secreted then degraded by endogenous proteases, because placental annexin 4 was not degraded when added to prostate fluid and incubated at 37°C. The collective concentrations of annexin 1, des-(1–29)-annexin 1, and annexin 5 in prostate fluid and seminal plasma were 1.3% and 0.2% of the total protein, respectively. This is consistent with their secretion by the prostate and subsequent dilution of seminal vesicle fluid.

Summary

The human prostate expresses very high concentrations of annexins 1, 4, and 5 and secretes high concentrations of annexins 1 and 5. Although the biological roles of these proteins in prostate secretions are not known, these studies emphasize the need to consider extracellular sites for physiological functions of annexins. The clear demonstration of secretion of proteins that have blocked N-termini and lack hydrophobic signal sequences raises the possibility that novel cellular secretory pathways exist. Preliminary immunohistochemical experiments in collaboration with Dr James Fallon indicate that both annexins 1 and 4 are expressed in prostate ductal secretory epithelium. Since annexin 1, but not annexin 4, is secreted, a comparison of the cellular fate of these two related proteins in the prostate may provide a useful model system for determining the structural elements that direct the secretion of proteins which lack conventional signal sequences.

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Tyrosine kinase substrate annexin II (p36) — biochemical characterization and conservation among species

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Introduction

Annexin II (p36) is a member of the annexin family of Ca^{2+}- and phospholipid-binding proteins (for review see [1–5]). Like other proteins which belong to this multigene family, annexin II shows a characteristic structural feature, the so-called annexin repeat. This term describes a segment 70–80 amino acids in length which is repeated four times along the annexin II polypeptide chain. The annexin repeats exhibit homologies with one another not only within annexin II but also with other members of the family [1–5]. Typically, a given repeat shows a higher degree of conservation between distinct members of the family than to other repeats in the same protein. The sum of the four repeats in annexin II forms the so-called protein core, which is resistant to mild proteolytic treatment. In the primary structure, this core is preceded by a proteinase-sensitive N-terminal tail, some 30 residues in length. Biochemical characterization of the proteolytic derivatives revealed that the protein core harbours binding sites for Ca^{2+}, phospholipids and cytoskeletal proteins, such as F-actin and non-erythroid spectrin. Since the ability to interact with phospholipids in a Ca^{2+}-dependent manner is shared with all other annexins, it is believed that a highly conserved stretch of 17 amino acids (the so-called endonexin fold), which is found in each annexin repeat of every member of the family, is involved in the interaction with these ligands. The N-terminal tail of annexin II is not required for Ca^{2+}-, phospholipid- or F-actin-binding, but harbours different phosphorylation sites and thus is believed to represent an important regulatory region of the molecule. Tyr-23 is phosphorylated by pp60^{src}, while Ser-25 is a site for protein kinase C phosphorylation. Consequently, annexin II was originally identified as a major cytoplasmic substrate for the *src*-encoded tyrosine kinase (for review see [6]).

Annexin II is so far the only annexin which has been shown to interact with a cellular protein ligand, p11, both *in vitro* and *in vivo* (for review see [3]). Interaction leads to the formation of a tetrameric complex, which comprises two annexin II and two p11 chains. p11 binding is probably an important regulatory event, since it modulates properties displayed by annexin II. While monomeric annexin II shows 50% binding to liposomes at 10 μM-free Ca^{2+}, annexin II complexed with p11 only needs 10 nM-Ca^{2+} for the same interaction [7]. Similarly, the annexin II–p11 complex is able to induce chromaffin granule aggregation and even fusion of these vesicles at much lower Ca^{2+} concentrations than monomeric annexin II [8]. The p11-binding site is also located within the N-terminal tail of annexin II. However, while the phosphorylation sites cluster between residues 20 and 30, only the 12 N-terminal amino acids are required for p11 binding. This region of the molecule forms an amphipathic helix with the hydrophilic face of this helix probably being the actual p11 contact site [9].

p11 itself belongs to a different multigene family showing sequence homologies to the S100 proteins and other related polypeptides like calcyclin or the calgranulins (for review see [10]). Members of this protein family are characterized by

the existence of two classical Ca^{2+}-binding loops, the so-called EF-hand structures. However, while S100, calcyclin and calgranulin bind Ca^{2+} *in vitro*, p11 has lost this property owing to crucial amino acid deletions or substitutions in the two putative Ca^{2+} loops.

To describe structural parameters which are involved in the binding of different ligands (Ca^{2+}, phospholipid, p11) to annexin II, we started to determine the conservation of annexin II and p11 sequences among species. Here, complete cDNA sequences for annexin II and p11 from two different non-mammalian species, *Gallus domesticus* and *Xenopus laevis*, are reported. Interestingly, annexin II shows a much higher degree of conservation than p11.

Identification of annexin II and its cellular ligand, p11, in different species

To identify annexin II and p11 in different non-mammalian species, total cellular proteins from various cells were analysed for the existence of cross-reacting polypeptides in immunoblots. As shown in Fig. 1 (lanes 1, 2), monoclonal antibodies raised against porcine annexin II and p11 ([11], kindly provided by Dr M. Osborn, Max Planck Institute, Göttingen, F.R.G.) clearly demonstrate the presence of the respective proteins in chicken embryo fibroblasts. In addition, earlier biochemical studies had revealed that a p36-p11 complex could be purified from chicken intestinal epithelium following a procedure developed for the mammalian proteins [12]. Owing to this conservation of biochemical properties and antigenic determinants, chicken annexin II and p11 are likely to be highly similar in primary structure to their mammalian counterparts.
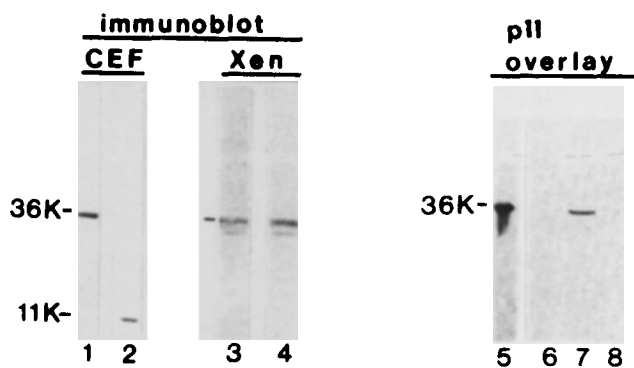

Fig. 1. *Identification of annexin II and p11 in different species*

Total cellular proteins from chicken embryo fibroblasts (lanes 1, 2), *X. laevis* A6 cells (lanes, 3, 4, 7), *Drosophila melanogaster* S3 cells (lane 6) or *Dictyostelium discoideum* Ax2 cells (lane 8) were separated in SDS gels, transferred to nitrocellulose and processed for immunoblotting (lanes 1-4) or p11 overlay (lanes 5-8). Western blots were performed using anti-p36 (lane 1) and anti-p11 (lane 2) monoclonals [11] or polyclonal antibodies directed against annexin consensus peptides (lanes 3, 4). The sequences of the peptides represent a consensus of the endonexin folds present in annexin repeats II (CP2, lane 3) or IV (CP4, lane 4), respectively [13]. The p11 overlay was carried out by incubating the nitrocellulose filters in a solution containing 5 μg of human p11/ml in 1% (w/v) bovine serum albumin, 150 mM-NaCl, 25 mM-Tris/HCl, pH 7.5. After washing, bound p11 was detected using the anti-p11 monoclonal and peroxidase-conjugated anti-mouse immunoglobulins as second antibodies. In the p11 overlay, purified porcine annexin II served as a positive control (lane 5).

While the monoclonal antibodies failed to detect cross-reacting polypeptides in *Xenopus*, polyclonal antibodies directed against annexin consensus peptides [13] recognize annexin-like proteins at around 32 and 36 kDa (Fig. 1, lanes 3, 4). To assess whether one of these polypeptides represents the *Xenopus* homologue of annexin II, we performed a p11-overlay experiment. Owing to the high specificity of the p11-annexin II interaction, the p11 overlay should unambiguously identify annexin II, once the p11-binding site in the N-terminal tail of annexin II is able to refold properly after SDS/gel electrophoresis and transfer to nitrocellulose. Figure 1 demonstrates that this is indeed the case. Immobilized procine annexin II binds human p11 which is present in the overlay solution and detected after binding by a p11 monoclonal antibody (lane 5). If the same experiment is performed on total *Xenopus* proteins which were separated in SDS gels and transferred to nitrocellulose, a single polypeptide which co-migrates in the SDS gel with human annexin II is decorated (lane 7). In contrast, when total cellular proteins from *Drosophila melanogaster* (lane 6) or *Dictyostelium discoideum* (lane 8) are treated in the identical way, no p11-binding protein is detected. Thus *X. laevis* cells contain a protein of around 36 kDa which is recognized by antibodies against two different annexin consensus peptides and interacts specifically with human p11. The data suggest that this *Xenopus* annexin II has a highly conserved p11-binding site, since it interacts with human p11. Based on this conservation it seems likely that *Xenopus* cells also express a p11 analogue which could bind to and thereby regulate the *Xenopus* annexin II.

Annexin II is highly conserved among species

Given the immunological and biochemical evidence for the existence of annexin II in chicken and *Xenopus*, we attempted to isolate the corresponding cDNA clones from λ phage libraries. A full-length cDNA clone for chicken annexin II was obtained by screening a chicken embryo fibroblast cDNA library in λgt 11 (kindly provided by Dr R. Hynes, M.I.T., Cambridge, U.S.A.) with both a monoclonal antibody directed against porcine annexin II (see above) and a human annexin II cDNA probe. The chicken cDNA clone has a length of 1370 nucleotides and contains a 1017 nucleotide open-reading frame which codes for 339 amino acids. Both the nucleotide and the translated amino acid sequence of the open reading frame are remarkably similar to the corresponding human [14], bovine [15] and murine [16], annexin II sequences. The identities range between 80 and 90% at the nucleotide level and are around 90% at the amino acid level, respectively.

A human annexin II cDNA probe was also used to screen at low stringency a λgt 10 cDNA library made from *Xenopus* kidney cell RNA (kindly provided by Dr I. Mattaj, E.M.B.L., Heidelberg, F.R.G.). This approach led to the isolation of a full-length cDNA clone for *Xenopus* annexin II. This clone (1210 nucleotides in length) has an open-reading frame of 1020 nucleotides encoding a polypeptide of 340 amino acids. When compared with annexin II from other species, the *Xenopus* protein also shows a high degree of sequence conservation, with amino acid identities ranging from 80 to 85%.

A close examination of the different protein sequences reveals that most of the annexin repeats, as well as the N-terminal 14 residues, i.e. the p11-binding domain, are particularly highly conserved. Species-specific differences in the primary structure cluster in a region between amino acids 15 and 41, i.e. the 'hinge' region connecting the p11-binding site and the first annexin repeat. Interestingly, this portion of the molecule is easily accessible to proteinases and contains all the major phosphorylation sites. Therefore, it seems that the hinge region resides on the outside of the protein showing no

extensive higher-order structure. Owing to the existence of various phosphorylation sites it might, however, represent an important regulatory region. This notion is supported by the finding that within the native annexin II, residues at positions around 26 and 65 are in close proximity and form the discontinuous epitope for a monoclonal antibody [17]. Thus, the hinge region is in close contact to the endonexin fold of the first repeat, which is believed to serve as an important site for the interaction with Ca^{2+} and/or phospholipids.

p11 shows a higher degree of sequence variations among species

To identify evolutionarily conserved portions within the p11 sequence which are likely to serve as structurally important regions and/or contact sites for annexin II, cDNA clones for p11 were isolated from different species. Using as probes synthetic oligonucleotides whose sequences were derived from the porcine p11 protein sequence [12], a cDNA clone for human p11 was isolated from a λgt 10 cDNA library made from HT 29 (a human adenocarcinoma cell line) mRNA (kindly provided by Dr D. Louvard, Pasteur Institute, Paris, France). The human p11 cDNA was in turn used as a hybridization probe to screen the above-mentioned chicken and *Xenopus* cDNA libraries at low stringency. Sequence analysis of the cDNA clones obtained revealed that both the human and chicken cDNAs encode a protein of 96 amino acids, whereas the *Xenopus* p11 clone codes for a protein which comprises only 95 residues. While mammalian [12, 18] and chicken p11 are highly homologous (around 90% identities at the protein level), *Xenopus* p11 shows a considerably larger degree of sequence variations with the identities limited to around 60% as compared with the other species. Most differences are found in the N-terminal-half of the molecule and none of them re-installs a functional EF-hand. Since *Xenopus* annexin II is able to bind human p11 and contains a highly conserved p11-binding site, it seems likely that the annexin II-binding site in *Xenopus* p11 is similarly highly conserved. This makes the second half of the p11 molecule a prime candidate for this binding site.

Taken together, the sequence data presented here point to an important physiological role of annexin II, since this polypeptide is highly conserved among different species. Within the protein sequence, the endonexin folds in all four annexin repeats are particularly well conserved. Mutational analysis should further clarify whether the endonexin fold indeed represents the binding site for Ca^{2+} and/or phospholipid.

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Annexins: a subcellular localization and reconstitution approach to elucidate cellular function

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The goal of our laboratory has been to elucidate the cellular pathways used to mediate and discriminate the intracellular Ca^{2+} signal. Our approach has been to identify, purify and characterize Ca^{2+} binding proteins with regard to tissue specificity, and cellular and subcellular localization. Using this collective information, biochemical reconstitution experiments can be designed to elucidate the cellular function of the individual Ca^{2+} mediator proteins. The fact that Ca^{2+} regulates a wide variety of cellular activities suggests that this complex mechanism of regulation would include multiple Ca^{2+} pathways with differential expression of the independent mediator proteins.

Several laboratories have independently identified a group of Ca^{2+}-binding proteins through their Ca^{2+}-dependent binding to cytoskeletal elements, isolated membranes, secre-

Abbreviation used: SR, sarcoplasmic reticulum.

tory granules or hydrophobic resins. Sequence data indicate that there are eight unique gene products, each of which is composed of four or eight repeats of a motif of 60–70 amino acids. These Ca^{2+}/phospholipid-binding proteins have been designated as annexins I–VIII [1, 2]. This superfamily is not related to calmodulin, troponin C and protein kinase C with regard to potential structural mechanisms of binding calcium and phospholipid. Although sequence information on these proteins is complete, their cellular roles have not been defined [3, 4]. We have utilized subcellular localization studies to focus on possible cellular functions of the 35 kDa and 67 kDa calcimedins, that is, annexins III–VI.

Annexins III, IV, V and VI (calcimedins 35α, 35β, 35γ and 67) were identified and purified by Ca^{2+}-dependent phenyl–Sepharose and DE-52 of Mono Q h.p.l.c. chromatography [5, 6]. The proteins were injected into sheep and the antibodies antigen-affinity purified. To identify whether the 35 kDa calcimedins were annexins, an antibody was produced against the annexin consensus sequence, K-A-M-K-G-L-G-T-D-E; this represents a sequence found in all members of the annexin family [2]. This consensus sequence antibody cross-reacts with annexins I, II, III, IV, V and VI, but not with 30 kDa and 32 kDa calcimedins or calmodulin [2].

Our initial step to identify annexin function was to use