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# The expression of different annexins in the fish embryo is developmentally regulated

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**Abstract** The expression of annexins, a family of $Ca^{2+}$/phospholipid-binding proteins, was analyzed by biochemical and immunological criteria in the fish *Misgurnus fossilis* (loach), which is a good model system to study embryonic events. Five different annexins (loach annexins A to E) are present as a maternal pool in the unfertilized eggs. Only annexin E is newly synthesized in the early embryo. Its synthesis, already apparent at mid-blastula, decreases in later stages when two additional annexins (F and G) appear. They are present among the newly synthesized polypeptides of mid-gastrula and later embryonic stages and are also found in loach larvae. The developmentally controlled expression of several annexins indicates a specific role of these proteins at certain embryonic stages.

*Key words:* Annexin; $Ca^{2+}$/lipid-binding protein; Embryonic development; Fish egg

## 1. Introduction

The annexins, members of a multigene family of $Ca^{2+}$ and phospholipid binding proteins, have been implicated in a number of membrane-related events, including exo- and endocytosis, cytoskeleton-membrane interactions, and the formation and regulation of $Ca^{2+}$ channels (for review see [1–3]). Structurally, the annexins show sequence homologies and share a common three-dimensional folding of their principal domain, the so-called annexin core [4,5]. This domain comprises four, in one case eight, repeated segments of 70–80 amino acids (annexin repeats) which harbor novel types of $Ca^{2+}$ binding sites [6,7]. The core domain mediates the $Ca^{2+}$-dependent phospholipid binding observed in vitro and is most likely involved in attaching annexins to cellular membranes [8–10]. An N-terminal head domain which precedes the core and harbors binding sites for specific protein ligands, as well as phosphorylation sites for signal transducing protein kinases, is specific for the individual members of the family and varies in sequence and length [1]. Annexins, originally described in vertebrates, have recently also been indentified in lower animals, in slime moulds, and in plants (for review see [11]).

At least some vertebrate annexins seem to show a regulated expression during development. The expression of annexin I, the EGF receptor/kinase substrate, seems to be connected to the development of commissures in the embryonic central nervous system of the rat [12]. The $pp60^{src}$ kinase substrate annexin II shows an increased expression during avian limb bud development [13] and is also regulated during embryogenesis of *Xenopus laevis*. While no or only minor amounts of annexin II mRNA are present in unfertilized eggs, the mRNA level increases markedly during neurulation, i.e. 24 h after fertilization, and remains high throughout later embryogenesis [14,15].

These results suggest that annexin expression could be developmentally regulated, although a specific function of annexins during certain stages of development is far from understood. It is also not known whether annexins are present as a maternal pool in unfertilized eggs and thus could be involved in some aspect of the $Ca^{2+}$ signalling pathway which is initiated upon fertilization and is thought to trigger development in the eggs of many species [16]. To study such possible annexin functions, we began to describe $Ca^{2+}$-dependent phospholipid binding proteins in the fresh-water fish *Misgurnus fossilis* (loach) [17], i.e. in an organism in which early embryonic development can be studied after artificial fertilization [18]. We show here that unfertilized *Misgurnus* eggs already contain five different members of the annexin family (tentatively designated as loach annexin A to E), which are characterized by their typical biochemical properties and their reaction with an annexin consensus antibody. Only one of the egg annexins (annexin E) is newly synthesized during early embryonic development, whereas two additional members of the family (annexins F and G) are specifically expressed in older embryos, indicative of different functions of the annexins during development.

## 2. Materials and methods

### 2.1. Fish, eggs and embryos

The loach (*Misgurnus fossilis* L.) were caught in natural ponds in Russia and kept in a dormant state at 4°C for several months. Loach eggs were obtained and processed for biochemical fractionation or artificial fertilization as described [18]. Following fertilization the embryos reached mid-blastula stage after 6.5 h and mid-gastrula after 14 h. Hatching of the larvae occurred after 50 h.

### 2.2. Purification of annexins from loach eggs

Preparation of EGTA extracts and $Ca^{2+}$-dependent liposome binding were performed essentially as described [19,20]. 20 ml of egg cytoplasm was homogenized in 180 ml of buffer A (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 0.25 mM PMSF) using a glass-Teflon homogenizer. Subsequently, E-64 (an inhibitor of $Ca^{2+}$-activated proteases; Sigma), and $CaCl_2$ were added to final concentrations of 5 $\mu$M and 10 mM, respectively. The mixture was left on ice for 30 min and then centrifuged for 45 min at 100,000 × *g*. The resulting pellet was washed in buffer A containing 5 $\mu$M E-64 and 2 mM $CaCl_2$, centrifuged as before, and resuspended in 50 ml of the same buffer containing 10 mM EGTA instead of $CaCl_2$. The suspension was kept on ice for 30 min and then centrifuged for 45 min at 100,000 × *g*. The supernatant (EGTA extract) was dialyzed against buffer A containing 0.5 mM EGTA and

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2 mM $NaN_3$, clarified by centrifugation for 1 h at 100,000 × g, and subjected to $Ca^{2+}$-dependent liposome binding. Similar results were obtained with liposomes prepared by sonication of either phosphatidylserine (Sigma) or chloroform-methanol extract of bovine brain (brain extract, type VII; Sigma). The clarified EGTA extract was adjusted to 5 μM E-64 and 5 mM $CaCl_2$, mixed with liposomes (1 mg/ml), and incubated for 20 min at room temperature. Subsequently, liposomes were pelleted (30 min at 100,000 × g) and washed with buffer A plus 5 μM E-64 and 2 mM $CaCl_2$. Proteins bound to the liposomes in a $Ca^{2+}$-dependent manner were eluted with 20 ml of buffer A containing 10 mM EGTA (liposome extract). For fractionation of the individual annexins, the liposome extract was dialyzed against buffer B (10 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.5 mM EGTA, 0.25 mM PMSF) and applied to DEAE-Sepharose (Pharmacia) equilibrated in the same buffer. The column was washed with 5 vols. of buffer B and elution was performed by increasing the salt concentration in a stepwise manner or by using a continuous salt gradient. Proteins present in the different fractions were concentrated [21], and analyzed by SDS-PAGE [22] followed by either Coomassie blue staining or immunoblotting.

### 2.3. Miscellaneous

The annexin consensus antibody (anti-CP2) was raised in rabbits against a synthetic peptide corresponding to a highly conserved sequence motive in the annexins. It has been characterized previously and was used after affinity purification on the immobilized peptide [19]. The same antibody has also been used successfully in another laboratory for the immunological identification of annexins in *Hydra vulgaris* [23]. Immunoblotting, carried out by standard procedures [24], used peroxidase-conjugated second antibodies (Dako). Isotopic labelling of loach embryos and fluorographic analysis of the annexin expression were carried out as described [25].

## 3. Results and discussion

### 3.1. Identification of five distinct annexins in fish eggs

*Misgurnus* eggs can be fertilized artificially and thus represent a useful experimental system to study the expression and function of a given protein during embryonic development [18]. To exploit this system for analyzing the developmental expression of annexins, we employed a combined biochemical and immunological approach [19,20] for the identification of egg annexins. Loach egg cytoplasm was subjected to a $Ca^{2+}$-dependent protein fractionation known to lead to a substantial

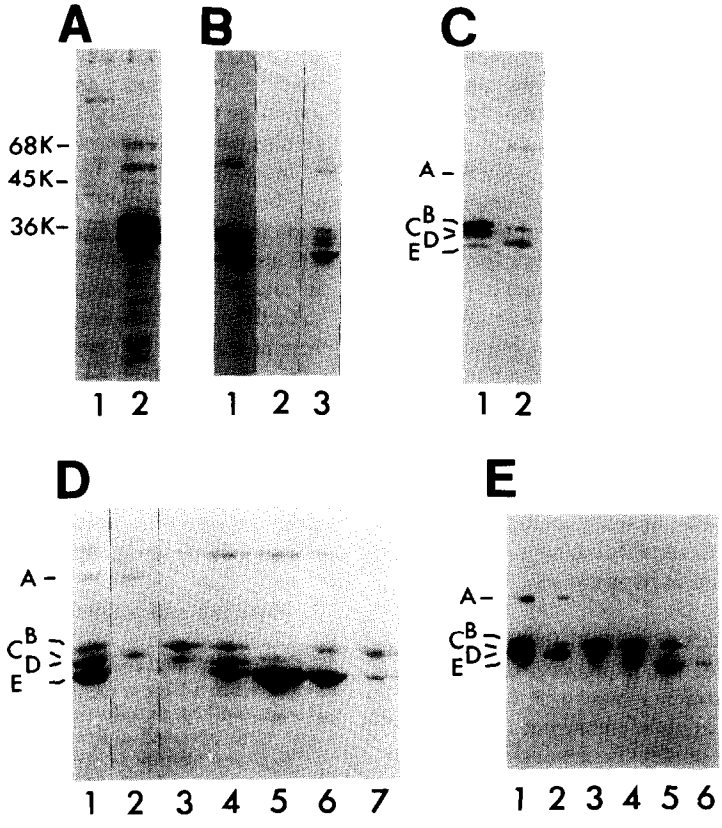


Fig. 1. Biochemical and immunological characterization of loach egg annexins. (A) Phosphatidylserine–liposome binding. Proteins present in the EGTA extract from loach eggs were subjected to liposome pelleting as described in section 2 and the different fractions were analyzed by SDS-PAGE. Lane 1 shows the unbound fraction, whereas proteins that bound to the liposomes in the presence of $Ca^{2+}$ and that were eluted by the addition of EGTA are given in lane 2. (B) Binding of loach egg annexins to lipsosomes prepared from a chloroform/methanol extract of bovine brain. Proteins present in the EGTA extract after phosphatidylserine–liposome pelleting (lane 1) were mixed with bovine brain–liposomes in the presence of $Ca^{2+}$. Liposomes were separated from the unbound material (lane 2) by centrifugation and $Ca^{2+}$-dependently bound proteins were released by the addition of EGTA (lane 3). (C) Immunoblot analysis. Egg proteins specifically bound to liposomes in the presence of $Ca^{2+}$, i.e. the material shown in Fig. 1B, lane 3, were subjected to immunoblotting using the anti-CP2 annexin consensus antibody (lane 1). Lane 2 shows an identical immunoblot analysis of purified porcine annexins II, IV and VI. The loach egg annexins are designated A–E according to their apparent molecular masses in this SDS-10% polyacrylamide gel. (D and E) Fractionation of loach egg annexins by ion-exchange chromatography. Proteins purified by liposome pelleting (lane 1) were mixed with DEAE-Sepharose. Lane 2 shows the material not bound to the matrix whereas lanes 3–7 show the fractions eluted by increasing the salt concentration to 30, 60, 100, 150, and 200 mM, respectively. Total proteins as revealed by SDS-PAGE and Coomassie staining are depicted in D. E shows the corresponding immunoblot with the anti-CP2 antibody. The fraction eluted in the presence of 150 mM salt has been omitted in E. Note that the five annexins show different elution profiles. Note also that they differ in their affinity towards the anti-CP2 antibody, with annexin A showing the strongest and annexin E the weakest reaction.

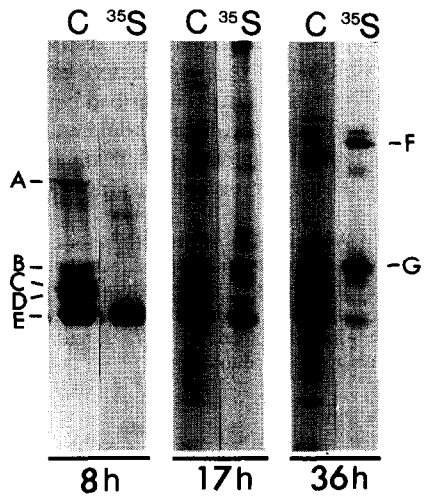


Fig. 2. De novo synthesis of loach annexins during embryonic development. Loach embryos were labelled with [$^{35}$S]methionine 8, 17 and 36 h after fertilization (see section 2). EGTA extracts were prepared from the differently staged embryos and subjected to SDS-PAGE using long 12.5% polyacrylamide gels. The gels were stained with Coomassie (lanes C) and then processed for fluorography to visualize metabolically labelled proteins (lanes $^{35}$S). Note that de novo synthesis of annexin E is already apparent 8 h after fertilization. Two additional annexins (F and G) are newly synthesized in older embryos. The longer gels employed here yielded a better resolution in the 30–40 kDa range than the shorter gels shown in Fig. 1. Annexin G can thus be separated from annexin B, which has a slightly greater electrophoretic mobility.

enrichment of putative annexins in the final EGTA extract [20]. SDS-PAGE of the proteins present in this EGTA extract revealed several prominent bands in the 30–40 kDa region, i.e. polypeptides with molecular masses similar to those of most known annexins (not shown). To verify the annexin nature of these proteins by biochemical criteria, the $Ca^{2+}$ concentration in the EGTA extract was adjusted to 5 mM and the extract was subjected to a phospholipid–liposome pelleting analysis. Several polypeptides specifically enriched in the EGTA extract interact with liposomes in a $Ca^{2+}$-dependent manner, and thus show a property generally considered to be the biochemical hallmark of the annexin family (Fig. 1A, lane 2). Moreover, the proteins released from the liposomes by the addition of EGTA will rebind in the presence of $Ca^{2+}$ to liposomes prepared from purified phosphatidylserine (not shown) or from a chloroform/methanol extract of bovine brain (Fig. 1B).

Further evidence that the liposome-binding polypeptides are indeed annexins was obtained with an annexin consensus antibody in immunoblot analyses. This antibody is directed against a synthetic peptide corresponding to the region of highest sequence conservation among vertebrate annexins I–VI and recognizes the major vertebrate annexins in immunoblots [19]. Fig. 1C shows that five polypeptides present in the initial EGTA extract and specifically enriched in the liposome-bound fraction react with the consensus antibody. Thus these proteins are identified as annexins not only by biochemical but also by immunological criteria. In addition, microsequencing of tryptic peptides from these polypeptides provided unique sequences which (i) are clearly related to known annexin sequences, and (ii) show that at least four of the five polypeptides (annexins B–E) are most likely novel members of the family and represent distinct molecules. Since this analysis also excludes the possibility that one or more of the smaller loach egg annexins has been generated by proteolysis of a larger molecule, the proteins are referred to as annexins A to E. Annexin A has the largest and annexin E the smallest apparent molecular mass in SDS-PAGE (50 kDa and 32 kDa, respectively). Ion-exchange chromatography on DEAE leads to partial separation of annexins A and C, which do not bind to the resin under the conditions chosen, from the more acidic annexins B, D and E. Annexins B and D elute at approximately 50 mM salt, and annexin E is released at about 80–150 mM salt (Fig. 1D and E).

Four of the five egg annexins have apparent molecular masses between 30 and 40 kDa, a value typical of four repeat annexins, while annexin A is considerably larger (around 50 kDa). As this mass is too small for a polypeptide with eight annexin repeats, it seems that annexin A has a large N-terminal domain preceding a protein core of four repeats. A similar situation has been described in two mammalian annexins, annexin VII and annexin XI, which contain N-terminal domains of 167 and 202 residues, respectively [26–28]. Interestingly, the annexin A band appears as a closely spaced doublet in some immunoblots (Fig. 1; c.f. Fig. 3). While this could reflect proteolysis, it is also reminiscent of the behaviour of mammalian annexin VII. The latter occurs in two splice variants differing by 22 amino acids encoded by a cassette exon inserted into the N-terminal domain [29].

### 3.2. *Expression of fish annexins during embryonic development and tissue distribution of the proteins in the adult animal*

The preceding analyses carried out with unfertilized loach eggs revealed the existence of five different annexins. These annexins therefore represent a maternal pool which could be required for certain, possibly $Ca^{2+}$-regulated processes occurring upon fertilization, e.g. the $Ca^{2+}$-triggered exocytosis of cortical granules [30,31]. Alternatively, the annexins may have a role to play in very early embryos since they contain considerable amounts of the maternally derived proteins. To analyze whether one or more of the annexins could be specifically required for certain later stages of development, we studied annexin expression during loach embryogenesis. Loach eggs were fertilized artificially [18] and newly synthesized proteins of differently staged embryos were labelled specifically by culti-

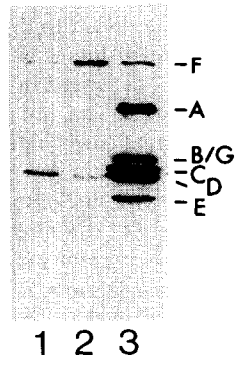


Fig. 3. Tissue distribution of loach annexins in the adult fish. EGTA extracts were prepared from loach intestine (lane 1), muscle (lane 2), and brain (lane 3), respectively, and subjected to immunoblotting with the anti-CP2 antibody. Note the differences in the tissue distribution of the different annexins. Annexins B and G have very similar apparent molecular masses and migrate almost identically in this SDS-12.5% polyacrylamide gel of normal length.

vation of the embryos in medium containing [$^{35}$S]methionine. Fig. 2 compares the metabolically labelled proteins with the total proteins present in EGTA extracts from embryos of the mid-blastula (8 h), the mid-gastrula (17 h), and a later stage when muscular movements of the embryo are initiated (36 h). While substantial amounts of the egg annexins are found in all embryonic stages analyzed (as also revealed by immunoblotting with the anti-CP2 antibody, data not shown), only annexin E is synthesized de novo at the earliest stage analyzed, i.e. at mid-blastula (8 h). The synthesis of annexin E seems clearly regulated throughout embryonic development, as it decreases again in later stages (compare for example the 8 h and 36 h analyses). In contrast, no, or only minor, de novo synthesis of the other four egg annexins (A–D) is observed in the early embryonic stages analyzed. Thus annexins A–D of early embryos reflect the maternal pool already documented in the unfertilized eggs.

Two proteins of 36 and 70 kDa are the major newly synthesized polypeptides found in EGTA extracts of older embryos (17 h and 36 h after fertilization, Fig. 2). Interestingly, both proteins, which are not present in the eggs, are also enriched in the EGTA extract of loach larvae and react with the annexin consensus antibody anti-CP2 (data not shown). They are referred to as loach annexins F (70 kDa) and G (36 kDa). The apparent molecular mass of annexin F (70 kDa) is identical to that of the only eight-repeat annexin of mammals, annexin VI, and immunoblotting experiments reveal that loach annexin F is indeed recognized by an antibody raised against mammalian annexin VI (V.V.I. and J. Dedman, unpublished results). Thus, at least seven distinct annexins have been identified in *Misgurnus fossilis* (annexins A–G), with annexin F being closely related to mammalian annexin VI.

The specific induction of the expression of annexins F and G at later stages of development contrasts with the early de novo synthesis of annexin E, and provides further support for the idea that at least some members of the annexin family fulfill specific functions, e.g. at certain developmental stages or in certain cells of the organism. To analyze a potential cell-type specific expression of the loach annexins we performed an anti-CP2 immunoblot using EGTA extracts from three different loach tissues. Fig. 3 reveals remarkable differences in the annexin content of these tissues. While annexins A,C,D,E,F and B and/or G (the latter two have very similar molecular masses in SDS-PAGE and thus are difficult to distinguish in this immunoblot) are expressed in the brain, only annexin F and a small amount of annexin D (as judged by co-migration of the band with annexin D of egg extracts, not shown) are found in muscle. The EGTA extract of loach intestine, on the other hand, contains annexin D as the major representative of the multigene family. On longer exposure of the immunoblot, some annexin F and some annexin C can also be identified in the intestinal EGTA extract. These results indicate that with the possible exception of annexin D, none of the loach annexins seems ubiquitously expressed in the different tissues and cell types of the adult animal. In addition, this experimental approach reveals for the first time the high level expression of several annexins in adult brain. Taken together, we can conclude from the tissue and developmental expression patterns that different members of the family are likely to have similar and at least partially overlapping functions in different cell types and embryos of different stages, respectively, or that individual annexins may have specific activities not required in every cell type. The model system described here, i.e. the loach egg that can be artificially fertilized, should enable us to study a potential function of annexins specifically associated with fertilization and/or early embryogenesis in more detail.

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