspended in SDS/PAGE sample buffer. After electrophoresis, proteins were detected by Western blotting as described above.

Protein sequence determination

After separation by SDS/PAGE, proteins were detected by staining with Coomassie Blue and excised. Gel pieces were digested with trypsin and applied directly to tandem h.p.l.c. separation using 2.1 mm AX-300 anion-exchange and OD-300 reverse-phase columns on a Hewlett-Packard 1090M with diode-array detection. Peak fractions were collected during elution with a linear acetonitrile concentration gradient in 0.1% trifluoroacetic acid and sequenced using fast cycle automated Edman chemistry on an Applied Biosystems 477A modified as described (Totter et al., 1992).

RESULTS

Sea urchin eggs have been extensively used as a model for the study of both exocytosis and the cell cycle (Vacquier, 1975; Baker and Whitaker, 1978). To investigate the possibility that this may be a suitable system for the study of annexin function we examined expression of annexins in *L. pictus* eggs. Western blot analysis of *L. pictus* whole egg lysates revealed the presence of several proteins as judged by immunoreactivity with antisera to human annexins I, V and VI (Figure 1a). Although the pattern of proteins detected by the antisera to annexin I was remarkably consistent, the protein profile seen with antisera to annexins V and VI varied considerably from season to season and within sea urchin batches. Thus, in the example shown in Figure 1, antisera to annexin I identified a major band at 34 kDa and a minor band at approximately 80 kDa. Antisera to annexin V recognized minor bands at 35 kDa and 55 kDa and a more prominent band at 68 kDa. Antisera to annexin VI revealed a major band at 68 kDa and a series of slightly smaller bands that may represent degradation products. The reason for blotting whole egg lysates with polyclonal anti-(human annexin) sera was simply to de-

Figure 1 Expression of annexins in *L. pictus* eggs

(a) Samples of *L. pictus* whole egg lysate (20 µg protein per track) were resolved by SDS/PAGE and transferred to Immobilon P by Western blotting. Immunoreactive proteins were detected using antisera to human annexins I, V and VI as indicated and the products visualized using either chemiluminescence (annexins I and V) or Western Blue (annexin VI). Arrow heads point to the major protein bands and the positions of molecular-mass standards are shown. (b) Annexins were partially purified from the detergent-insoluble residue of sea urchin egg membranes. A Coomassie Blue stained gel of the residue before (lane 2) and after (lane 3) extraction with EGTA is shown with the EGTA-extracted material (lane 4). Molecular mass markers are in lane 1 and are indicated to the left in kDa. A sample identical to lane 4 was Western blotted with the pan-reactive annexin antisera CP2 (Gerke, 1989) and visualized using ECL. Proteins visible by Coomassie staining in lane 4 of approximately 34 kDa and 160 kDa were also recognized by the CP2 antibody.

termine whether or not annexins were present in sea urchin eggs, and the results shown in Figure 1 appeared to confirm this.

To examine the properties of and attempt to obtain protein sequences from the sea urchin annexins, a purification strategy was devised based on the ability of all annexins to bind calcium-dependently to the detergent-insoluble residue of plasma-membranes. Figure 1(b) shows that following extraction of the detergent-insoluble residue with EGTA (lane 3), four major polypeptides of approximately 34 kDa, 55 kDa, 96 kDa and 160 kDa were detectable by Coomassie Blue staining, the first and last of which were also recognized by the consensus peptide antibody CP2. The antibody also detected a protein of ~ 68 kDa which was not stained by Coomassie Blue. A preparation such as this, enriched in annexins, was resolved by ion-exchange chromatography on Mono Q and fractions collected were examined by Western blotting (Figure 2). Using antisera to annexin I, a major protein of ~ 34 kDa was detected in the flow-through fraction. This antibody failed to detect immunoreactive protein in any of the remaining fractions. Antisera to annexin V detected a major protein band of approximately 35 kDa eluting from the Mono Q column at ~ 0.2 M NaCl. This antibody also cross-reacted weakly with a 35 kDa protein in the flow-through. The anti-annexin VI serum identified a 68 kDa protein eluting at ~ 0.25 M NaCl.

The partially purified sea urchin annexins were analysed by two-dimensional gel electrophoresis followed by Western blotting (Figure 3). The protein identified in the flow-through (see above and Figure 3a) by antisera to annexin I displayed considerable heterogeneity with several overlapping protein spots with isoelectric points ranging from 7.0 to 7.5. Under these conditions this protein appeared as two distinct molecular mass forms. The

Figure 2 Separation of *L. pictus* annexins by f.p.l.c.

An annexin-enriched mixture of proteins extracted from *L. pictus* eggs was applied to a Mono Q f.p.l.c. ion-exchange column. A linear salt gradient of 0 M–0.5 M NaCl was used to elute retained proteins. The flow-through (FT) and fractions 17–29 were examined by Western blotting for annexins I, V and VI. The anti-annexin I sera (Anx I) detected a single protein in the flow-through, anti-annexin V sera (Anx V) a minor protein in the flow-through and a major protein in fractions 21 and 22 (corresponding to ~ 0.2 M NaCl) and anti-annexin VI sera (Anx VI) a protein eluting in fractions 24 and 25 (corresponding to ~ 0.25 M NaCl). The positions of molecular mass markers for each gel are shown to the left.

lower form probably arose through limited proteolysis at the N-terminus, a region known to be especially susceptible to protease attack in several annexins. Similar lower-molecular-mass forms of this protein are also visible in Figure 4. The 35 kDa protein identified with antisera to annexin V (Figure 3b) migrated as a single protein spot with a pI of ~ 6.2 and the 68 kDa sea urchin annexin appeared as a single spot with a pI of ~ 6.0 (Figure 3c). Closer examination of the 68 kDa sea urchin annexin by one-dimensional SDS/PAGE and Western blotting revealed the closely-spaced polypeptide doublet characteristic of mammalian annexin VI (Figure 3d).

Since annexins share the unifying ability to bind calcium-dependently to phospholipids, we investigated the interaction between the partially purified sea urchin annexins and a variety of phospholipids (Figure 4). All of the sea urchin annexins bound to phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine in the presence (but not in the absence) of 1 mM CaCl_2 . The appearance of the *L. pictus* annexin V homologue as a protein doublet (Figure 4) is an artefact due to overloading. In contrast, the three annexins all failed to bind to the only cationic phospholipid, namely phosphatidylcholine. The observed calcium-dependent association of the sea urchin annexins with only the negatively charged phospholipids confirms that these proteins exhibit a key biochemical property of the annexin family. These results, considered together with the approximate molecular masses, isoelectric points, affinities for Mono Q and immunological properties of the three sea urchin annexins, are consistent with their being homologues of mammalian annexins I, V and VI.

To designate the sea urchin proteins as annexins unequivocally, the partially purified proteins were resolved by SDS/PAGE, stained briefly with Coomassie Blue (see Figure 1b for example)

Figure 3 Two-dimensional gel electrophoresis of *L. pictus* annexins

Partially purified annexins were resolved by two-dimensional electrophoresis: isoelectric focusing in the first dimension and SDS/PAGE in the second as indicated. Proteins were transferred to Immobilon-P by Western blotting and detected using antisera to annexin I (a), annexin V (b) and annexin VI (c); (d) Shows a Western blot of annexin VI from sea urchin egg cortices (lane 1) and human placenta (lane 2) after one-dimensional SDS/PAGE. Molecular mass markers are indicated to the right.

Figure 4 Calcium-dependent phospholipid-binding

The three sea urchin annexins partially purified in Figure 3 were passed over mini-columns of (a) phosphatidylserine, (b) phosphatidylethanolamine, (c) phosphatidylinositol and (d) phosphatidylcholine bound to phenyl-Sepharose in the presence of 1 mM CaCl_2 . Protein that bound to the columns was eluted with 8 mM EGTA (R) and compared with that which failed to bind (U). Following SDS/PAGE, proteins were detected by Western blotting using antisera to annexins I, V and VI as indicated.

and gel slices containing the relevant protein bands excised and subject to amino acid sequence determination following proteolysis (Figure 5). Although the annexin V and VI homologues were not expressed at sufficiently high levels to permit protein sequence analysis, adequate protein was obtained for the annexin I homologue. Densitometric analysis (calibrated against a known range of BSA standards) of the staining intensity of the annexin I homologue indicated a yield of approximately 1 μg –2 μg of protein from 10 ml of whole sea urchin eggs. Peptide sequencing yielded six different amino acid sequences, comprising a total of 83 residues, which probably corresponds to approximately one quarter of the intact protein. Alignment of five of these peptides with the complete sequence of human annexin I revealed substantial sequence identity within the highly conserved four repeats

(Figure 5). However, one of the six peptides (ALYDA...etc.) exhibited only weak similarity to a region near the start of human annexin I repeat 3. This peptide was omitted from the alignment because the region of closest similarity with annexin I overlapped with that of another peptide (AQAEA...etc.). The peptide aligned at the N-terminus (MALTD...etc.) bears virtually no similarity to the corresponding part of annexin I. The reason for placing it in this position is because all annexins, despite having unique N-termini, share certain diagnostic sequence elements close to the start of the first repeat. In particular, the motif TVK/R... (4 residues)...F, occurs in the N-terminus of almost all annexins, indeed the phenylalanine is invariant. These data show that although the 34 kDa sea urchin annexin is sufficiently similar to annexin I to account for immunological cross-reactivity, the unique N-terminus indicates that this is a novel member of the family.

DISCUSSION

The pattern of expression of annexins in lower eukaryotes and invertebrates is enigmatic (Smith and Moss, 1994). Although homologues of annexins I and VII have been discovered in sponge and *Dictyostelium* respectively (Robitzki et al., 1990; Döring et al., 1991), in *Hydra* the major identified annexin has no apparent counterpart in higher eukaryotes (Schlaepfer et al., 1992a). Similarly, the fruitfly *Drosophila* expresses at least two annexins (IX and X) for which homologues have not been identified in any other species (Johnstone et al., 1990). Among vertebrates, columbids appear unusual in that they have two types of annexin I gene, one characterized by its exquisite sensitivity to prolactin and the other by its closer similarity to mammalian annexin I (Hitti and Horseman, 1991). Recent structural analyses of the genes for annexins I, II, III and VI (Kovacic et al., 1991; Spano et al., 1990; Tait et al., 1993; Smith et al., 1994) revealed close similarity in the positions of the intron/exon boundaries, suggesting that the structural organization of the annexin genes may have stabilized relatively early in evolutionary terms.

In this report we have shown that the echinoderm *L. pictus* expresses several members of the annexin family. The sea urchin annexins were identified by virtue of their biochemical and immunological properties and one of them was purified in sufficient quantities for partial peptide sequence analysis. Given that only three annexin antisera were tested in this study and all identified proteins in *L. pictus* eggs, it seems likely that other

Figure 5 Alignment of *L. pictus* and mammalian annexin sequences

Peptides derived by tryptic digestion of the *L. pictus* annexin (LP-Anx) were sequenced by automated Edman degradation. The amino acid sequences were aligned with the previously published sequence of human annexin I. Sea urchin annexin sequences are underlined. Amino acid identities are bridged by double dots, conservative changes by single dots. The sequence is arranged to illustrate the internally repetitive structure typical of annexins. An additional peptide with the sequence ALYDAGAGQLGDESEFQR is not included in the alignment because the region of closest similarity overlaps that of the peptide in the third repeat.

members of the annexin family will also be found in these cells. Indeed, an antibody raised against an annexin consensus peptide recognized a protein of ~ 160 kDa. Although no annexins of this size have yet been described, this could represent a multimeric form of a smaller annexin or perhaps a sixteen-repeat duplicated form of annexin VI. The presence of an apparent homologue of annexin VI raises the question as to how many mammalian-type annexins are expressed in *L. pictus* eggs. The observation that *L. pictus* annexin VI migrated as a polypeptide doublet with the same mobility on gel electrophoresis as purified placental annexin VI, strongly argues that this is a genuine homologue. This notion is supported by the observation that PCR products obtained from *L. pictus* RNA using human annexin VI specific primers, yielded products of the correct predicted sizes which hybridized with a human annexin VI cDNA probe on Southern blotting (results not shown). It has been suggested that the annexin VI gene arose by duplication of a four-repeat annexin gene and that this event is likely to have been comparatively recent in evolutionary terms (Crompton et al., 1988). However, the identification of annexin VI in an invertebrate, albeit one of the closest phyla to vertebrates, might indicate that annexin VI in fact predates some members of the modern annexin family. The presence of a possible homologue of annexin I in *L. pictus* eggs is less surprising since annexin I has been cloned in the more primitive sponge. Annexin V on the other hand has so far only been reported in chordates. If the 35 kDa *L. pictus* protein described here is shown to be a homologue of mammalian annexin V then this might have important implications concerning the proposed functions of this protein as either a calcium channel (Huber et al., 1990) or cellular inhibitor of protein kinase C (Schlaepfer et al., 1992b).

Of the three sea urchin annexins, one was expressed at relatively high levels and sufficient protein was purified for partial peptide sequence analysis. In many respects, the 34 kDa *L. pictus* annexin exhibited properties similar to those of annexin I, such as isoelectric point, failure to bind to Mono Q during purification, and immunological cross-reactivity with antisera against human placental annexin I. The average sequence similarity (including conservative changes) between annexin I and *L. pictus* annexin fragments was 75 % within the repeated domains, certainly high enough to account for common epitopes. However, this protein is clearly not a simple sea urchin homologue of annexin I because the N-terminus is no more similar to annexin I than to any other annexin. The designation of the N-terminal fragment was based

on the presence of a small but diagnostic sequence, part of which is a phenylalanine residue present in all annexins. Whether or not this fragment represents the complete N-terminus is unclear. The presence of a methionine residue at the start of this peptide may be misleading since little is known about post-translational removal of methionine or N-terminal modification in sea urchin eggs.

It should also be noted that the choice of annexin I to illustrate sequence similarity with the sea urchin peptides (Figure 5) does not imply that the *L. pictus* annexin is more similar to annexin I than to any other annexin. Thus, the *L. pictus* annexin peptides can be equally persuasively aligned with several other members of the mammalian annexin family, particularly annexins V, VII, IX and XI. One unanswered question concerns the origin of the peptide omitted from the alignment (ALYDA... etc.) in Figure 5. This may have been derived from a second annexin present as a minor contaminant in the purified annexin preparation, or more likely, since this is a novel annexin, it could simply be derived from another part of the parent molecule. In conclusion, we have described the presence of several annexins in *L. pictus* eggs and suggest that two of these may be homologues of mammalian annexins V and VI. In addition, we have obtained amino acid sequence data from a novel annexin related to, but distinct from, annexin I. Our data raise the possibility of using sea urchin eggs as a model system in which to study annexin function.

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