

Isolation, characterization and localization of annexin V from chicken liver

Catherine M. BOUSTEAD,*‡ Robyn BROWN† and John H. WALKER*

Departments of *Biochemistry and Molecular Biology, and †Anatomy, University of Leeds, Leeds LS2 9JT, U.K.

Annexin V has been purified from chicken liver; 40 mg of annexin V was obtained per kg of tissue. In contrast with mammalian liver, very little annexin VI was obtained. Surprisingly, chicken liver annexin V resembles mammalian annexin IV in its M_r (32 500) and its isoelectric point (5.6), but amino-acid-sequence analysis demonstrates identity with chicken annexin V (anchoring II). It binds to phospholipids in a Ca^{2+} -dependent manner with free- Ca^{2+} concentrations for half-maximal binding to phosphatidylserine and phosphatidic acid of 10 μM ; phosphatidylethanolamine of 32 μM and phosphatidylinositol of 90 μM . No binding to phosphatidylcholine was observed at Ca^{2+} concen-

trations up to 300 μM . In isolated liver membranes a significant proportion of annexin V was not extractable with EGTA but could only be extracted with Triton X-100, suggesting the existence of a tightly membrane-associated form of annexin V. A specific antiserum to chicken annexin V was used to localize the protein in adult and embryonic chicken liver. In the adult, annexin V was highly concentrated in epithelial cells lining the bile ducts, and along the bile canaliculi. In embryonic liver, strong staining of the bile-duct epithelial cells was again evident, and in addition, endothelial cells were strongly immunoreactive.

INTRODUCTION

Annexins are a highly conserved family of major cellular proteins which are widely distributed in mammalian and avian species (for reviews see [1–4]; for nomenclature see [5]). They have also been found in fish [6], insects [7], simple eukaryotes such as sponges [8], hydra [9] and slime-moulds [10], and in higher plants [11,12]. At least 12 members of the family have been identified to date. The annexins are Ca^{2+} -binding proteins which bind to phospholipids in a Ca^{2+} -dependent manner. Annexin V is a 32 500- M_r member of the annexin family. The complete amino acid sequences of human [13] and chicken [14,15] annexin V have been determined, and show 80% identity. In common with other annexins, annexin V consists of a non-conserved N-terminal region, followed by four repeats of a highly conserved sequence of approx. 70 amino acid residues. Recently the molecular structures of human [16–18] and chicken [18a] annexin V have been solved by X-ray crystallography.

Annexin V is implicated in a variety of important cellular functions. The three-dimensional structure of human annexin V shows features which are characteristic of channel-forming proteins, and the protein has activity as a Ca^{2+} channel *in vitro* [19]. Other proposed functions for which there is evidence *in vitro* are anti-coagulant activity [20], and inhibition of protein kinase C [21]. There is also some evidence for an extracellular location for annexin V, despite its lack of a conventional signal sequence [22–24]. However, its precise physiological role is not yet known. In this paper we present results on the biochemical properties and localization of annexin V. We demonstrate that large amounts of highly purified annexin V can be isolated from chicken liver. We have analysed some of the molecular properties of the pure protein and have used immunohistochemistry to localize annexin V in embryonic and adult chicken liver.

MATERIALS AND METHODS

Materials

Chicken tissues were obtained from Animal Services, Leeds

University and were either processed within 15 min or stored frozen at $-70^\circ C$. Ampholines were obtained from Pharmacia (Uppsala, Sweden). DEAE-cellulose (DE52) was from Whatman, (Maidstone, Kent, U.K.). Bicinchoninic acid (BCA) reagent was from Pierce (Rockford, IL, U.S.A.). Horseradish peroxidase-conjugated antibodies were obtained from ICN Biomedicals (High Wycombe, Bucks., U.K.). All other reagents were of the highest purity available from Sigma Chemical Co. (Poole, Dorset, U.K.), or BDH Chemicals (Poole, Dorset, U.K.).

Isolation of annexins

Annexins were purified from chicken liver using a method modified from [25]. Tissue (50 g) was homogenized in a Waring blender with 250 ml of 0.15 M NaCl/5 mM EGTA/0.25 mM phenylmethanesulphonyl fluoride (PMSF)/10 mM Hepes, pH 7.4, and centrifuged in a Sorvall SS-34 rotor at 25000 g_{av} for 30 min. The supernatant was removed and $CaCl_2$ added to it to a final concentration of 6 mM (1 mM excess). After 15 min on ice, the fraction was centrifuged for 30 min at 25000 g_{av} . The pellet was washed twice with 100 ml of 0.15 M NaCl/1 mM $CaCl_2$ /10 mM Hepes, pH 7.4, by resuspension and centrifugation (Sorvall SS-34 rotor, 25000 g_{av} for 30 min), and then twice with 100 ml of 1 mM $CaCl_2$ /10 mM Hepes, pH 7.4. The pellet was then resuspended in 15 ml of 10 mM EGTA/10 mM Hepes, pH 7.4, and centrifuged for 30 min at 100000 g_{av} (Beckman Ti50 rotor). All steps were performed at $4^\circ C$. The same method was used for the isolation of annexins from chicken gizzard, except that EDTA was used in place of EGTA.

The final supernatant in each case was dialysed against 20 mM Hepes, pH 7.4, and further purified using ion-exchange chromatography on a DEAE-cellulose column equilibrated in the same buffer. The conditions were essentially as described in [25], except that a linear gradient of 0–0.6 M NaCl in 20 mM Hepes, pH 7.4, was used for elution. Isolation of annexins from chicken liver yielded annexin V as the major protein peak, eluting at approx. 0.1 M NaCl. For dot-blot analysis, annexins were isolated from

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; BCA, bicinchoninic acid; TBS, Tris-buffered saline; HBS, Hepes-buffered saline; PC, phosphatidylcholine.

‡ To whom correspondence should be addressed.

chicken gizzard and separated by ion-exchange chromatography as described above.

Protein concentrations were measured using either the method of Bradford [26], or that using BCA reagent [27].

Electrophoresis

SDS/PAGE was performed essentially according to [28] using 10% (w/v) polyacrylamide gels. The running buffer was 188 mM glycine/188 mM Tris/0.1% SDS. Two-dimensional gel electrophoresis was performed following the method of O'Farrell [29] as modified by Anderson and Anderson [30]. Ampholines were 2D Pharmalytes, pH 3–10 (Pharmacia). Gels were stained with Coomassie Brilliant Blue.

Amino acid sequencing

Peptides were produced from the purified chicken annexin either by fragmentation by cyanogen bromide [31] or by treatment with chymotrypsin [32]. The peptides were separated by reverse-phase h.p.l.c. (chymotrypsin-treated peptides), or by electrophoresis on SDS/15% (w/v) polyacrylamide gels followed by electrophoretic transfer to Teflon membranes (cyanogen bromide-treated peptides). Amino acid sequencing of the peptides was performed using an Applied Biosystems Model 477A amino acid sequencer.

Immunological reagents

Rabbit antisera to *Torpedo* calectrin [6], bovine annexin IV [33], and bovine annexin V (p32/34) [34] have been described previously. The rabbit antiserum to chicken annexin V was prepared as described previously for annexin VI [33].

Immunoblotting

Proteins separated by SDS/PAGE were electrophoretically transferred to nitrocellulose [35]. For dot-blotting, 2 μ l of a 0.25 mg/ml solution (i.e. 0.5 μ g) of protein was spotted on to nitrocellulose. Non-specific binding sites were blocked in 3% (w/v) low-fat dried milk powder in Tris-buffered saline (TBS: 0.15 M NaCl/10 mM Tris/HCl, pH 7.4) for 1 h at 20 °C. The nitrocellulose was then further processed to reveal antigens as described in [36]. Primary antisera and non-immune control sera were used at a dilution of 1:50 for 2 h at 20 °C, unless otherwise specified. Peroxidase-conjugated antibodies raised to rabbit IgG were used at a dilution of 1:500 for 1 h at 20 °C. Peroxidase activity was visualized using either 0.05% (w/v) 3,3'-diaminobenzidine and 0.01% (v/v) H₂O₂ in 50 mM Tris/HCl (pH 7.4), or 0.05% (w/v) 4-chloronaphthol and 0.03% (v/v) H₂O₂ in 50 mM Tris/HCl (pH 7.4).

Phospholipid binding

Phospholipid vesicles were made in the presence of 240 mM sucrose from phosphatidylcholine (PC) alone, or 1:1 mixtures of PC and phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol or phosphatidic acid [37]. The liposomes were collected by adding 2 volumes of buffer (100 mM KCl/2 mM MgCl₂/1 mM EGTA/20 mM Hepes, pH 7.4, plus CaCl₂ to give a range of free-Ca²⁺ concentrations from 0 to

300 μ M), and centrifuging for 10 min at 12000 g_{max} . Free concentrations of Ca²⁺ were calculated on a BBC microcomputer using a computer program adapted from that of Feldman et al. [38] as described by Denton et al. [39]. Apparent dissociation constants for the binding of Ca²⁺ and Mg²⁺ to EGTA at pH 7.4 were obtained using the stability constants given in [40]. After two further washes in the appropriate Ca²⁺ buffer, binding of the proteins to liposomes was measured. A sample (10 μ g) of protein was incubated for 15 min at 20 °C with liposomes (150 μ g of phospholipid) in a total volume of 500 μ l of the appropriate Ca²⁺ buffer. After centrifugation for 10 min at 12000 g_{max} , the supernatants were removed and protein precipitated with an equal volume of 20% (v/v) trichloroacetic acid. The pellets were washed once in 500 μ l of the appropriate buffer, extracted with 300 μ l of acetone at -20 °C for 30 min, and centrifuged for 10 min at 12000 g_{max} . Equal proportions of the supernatants and pellets were analysed by SDS/10% (w/v) PAGE.

Sequential extraction of annexin V

Frozen chicken liver (5 g) was homogenized in 25 ml of Hepes-buffered saline containing EGTA (HBS/EGTA; 0.15 M NaCl/5 mM EGTA/10 mM Hepes, pH 7.4, plus PMSF to a final concentration of 0.25 mM). The homogenate was then treated as follows, with a sample of each supernatant and resuspended pellet being retained following each centrifugation step for analysis by SDS/PAGE. The homogenate was filtered through muslin and centrifuged for 15 min at 18000 rev./min in an SS-34 rotor (25000 g_{av}). The supernatant was retained and the pellet given four washes by repeated resuspension in 25 ml of HBS/EGTA and re-centrifugation for 15 min at 25000 g_{av} . Following the last wash, the pellet (P5) was resuspended in 25 ml of the same buffer, frozen using solid CO₂, thawed and centrifuged for 15 min at 25000 g_{av} to yield P6 and S6. The freeze/thaw process was repeated once to give P7 and S7. P7 was resuspended in 25 ml of HBS/EGTA/1.0% Triton X-100 and centrifuged for 15 min at 25000 g_{av} to produce P8 and S8. The HBS/EGTA/Triton X-100 extraction was repeated on P8 to give P9 and S9. The samples of each supernatant and pellet were then separated by SDS/10% (w/v) PAGE, transferred to nitrocellulose, and probed with a 1:100 dilution of antiserum to chicken annexin V, followed by peroxidase-labelled antibodies to rabbit IgG and staining with 4-chloronaphthol as described above.

Tissue-distribution studies

Samples of freshly excised chicken tissues were prepared for PAGE by homogenizing 0.1 g of tissue with 1.0 ml of sample buffer [3% (w/v) SDS, 10 mM dithiothreitol, 4 mM EDTA, 10% (w/v) glycerol in 0.125 M Tris/HCl, pH 6.8]. After heating in a boiling-water bath for 5 min, the sample was further diluted by the addition of an equal volume of sample buffer. Samples (2 μ l) of the tissue extracts were loaded in each channel of SDS/10% (w/v) polyacrylamide gel. After separation by SDS/PAGE, proteins were transferred to nitrocellulose and immunoblotted using a 1:500 dilution of the antiserum to chicken annexin V, peroxidase-conjugated antibody raised to rabbit IgG, and diaminobenzidine, as described above.

Immunohistochemistry

Immunoperoxidase staining was performed essentially as described by Woolgar et al. [34]. Small pieces of tissue were fixed in Carnoy fixative or in 3.7% (w/v) formaldehyde for 24 h at 20 °C. The same results were obtained under both fixation

conditions. The fixed tissue was then dehydrated, embedded in paraffin, sectioned and mounted on slides using routine histological procedures. Sections 8 μm thick were dewaxed with xylene (2 \times 15 min), then treated with absolute ethanol (2 \times 15 min), 50% ethanol (1 \times 15 min), water (1 \times 5 min), 6% (w/v) H_2O_2 (1 \times 5 min) and TBS (3 \times 5 min). Non-specific staining was prevented by incubation for 1 h at 20 $^\circ\text{C}$ in TBS containing 0.05% Tween-20 and 5% (v/v) normal goat serum. Sections were incubated with antiserum diluted 1:50 in TBS-Tween (0.15 M NaCl/0.05% Tween 20/10 mM Tris/HCl, pH 7.4) containing 5% (v/v) normal goat serum at 20 $^\circ\text{C}$ for 2 h. Pre-immune rabbit serum and antigen-adsorbed antiserum were also processed as controls. Sections were washed over a period of 15 min in several changes of TBS-Tween and then incubated at 20 $^\circ\text{C}$ for 1 h with peroxidase-conjugated goat anti-(rabbit IgG) antibody diluted 1:100 in TBS-Tween containing 5% (v/v) normal goat serum. After washing with TBS-Tween as before, peroxidase activity was visualized with 0.05% 3,3'-diaminobenzidine and 0.01% H_2O_2 in 50 mM Tris/HCl buffer, pH 7.4. After 5 min at 20 $^\circ\text{C}$, the slides were washed in several changes of TBS-Tween. The sections were then dehydrated with 50% ethanol (2 \times 5 min), absolute ethanol (2 \times 5 min) and xylene (2 \times 5 min), and mounted in DPX mountant.

RESULTS

Isolation and purification of chicken annexins

Annexins were isolated from chicken tissues using a method which has previously been used on various mammalian tissues. The proteins isolated from chicken gizzard and liver were analysed by SDS/10% (w/v) PAGE (Figures 1a and 1b). Figure 1(a) shows a preparation of annexins from chicken gizzard. The chicken gizzard proteins of M_r 70000 and M_r 32500 have isoelectric points of approx. 5.8 and 5.6 respectively (shown in Figure 5c). The 70000- M_r protein is likely to be the chicken

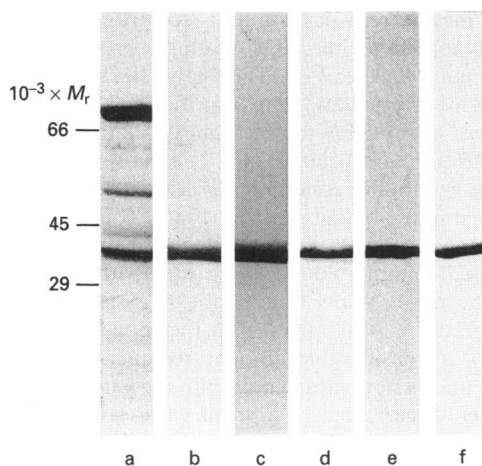


Figure 1 SDS/10% (w/v) PAGE and immunoblotting of chicken annexins

SDS/10% (w/v) PAGE of annexins: lane a, annexin preparation from chicken gizzard; lane b, annexin preparation from chicken liver; and lane c, 32500- M_r annexin purified from chicken liver preparation by anion-exchange chromatography. Protein bands are stained with Coomassie Brilliant Blue. Also shown are immunoblots on purified chicken 32500- M_r annexin using antisera raised to: lane d, *Torpedo* calelectrin; lane e, bovine annexin IV; and lane f, bovine annexin V. Antibody binding was detected using peroxidase-labelled second antibody and 3,3'-diaminobenzidine.

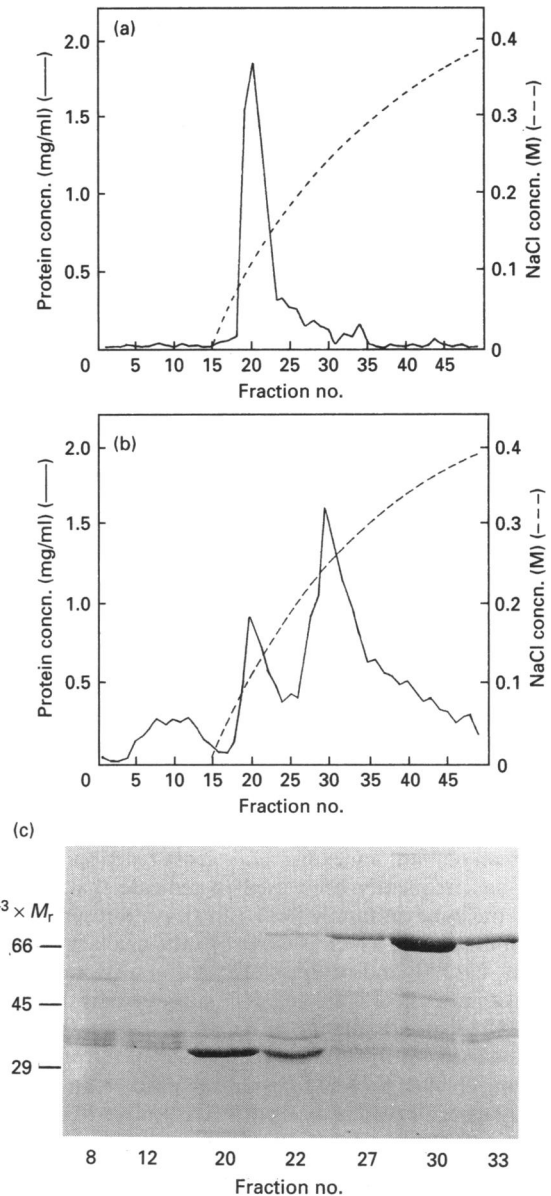


Figure 2 Ion-exchange chromatography on DEAE-cellulose of annexin preparations

Annexins were isolated from (a) chicken liver, and (b) chicken gizzard. (c) SDS/10% (w/v) PAGE of fractions from ion-exchange chromatography of the chicken gizzard preparation. Numbers correspond to fraction numbers in (b). Protein bands are stained with Coomassie Brilliant Blue.

homologue of annexin VI, which it resembles in M_r and pI [33,41,42]. The 32500- M_r , pI 5.6 protein resembles the major protein isolated from the chicken liver preparation as described below.

Figure 1(b) shows the final supernatant of the chicken liver preparation, which contains one major polypeptide of M_r 32500. The 32500- M_r chicken annexin was purified to homogeneity by ion-exchange chromatography on DEAE-cellulose (Figures 1c and 2a). A single major peak of protein eluted from the column at approx. 0.1 M NaCl (Figure 2a) and on two-dimensional gel electrophoresis migrated as a single spot, with a pI value of 5.6 [not shown, but for position of protein see Figures 5(b) and 5(d)]. The amount of protein obtained was equivalent to approx. 40 mg per kg wet wt. of tissue. Annexins were also purified from the

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Anchorin CII (ACII):          MAKYTRGTVTAFSFF
Chicken liver annexin V (V): RGTVTAFSFF

(ACII): DARADAEALRKAMKMGTDDEETILKILTSRNNARQREIASAFKTLFGRDLVDLQKSELTKGFETLMVSLMRP
(V):     DARAD (Chymotrypsin)                                MXP

(ACII): ARIFDAHALKHAIKGAGTNEKVLTEILASRTPAEVQNIKQVVMQYEANLEDKITGETSGHFQRLLVLLQA
(V):     AXIFDAXALXXAIXGAXXNE (CNBr)                       MQEYEANL (CNBr)

(ACII): NRDPDGRVDEALVERDAQVLFragELKWTDEETFITILGTRSVSHLRRVDFKYMVISGFIETIDRETSG
(ACII): DLEKLLAVVKCIRSVPAYFAETLYSMKAGTDDDDTLIRVMVSRSEIDLLDIRHFERNKFAKSLYQMIQKD
(ACII): TSGDYRKALLLCCGGDDE

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Figure 3 Amino acid sequences of peptides derived from chicken liver annexin V compared with the predicted sequence of chicken anchorin CII

chicken-gizzard preparation using ion-exchange chromatography on DEAE-cellulose (Figure 2b). Polypeptides of M_r 30000–38000 were present in the flow-through fraction and are likely to include annexins I and II, which are known to be present in avian species [43,44]. A salt gradient sequentially eluted annexins of M_r 32500 and M_r 70000 (Figure 2c).

Immunochemical studies on the purified chicken liver protein

The identity of the purified chicken liver protein, M_r 32500, pI 5.6, was investigated by immunoblotting with antisera raised to bovine and *Torpedo* annexins. As shown in Figure 1(d) the chicken protein cross-reacts with antiserum raised to the *Torpedo* annexin, calelectrin. This antiserum recognizes many mammalian and non-mammalian annexins, and cross-reaction with this antiserum has frequently been used to indicate that a protein belongs to the annexin family [8,11,12,33]. An antiserum raised to bovine annexin IV, which is closest to the chicken protein in terms of M_r (32500) and pI (5.6), also shows a strong response to the chicken protein (Figure 1e). This suggests that the chicken protein is the avian homologue of mammalian annexin IV, and provided the basis for the initial identification of the protein [45]. However, the chicken protein is also recognized by an antiserum to bovine annexin V (which exists in two isoforms of M_r 32000, pI 5.1 and M_r 34000, pI 5.0 [25,46] (Figure 1f)). In order to establish unambiguously the identity of the chicken protein, amino-acid-sequence information was therefore necessary.

Determination of a partial sequence of chicken annexin V

The purified 32500- M_r chicken annexin was found to be blocked to Edman degradation. Peptides were therefore prepared by cleavage with either chymotrypsin or cyanogen bromide (CNBr), and their amino acid sequences determined (Figure 3). All peptide sequences show 100% identity with sequences found in chicken anchorin CII, now identified as the chicken homologue of mammalian annexin V [47]. Thus, despite the similarity in M_r and pI of the chicken liver protein to mammalian annexin IV, and its cross-reaction with an antiserum raised to bovine annexin IV, the amino acid sequences unambiguously identify the chicken protein as anchorin CII, and therefore the homologue of mammalian annexin V.

Phospholipid-binding studies

A key property of annexins is their Ca^{2+} -dependent binding to acidic phospholipids. We therefore investigated the Ca^{2+} -requirements and phospholipid-binding specificity of chicken liver

annexin V (Figure 4). In the presence of Ca^{2+} , chicken annexin V binds to vesicles containing phosphatidic acid or phosphatidylserine with half-maximal binding occurring at approx. $10 \mu M$ free Ca^{2+} . It also binds to vesicles containing phosphatidylethanolamine or phosphatidylinositol, but higher free- Ca^{2+} concentrations are required, half-maximal binding occurring at approx. $32 \mu M$ and $90 \mu M$ free Ca^{2+} respectively. The protein did not bind to vesicles formed from PC alone at free- Ca^{2+} concentrations up to $300 \mu M$.

Antiserum to chicken annexin V

An antiserum was raised to chicken annexin V. To test the specificity of the antiserum, a total homogenate of chicken liver was separated by two-dimensional gel electrophoresis (Figure 5a). After electrophoretic transfer of the proteins from the same gel to nitrocellulose, immunoblotting was performed using the antiserum to chicken annexin V. As shown in Figure 5(b), the antiserum is specific to annexin V and does not cross-react with any other chicken liver proteins. The specificity of the antiserum to annexin V compared with other proteins co-purifying was tested by isolating annexins from chicken gizzard, separating them by two-dimensional gel electrophoresis (Figure 5c), and immunoblotting with the antiserum to chicken annexin V (Figure 5d). The antiserum cross-reacts only with annexin V. To ensure that faint cross-reactivity was not present, in particular to annexins I and II (which are likely to be present in the unbound fraction of the ion-exchange column [43,44]), peak fractions from ion-exchange chromatography as described in Figure 2(b) were concentrated to equivalent concentrations and dot-blotted. No cross-reactivity was seen except in the fraction containing annexin V (results not shown).

Sequential extraction of annexin V

The antiserum to chicken annexin V was used to study the solubility properties of the chicken liver protein to provide information on its subcellular distribution. Chicken liver tissue was homogenized in an EGTA-containing buffer and membranes were collected by centrifugation. The pelleted membranes were washed four times by resuspension and recentrifugation in the same buffer to release all the EGTA-soluble protein into the supernatant (Figure 6, lanes 1–5). The final pellet was subjected to two rounds of freeze-thaw and centrifugation in the same buffer to extract any EGTA-soluble annexin V trapped inside membrane vesicles (Figure 6, lanes 6 and 7). Finally the pellet was extracted twice with buffer containing EGTA and Triton X-100 to solubilize membrane-bound proteins (Figure 6, lanes 8 and 9). Samples of each supernatant and pellet were separated by SDS/PAGE and immunoblotted using antiserum to chicken annexin V. The results show that although a significant proportion of annexin V is extracted into the initial supernatant (Figure 6, S1), a considerable amount remains in the pellet (Figure 6, P1) and is not extracted despite repeated washing in the same buffer. Freeze-thaw treatment extracts only a small amount of trapped annexin V (Figure 6, S6), and a repeat of the treatment does not extract further detectable amounts (Figure 6, S7). However, resuspension of the pellet in Triton X-100-containing buffer extracts all the remaining annexin V into the supernatant (Figure 6, S8).

Tissue distribution of annexin V in chicken

The antiserum to chicken annexin V was used to test for the presence of this protein in various tissues of the adult chicken,

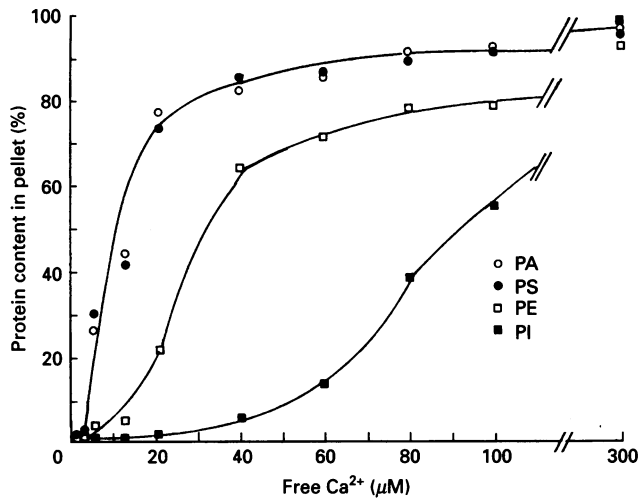


Figure 4 Binding of chicken liver annexin V to phospholipid vesicles at various free-Ca²⁺ concentrations

Phospholipid vesicles containing equimolar amounts of PC and either phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylethanolamine (PE) or phosphatidylinositol (PI) were incubated with chicken annexin V at various free-Ca²⁺ concentrations. Following centrifugation the amount of protein in the supernatants and pellets was measured. No binding occurred to vesicles of PC alone.

using immunoblotting of tissue homogenates (Table 1). Annexin V was detected in all tissues tested except whole blood. The highest concentrations of the protein were found in spleen, liver, intestine and lung, with intermediate amounts in skin and gizzard. Neural tissues, bone marrow, pancreas, adipose tissue, heart and breast muscle also contain detectable amounts of annexin V.

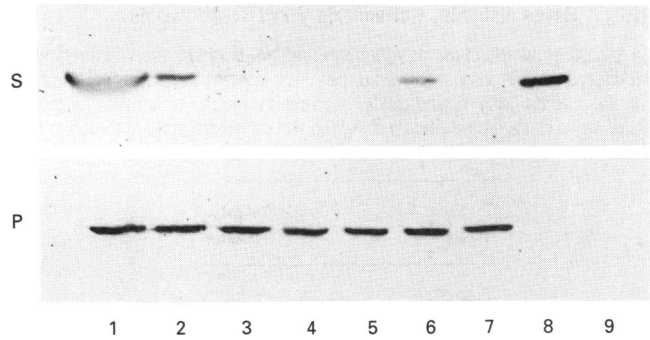


Figure 6 Sequential extraction of annexin V

Immunoblot of samples of supernatants (S) and pellets (P) following sequential washing of a homogenate of chicken liver with EGTA-containing buffer (lanes 1–5); freeze–thaw treatment in EGTA-containing buffer (lanes 6 and 7); EGTA and Triton X-100 containing buffer (lanes 8 and 9). Samples were separated by SDS/10% PAGE, and immunoblotted using antiserum to chicken annexin V, followed by peroxidase-labelled second antibody and staining with 4-chloronaphthol.

Immunohistochemical localization of annexin V in chicken liver

The specific antiserum to chicken annexin V was used to determine its localization in paraffin sections of adult and embryonic chicken liver. Figure 7 shows the results of the immunohistochemistry. In embryonic liver, strong staining is seen along the periphery of the hepatocytes (Figure 7a, arrow-head). Examination at higher resolution allowed this staining to be localized to the endothelial cells, rather than the hepatocyte plasma membrane (results not shown). The epithelial cells lining the bile ducts are also strongly immunoreactive (Figure 7b, arrow). Very little staining of the hepatocytes is seen in the embryonic tissue.

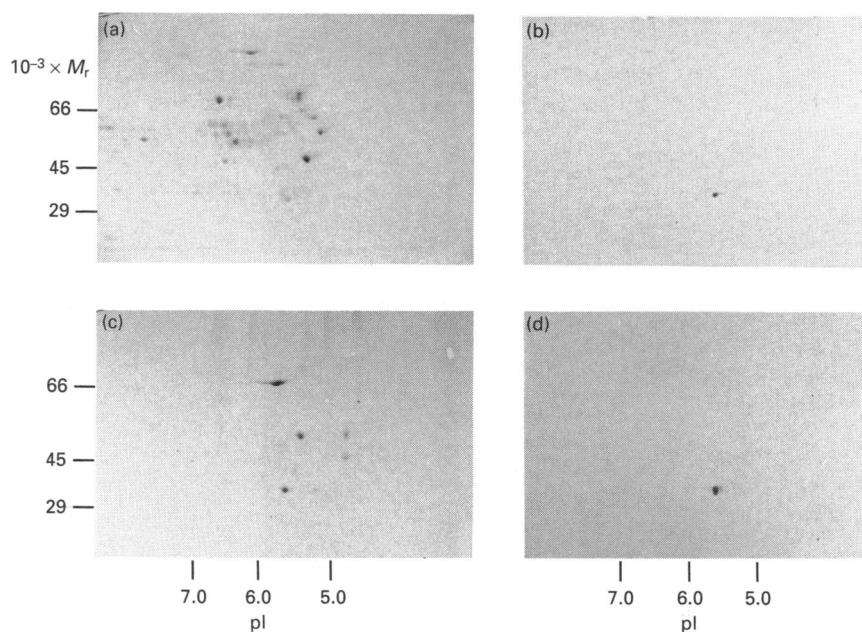


Figure 5 Immunoblots of proteins separated by two-dimensional gel electrophoresis using antiserum to chicken annexin V

(a) Coomassie Blue-stained two-dimensional gel electrophoresis of homogenate of chicken liver. (b) An immunoblot of (a) using antiserum to chicken annexin V. (c) Coomassie Blue-stained two-dimensional gel electrophoresis of a preparation of annexins from chicken gizzard. (d) An immunoblot of (c) using antiserum to chicken annexin V.

Table 1 Tissue distribution of annexin V in chicken tissues

Equal amounts of extracts of tissues were fractionated by SDS/PAGE and blotted with rabbit anti-(chicken annexin V) antibody followed by peroxidase-labelled second antibody. Peroxidase activity was detected using diaminobenzidine. Approximate amounts of annexin V content are indicated, based on the relative intensity of staining with diaminobenzidine. Abbreviation: N.D., not detectable.

Tissue	Presence of annexin V
Brain stem	+
Cerebellum	+
Forebrain	+
Bone marrow	+
Spleen	+++
Liver	+++
Pancreas	+
Skin	++
Intestinal wall	+++
Intestinal mucosa	++
Blood	N.D.
Adipose tissue	+
Breast muscle	+
Gizzard	++
Heart	+
Lung	+++

In the adult, annexin V is also highly concentrated in the epithelial cells lining the bile ducts. Strong staining is also visible along the bile canaliculi (Figure 7d and 7e, arrowheads). In some

of the hepatocytes, staining of the cytosol is also visible (Figure 7d). No staining of either embryonic or adult tissue is seen using pre-immune serum (results not shown) or antigen-adsorbed serum (Figures 7c and 7f).

DISCUSSION

Comparison of chicken annexin V with mammalian annexins IV and V

Using standard procedures, annexins have been isolated from chicken gizzard and liver. The major component of the chicken liver preparation, a 32500- M_r protein, has been purified to homogeneity as demonstrated by three lines of evidence. First, the protein migrated as a single spot on two-dimensional gel electrophoresis; secondly, the protein was sufficiently pure to allow its crystallization [18a] and finally, all amino acid sequences determined showed 100% identity to chicken anchorin CII. This protein has been identified as the chicken homologue of mammalian annexin V on the basis of 80% overall amino-acid-sequence identity [14,15]. The degree of sequence conservation of chicken and mammalian annexin V (80%) falls between the very high amino-acid-sequence identity found between mammalian species (95–98% [48,49]) and the lesser degree of conservation (40–50% identity to mammalian forms) observed in more distantly related species such as insects and lower eukaryotes [7,50]. Interestingly, the M_r , pI and elution position on DEAE-cellulose chromatography of chicken annexin V more closely resemble mammalian annexin IV than mammalian annexin V. This demonstrates the importance of amino-acid-sequence information in assigning homology of non-mammalian annexins to mammalian forms.

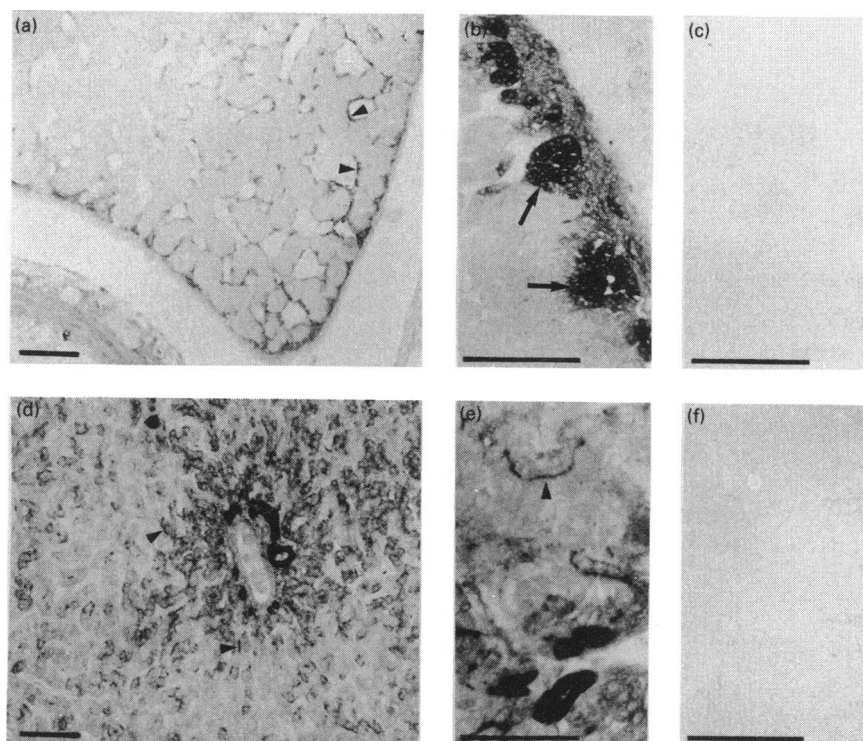


Figure 7 Immunohistochemical localization of annexin V in chicken liver

Eight-day-old chick-embryo samples: (a) liver showing positively stained endothelial cells; (b) bile ducts bordering the hepatic vein; and (c) control serum. Panels d–f show adult chicken samples: (d) portal-tract region of adult liver showing strongly stained bile ducts and bile canaliculi; (e) bile ducts and canaliculi; and (f) control serum. Scale bars indicate 50 μ m.

We have now solved the three-dimensional structure of chicken annexin V [18a] and shown it to be almost identical to human annexin V [18]. Consistent with the similarity in structure, avian and mammalian annexin V show very similar phospholipid-binding properties including a preference for acidic phospholipids and micromolar free-Ca²⁺ concentrations for binding [25,34,51].

Sequential extraction of annexin V

The results on annexin solubility suggest that a significant proportion of annexin V can associate with membranes in a manner which is not reversible with EGTA but can be solubilized with Triton X-100. Similar results showing detergent-extractable forms of annexins have been obtained for human annexin V [52] and for annexin X from *Drosophila* [7]. There is also evidence for relocation of annexins to membranes following physiological stimulation of cells [53,54]. In the case of neutrophils, annexin III associates with membranes at sites of phagocytosis, and the membrane location persists beyond the transient rise in free-Ca²⁺ concentration [55]. It is therefore possible that stimulation of cells may lead to the formation of tightly membrane-associated forms of annexins, which no longer require elevated levels of Ca²⁺ for their attachment.

Tissue distribution and localization of annexin V in chicken

The distribution of annexin V in chicken tissues is wide but not ubiquitous. High concentrations can be detected in liver, spleen and lung, whereas much smaller amounts are found in neural tissues, some muscular tissues and pancreas. This suggests that the protein is not involved in a general 'housekeeping' function.

In chicken liver some annexin V is found throughout the hepatocytes, consistent with a cytosolic pool of the protein. This is similar to the results obtained elsewhere for annexin V in rat liver [56]. Additionally, in chicken, we find strong association of annexin V with bile-duct epithelial cells and with bile canaliculi. Further studies at the level of resolution of the electron microscope will be needed to identify the precise location of annexin V. The association of annexin V with the bile canaliculi is particularly interesting since Ca²⁺ is known to mediate pericanalicular functions such as bile secretion [57], canalicular contraction [58] and tight-junction permeability [59]. Our future work will be aimed at testing the hypothesis that annexin V functions as a regulatory element in intracellular signalling.

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