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Consensus peptide antibodies reveal a widespread occurrence of Ca^{2+}/lipid-binding proteins of the annexin family

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Antibodies generated against synthetic peptides that correspond to highly conserved sequence motifs in the annexins reacted with a variety of annexins from different species These include *Xenopus laevis* and *Drosophila melanogaster*, which contain cross-reacting polypeptides of apparent M_r 34000 and 30000 As expected for typical annexins, the two *Drosophila* proteins interact in a Ca^{2+}-dependent manner with phosphatidylserine liposomes

Ca^{2+}/phospholipid-binding protein, Calpactin, Endonexin fold, Lipocortin

1. INTRODUCTION

In the recent past, several proteins have been identified in higher animal cells that interact with phospholipids in a Ca^{2+}-dependent manner and are thought to be involved in membrane fusion events and/or membrane-cytoskeletal linkage (for review see [1-3]). This new protein family, known as the annexins or lipocortins, is of widespread interest, since several members are substrates for various protein kinases that are thought to regulate processes of cellular growth and differentiation. All annexins sequenced to date are composed of a conserved segment of 70-80 amino acids, which is repeated either 4-fold (annexins of the 30-40 kDa class) or 8-fold (annexins of the 65-70 kDa class) along the polypeptide chain. These repeats exhibit a high degree of sequence similarity within any one molecule, and also between different members of the protein family. The repeat segments comprise some 90% of each annexin molecule (also known as protein core) and are preceeded by an aminoterminal extension (tail) of variable sequence and length. The tail region contains all phosphorylation sites whereas the protein core harbors the binding sites for Ca^{2+} and phospholipid. Although the molecular structure of these binding sites is not known to date, it is generally believed that a highly conserved stretch of 17 amino acids, which is present in each repeat segment and known as the endonexin fold, is involved in Ca^{2+} and/or phospholipid binding [4-7].

Based on immunological cross-reactivity, primary structure, and their Ca^{2+}-dependent association with membranes, annexins have been identified in several animal species. The protein family includes p36 (calpactin I heavy chain) [7,8-10], p35 (lipocortin I) [11,12], p68 [13,14], endonexin II [15], protein II [8,16], and lipocortin III [17], all purified from mammalian cells, as well as anchorin CII [18] and calectrin [19], which have been isolated from chicken chondrocytes and the ray *Torpedo marmorata*, respectively. An immunological approach is described here that led to the identification of annexins in several other species. In particular, *Drosophila melanogaster* was shown to contain two annexin-like proteins that interact with phosphatidylserine liposomes in a Ca^{2+}-dependent manner.

2. MATERIALS AND METHODS

2.1. *Cells*

Drosophila melanogaster cells (Schneider, line 3, kindly provided by A. von Bogen and Dr D Jovin) were maintained in Schneider's medium (Gibco) containing 10% fetal calf serum (FCS) Chicken embryo fibroblasts and *Xenopus laevis* A6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS

2 2 *Peptide synthesis and preparation of antibodies*

Peptides CP2 and CP4 (see fig 1A) were synthesized on a Milligen model 9050 synthesizer and purified by high-pressure liquid chromatography on a C_4 resin using a 0-100% acetonitrile gradient in 0 1% trifluoroacetic acid The accuracy of the peptide synthesis was verified by automated gas-phase sequencing (Applied Biosystems model 470A). For immunization, purified peptides were crosslinked via their N-terminal cysteine residue to either keyhole limpet hemocyanine or bovine serum albumin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS, Pierce) as the coupling reagent [20]. Peptide-carrier conjugates were injected into rabbits, which were bled after two booster injections For the preparation of antigen-affinity purified antibodies, the peptides were coupled to AH Sepharose (Pharmacia) using again MBS as bifunctional crosslinker. Antisera were passed over the affinity matrix and the column was washed with

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phosphate-buffered saline (PBS) containing 0 5 M NaCl followed by 0 1 M Na-acetate, 0 5 M NaCl, pH 4 8 Peptide-specific IgGs were eluted with 0.2 M acetic acid, 0 5 M NaCl, pH 2 7, immediately neutralized, and dialyzed against PBS for storage Immunoblotting was carried out following standard procedures [21], using peroxidase-conjugated second antibodies (Dako)

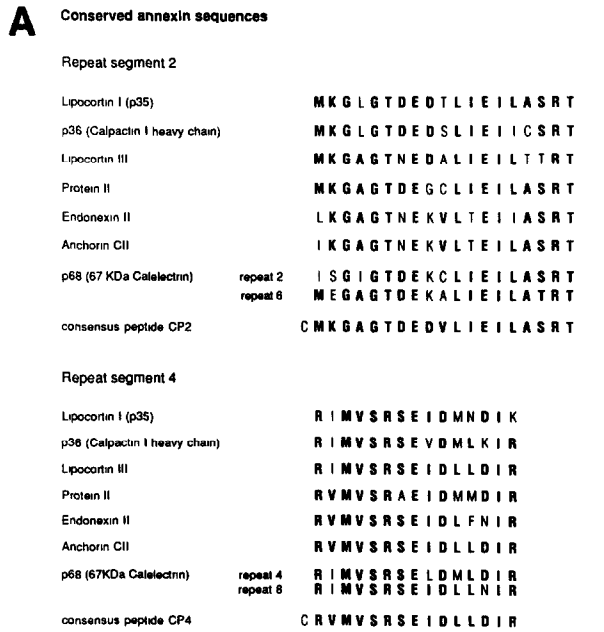
2 3 *Preparation of EGTA extracts and liposome binding*

Cells were washed with PBS, frozen in liquid N_2 and ground to powder in a mortar 1 g of powder was homogenized in 10 ml HN buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM DTT), using a glass/Teflon homogenizer. Subsequently, $CaCl_2$ was added to 15 mM and the mixture was kept on ice for 5 min After centrifugation (30 min at 25 000 × *g*), the pellet was washed twice in HN buffer containing 3 mM $CaCl_2$, and finally extracted with HN buffer plus 10 mM EGTA For liposome binding, the EGTA extract was dialyzed against TN buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT), mixed with phosphatidylserine (PS)-containing liposomes (1 mg/ml) in the presence of 5 mM $CaCl_2$, and incubated for 20 min at 4°C Subsequently, liposomes were pelleted (20 min at 100 000 × *g*) and washed with TN buffer plus 2 mM $CaCl_2$ Proteins bound to the PS liposomes in a Ca^{2+}-dependent manner were eluted in TN buffer containing 2 mM EGTA

3. RESULTS

In order to obtain antibodies that recognize conserved sequence elements present in all annexins, synthetic peptides corresponding to the regions of highest sequence conservation were synthesized and used for immunization. Two consensus peptides, CP2 and CP4, were constructed based on two highly conserved stretches of amino acids present in repeat segments 2 and 4 of all annexins sequenced so far (fig.1A). The peptides cover at least part of the endonexin folds in these repeat segments. Polyclonal antibodies against CP2 and CP4 were elicited in rabbits, affinity purified, and characterized on several purified annexins. Fig.1B shows that both CP2 and CP4 antibodies recognize different annexins, including p68 and p36.

The specificity of the two consensus peptide antibodies was demonstrated in Western blots of total cellular proteins from different mammalian and avian cells. Fig.2 (lanes 1,2) shows an example of porcine intestinal epithelial cells. Both antibodies only react with the annexins present in these cells, i.e. p36 and protein II (32 kDa). Similar results were obtained with several murine cell lines and chicken embryo fibroblasts (not shown). Thus, the CP2 and CP4 antibodies are specific for proteins of the annexin family. The consensus peptide antibodies were then employed to search for annexins or annexin-like proteins in species of distant evolutionary origin. Cross-reacting polypeptides were identified in *Xenopus laevis* (fig.2, lanes 3,4) and *Drosophila melanogaster* (fig.2, lanes 5,6). In *Xenopus*, two proteins of apparent M_r 34 000 and 30 000 are



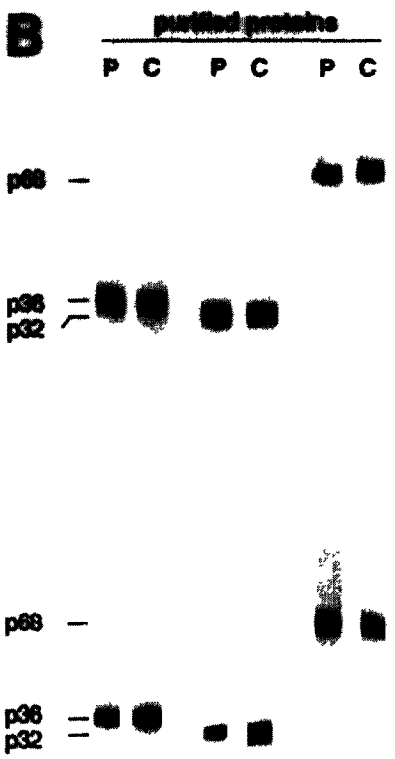


Fig.1 Sequences of consensus peptides and characterization of peptide antibodies (A) Conserved sequences present in repeat segments 2 and 4 of various annexins Sequences were obtained from [10,12–18]. In lipocortin I, the sequence blocks start with Met 126 (repeat 2) and Arg 297 (repeat 4), respectively. Bold lettering denotes exact residue match with sequences of the consensus peptides given at the bottom (B) Characterization of consensus peptide antibodies CP2 (upper panel) and CP4 (lower panel) in Western blots of purified porcine (P) or chicken (C) annexins (p68, p36, protein II)

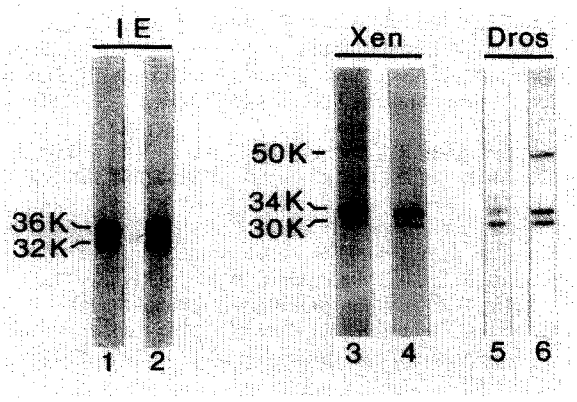


Fig.2. Characterization of consensus peptide antibodies in Western blots of total cellular proteins. Proteins were from porcine intestinal epithelium (IE), and cultured cells of *Xenopus* (Xen), or *Drosophila* (Dros), and were probed with CP2 (lanes 1,3,5) or CP4 (lanes 2,4,6) antibodies, respectively.

recognized by both the CP2 and CP4 antibodies. Two proteins of similar apparent M_r are also present in *Drosophila*, which react strongly with the CP4 and somewhat weaker with the CP2 antibody. In addition, *Drosophila* contains a 50 kDa band, which is identified only by the CP4 antibody.

To establish whether the immunoreactive proteins from *Drosophila* also exhibit annexin-like biochemical properties, a series of Ca^{2+} and EGTA extractions were performed. Annexins in higher mammalian cells typically remain insoluble if cells are lysed in the presence of Ca^{2+}, but are specifically extracted after chelation of the divalent cation. When *Drosophila* cells are first extracted in the presence of Ca^{2+}, and the Ca^{2+} precipitate is subsequently treated with EGTA-containing buffers, two of the cross-reacting polypeptides behave like typical annexins, i.e. they remain insoluble unless Ca^{2+} is chelated with EGTA. Both the 34 kDa and the 30 kDa species are found to be enriched in the EGTA extract, whereas the 50 kDa protein (which only reacts with the CP4 antibody) is extracted even in the presence of Ca^{2+} (fig.3A). Thus, based on these biochemical characteristics, the 50 kDa polypeptide most likely does not belong to the annexin family. To further prove the annexin nature of the 34 kDa and 30 kDa *Drosophila* proteins, they were assayed for Ca^{2+}-dependent interaction with phospholipids. The EGTA extract from *Drosophila* cells was incubated with phosphatidylserine liposomes in the presence of Ca^{2+}. Fig.3B shows that both the 34 kDa and the 30 kDa proteins bind to the liposomes under these conditions, and are specifically eluted when Ca^{2+} is chelated by the addition of EGTA. A Coomassie stain of the EGTA-eluted material reveals that the two proteins reacting with the consensus peptide antibodies are the major bands present in this sample. Hence, the two *Drosophila* proteins exhibit properties typical of annexins.

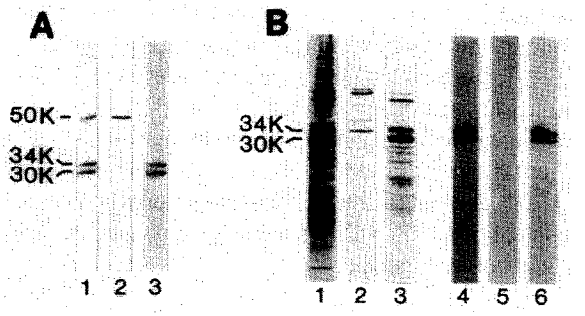


Fig.3. Ca^{2+}-dependent interaction of *Drosophila* annexins with subcellular structures (A) and phospholipids (B). (A) Consecutive Ca^{2+} and EGTA extractions. Total *Drosophila* proteins (lane 1) and proteins present in the Ca^{2+} (lane 2) or EGTA (lane 3) extracts were separated in 10% SDS gels and analyzed in Western blots using the CP4 antibody. (B) *Drosophila* proteins present in the EGTA extract (lanes 1,4) were incubated with PS liposomes in the presence of Ca^{2+}. Lanes 2, 5 and 3, 6 show the unbound fraction and proteins, respectively, which were specifically eluted with EGTA. Polypeptides were separated in 15% SDS gels and analyzed by Coomassie blue staining (lanes 1–3) or Western blotting using the CP4 antibody (lanes 4–6).

4. DISCUSSION

The immunological approach presented here demonstrates that Ca^{2+}/phospolipid-binding proteins are present in a variety of different species including *Drosophila melanogaster*. As expected for true annexins, the 34 kDa and 30 kDa *Drosophila* proteins bind to phospholipid in a Ca^{2+}-dependent manner and cross-react with antibodies directed against annexin consensus peptides. The sequences of these peptides represent a best-fit of the most highly conserved sequence blocks found in mammalian annexins, i.e. sequences in or close to the endonexin folds of repeat segments 2 and 4. Despite their widespread occurrence and high degree of conservation, the structural and functional role of these sequences within the annexins is not yet understood. It has been suggested that the endonexin folds are directly involved in the binding of Ca^{2+} and/or phospholipids [4–6]. Experimental evidence supporting this hypothesis stems from membrane binding studies. Ali et al. [22] showed that a synthetic peptide corresponding to the consensus sequence of the endonexin fold in repeat segment 2 interferes with the Ca^{2+}-dependent binding of p36 to chromaffin granule membranes. While this points to a direct interaction of the consensus peptide with the p36 binding site on chromaffin granule membranes, I did not observe a similar interference in PS binding assays. Neither consensus peptide (CP2 or CP4) inhibited the Ca^{2+}-dependent interaction of p36 with PS liposomes or a PS affinity matrix even in a 30- to 50-fold molar excess of p36 (not shown). Whether this reflects a characteristic difference between PS vesicles and chromaffin granule membranes used in the assay or is due to some slight variations in the peptide sequences remains to be seen.

Annexins have now been shown to occur in a variety of eucaryotic species including mammals [7–17,23,24], fish [19], plants [25], and insects (this study). Although their exact physiological role is not known, the ubiquitous expression of the annexins points to a fundamental function. Most likely, this function involves the Ca^{2+}-dependent interaction with negatively charged phospholipids since this property is conserved among the various annexins.

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