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Characterization and immunolocalization of rat liver annexin VI

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Abstract

Annexin VI has been purified to homogeneity from rat liver and monospecific antibodies have been produced. The antibodies have been used for immunoblot analysis of rat tissues. Annexin VI is present in most tissues, with particularly high concentrations in liver, spleen, muscle, and intestine. In liver, annexin VI constitutes approximately 0.25% of total cellular protein. Immunohistochemical studies have located annexin VI on plasma membranes of hepatocytes with enhanced concentration on bile canaliculi. Annexin VI binds in a Ca^{2+}-dependent manner to a sub-cellular fraction containing membranes. In the presence of physiological concentrations of ATP, the free Ca^{2+} concentration required for half-maximal binding of annexin VI to membranes is significantly reduced. While annexin VI binds in vitro to membranes in the presence of Ca^{2+}, in rat liver about 31% of the annexin VI is associated with membranes in a Ca^{2+}-independent manner and its solubilization requires the presence of Triton X-100. However, studies using Triton X-114 showed no increase in the hydrophobicity of this fraction of the protein compared to the purified EGTA-soluble annexin VI.

Key words: Annexin VI; Membrane-binding; Calcium ion dependence; (Rat liver)

1. Introduction

Annexins are a family of about thirteen structurally related calcium-dependent phospholipid-binding proteins with as yet undefined physiological roles [1]. The annexins constitute a significant proportion of total cellular protein and have been highly conserved throughout evolution [2]. They contain a core domain formed by a four- or eight-fold repeat of a conserved sequence of approximately 70 amino acid residues. A variable N-terminal domain is thought to confer functional diversity [3]. Suggested functions of the annexins include mediation of cytoskeletal-membrane interaction [4,5], regulation of membrane traffic and exocytosis [6–8], inhibition of phospholipase A_2 and protein kinase C activities [9,10], and roles in mitogenic signal transduction [11], transmembrane channel activity [12], inhibition of blood coagulation [13], tissue differentiation [14] and anti-inflammation [15]. Much effort is now being directed at elucidating the specific physiological roles of the annexins.

The hepatocyte plasma membrane consists of discrete functional domains [16]. The canalicular domain is important in the formation and secretion of bile. The blood-bathed sinusoidal membrane is involved in secretion and signal transduction. Proteins of the intercellular junctions are localized to the lateral plasma membrane. The specialization of hepatocyte membranes into regions with distinct functions provides a useful means of investigating specific membrane-associated proteins of unknown function. An association of annexin VI with membranes has been reported [17], and roles in secretion and in the modulation of Ca^{2+}-channel activity [18,19] have been suggested for this annexin. In some cell types however, a clear relationship with membranes has been difficult to demonstrate and significant amounts of the annexin have been found at non-membrane sites [20,21]. It is also becoming increasingly obvious that some annexin VI may exist in association with membranes in a Ca^{2+}-independent

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manner [22]. We report here the first localization of annexin VI in rat liver, and an investigation of the association of this annexin with membranes.

2. Materials and methods

Materials

Rat livers were obtained from mature Wistar rats, frozen in liquid nitrogen immediately after removal, and then kept at −70°C until required. Anti-clathrin antibody was from ICN Biomedicals (High Wycombe). Unless otherwise stated, all other reagents were from Sigma (Poole, Dorset, U.K.) or BDH Chemicals (Poole, Dorset, U.K.).

Purification of annexin VI

The isolation of annexin VI and its further purification by ion-exchange chromatography and gel filtration chromatography were performed as described [23]. For use as antigen, annexin VI from pooled ion-exchange fractions was electroeluted from SDS-polyacrylamide gels [24].

Preparation of clathrin-enriched brain microsome fraction

A clathrin-enriched pig brain microsome fraction was prepared by a modification of the method of Pearse [25] as described in Ref. [26].

Protein determination

Protein concentrations were determined using BCA reagent (Pierce, Rockford, IL) according to the manufacturer's instructions, or by using the Bradford method [27].

Determination of amino acid composition of annexin VI

Annexin VI (150 μg) was dialysed against 0.1 M NH_4HCO_3 (four batches each of 2 litres) at 4°C. The protein was lyophilised and aliquots subjected to vapour phase hydrolysis in 6 M HCl/ 1% (w/v) phenol at 110°C for 24 h in vacuo. The acid was removed by drying over NaOH in vacuo. The hydrolysate was treated with phenylisothiocyanate (PITC) and the PITC-amino acids were separated by reverse-phase HPLC on a Waters NovaPak™ C_{18} column (300 × 3.9 mm) using a gradient of acetonitrile in 140 mM acetic acid/7 mM triethylamine (pH 6.5 with NH_4OH). Elution was monitored at 254 nm using an Applied Biosystems (Kratos) Spectroflow 757 UV detector.

Peptide sequencing

Tryptic peptides were isolated by reverse-phase HPLC with a Waters Model 510 (Millipore) using a 300 × 3.9 mm C_{18} column (μ Bondapak) and a gradient of 0–70% (v/v) acetonitrile (Fisons) in 0.1% trifluoroacetic acid. Peptides selected at random were sequenced using an Applied Biosystems model 477A protein sequencer or a Milligen Biosearch 6600 prosequencer, by the Sequencing Unit, Department of Biochemistry and Molecular Biology, University of Leeds.

Electrophoresis and immunoblotting

SDS-PAGE was performed according to Laemmli [28], using 10% (w/v) polyacrylamide. Silver staining was by the method of Oakley et al. [29]. Two-dimensional gel electrophoresis was according to O'Farrell [30] as modified by Anderson and Anderson [31]. Immunoblotting was performed essentially as described in [26], using 4-chloro-1-naphthol for visualization of peroxidase activity.

Immunoassay for annexin VI

Annexin VI was quantified using immunoblotting as described [26]. The calibration curve was linear for values of annexin VI between 5 ng and 120 ng. The values are the mean of three determinations.

Antiserum preparation

Antibodies to SDS-denatured rat liver annexin VI were raised in male New Zealand White rabbits as described previously for bovine 32.5 kDa calelectrin (annexin IV) [32].

Adsorption purification of antibodies

On immunoblotting the initial antiserum to rat annexin VI against rat liver homogenate a cross-reacting protein of 55 kDa was observed in addition to the annexin VI (70 kDa). This cross-reacting activity was removed by adsorption purification of the antiserum with a rat liver fraction enriched in the 55 kDa protein but devoid of annexin VI. The fraction was prepared by binding a rat liver cytosol fraction to DEAE-cellulose and washing it with 20 mM Hepes-NaOH pH 7.4 until the absorbance at 280 nm was < 0.05. The column was then eluted with 20 mM Hepes-NaOH pH 7.4 containing 0.1M NaCl. The eluant contained the 55 kDa protein but no annexin VI. This protein preparation was coupled to CNBr-activated Sepharose 4B (Sigma) using ~ 0.5 mg protein per ml of swollen gel, and following the manufacturer's instructions.

Immunohistochemistry

Rat liver and intestine from freshly-killed animals were fixed by immersion in 3% (v/v) paraformaldehyde in phosphate-buffered saline (0.15M NaCl, 10 mM sodium phosphate buffer pH 7.4), then embedded in paraffin and sectioned (4 μm thick). Immunoperoxidase staining was then performed essentially as described in Ref. 33. Control sections were treated either with pre-immune rabbit serum or with antibodies to rat annexin VI pre-adsorbed with the purified antigen. All

control sections were blank. Nuclei were counterstained in some experiments with Mayer's haematoxylin.

Effects of Ca^{2+} and ATP on membrane-binding by annexin VI

Fresh rat liver was homogenized using a Polytron homogenizer in 10 volumes of buffer containing 20 mM Hepes-KOH, 100 mM KCl, 2 mM $MgCl_2$ and either 5 mM EGTA (pH 7.4) or EGTA and $CaCl_2$ mixtures to give final free [Ca^{2+}] from 0 to 5 mM. Free Ca^{2+} concentrations were calculated using the Metlig programme adapted from Feldman et al. [34] as described by Denton et al. [35]. Apparent dissociation constants for the binding of Ca^{2+} to EGTA at pH 7.3 were obtained using the stability constants given by Martell and Smith [36]. After the tissue had been homogenized, the homogenate was centrifuged at 120 000 × *g* for 30 min. The pellet (P_1) was resuspended in Ca^{2+}-EGTA buffer (same volume as the original homogenate) and centrifuged at 120 000 × *g* for 30 min. The process was repeated twice with Ca^{2+}-free buffer. In some experiments ATP was added to a concentration of 5 mM for the initial homogenization step. Aliquots of supernatants and the final pellet were analysed by SDS-PAGE and immunoblotting. All steps were performed at 4°C unless otherwise stated.

Ca^{2+}-independent association of annexin VI with membranes

Liver tissue (0.2 g) from 5 day old rats was excised and immediately homogenised in a glass/glass hand homogeniser in 2 ml buffer containing 50 mM KCl, 2 mM $MgCl_2$, 5 mM ATP, 20 mM Hepes pH 7.4 and either 1 mM $CaCl_2$ or 10 mM EGTA. The homogenates were centrifuged for 15 min at 100 000 × *g* at 25°C. The pellets were resuspended in each case in the EGTA-containing buffer, re-centrifuged, and this process repeated once more. Pellets were then either (1) resuspended in Triton X-100-containing buffer (100 mM KCl, 0.5 mM DTT, 5 mM EGTA, 1 mM NaN_3, 10 mM imidazole pH 7.4 and 1% Triton X-100) and recentrifuged; or (2) treated with Triton X-114 as described below. Samples of supernatants were separated by SDS-PAGE [28], transferred to nitrocellulose and immunoblotted with antibodies to rat annexin VI.

Triton X-114 phase separation studies

The method of Bordier [37] was used with 5 μg of purified annexin VI, or with 0.1 mg of pelleted protein obtained as described above. Aqueous and detergent phases were obtained by incubation at 30°C and centrifugation [37]. The fractions were analysed for annexin VI content by immunoblotting with the antiserum to annexin VI as described above.

3. Results

3.1. Isolation and characterization of rat liver annexin VI

Several proteins were isolated from rat liver using the annexin purification method of Boustead et al. [23] (Fig. 1). They included proteins with molecular weights of 70 kDa and 35 kDa, and a protein of 180 kDa (Fig. 1b). From 100 g of rat liver, approximately 11 mg of protein were obtained. Following ion-exchange chromatography of the preparation, the pooled fractions containing annexin VI (Fig. 1c) were further purified by electroelution (Fig. 1d) or by gel filtration chromatography. This resulted in the separation of annexin VI from the 180 kDa protein as well as from other proteins of 30–35 kDa (Fig. 1, c and d).

The purified protein could be recognized on immunoblotting by a well-characterized antiserum against bovine annexin VI [26] (not shown). The antibodies raised to purified rat annexin VI (described in Section 2) were shown to be monospecific for annexin VI by immunoblotting rat liver homogenate (Fig. 1, e and f). The 180 kDa protein which was also present in the annexin preparation was identified as clathrin heavy chain by immunoblotting with a specific monoclonal antibody (not shown).

On SDS-PAGE, rat liver annexin VI has a molecular weight of 70 kDa (Fig. 1d). On two-dimensional gel

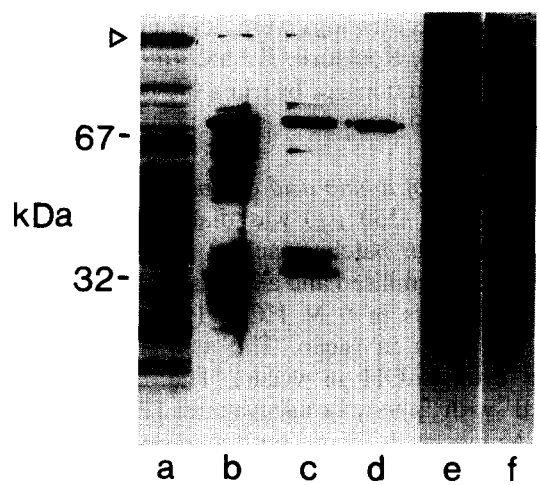

Fig. 1. Purification of rat liver annexin VI. Lanes a–d: SDS-polyacrylamide gel electrophoresis of fractions from the purification of annexin VI: (a) rat liver homogenate; (b) rat liver annexins isolated as described in Section 2; (c) fractions from ion-exchange chromatography (NaCl concentration > 0.2M) containing annexin VI; (d) electroeluted annexin VI. Lane (a) is stained with Coomassie brilliant blue; lanes (b–d) are silver-stained. The arrowhead marks the postion of the 180 kDa polypeptide referred to in the text. Lanes e and f: Immunoblots of: (e) antiserum to rat liver annexin VI on sample (approx. 50 μg) of rat liver homogenate similar to lane (a); (f) antiserum to rat liver annexin VI on sample (approx. 150 ng) of purified rat liver annexin VI similar to lane (d).

Table 1
Amino acid composition of rat liver annexin VI

	Amino acid composition (mol%)		
	rat liver annexin VI	pig lung annexin VI [a]	bovine brain annexin VI [b]
Asx	11.43	9.6	10.5
Thr	5.81	5.1	5.3
Ser	8.00	8.9	8.3
Glx	15.76	11.8	13.2
Pro	4.28	n.d.	2.6
Gly	9.74	15.0	9.0
Ala	9.50	7.0	8.3
Val	3.84	3.8	2.6
Cys	n.d.	n.d.	1.1
Met	1.97	2.5	2.6
Ile	4.55	5.3	4.9
Leu	6.82	9.1	9.8
Tyr	2.72	4.1	2.6
Phe	3.26	3.3	3.0
Lys	5.68	6.8	9.0
His	1.41	2.1	1.5
Arg	5.22	5.6	5.6
Trp	n.d.	n.d.	n.d.

The amino acid composition of rat liver annexin VI is shown. Pig lung annexin VI and bovine brain annexin VI compositions are included for comparison.
n.d., not determined.
[a] From Martin et al. [38]. [b] From Fauvel et al. [39].

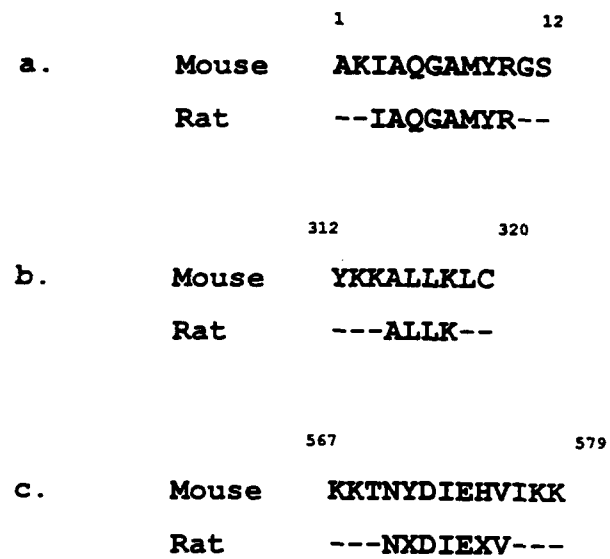

Fig. 2. Partial amino acid sequence analysis of rat liver annexin VI. Amino acid sequence comparison of three tryptic peptides, (a), (b) and (c) from rat annexin VI, and portions of the mouse annexin VI sequence [40]. Peptide (a) shows sequence identity to the N-terminal region of the mouse protein. Peptides (b) and (c) show sequence homology to internal regions of mouse annexin VI. X represents an undetermined residue. The numbers refer to amino acid residues in mouse annexin VI.

electrophoresis the protein was found to have a pI of 5.9 (not shown). Results of amino acid composition analysis (Table 1) were very similar to published data for other mammalian annexin VI proteins [38,39]. Amino acid sequence analysis showed complete identity between an eight amino acid peptide from rat annexin VI and a sequence close to the N-terminal of murine annexin VI [40] (Fig. 2). Two further peptides from rat annexin VI could also be aligned closely with the murine sequence.

3.2. Determination of the yield of annexin VI

To assay for amounts of annexin VI, samples were immunoblotted with the monospecific antibodies to annexin VI, together with known amounts of pure annexin VI, and the intensity of staining determined by densitometry. The calibration curve obtained was linear for values of annexin VI between 5 ng and 120 ng (not shown). Calculations showed that approximately 2 mg of annexin VI were isolated from 100 g of rat liver using the method described.

3.3. Tissue distribution of annexin VI

Table 2 shows the amounts of annexin VI in a variety of tissues. Rat liver annexin VI was shown by immunoblot analysis to constitute 0.25 ± 0.04% of total cellular protein. Significant amounts of annexin VI were also found in heart, skeletal muscle (gastrocnemius), small intestine, ventral prostate, spleen and brain, whereas lung and kidney were relatively deficient in annexin VI. Annexin VI was present in rat seminal vesicles but was absent from seminal vesicle

Table 2
Tissue distribution of rat annexin VI

Rat tissue	Annexin VI as a percentage of total cellular protein
Seminal vesicle	0.13
Lung	0.02
Anterior prostate	0.08
Spleen	0.21
Ventral prostate	0.26
Cerebellum	0.09
Kidney	0.03
Cerebrum	0.10
Small intestine	0.23
Heart	0.21
Liver	0.25
Skeletal muscle	0.20

Annexin VI in various tissues of the rat was quantified by densitometric scanning of immuno blots performed with a monospecific antiserum against rat liver annexin VI, using purified annexin VI to obtain a calibration curve. The numbers represent the percentage of cellular protein corresponding to annexin VI. The annexin is abundant in liver, all forms of muscle including skeletal muscle (gastrocnemius) and in spleen, and is deficient in lung and kidney. Annexin VI is also relatively abundant in cerebrum, cerebellum and in the accessory reproductive organs, namely the seminal vesicle, anterior prostate and ventral prostate. The values are the average of three determinations.

secretion. The annexin was also present in the anterior prostate.

3.4. Localization of annexin VI

Immunohistochemical localization of annexin VI in rat liver (Fig. 3a) showed the annexin to be predominantly located on the sinusoidal membrane of the hepatocyte with some cytoplasmic staining and enhanced concentration on bile canalicular membranes. There was little staining of the lateral membranes. The overall staining of liver was uneven (not shown), with some areas of the tissue relatively unstained, despite their apparent morphological identity to the region shown in Fig. 3a. Sections treated with pre-immune serum (Fig. 3b), or with antibodies to rat annexin VI pre-adsorbed with the purified antigen (not shown) were unstained.

In rat small intestine (Fig. 3c), antibodies to annexin VI stained the vascular tissue in the core of the villi (lamina propria) and the smooth muscle layers, including the muscularis mucosae. The enterocytes and goblet cells of the epithelium were unstained. Unlike liver, intestinal tissue exhibited the same staining pattern throughout the section. Control sections treated as for rat liver were unstained. (pre-immune serum Fig. 3d; antibodies adsorbed with annexin VI not shown).

3.5. Characterization of the association of annexin VI with membranes

Annexin VI binds to a membrane fraction derived from liver in a Ca^{2+}-dependent manner in 20 mM Hepes-KOH, 100 mM KCl, 2 mM $MgCl_2$ at pH 7.4. The free Ca^{2+} concentration required for the binding of annexin VI to the membrane fraction in the presence of 5 mM ATP was significantly lower than that observed with no nucleotide present, being half-maximal at ~ 3 μM free Ca^{2+} and maximal at 100 μM free Ca^{2+} (Fig. 4).

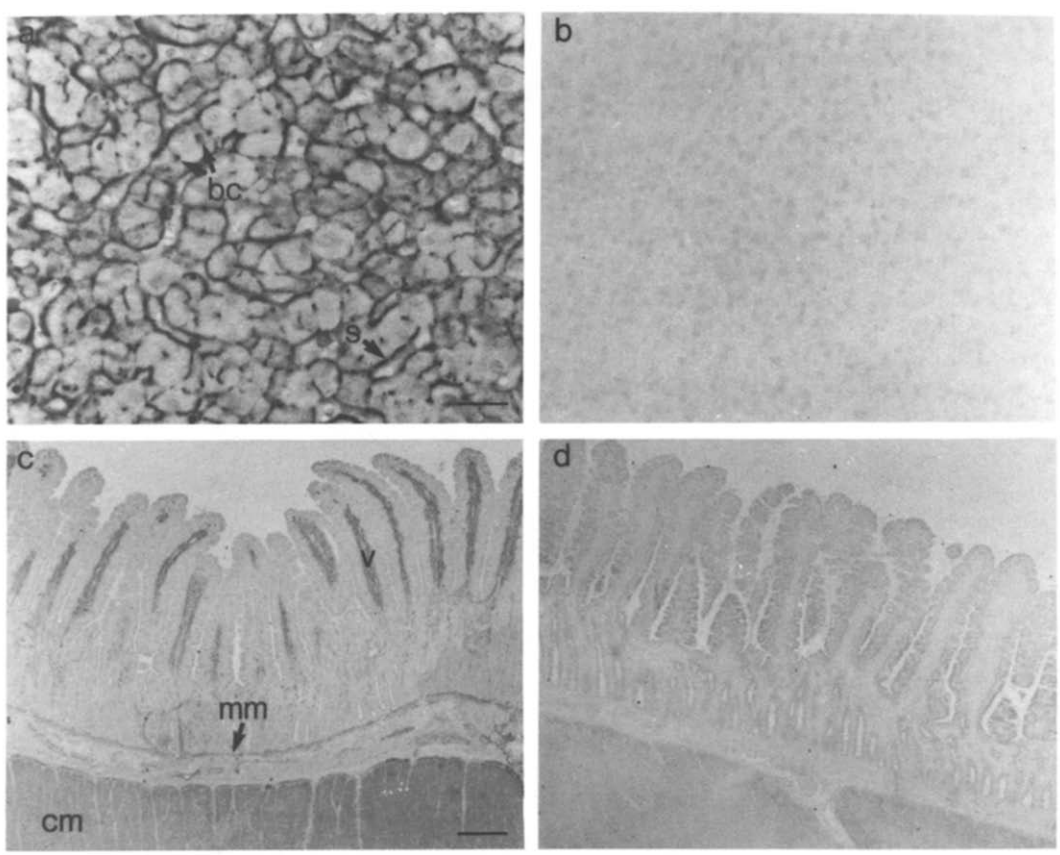

Fig. 3. Immunoperoxidase localization of annexin VI. Annexin VI localization in rat liver and intestine demonstrated by immunoperoxidase histochemistry. (a) Rat liver treated with antibodies to annexin VI shows prominent membrane staining of the sinusoidal phase of the hepatocyte (s). Bile canaliculi are also prominently stained (bc). Nuclei are counterstained with haematoxylin. (b) Rat liver treated with pre-immune serum. Nuclei are counterstained with haematoxylin. (c) Rat intestine treated with antibodies to annexin VI. Vascular tissue in the core of the villi (v) and smooth muscle tissue are stained. cm, circular muscle; mm, muscularis mucosae. (d) Rat intestine treated with pre-immune serum. Bars: (a,b) = 25 μm; (c,d) = 250 μm.

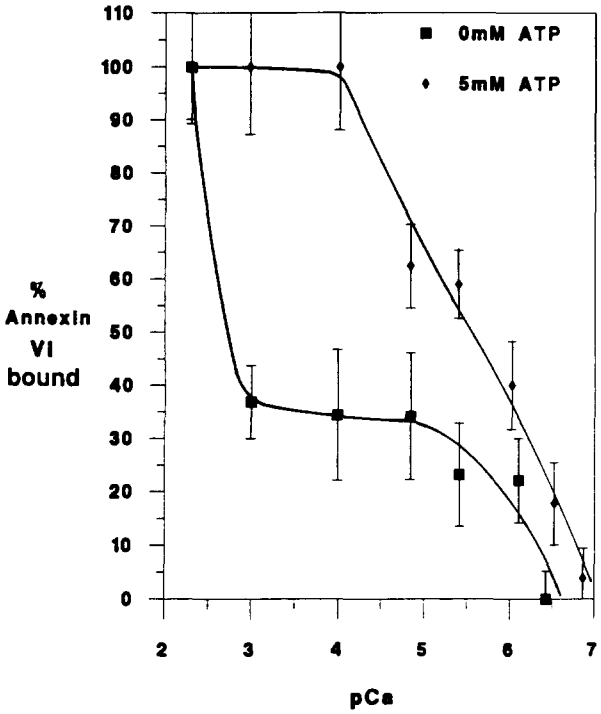

Fig. 4. Calcium-dependent association of rat liver annexin VI with a membrane fraction in the presence and absence of ATP. The graph shows the percentage of annexin VI in rat liver that is bound to the particulate fraction at varying free calcium concentrations (pCa) in the absence of ATP (■), and in the presence of 5 mM ATP (♦). The percentage of bound annexin VI was estimated by subtraction of the non-pelletable annexin VI at the various concentrations of free Ca^{2+} from the total amount of annexin VI in the first supernatant in the absence of Ca^{2+} and ATP. Bars represent mean ± S.D. ($n = 3$).

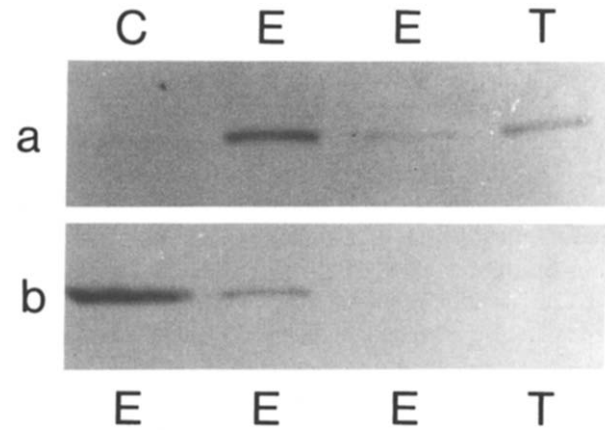

Fig. 5. Ca^{2+}-independent association of rat liver annexin VI with membranes. Immunoblots of samples of supernatants following homogenization and centrifugation of rat liver in buffer containing either: (a) Ca^{2+} (lane C), followed by two washes in EGTA-containing buffer (lanes E), and one wash in Triton X-100-containing buffer (lane T) or (b) EGTA, followed by two further washes in EGTA-containing buffer (lanes E), and one wash in Triton X-100-containing buffer (lane T).

The yield of annexin VI in the purification procedure suggested that a significant proportion of the annexin was not extracted from membranes by EGTA after calcium-dependent binding. We therefore subfractionated liver in the presence or absence of calcium and looked for the presence of annexin VI in membrane and supernatant fractions. Rat liver was homogenised in the presence of Ca^{2+} and ATP, and the pellet washed twice with EGTA until no further annexin VI could be extracted into the supernatant (Fig. 5a, lanes C and E). Addition of Triton X-100 solubilized a further fraction of annexin VI, amounting to approximately 31% of the total (Fig. 5a, lane T). However, in the case of rat liver homogenised immediately in buffer containing EGTA and ATP (Fig. 5b), all the annexin VI was extracted by three washes in the buffer (lanes E), and no further annexin VI was released on addition of Triton X-100 (lane T).

The irreversible association of annexin VI with membranes after Ca^{2+}-dependent binding could be due to an increase in hydrophobicity of the protein leading to integration into the membrane. To investigate the hydrophobicity of rat liver annexin VI bound irreversibly to membranes after calcium-dependent association, Triton X-114 phase separation studies were performed according to the method of Bordier [37], on membranes prepared in buffer containing either

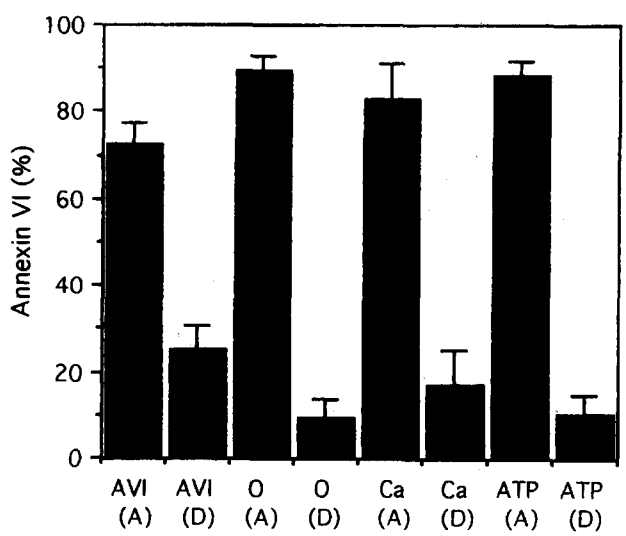

Fig. 6. Triton X-114 phase separation of purified and membrane-bound annexin VI. Proportions of annexin VI in the detergent (D) and aqueous (A) phases of Triton X-114 phase separation performed on various fractions of rat liver. AVI represents purified annexin VI, O represents annexin VI in the rat liver membrane pellet washed with buffer containing EGTA, Ca represents the annexin in the membrane pellet prepared in the presence of buffer containing 1 mM Ca^{2+} and ATP represents the annexin in the membrane pellet prepared in the presence of 1 mM Ca^{2+} and 5 mM ATP. Error bars represent mean ± S.D. ($n = 3$).

EGTA, Ca^{2+}, or Ca^{2+} and ATP. The results showed that there was no significant difference in the proportion of annexin VI partitioning into the detergent or aqueous phases for membranes prepared in buffer containing 10 mM EGTA (Fig. 6, O), membranes prepared in the presence of buffer containing 1 mM calcium (Fig. 6, Ca) or in buffer containing 1 mM calcium plus 5 mM ATP (Fig. 6, ATP) at $P < 0.05$ using Student's t-test (Fig. 6). These results indicate that Ca^{2+}-independent binding of annexin VI to membranes in the absence of Ca^{2+}, in the presence of 1 mM Ca^{2+}, and in the presence of 1 mM Ca^{2+} plus 5 mM ATP does not involve an increase in hydrophobicity of the protein above that of the purified annexin (Fig. 6, AVI).

4. Discussion

This paper has described the purification and characterization of annexin VI from rat liver. As reported previously for porcine cerebrum [26], significant amounts of clathrin heavy chain were also present in the rat liver annexin preparation. There is therefore a possibility of some interaction between annexins or some other protein in the final EGTA elution wash, and clathrin heavy chain. Signficantly, Lin and coworkers [41] have shown that annexin VI is required, in the presence of ATP and Ca^{2+}, for the budding of clathrin-coated pits from isolated cell membranes.

A wide distribution of annexin VI has been found in rat tissues. Annexin VI is particularly abundant in liver, muscle and heart, and is also present in spleen, brain, lung and kidney. This agrees well with the results of Smith and Dedman [42], but differs from those of Giambanco et al. on 1- to 60-day-old rats [43]. The abundance of annexin VI in muscle is consistent with its proposed function as a modulator of Ca^{2+}-channel activity in sarcoplasmic reticulum [18]. This study has also demonstrated the presence of annexin VI in small intestine, and in the accessory sexual organs of the male rat. The relatively large amounts in the latter, namely the seminal vesicles, anterior prostate and ventral prostate, may also indicate a role of annexin VI in Ca^{2+}-dependent processes involved in secretion of seminal fluid by these organs. However, we found no annexin VI in rat seminal vesicle secretory fluid. Consistent with these results, Pepinsky et al. [44] found annexins I, III and V, but not II, IV or VI in another extracellular fluid, namely rat peritoneal exudates. Similarly, Christmas et al. [45] reported the secretion of annexins I and V, but not IV or VI, in human seminal plasma.

The immunohistochemical studies on annexin VI in rat liver show it to be predominantly plasma membrane-associated, localizing to the sinusoidal and canalicular domains. A plasma membrane location has been reported previously for annexin VI [17,26,46]. The concentration of annexin VI in these domains may be linked to a role in cation transport across membranes not dissimilar to the modulation of the sarcoplasmic reticulum Ca^{2+}-release channel described in muscle [18,47]. It is also of interest that calcium plays a role in several bile canalicular functions, including secretion, canalicular contraction and tight-junction permeability [48–50]. The pattern of annexin VI staining in rat liver is distinct from the mainly cytoplasmic staining pattern of annexin V in rat [51] and chicken [33] livers. However, both annexins show uneven levels of staining in liver, despite the morphological similarity of the stained and unstained areas of tissue. The reason for this non-uniform staining is not clear, but it has also been observed for annexin VI in several human tissues, including the zona fasciculata of adrenal cortex, and thyroid cells [52], where it may be due to different functional states of the cells at the time the tissue was removed. Clark et al. [52] were unable to find annexin VI immunoreactivity in immunohistochemical studies of human liver. The reason for this is not clear.

The localization of annexin VI in the lamina propria and smooth muscle layers of rat intestine is similar to that reported by Glenney [53] for annexin VI in bovine intestine. The lack of staining of the epithelial cells agrees with the results of Glenney [53] and Clark et al. [52]. The uniform nature of the staining in intestine is in contrast to the uneven appearance of annexin VI in liver.

After homogenization in the presence of Ca^{2+}, approximately 31% of rat liver annexin VI was soluble only in the presence of 1% (w/v) Triton X-100. Bianchi et al. [22] reported a similar finding in bovine heart, lung and brain where about 40% of annexin protein partitioned in the detergent phase after Triton X-114 solubilization of an EGTA-insoluble form. In our work, no significant difference in the hydrophobicity of the EGTA-soluble and EGTA-insoluble forms of annexin VI was observed using Triton X-114 phase separation. Annexin VI binding to Ca^{2+} does not expose hydrophobic domains that would allow the protein to integrate into membranes [54], and there is no evidence to date of a lipid anchor. The binding of annexin VI to membranes may therefore involve an association with some other membrane component.

The binding of annexin VI to a membrane fraction (Fig. 4) demonstrates a pronounced decrease in the free calcium concentration required for binding at physiological concentrations of ATP. Burgoyne and Geisow [55] reported that the Ca^{2+}-dependent binding of annexin VI to chromaffin granule membranes was increased by ATP, and that separation from other

annexins could be achieved using ATP-agarose affinity chromatography. The means by which Ca^{2+} and ATP synergistically increase the association of annexin VI with membranes in a Ca^{2+}-dependent manner, both reversibly and irreversibly, is unknown. It is also at present unclear whether this occurs to any significant extent in vivo. Previous studies of the binding of annexin VI to phospholipid vesicles and chromaffin granules have demonstrated half-maximal binding in the μM range of free Ca^{2+} concentrations [32]. It has been difficult to explain the subcellular localization of this annexin in the light of the existing binding data since even in stimulated cells free Ca^{2+} levels are rarely in the μM range. Our present study demonstrates that under physiological conditions and concentrations of ATP there is potentiation of association to the particulate fraction generally and to membranes in particular. We therefore suggest that factors other than free Ca^{2+} concentration alone may be important in the binding of annexin VI to specific cell membranes. The possibility of different forms of annexin VI with different affinities for various components of the membrane fraction must also be considered.

Subfractionation in the presence of calcium (Fig. 5) shows the formation of a tightly membrane-associated form of annexin VI which resists extensive extraction with EGTA and requires Triton X-100 for its solubilisation. Physiological relocation of annexin V [56] and annexin II (Trotter, P.J., Orchard, M.A. and Walker, J.H., unpublished data) to membranes in a manner which resists extraction by EGTA has been observed in platelets. This suggests that annexins can interact with membranes in at least two distinct ways: a reversible Ca^{2+}-dependent mechanism, and a second manner requiring Ca^{2+} initially but subsequently independent of Ca^{2+}. The membrane-associated form does not show increased hydrophobicity as judged by Triton X-114 partitioning, which could argue for binding of annexin VI to a membrane component other than phospholipid.

In conclusion, annexin VI has been shown to be a major cellular component of the rat hepatocyte. It has been localized to the plasma membrane and, in keeping with the findings of other workers, we have found that significant amounts can be associated with membranes in an EGTA-insoluble, Triton X-100-extractable form [22,57]. The EGTA-resistant membrane-associated form of annexin VI can be created by homogenization of tissue in Ca^{2+}-containing buffer. The membrane localization of annexin VI may indicate a possible role in membrane trafficking and/or cation transport processes in both the sinusoidal and canalicular domains of the rat hepatocyte membrane. The presence of ATP has been found to alter significantly the binding of annexin VI to the particulate fraction. Further investigation of the Ca^{2+}-dependent and Ca^{2+}-independent association of annexin VI with membrane elements is underway.

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