Biochimica et Biophysica Acta, 1105 (1992) 201–206

BBAMEM 75583

Annexins IV (p32) and VI (p68) interact with erythrocyte membrane in a calcium-dependent manner

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(Received 13 August 1991)
(Revised manuscript received 6 January 1992)

Key words: Annexin; Erythrocyte plasma membrane; Membrane–protein interaction; (Porcine liver)

Purification of annexin IV and VI from porcine liver was achieved by Mono Q ion exchange chromatography at pH 8.9 and pH 7.5, respectively. The isolated proteins interacted with erythrocyte membrane as function of calcium ion and the protein concentration. Half-maximal binding of annexin VI to erythrocyte membrane was found to occur at 8 μM Ca^{2+}. The maximal binding was estimated as 2 μg of annexin VI per 1 μg or erythrocyte membrane protein, in the presence of 100 μM Ca^{2+}. The property of erythrocyte membrane to interact with annexins was utilized in preparation of a affinity-column with polyacrylamide-immobilized erythrocyte membrane.

Introduction

Recently, a new family of Ca^{2+}-binding proteins, interacting with the anionic phospholipids of cell membranes, has been isolated from various cells and tissues [1–6]. While originally described under different names (lipocortins, calpactins I and II, calelectrins, annexins, chromobindins or, because of their molecular weight, p32, p35, p36 and p68) [7,8], they are now classified as one superfamily of annexins [9].

Annexins are localized on the cytoplasmic side of plasma membrane sometimes in association with the cytoskeleton [1,5,10–16]. They have been shown to be the substrates of tyrosine kinase [6,17]. However, little is known concerning their physiological role in living cells. There are suggestions that annexins might be involved in regulation of membrane traffic, during endo- and exocytosis, in interactions of membrane with cytoskeleton components, and in mitogenic signal transduction [1,5,6,11,17–20].

Abbreviations: DTT, dithiothreitol; EGTA, ethyleneglycol bis(2-aminoethyl ether)-*N*,*N*′-tetraacetic acid; M_r, relative molecular mass; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane.

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Despite their different molecular mass (from 32 to 68 kDa), annexins are structurally related. Their primary structure is characterized by the presence of four to eight conservative domains (72 amino acid residues) essential for binding of the protein to anionic phospholipids in a calcium-dependent manner [21,22]. This property has been successfully used in time purification of annexins.

In this report we describe a method for the purification of annexins IV (p32) and VI (p68) by ion exchange chromatography on a Mono Q resin or by affinity column chromatography using erythrocyte ghosts 'embedded' in polyacrylamide gel. The Ca^{2+}-dependent interaction of annexins with erythrocyte membrane is also shown.

Materials and methods

Chemicals

Aprotinin, DTT and Tris were purchased from Sigma. EGTA and SDS were obtained from Serva. Mono Q (HR 5/5) column was from Pharmacia. PMSF was purchased from Calbiochem. Acrylamide and bisacrylamide were obtained from Bio-Rad. TEMED was from Fluka AG, Buchs SG. All other chemicals the highest purity were purchased from POCH (Poland).

Isolation of annexins

The proteins were isolated from fresh or frozen porcine liver by the similar procedure for purification

of the 32 kDa protein from human monocytes [23]. Briefly, 100 g wet liver tissues was cut into small pieces and homogenized in 200 ml buffer solution A (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.2 mM PMSF, 5 TIU/l aprotinin and 5 mM EGTA) using a Polytron homogenizer (3 × 20 s, setting 3) at 0°C. The homogenate was filtered through cheese-cloth and then centrifuged at 15000 × *g* for 15 min. This was followed by centrifugation of supernatant at 39000 × *g* for 30 min. The supernatant was supplemented with 6.5 mM $CaCl_2$, gently stirred for 30 min at 0°C and centrifuged at 39000 × *g* for 30 min. The calcium-precipitated proteins were washed with buffer solution A in which EGTA was replaced by 1 mM $CaCl_2$, and spun down at 39000 × *g* for 30 min. The pelleted material was resuspended in buffer solution A with 10 mM EGTA and, after 30 min the suspension was centrifuged at 100000 × *g* for 1 h. The 100000 × *g* supernatant was dialyzed overnight against of 20 mM Tris-HCl (pH 7.5), 10 mM NaCl (buffer B). A further purification of the annexins was achieved by either ion exchange or affinity column chromatography.

Ion exchange chromatography was carried out at room temperature on a Mono Q (HR 5/5) column using the Pharmacia FPLC equipment. The column was equilibrated with either 20 mM Tris-HCl (pH 7.5) or 20 mM ethanolamine (pH 8.9). The 5 ml samples (10 mg protein) in either Tris-HCl or ethanolamine buffers were loaded onto the column. The proteins bound to the Mono Q were eluted with NaCl (gradient 0 to 0.4 M, flow rate 1 ml/min) in the same buffer solution as used for equilibration of the samples.

The affinity chromatography column was prepared according to the procedure described for immobilization of phosphatidylserine liposomes in polyacrylamide gels [24]. Briefly, 2 ml of erythrocyte ghosts (4.5 mg of membrane protein per ml), prepared as in Ref. 25, were vigorously mixed with 10 ml of 15% acrylamide/5% bisacrylamide and with 100 μl of 14% ammonium persulfate and 5 μl of TEMED. The mixture was left for 2 h at room temperature. The obtained gel was rinsed and homogenized in water with a loose-fitting homogenizer and then resuspended in 50 ml solution of 20 mM Tris-HCl (pH 7.5)/0.2 mM DTT/100 mM NaCl/2 mM $CaCl_2$/0.1 mM PMSF.

5 ml of crude annexin fraction (10 mg protein; 100000 × *g* supernatant) was mixed with an equal volume of buffer solution B supplemented with 4 mM $CaCl_2$ and 1 ml portions were loaded onto the affinity chromatography column. After the last portion was loaded, the column was washed with 20 ml of buffer solution B supplemented with 2 mM $CaCl_2$. This was followed by a further washing with 30 ml of the same solution but with $CaCl_2$ concentration reduced to 0.1 mM. The proteins bound to the immobilized erythrocyte membrane were eluted with buffer solution B containing 2 mM EGTA. 1-ml fractions were collected and analysed by SDS-PAGE.

Binding of annexins to erythrocyte membrane

5–10 μg protein of purified annexins (32 and 68 kDa) or crude fraction (100000 × *g* supernatant), were incubated for 20 min at 20°C with human erythrocyte membrane (130 μg protein) in 20 mM Tris-HCl (pH 7.5)/100 mM NaCl (buffer solution C) in the presence of either 0.5 mM $CaCl_2$ or 2 mM EGTA. Then, the erythrocyte ghosts were spun down at 10000 × *g* for 10 min (the first supernatant) and resuspended in buffer solution C supplemented with 2 mM EGTA. After 20 min incubation at room temperature, the samples were again centrifuged for 10 min at 10000 × *g* (the second supernatant). The protein composition of both supernatants was analysed by SDS-PAGE. The amount of proteins in supernatants was quantified by laser scanning densitometry. When the binding of annexins to the membrane was studied as a function of Ca^{2+} concentration, the level of free calcium ions in annexin/erythrocyte membrane mixture was monitored by a Ca^{2+}-selective electrode (Orion Res. Inc., USA).

Other procedures

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 10% separating acrylamide gel with 3% stacking gel, according to Laemmli [26]. Protein concentration was determined by the Bradford method [27].

Results and discussion

Homogenization and extraction of liver tissue in solution containing 5 mM EGTA followed by precipitation of soluble proteins in the presence of Ca^{2+} (see Materials and Methods) resulted in isolation of a group of proteins in which two with molecular masses of 32 kDa and 68 kDa were found in largest amount (Fig. 1, lane S). Further purification of these proteins was achieved by strong anion exchange chromatography on a Mono Q resin developed with a linear NaCl gradient. At pH 7.5, the proteins were eluted as three major peaks (peaks A-C, Fig. 1A): peak C contained a single polypeptide of M_r 68 kDa, as determined by SDS-PAGE, while protein of M_r 32 kDa together with a band of M_r 36 kDa were identified in peak A (Fig. 1B). At pH 8.9, a similar elution profile as at pH 7.5 was observed (Fig. 2A). In this case, however, peak A comprised of a single polypeptide of M_r 32 kDa. In peak C, in addition a polypeptide of M_r 68 kDa, a 36 kDa protein was found (Fig. 2B). Therefore, it is worth noting that by altering pH values of the elution buffers it is possible to purify either the protein of M_r 32 kDa or 68 kDa. Overolading of 10% polyacrylamide gels with the purified proteins did not reveal any other

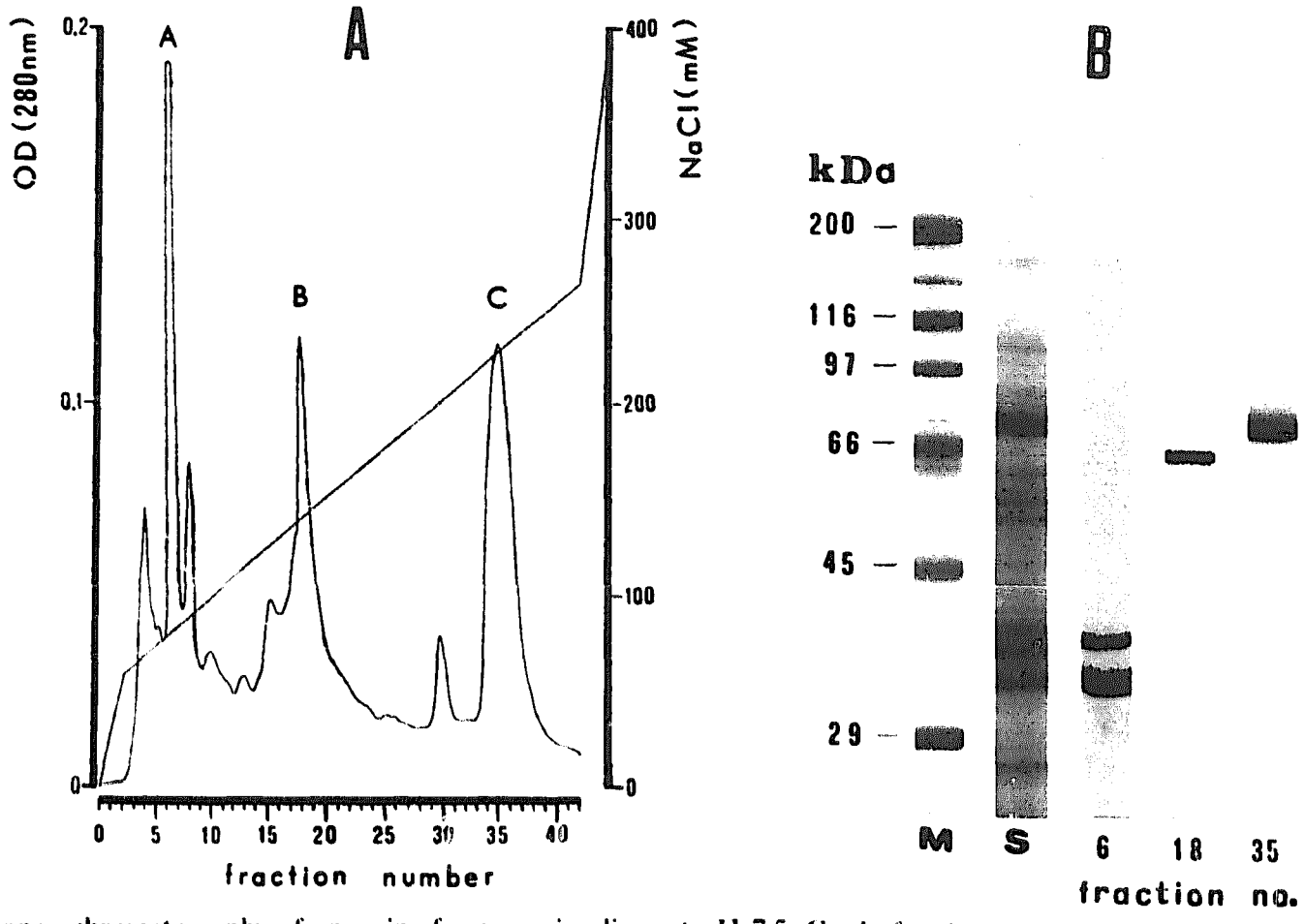

Fig. 1. (A) Ion exchange chromatography of annexins from porcine liver at pH 7.5. Crude fraction of annexins (100000 × *g* supernatant) was loaded onto a Mono Q column and the proteins were eluted with gradient of 0–400 mM NaCl (see Methods). (B) Fractions were analyzed by SDS-PAGE. Lane M, molecular weight standards: myosin (M_r 200000), β-galactosidase (M_r 116000), phosphorylase *b* (M_r 97400), bovine serum albumin (M_r 66200), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000); lane S, starting material.

lower or higher molecular weight polypeptides (Figs. 1 and 2). To check the homogenity of 32 kDa and 68 kDa polypeptides, their minute amounts were analyzed on 14% polyacrylamide gels. In this case, only single tiny bands with no commigrating polypeptides were observed. Also a single spot was detected when 32 kDa

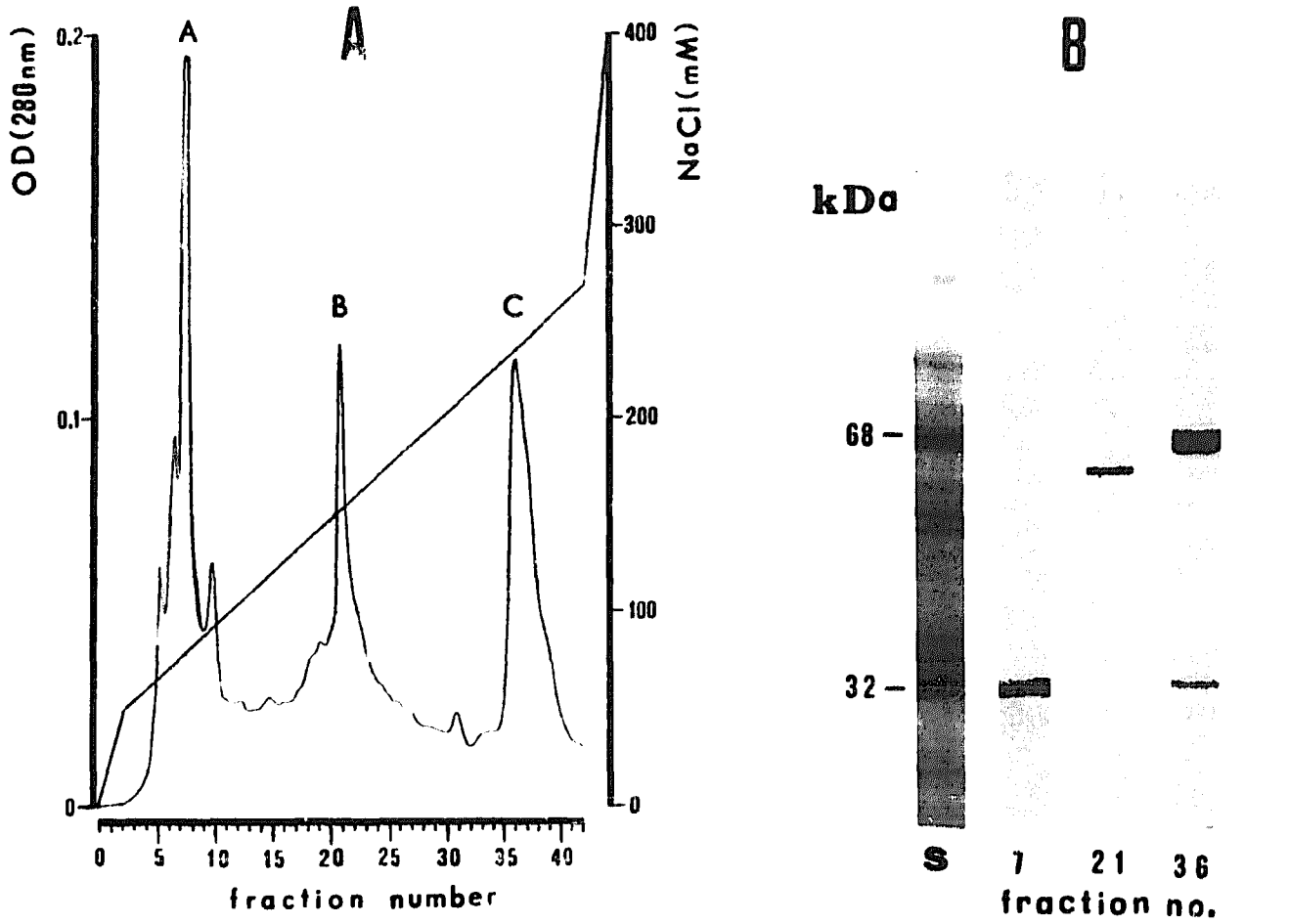

Fig. 2. (A) Ion exchange chromatography of annexins from porcine liver at pH 8.9. Crude fraction of annexins (100000 × *g* supernatant) was loaded onto a Mono Q column and the proteins were eluted with a gradient of 0–400 mM NaCl. (B) SDS-PAGE analysis of some of the column fractions. Lane S, starting material.

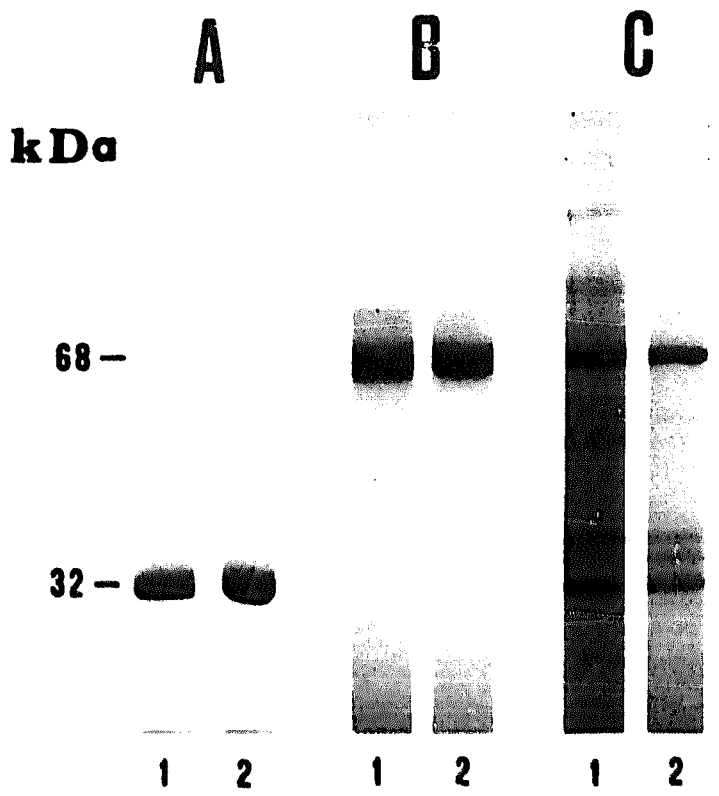

Fig. 3. Binding of annexins to erythrocyte ghost membranes. 5 μg of the purified proteins: (32 kDa (A) and 68 kDa (B)) or 10 μg of crude annexins (100000 $\times$ g supernatant) (C), were supplemented with either 0.5 mM $CaCl_2$ or 2 mM EGTA and were analyzed by SDS-PAGE. Lane 1, represents the protein pattern of supernatant obtained after incubation of sample containing annexins and erythrocyte ghosts (130 μg of membrane protein) in the presence of 2 mM EGTA. Lane 2, the annexins were mixed with erythrocyte membrane (130 μg of membrane protein) in the presence of 0.5 mM $CaCl_2$. After incubation the pellet was extracted with 2 mM EGTA and proteins of the final supernatant were analyzed.

protein was analyzed by two-dimension gel electrophoresis (data not shown). The purified proteins of 32 kDa and 68 kDa were similar to those found in liver by Südhof et al. [2] and by Shadle et al. [4], being Ca^{2+}-sensitive, phospholipid- (membrane-) binding proteins (see also our further data). Therefore, we classified them as annexin IV and annexin VI according to current annexin nomenclature [28].

To examine the ability of purified proteins or crude fraction of annexins (100000 $\times$ g supernatant) to bind to the plasma membrane, they were incubated with human erythrocyte ghosts, which were shown to contain no annexins neither in cytosol nor attached to plasma membrane [4].

Sedimentation of the annexins with erythrocyte membrane revealed that both annexin IV and VI bound to the membrane in a Ca^{2+}-dependent manner (Fig. 3). The membrane-bound annexins could be detached from ghosts by treatment with 2 mM EGTA (Fig. 3, lines 2). The interaction of erythrocyte ghosts with annexin VI was further studied. Incubation of annexin VI in the presence of increasing concentrations of $CaCl_2$ (0–100 μM) demonstrated that at 40 μM $CaCl_2$ the whole amount of the protein cosedimented with the ghost membranes (Fig. 4A). Half-maximal binding of annexin VI to erythrocyte ghost was observed at 8 μM $CaCl_2$ (Fig. 4A).

Binding of annexin VI to erythrocyte membrane was also studied as a function of protein concentration. Binding was saturable in the presence of 200 μg of annexin (maximal binding was 2 μg annexin per 1 μg of ghost protein, Fig. 4B).

This property of annexins was utilized in their purification by affinity-column chromatography. Erythrocyte ghosts, immobilized in a polyacrylamide gel, were able to bind annexin IV and VI in the presence of Ca^{2+}. Elution of the column with buffer containing EGTA released a fraction of proteins mainly of M_r 32 and 68 kDa but also of 36 kDa (Fig. 5).

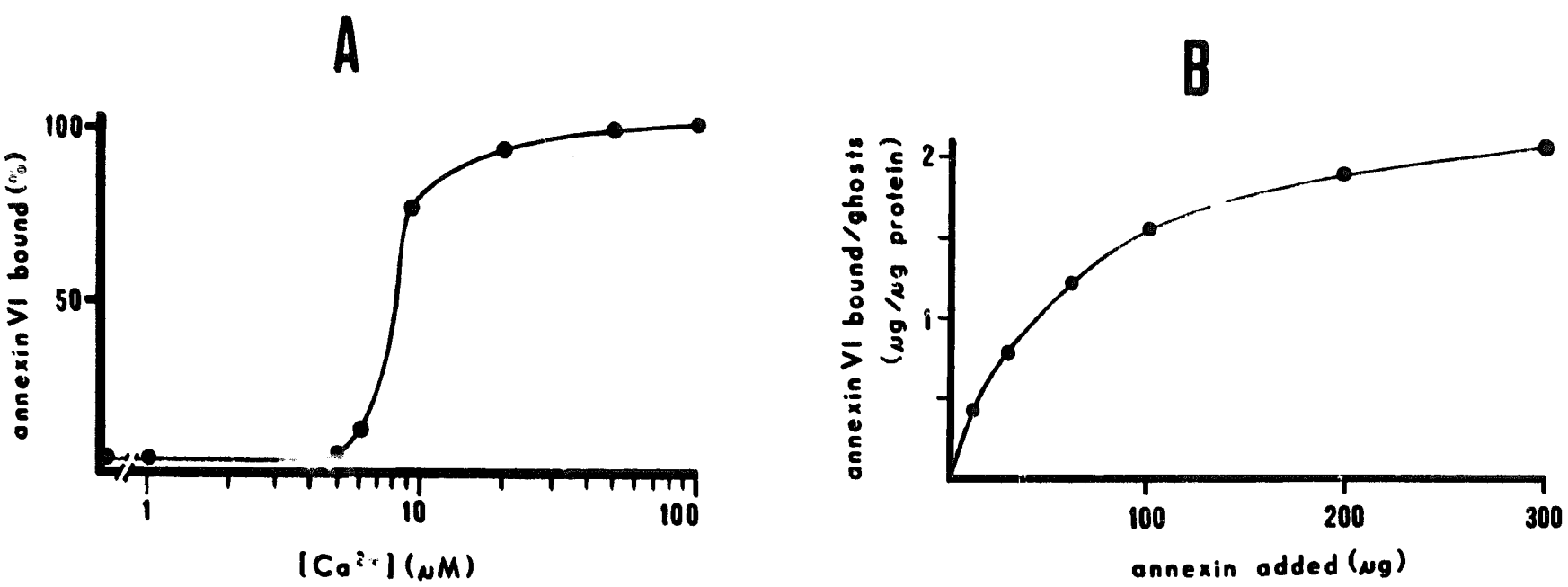

Fig. 4. (A) Binding of annexin VI to erythrocyte ghost as a function of $CaCl_2$ concentration. 20 μg of annexin VI was incubated with erythrocyte ghosts (130 μg of membrane protein) in the presence of 0–100 μM $CaCl_2$. (B) Binding of annexin VI to erythrocyte ghosts as a function of protein concentration. 10–300 μg of annexin VI was incubated with erythrocyte membrane (130 μg of membrane protein) in the presence of 0.5 mM $CaCl_2$. (A and B) The protein was incubated with erthrocyte membranes in the presence of $CaCl_2$. 30 min later the membranes were separated from unbound proteins by centrifugation and the resulting pellets were treated with 2 mM EGTA. The EGTA-extracted proteins were analyzed by SDS-PAGE. The amount of protein (presented in relative units) was quantified by laser scanning densitometry.

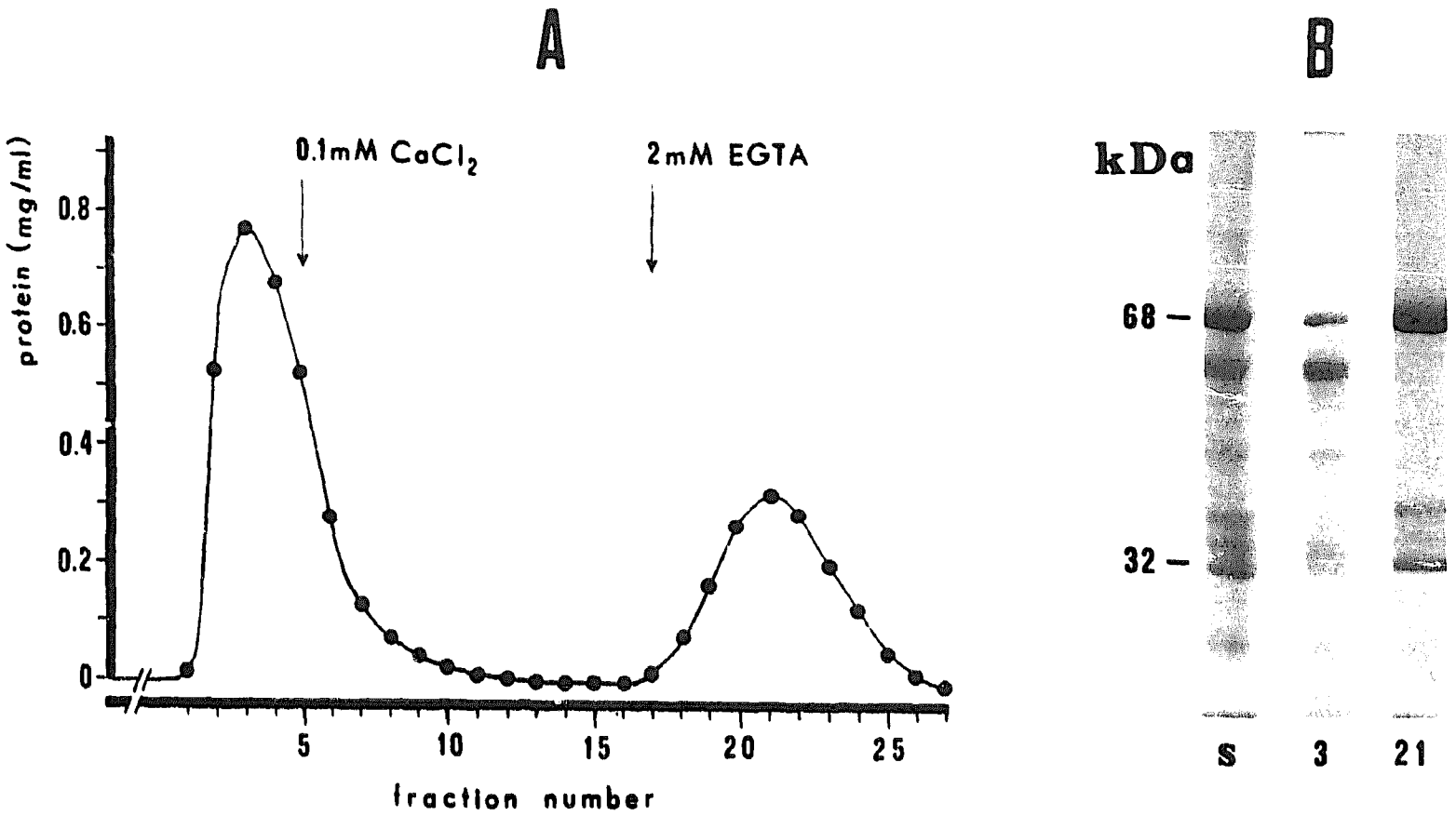

Fig. 5. Affinity chromatography of annexins. (A) Crude fraction of annexins (100000× *g* supernatant, see Material and Methods) was loaded onto polyacrylamide-immobilized erythrocyte membranes in the presence of 2 mM $CaCl_2$. At the points indicated by arrows, $CaCl_2$ in the column solution was reduced to 0.1 mM and then replaced by 2 mM EGTA (as indicated). (B) SDS-PAGE of the eluted fractions. Lane S, the crude annexin sample before the column; fraction 3, flow-through the affinity column; fraction 21, proteins eluted by the addition of 2 mM EGTA.

In this report we describe a simple procedure for isolation of annexins IV and VI from porcine liver. The annexins bind to erythrocyte membrane in a calcium-dependent manner. For annexin-plasma membrane interactions the anionic phospholipids such as phosphatidylserine, phosphatidic acid, phosphatidylinositol, are crucial [10]. These phospholipids, being the components of erythrocyte membrane, seem to be involved in binding of the annexins. However, a high level of annexin VI bound by erythrocyte membrane (2 μg annexin VI per 1 μg of ghost membrane protein) suggests that beside annexin–phospholipids (–membrane) interactions, self-association/aggregation of annexin VI in the presence of Ca^{2+} may take place on the membrane as reported by Mosser et al. [29], Newman et al. [30], and Zaks and Creutz [31]. Nothing is still known about the ability of annexin IV and VI to interact with membrane proteins.

Most of the studies on annexin–membrane interactions were performed on liposomes as a model system of biological membranes. It was found, for example, that annexin I (p35), II (p36), and IV (p32) might promote aggregation and fusion of liposomes [32], while annexin VI (p68) inhibited this process [33,34]. The differences between members of annexin family to their ability to induce aggregation and fusion of liposomes may reflect the biological functions of these proteins during Ca^{2+}-regulated membrane traffic processes. The exocytosis and endocytosis can be considered as model systems for such studies. There are observations indicating the requirement of annexin II in aggregation of chromaffin granules at physiological concentrations of calcium ions [20]. The immunoelectron microscopy of frozen sections of chromaffin cells revealed that annexin II was indeed present on the cytoplasmic surface of plasma membrane forming a linker between membrane and adjacent chromaffin granule [11]. However, the question of the role of annexins in regulation of membrane traffic processes remains open. Studies of interactions of erythrocyte ghosts with annexins may help in future to solve the problem of a possible mechanism of aggregation and fusion of natural membranes.

Acknowledgement

This study was performed under the project CPBP 04.01 from the Polish Academy of Sciences.

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