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Purification and Characterization of Annexin Proteins from Bovine Lung†

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ABSTRACT: Calcium-dependent association with a detergent-extracted particulate fraction was used as the first step in the purification of a group of phospholipid binding proteins. Elution of the detergent-insoluble fraction with excess ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) resulted in the release of several soluble proteins, termed calcium-activated proteins or CAPs. In the present paper, we describe the simultaneous purification of these CAPs and characterize their interaction with phospholipid, actin, and calmodulin. Partial sequence analysis has identified the majority of the CAPs as members of the annexin family of calcium and phospholipid binding proteins. Two additional CAPs may be novel proteins, one of which appears to be an annexin protein. All CAPs demonstrated Ca^{2+}-dependent binding to phosphatidylserine vesicles but did not bind to phosphatidylcholine vesicles. The majority of CAPs exhibited Ca^{2+}-dependent binding to F-actin; however, only CAP-III affected the rate of conversion of G-actin to F-actin. The interaction of CAP-III and lipocortin-85 with F-actin resulted in a Ca^{2+}-dependent increase in both light scattering and sedimentation of F-actin under comparatively low centrifugal force. In contrast, only lipocortin-85 caused the formation of F-actin bundles. Although all of the CAPs bound to a calmodulin affinity column in a Ca^{2+}-dependent manner, attempts to demonstrate binding of CAPs to native calmodulin were unsuccessful. These studies therefore document the similar behavior of the CAPs toward phospholipid and calmodulin but clearly show that F-actin binding or bundling is not a general property of these proteins. The reported purification procedure should allow further comparative studies of these proteins.

Over the last few years, several laboratories interested in the role of calcium in the regulation of secretion and cell motility have identified a family of Ca^{2+} binding proteins distinct from the "EF domain" group of proteins [for reviews, see Klee (1988), Khanna et al. (1988), Crompton et al. (1988a), Dedman (1986), Geisow et al. (1988), and Glenney (1988)]. These proteins have been identified on the basis of their Ca^{2+}-dependent binding to membrane fractions and elution in the absence of Ca^{2+}. The membrane fractions used for isolation of these proteins have included chromaffin granule membranes [the ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)[1]-eluted proteins were called chromobindins; Creutz, 1981; Geisow & Burgoyne, 1982], the intestinal epithelial cell (the EGTA-eluted proteins were called proteins I-III; Gerke & Weber, 1985), smooth muscle membranes (Raeymaekers et al., 1985), liver (one of the mem-

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; CAP, calcium-activated protein; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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brane-associated proteins was named endonexin; Giesow et al., 1984), the electric organ membrane from the ray *Torpedo marmorata* (the EGTA-eluted protein was called calelectrin; Walker, 1982), detergent-extracted actin-rich cytoskeletons of lymphocytes (Owens et al., 1984), detergent-extracted bovine lung membranes (Khanna et al., 1986, 1987), or precipitated from bovine lung 100000*g* supernatants (the purified proteins were called calpactins; Glenney et al., 1987). Independently, Dedman (Moore & Dedman, 1982; Dedman, 1986) used a phenothiazine–Sepharose column to purify several proteins from smooth muscle homogenates that bound to this resin in a Ca^{2+}-dependent manner (the EGTA-eluted proteins were called calcimedins).

While a variety of these proteins have been purified from many tissues and referred to by many names, sequence information has suggested that all these proteins contain a 17 amino acid consensus sequence which is highly conserved and present in multiple copies (Giesow et al., 1986a,b). The consensus sequence has been named the annexin fold and the family of related proteins called the annexins. The sequence information available suggests that on average the annexins share about 40–50% amino acid sequence homology.

Because individual proteins have been purified from a variety of tissues and have been studied by many different laboratories, a unifying picture of the overall similarities and differences in the properties of these proteins has been difficult. Generally, the annexin family is characterized by their Ca^{2+}-dependent interaction with certain phospholipids including PS, PE, PI, and PA, but not PC (Gerke & Weber, 1984; Glenney, 1986; Creutz et al., 1983; Geisow et al., 1986a,b). The lipocortins (calpactins) are three annexin proteins which have been the most extensively characterized. The lipocortins have been shown to be substrates of protein tyrosine kinases; lipocortin II is a major substrate of $pp60^{src}$ (Gerke & Weber, 1984; Glenney, 1985) while lipocortin I is a substrate of the EGF receptor kinase (Fava & Cohen, 1984; De et al., 1986). The lipocortins are also phosphorylated in vivo by protein kinase C (Gould et al., 1986) with a stoichiometry nearing 1 mol of phosphate per mole of protein in vitro (Khanna et al., 1986, 1987).

Phospholipid binding has been demonstrated to increase the affinity of Ca^{2+} binding (Glenney, 1986a; Geisow et al., 1986a,b; Sudhof et al., 1984; Johnsson et al., 1986; Glenney et al., 1987; Schlaepfer & Haigler, 1987; Shadle & Weber, 1987) of several of the annexins. Furthermore, lipocortin-85 has been suggested to bind to calmodulin (Martin et al., 1988) and either bundle (Gerke & Weber, 1984) or sever F-actin (Martin et al., 1988). It has also been suggested that lipocortin I and lipocortin II are capable of bundling F-actin (Glenney et al., 1987). Whether or not F-actin binding or calmodulin binding is a general property of the annexins has not been resolved.

Bovine lung is a tissue especially rich in the annexin proteins. In the present paper, we report a simple and rapid purification procedure for several calcium-activated proteins (termed CAPs) present in this tissue. These proteins have been partially sequenced and compared with amino acid sequences of other purified annexin proteins. The majority of CAPs have been identified as annexin proteins while two CAPs may be novel proteins. The simultaneous purification of these proteins from a single tissue has allowed comparison of their interaction with phospholipid, actin, and calmodulin.

Experimental Procedures

Materials. Cyclic nucleotide phosphodiesterase and bovine brain calmodulin were a generous gift from Dr. R. K. Sharma (Department of Medical Biochemistry, University of Calgary).

Phospholipid Binding. Large unilamellar vesicles (LUV) used to determine the phospholipid binding characteristics of the CAPs were prepared according to Reeves and Dowben (1969). The vesicles (typically 200–300 μM) and CAP (typically 2–10 μg of protein) were suspended in 20 mM HEPES (pH 7.4), 0.2 mM EGTA, 2 mM $MgCl_2$, 0.1 M NaCl, and, where indicated, 1.2 mM Ca^{2+} at a final volume of 0.1 mL. Samples were incubated for 10 min at room temperature and centrifuged (20 min, 15600*g*). The supernatant (fraction S) was removed and the pellet adjusted to 0.1 mL in incubation buffer, vortexed, and recentrifuged. The supernatant (fraction W) was removed and the pellet adjusted to 0.1 mL (fraction P). Fractions S, W, and P were lyophilized, adjusted to 0.02 mL in SDS sample buffer, heated in boiling water for 2 min, and subjected to SDS–PAGE (Laemmli, 1970).

Interaction with Actin. Actin was purified from fresh rabbit skeletal muscle by the method of Pardee and Spudich (1982) and MacLean-Fletcher and Pollard (1981). After chromatography through Sephadex G-100 (Pharmacia), fractions were examined on polyacrylamide gels to establish purity. G-Actin was dialyzed against 1 L of G-actin buffer [2 mM Tris (pH 8.0), 0.33 mM ATP, and 0.5 mM DTT] without DTT for 12 h. A 10-fold molar excess of *N*,*N*-pyrenyliodoacetamide (Molecular Probes, Eugene, OR) was mixed in 0.2 mL of dimethylformamide (Fisher) and sonicated in a Braun water bath until it formed an even suspension. The mixture was then added to the rapidly stirring actin solution. KCl and $MgCl_2$ were immediately added to final concentrations of 100 and 2 mM, respectively. The pyrene/actin solution was then wrapped in foil to protect the solution from light and gently rotated end-over-end at room temperature to allow complete mixing of the pyrenyliodoacetamide. After 18 h, the mixture was centrifuged at 150000*g* for 90 min; the pellet was carefully washed with G-actin buffer and resuspended with a glass/Teflon homogenizer in a 5-mL volume of G-actin buffer. The pyrene-labeled F-actin was then dialyzed against 4 × 1 L of G-actin buffer for 48 h. The G-actin was clarified by centrifugation at 150000*g* for 90 min. It was then passed through a 2.5 cm × 30 cm Sephadex G-100 column equilibrated with G-actin buffer to remove unbound pyrene. The concentration of pyrene-labeled actin and the molar ratio of pyrene to actin were calculated by the method of Cooper et al. (1983). Typically, 80–90% of actin was labeled.

Kinetics of Actin Polymerization. Fluorescence studies were performed in a Perkin-Elmer spectrofluorometer. Excitation was set at 365 nm, and emission was detected at 407 nm; slit widths were set at 2 and 10 nm, respectively (Kouyama & Mihashi, 1981). The final actin concentration was 2–10 μM, of which 5–20% was pyrene-labeled actin. The actin was taken from stock solutions of 2–3 mg/mL and diluted with G-actin buffer containing 50 μM $MgCl_2$ and 50 μM EGTA, pH 8.0, to a final volume of 1 mL. After incubation at room temperature for 8 min, polymerization was induced by addition of KCl to 100 mM and $MgCl_2$ to 1 mM. The monomeric actin concentration was determined by centrifugation of samples at 100000*g* for 60 min, followed by measurement of actin in supernatants. Under these conditions, 85–90% of monomeric actin was polymerized by addition of 100 mM KCl and 1 mM $MgCl_2$.

Interaction with F-Actin. Actin bundling was determined by light scattering at 400 nm (2-nm slit width) by spectrofluorometry or by low-speed centrifugation (15600*g*, 10 min). Each bundling reaction (0.1 mL) was incubated at room

temperature for 30 min with 1.16 μM F-actin in buffer A (2 mM Tris-HCl, pH 7.5, 0.3 mM ATP, 0.01% NaN_3, 0.5 mM DTT, 100 mM KCl, 1 mM $MgCl_2$, and 0.2 mM EGTA) and where indicated 1.2 mM Ca^{2+}. For centrifugation assays, the 15600*g* pellet was adjusted to 0.1 mL with buffer A, and the supernatant and pellet were analyzed by SDS–PAGE.

Actin binding was performed in buffer A. Following incubation with 2.3 μM F-actin at room temperature for 2 h, the binding reaction (0.1 mL) was centrifuged (100000*g*, 60 min) the supernatant removed, and the pellet adjusted to 0.1 mL in buffer A. Both pellet and supernatant were lyophilized and analyzed by SDS–PAGE.

Interaction with Calmodulin. CAPs (EGTA-eluted fraction) were applied to a calmodulin affinity column (a generous gift of Dr. R. K. Sharma, Department of Medical Biochemistry, University of Calgary) equilibrated with buffer B (40 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.25 M NaCl, and 0.5 mM DTT) containing 0.5 mM Ca^{2+}. The column was extensively washed with this buffer followed by washes with buffer B containing 0.1 mM Ca^{2+} and buffer B containing 5.0 mM EGTA. Fractions were collected and analyzed by SDS–PAGE.

The potential interaction of CAPs with calmodulin was tested by examining if the CAPs could compete with cyclic nucleotide phosphodiesterase for calmodulin. Cyclic nucleotide phosphodiesterase was assayed according to Teo et al. (1973) in the presence of 20 ng/mL calmodulin (sufficient for 75% activation of phosphodiesterase) and in the absence or presence of 10 μg/mL CAPs.

The interaction of CAPs with calmodulin was also tested by gel permeation chromatography. An HR 10/30 analytical column of Superose 12 (Pharmacia) was equilibrated with buffer B containing 0.5 mM Ca^{2+}. Calmodulin (100 μg) and individual purified CAPs (100 μg) were adjusted with buffer B and 0.5 mM Ca^{2+}, incubated for 1 h at 4 °C, and injected separately or in combination onto the column in a volume of 0.2 mL. Protein concentration was monitored at 280 nm.

Protein Sequencing. Since all CAPs tested were N-terminally blocked, the purified proteins were subjected to CNBr (CAP-IV, CAP-VI, CAP-III) or tryptic cleavage (CAP-II, CAP-V, CAP-I). CAP (10 nmol) was incubated with 10% CNBr, 6 M guanidine hydrochloride, and 0.12 M HCl at room temperature for 48 h in the dark. Peptides were isolated by reverse-phase chromatography on a C-18 column equilibrated with 0.05% TFA in methanol, and eluted with a linear gradient (0–80%) of 0.05% TFA in methanol at 30 °C and at a flow rate of 1.0 mL/min. Isolated peptides were characterized by automated sequencing using an Applied Biosystems 470A protein sequencer (Hewick et al., 1981) with PTH-amino acid identification by reverse-phase HPLC (McKay et al., 1985). For digestion with TPCK-trypsin, CAPs were incubated in 0.2 M NH_4HCO_3 for 60 h at 37 °C at an enzyme to substrate ratio of 1:300 (w/w). Peptides were isolated by reversed-phase HPLC as indicated above.

Amino Acid Analysis. CAPs were dialyzed overnight against two changes of 1000 volumes of 0.04 M $NaHCO_3$ and portions of the protein solution transferred to hydrolysis tubes containing 0.1% phenol and 0.02% 2-mercaptoethanol. Norleucine (10 nmol) was used as an internal standard to correct for losses of protein. Hydrolysis was carried out at 110 °C for 24, 48, and 72 h. The calculated values were based on mean values for three determinations.

Peptide Mapping. Peptide mapping by limited proteolysis was performed by the method of Cleveland et al. (1977). Lyophilized protein was dissolved at approximately 0.5 mg/mL in sample buffer containing 0.1 M Tris-HCl, pH 6.8, 0.5% SDS, 10% (v/v) glycerol, and 0.001% bromophenol blue. The samples were then heated for 2 min in boiling water. Proteolytic digestion were carried out at 37 °C for 30 min by addition of *Staphylococcus aureus* V8 protease (10 μg/mL). After addition of 2-mercaptoethanol and SDS to final concentrations of 10% and 2% (v/v), respectively, proteolysis was terminated by heating the samples in boiling water for 2 min. The samples were then analyzed by 15.0% SDS–PAGE. Time course experiments were initially performed to ensure that proteolysis had reached completion.

Gel Electrophoresis. Electrophoresis in gels containing SDS was performed in a slab gel apparatus using the system described by Laemmli (1970). Isoelectric focusing in 4 M urea was performed on horizontal slabs as outlined by Saravis et al. (1979).

Protein Determination. Protein concentration was determined according to Bradford (1976).

Purification of Proteins. All steps were carried out at 4 °C. A fresh bovine lung was cleaned and homogenized in 10 mM imidazole hydrochloride (pH 7.3) containing 150 mM NaCl, 1 mM DTT, 100 μg/mL soybean trypsin inhibitor, 1 mM DIFP, and 10 mM Ca^{2+}. After standing for 20 min at 4 °C, the homogenate was centrifuged first for 15 min at 2900*g* and the resultant supernatant for 40 min at 27000*g*. The pellet was resuspended in buffer C (150 mM NaCl, 1 mM DTT, 1 mM Ca^{2+}, 1% Triton X-100, and 10 mM imidazole hydrochloride, pH 7.4), stirred for 20 min, and centrifuged (25000*g*, 30 min). The pellet was washed once in buffer C and then twice with buffer C without Triton and resuspended in buffer D (100 mM NaCl, 1 mM DTT, 5 mM EGTA, and 10 mM imidazole, pH 7.4) by Dounce homogenization. After centrifugation at 100000*g* for 1 h, the supernatant was extensively dialyzed against buffer E (10 mM imidazole, pH 7.4, 1 mM DTT, and 0.2 mM EGTA), clarified by high-speed centrifugation, and concentrated by pressure filtration (PM-10, Amicon). This protein fraction, referred to as the "EGTA-eluted fraction", was applied to an HPLC ion-exchange column (TSK-DEAE, Pharmacia) at a flow rate of 1 mL/min previously equilibrated with 40 mM MOPS (pH 6.8)/0.5 mM DTT. The flow-through fractions were collected, and lipocortin I, lipocortin II, and lipocortin-85 were purified from this fraction as detailed previously (Khanna et al., 1987). The column was then developed with 500 mL of a linear salt gradient (0–0.3 M NaCl) of equilibration buffer. Five-milliliter fractions were collected. Fractions were analyzed by 12.5% SDS–PAGE, and pooled fractions were assigned the indicated letters (Figure 1). For subsequent purification, pooled fractions were divided into three fractions: the flow-through fraction (Figure 1, pool a), the low-salt fraction (Figure 1, pools b–e), and the high-salt fraction (Figure 1, pools f–i). Purification of these fractions is indicated diagrammatically in Figure 2. The low-salt peak was concentrated by pressure filtration (PM-10, Amicon) and applied to a 2.6 × 90 cm column of AcA 44 equilibrated with 40 mM Tris (pH 7.5), 0.15 M NaCl, and 0.5 mM DTT at a flow rate of 30 mL/h. Four-milliliter fractions were collected and analyzed by SDS–PAGE (data not shown). Two peaks were resolved; the high molecular weight peak was identified as lipocortin-85 by V8 peptide mapping (Cleveland et al., 1977), and the single lower molecular weight peak was pooled, dialyzed against 40 mM Tris (pH 8.5)/0.5 mM DTT, and applied at a flow rate of 1 mL/min to a column of Mono Q (HR 10/10, Pharmacia) previously equilibrated with dialysis buffer. The column was developed with a 200-mL linear salt gradient

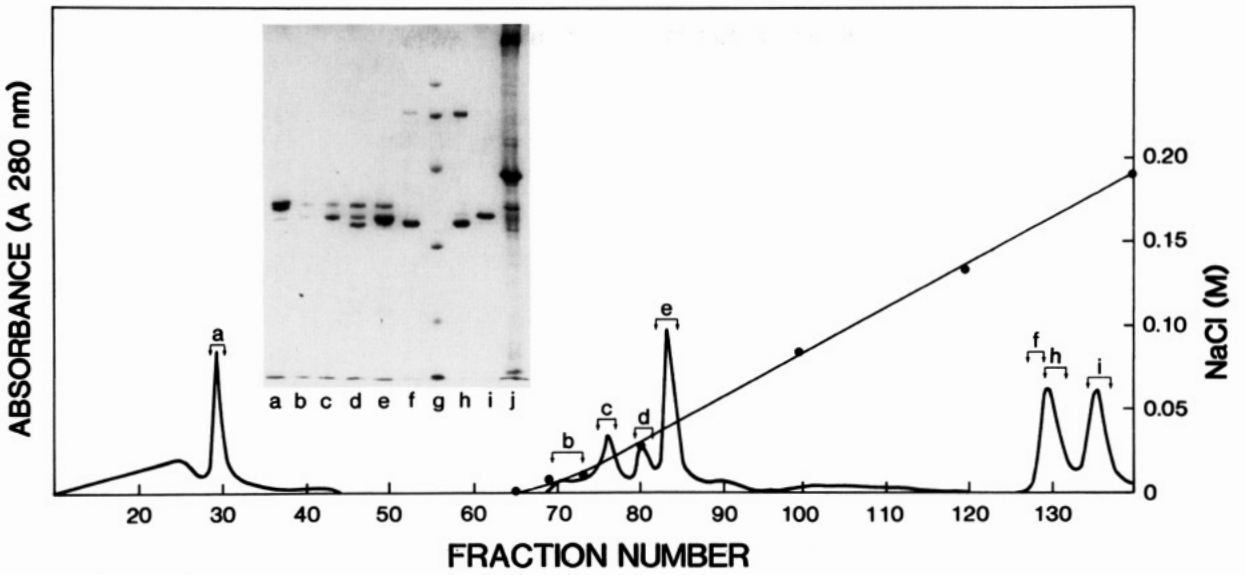

FIGURE 1: Fractionation of bovine lung EGTA-eluted proteins on HPLC TSK-DEAE. The protein fraction which bound to the detergent-extracted 29000*g* pellet in the presence of Ca^{2+} and eluted with excess EGTA was applied to TSK-DEAE (2.5 × 30 cm) as outlined under Experimental Procedures. The fractions were pooled after analysis by SDS–PAGE (inset) and assigned the indicated letters. The letter j represents the SDS–PAGE analysis of the detergent-extracted particulate fraction of bovine lung. Molecular weight markers are shown in lane g.

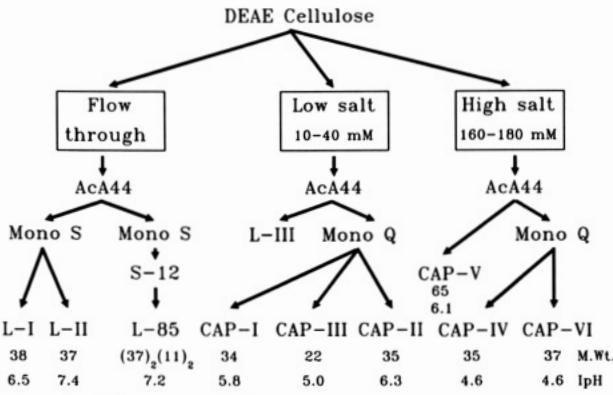

FIGURE 2: Diagrammatic illustration of the purification of CAPs. Molecular weights (determined by SDS–PAGE) and isoelectric points are also detailed. Specific details are provided under Experimental Procedures.

(0–0.15 M NaCl) in dialysis buffer. As shown in Figure 3, three proteins were resolved by this procedure. The proteins were pooled and named as follows: CAP-I (fractions 22–24), CAP-III (fractions 27–29), and CAP-II (fractions 30–34). Rechromatography of CAP-III on Mono Q was required to yield a homogeneous protein. Typically, 0.5 mg of CAP-III, 25.0 mg of CAP-I, and 45 mg of CAP-II were recovered after purification.

The high-salt peak (Figure 1, lanes f–i) was pooled, concentrated by pressure filtration (PM-10, Amicon), and applied to a 2.6 × 90 cm column of AcA 44 and operated under identical conditions with the low-salt peak (above). Two major protein peaks were resolved (Figure 4); fractions 50–53 contained a single protein, CAP-V, while the second protein peak (fractions 56–60) contained two proteins. The latter fraction was pooled, dialyzed against 40 mM Tris (pH 8.5)/0.5 mM DTT, and applied to a column of Mono Q (HR 10/10) which had been equilibrated with dialysis buffer. The column was washed with 20 mL of dialysis buffer containing 0.1 M NaCl and developed with a 200-mL linear salt gradient (0.1–0.3 M NaCl). As shown in Figure 5, two proteins were resolved. These proteins were pooled as follows: CAP-IV (fractions 36–46) and CAP-VI (fractions 59–66). Yields of proteins after purification were as follows: CAP-V, 55 mg; CAP-IV, 45 mg;

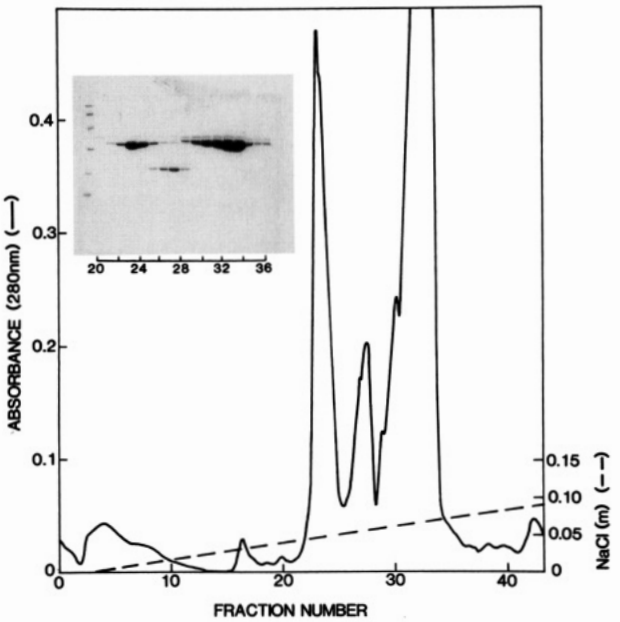

FIGURE 3: Purification of CAP-I, CAP-II, and CAP-III on MONO Q. The lower molecular weight peak from gel permeation chromatography of fractions b–e (Figure 1) was applied to Mono Q (HR 10/10) as outlined under Experimental Procedures. Individual proteins were pooled as indicated: CAP-I (fractions 22–24), CAP-III (fractions 27–29), CAP-II (fractions 30–34). Inset: 12.5% SDS–PAGE analysis of resolved proteins.

CAP-VI, 60 mg. CAPs were typically concentrated to about 1–2 mg/mL and stored at −70 °C.

Results

Purification of EGTA-Eluted Proteins from Bovine Lung Particulate Fraction. We have extended a protocol originally developed for the purification of the lipocortins (Khanna et al., 1987) and resolved a total of nine proteins. These proteins remain associated with the detergent-extracted particulate fraction of bovine lung in the presence of Ca^{2+}. Subsequent addition of EGTA released them from the detergent-extracted particulate fraction, and they were purified by ion-exchange chromatography and gel permeation chromatography (Figure 2). The procedure is extremely rapid and allows purification

Table I: Amino Acid Composition (Mole Percent) of Calcium-Activated Proteins[a]

amino acid	CAP-I	CAP-II	CAP-III	CAP-IV	CAP-V	CAP-VI	L-I	L-85
Asx	11.2	10.8	10.9	9.6	11.5	9.8	10.1	11.4
Thr	6.1	6.2	5.5	6.7	5.9	6.5	5.7	5.3
Ser	8.9	6.5	9.6	7.7	6.8	5.3	5.8	7.7
Glx	11.1	10.6	10.7	12.7	12.8	13.5	13.4	11.5
Pro	2.4	1.8	4.9	2.3	2.4	1.7	2.4	1.9
Gly	11.0	8.8	15.1	10.3	7.9	8.0	6.2	6.6
Ala	7.5	8.6	7.6	7.5	9.1	8.0	8.6	6.5
Val	4.0	2.3	4.4	4.5	2.9	5.4	4.8	5.2
Met	2.2	1.3	1.9	1.4	2.6	1.5	1.9	2.2
Ile	5.1	7.6	3.8	4.9	5.3	5.7	6.1	5.8
Leu	9.3	11.9	7.0	10.9	10.0	12.3	11.3	10.5
Tyr	3.7	3.6	4.2	4.0	3.5	4.1	3.5	5.5
Phe	3.7	4.0	5.0	4.0	3.7	3.9	2.3	2.4
His	1.2	1.4	1.2	1.3	1.7	1.0	1.8	1.2
Lys	6.0	7.9	3.6	5.9	7.5	6.2	8.8	10.0
Arg	6.5	6.9	4.6	6.3	6.3	7.0	6.3	6.1

[a] The results are reported as mean values for the 24-, 48-, and 72-h hydrolysis. Ser and Thr values were determined from interpolation to 0 time.

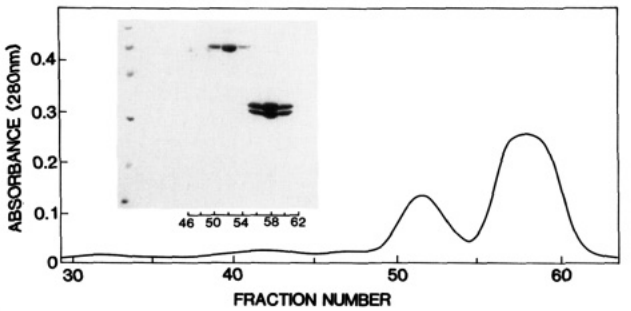

FIGURE 4: Chromatography of the high-salt peak on AcA 44. Fractions from HPLC TSK DEAE (Figure 1, f–i) were pooled, concentrated, and applied to AcA 44 as outlined under Experimental Procedures. The fractions were analyzed by SDS–PAGE (inset), and fractions 50–53 (CAP-V) and fractions 56–60 (CAP-IV and CAP-VI) were pooled.

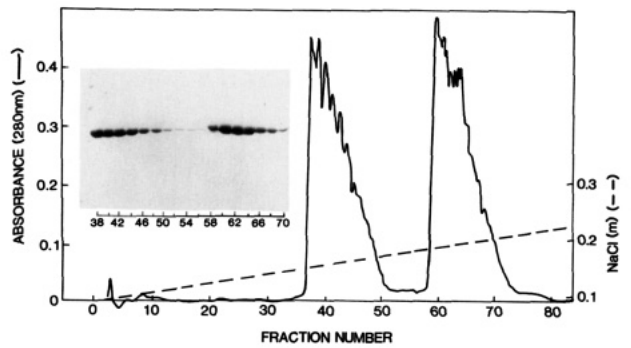

FIGURE 5: Chromatography of the AcA 44 pool (fractions 56–60) on Mono Q. Fractions from AcA44 (Figure 4) were pooled and applied to Mono Q (HR 10/10) as outlined under Experimental Procedures. Fractions were analyzed by 12.5% SDS–PAGE (inset). Fractions 37–46 (CAP-IV) and 59–65 (CAP-VI) were pooled.

of milligram quantities of these proteins. Since these proteins bound to the detergent-extracted cytoskeleton in the presence but not in the absence of Ca^{2+} (Figure 7), they were named Ca^{2+}-activated proteins or CAPs.

As shown in Figure 1, chromatography of the EGTA-eluted CAPs by ion-exchange resolved a flow-through fraction consisting of the three lipocortins (Khanna et al., 1987). A linear salt gradient was also used to resolve several proteins which bound to the HPLC–DEAE column. SDS–PAGE analysis of the proteins eluted from HPLC–DEAE suggested the presence of proteins of 34–38 kDa as well as a proteins of 65 and 22 kDa (visible only when fractions were concentrated). It was therefore important to determine if any of the purified proteins were proteolytic fragments of larger proteins or whether protein bands of identical molecular weight by

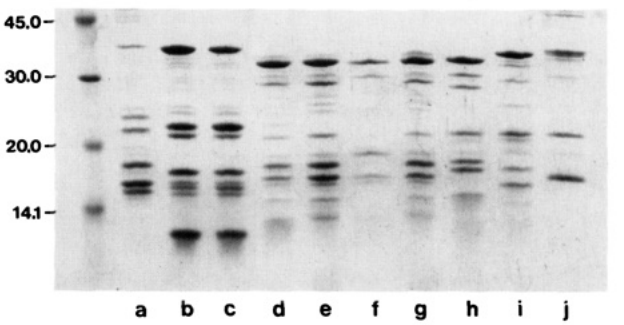

FIGURE 6: Peptide maps of CAPs. Purified CAPs from HPLC–DEAE pools (Figure 1) were analyzed by V8 peptide mapping as outlined under Experimental Procedures. Lane a, lipocortin I purified from HPLC–DEAE flow-through fraction; lane b, lipocortin-85 purified from HPLC–DEAE flow-through fraction; lane c, protein present in HPLC–DEAE pool (b–e) purified by AcA 44 chromatography of this pool; lanes d, e, and g, 35-kDa protein present in pool HPLC–DEAE pool (e) as the major protein, purified separately from pools c–e of HPLC–DEAE; lane f, 34-kDa protein purified from HPLC–DEAE pool c (lowest molecular weight protein in this pool); lane h, 35-kDa protein purified from HPLC–DEAE pool f (Figure 1); lane h, 35-kDa protein purified from HPLC–DEAE pool h (Figure 1); lane c, 37-kDa protein purified from HPLC–DEAE pool i; lane j, 65-kDa protein purified from HPLC-DEAE pool h.

SDS–PAGE were identical. Accordingly, the pooled fractions from HPLC–DEAE were subjected to further purification (see Experimental Procedures). The data presented in Figure 6 suggest that the highest molecular weight band present in pools b–e (Figure 1, inset) is lipocortin-85 (compare lanes b and c of Figure 6). Furthermore, the 35-kDa protein present as a major band in pool e (Figure 1, inset) appears identical with the protein present in HPLC–DEAE pools c–e (Figure 1, inset). This result was also confirmed by tryptic peptide analysis (data not shown). Comparing the peptide maps of the other CAPs eluted from HPLC–DEAE suggests that these proteins are distinct proteins and not proteolytic fragments of other proteins (compare Figure 6, lanes h–j). Unfortunately, CAP-III was resistant to the V8 protease, so a comparison of this protein with the other CAPs could not be undertaken.

Comparison of CAPs with Annexins. The amino acid compositions of the purified CAPs are presented in Table I. The overall similarity of the amino acid compositions of these proteins did not allow a helpful comparison with other annexin proteins. However, the significantly higher content of Gly and Phe in CAP-III suggested that this protein might not be a proteolytic fragment of other CAPs. It is also interesting to note that the amino acid compositions of CAP-IV and CAP-VI are almost identical.

Table II: Identification of CAPs[a]

name[b]	mol wt ($\times 10^{-3}$)	p*I*	possible identity	synonyms
lipocortin I	38.0	6.5	calpactin II	
lipocortin II	37.0	7.4	calpactin I	
lipocortin II	$(37)_2(10)_2$	7.2	calpactin I	lipocortin-85[i]
CAP-I	34.0	5.8	protein II[b]	endonexin I[c]
CAP-II	35.0	6.3	lipocortin III[d]	
CAP-III	22.0	5.0	unknown	
CAP-IV	35.0	4.6	unknown	
CAP-V	65.0	6.1	lipocortin VI[e]	p68[f]
CAP-VI	37.0	4.6	lipocortin V[g]	endonexin II[h]

[a] The identity of the lipocortins was confirmed by sequence analysis and antibody cross-reactivity (Khanna et al., 1986). CAPs were subjected to CNBr or tryptic cleavage and peptide-purified (see Experimental Procedures). Sequences were compared with published sequences of annexin proteins from the following sources: [b] Weber et al. (1987). [c] Giesow et al. (19863a,b). [d] Pepinsky et al. (1988). [e] Pepinsky et al. (1988). [f] Crompton et al. (1988). [g] Pepinsky et al. (1988). [h] Kaplan et al. (1988) and Boustead et al. (1988). [i] Khanna et al. (1987).

Since the purified CAPs were blocked at the NH_2 terminus, the proteins were subjected to cleavage by CNBr or TPCK-trypsin and the fragments purified by reversed-phase HPLC (Experimental Procedures). Isolated peaks were randomly chosen for protein sequencing. A summary of the comparison of CAPs with annexins, on the basis of amino acid sequence comparison, is presented in Table II. For brevity, a detailed comparison is not presented. The tryptic cleavage of CAP-I yielded two peptides which matched the sequence of bovine protein II (residues 275–279 and 31–42; Weber et al., 1987). Five peptides were isolated after the tryptic cleavage of CAP-II and showed the closest homology to human lipocortin-III (Pepinski et al., 1988). These were homologous to residues 305–316, 190–200, 110–118, 88–92, and 139–143, and of these peptides, only three amino acids were not identical with the published sequence. An amino acid sequence, obtained for CAP-III after digestion with trypsin (A-G-G-Y-G-G-A-T-G-G-D-A), could not be matched with the sequence of other annexins. A search of the protein database (PIR-release 19.0, December 31, 1988) was unsuccessful in the identification of this protein. This result presents the possibility that CAP-III is a novel protein. CAP-IV and CAP-VI were cleaved with CNBr, and the digest was separated by reverse-phase HPLC. The chromatogram of both proteins was virtually indistinguishable. Peptides which eluted at identical positions on each of the chromatograms were selected for sequence analysis. These peptides, obtained from the CAP-IV and CAP-VI digest, were sequenced and found to be identical. The peptides were identical with residues 29–41 (G-L-G-T-D-E-E-S-I-L-T-L-L) of the bovine endonexin II sequence (Boustead et al., 1988). In contrast, the sequences of a second set of CNBr peptides which eluted at identical positions on the chromatograms of CAP-IV (I-K-G-D-T-S-G-D-V-K-K-A-L-L-Y) and CAP-VI (I-K-G-D-T-S-G-D-Y-K-K-A-L-L-L) were found not to be identical. Although the sequence of the second peptide produced from digestion of CAP-VI was identical with residues 300–314 of the human lipocortin V sequence (Pepinsky et al., 1988), the second peptide produced from digestion of CAP-IV contains substitutions of a valine for Tyr-308 and a tyrosine for Leu-314. Clearly, CAP-IV and CAP-VI are homologous proteins, and CAP-VI best fits the sequence of endonexin II (lipocortin V).

The identity of CAP-V as lipocortin VI (Pepinsky et al., 1988) was suggested by the perfect match of the sequences of two tryptic peptides of CAP-V with the human protein sequence (residues 5–14 and 648–662).

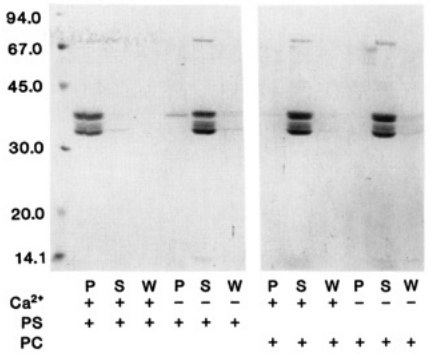

FIGURE 7: Phospholipid binding by CAPs. The EGTA eluate of detergent-extracted membranes was incubated with phosphatidylserine or phosphatidylcholine vesicles in buffer containing 20 mM HEPES (pH 7.4), 0.2 mM EGTA, 2 mM $MgCl_2$, 0.1 M NaCl, and where indicated 1.2 mM Ca^{2+}, as outlined under Experimental Procedures. The incubation mixtures were centrifuged to separate the free (S) from the vesicle-associated protein. The pellets were then washed with incubation buffer and centrifuged, and the free (W) and vesicle-associated proteins (P) were subjected to analysis by SDS–PAGE.

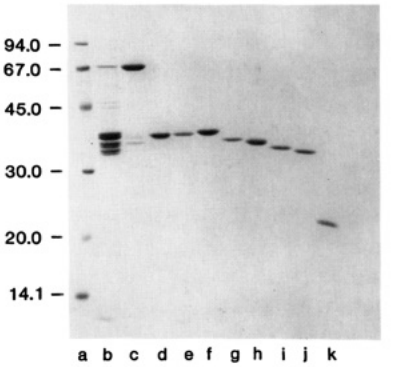

FIGURE 8: SDS–PAGE of purified CAPs. The purified proteins are (a) molecular weight markers, (b) proteins bound to detergent-extracted membranes in the presence of Ca^{2+} and eluted with excess EGTA, (c) CAP-V, (d) lipocortin II, (e) lipocortin-85, (f) lipocortin I, (g) CAP-VI, (h) CAP-II, (i) CAP-IV, (j) CAP-I, and (k) CAP-III.

F-Actin Binding. Ca^{2+}-dependent binding to F-actin by the lipocortins has been suggested by several laboratories (Gerke & Weber, 1984, 1985; Glenney & Glenney, 1985; Glenney et al., 1987; Burgoyne, 1987; Schlaepfer & Haigler, 1987); however, quantitation of the binding event has not been documented. Burgoyne (1987) has reported that the lipocortins and gelsolin share a homologous domain of 20 residues bounded by conserved leucine residues that is distinct from and carboxyl terminal to the consensus sequence of the lipocortins (Geisow et al., 1986a,b). The presence of a conserved domain in the lipocortins and in the actin binding protein gelsolin was interpreted by Burgoyne (1987) to suggest that the lipocortins might share the actin binding or F-actin severing properties of gelsolin. Other annexin proteins have been observed to associate with F-actin. Schlaepfer et al. (1987) reported that endonexin II (CAP-VI) associated with F-actin in a Ca^{2+}-dependent manner, although 50% binding required extremely high Ca^{2+} concentration (>2.0 mM Ca^{2+}). In contrast, Weber et al. (1987) reported that protein II (CAP-I) did not bind to F-actin.

In order to investigate the possibility that the annexins are actin binding proteins we have examined the possible binding of these proteins to F-actin by high-speed centrifugation (see Experimental Procedures). Under these experimental conditions, actin binding proteins will cosediment with F-actin. As shown in Figure 9, only lipocortin I, lipocortin II, lipocortin-85, CAP-IV, and CAP-VI demonstrated significant

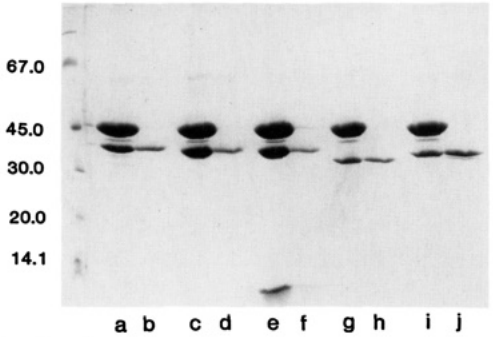

FIGURE 9: Cosedimentation of CAPs with F-actin. The interaction of CAPs with F-actin (see Experimental Procedures) was determined at 400 μM Ca^{2+}, 2.3 μM F-actin, and at 7.8 μM lipocortin I (a), 6.7 μM lipocortin II (c), 1.1 μM lipocortin-85 (e), 14.0 μM CAP-IV (g), and 14.0 μM CAP-VI (i). Mixtures of protein and F-actin were incubated at room temperature for 2 h in 2 mM Tris (pH 7.5), 0.33 mM ATP, 0.01% NaN_3, 0.5 mM DTT, 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, and 0.7 mM $CaCl_2$ ($[Ca^{2+}]_f$ = 400 μM). In control experiments, incubations were performed as indicated except that F-actin was omitted (lanes b, d, f, h, and j).

binding to F-actin when compared to control experiments in which centrifugation was performed in the absence of F-actin. In the absence of Ca^{2+}, these proteins did not interact with F-actin. In contrast, the amount of CAP-I, CAP-II, and CAP-V cosedimented with F-actin was identical with the amount of these proteins sedimented in the absence of F-actin (data not shown). Similarly, we were unable to demonstrate F-actin binding by CAP-III with this technique since CAP-III was sedimented in the absence of F-actin when Ca^{2+} was present in the incubation media (data not shown). It should be pointed out, however, that certain actin binding proteins, such as brevin (Doi & Frieden, 1984), can regulate actin polymerization when bound at molar ratios of 1:1000 (brevin/actin). This low level of binding would not be detected in our cosedimentation assay. It is therefore possible that the proteins could be involved in the regulation of actin polymerization if they interacted with F-actin at low molar ratios.

F-Actin Bundling. Gerke and Weber (1984) first demonstrated that lipocortin-85 could form F-actin bundles in the presence of Ca^{2+}. This observation was extended to lipocortin I and lipocortin II by Schlaepfer and Haigler (1987) and by Glenney et al. (1987). Considering the sequence homology between the various annexins (Pepinsky et al., 1988), it was reasonable to suspect that the annexins might, in general, represent F-actin bundling proteins. In order to test the potential role of the CAPs in promoting the formation of supramolecular structures of F-actin, the EGTA-eluted fraction (containing the entire complement of CAPs) was chromatographed on HPLC–DEAE (see Experimental Procedures), and the resultant fractions were analyzed for their ability to increase the light scattering of F-actin solutions. As shown in Figure 10, two peaks of light scattering were detected by using this procedure. Light scattering in the absence of Ca^{2+} or in the absence of both Ca^{2+} and F-actin was insignificant, which suggested that the bundling of F-actin was Ca^{2+} dependent. To confirm that the increase in light scattering was due to the formation of supramolecular structures (bundles) of F-actin, we took advantage of the observation that F-actin does not sediment at 15600*g* but that F-actin bundles can be harvested at this centrifugation force. As shown in inset A of Figure 10, the proteins of the larger peak of light-scattering activity (fractions 68–78) did not pellet in the absence of F-actin either with or without Ca^{2+}, but in the presence of both, F-actin and Ca^{2+} proteins of 37 and 10 kDa cosedimented with F-actin. Electron microscopy of this solution prior to centrifugation revealed closely packed anisotropic filaments similar to those reported by Gerke and Weber (1984) (data not shown). Subsequent purification has identified the protein that cosedimented with F-actin as lipocortin-85. In the absence of Ca^{2+}, neither actin nor lipocortin-85 sedimented. In contrast, in the presence, but not in the absence, of Ca^{2+}, low-speed centrifugation of the proteins of the smaller peak of light-scattering activity (Figure 10, fractions 87–91) pelleted a single protein of 22 kDa (CAP-III). When these pooled fractions were incubated with both Ca^{2+} and F-actin, the majority of F-actin was pelleted after low-speed centrifugation along with proteins of 37 kDa (lipocortin-85 subunit) and 22 kDa (CAP-III). In the absence of Ca^{2+}, none of these proteins were pelleted. The presence of both lipocortin-85 and CAP-III with pelleted F-actin did not answer the question of whether or not CAP-III cosedimented with F-actin, since it was possible that CAP-III sedimented independently of the lipocortin-85/F-actin complex. Accordingly, purified CAP-III was subjected to low-speed centrifugation in the presence or absence of both Ca^{2+} and F-actin (Figure 11). Although CAP-III demonstrated Ca^{2+}-dependent pelleting at low centrifugation force in the absence of F-actin, CAP-III also demonstrated Ca^{2+}-dependent cosedimentation with F-actin at low centrifugation force. This experiment suggested that CAP-III bound to F-actin, which was then pelleted with the aggregated CAP-III at the low centrifugation force. Although the interaction of F-actin and CAP-III resulted in a Ca^{2+}-dependent increase in light scattering, these experiments did not provide any evidence that CAP-III causes bundling of F-actin. Furthermore, analysis of the CAP-III/F-actin complex by electron microscopy has suggested that F-actin bundles were not formed. Densitometric analysis (Figure 11) suggested that a one to one molar complex of CAP-III to F-actin was formed at micromolar concentrations of CAP-III and F-actin, therefore suggesting that the interaction of these proteins is of moderate affinity.

In addition to testing the F-actin bundling activity of CAPs resolved by HPLC–DEAE, we have also examined the HPLC–DEAE flow-through fractions. The flow-through fraction of HPLC–DEAE, a fraction especially rich in the lipocortins (Khanna et al., 1987), was concentrated and applied to AcA 44 (Figure 12). Two protein peaks were resolved and tested for F-actin bundling activity. The higher molecular weight peak, which consists of lipocortin-85 (Khanna et al., 1987), showed both Ca^{2+}-dependent light scattering in the presence, but not absence, of F-actin and Ca^{2+}-dependent cosedimentation with F-actin. In contrast, the lower molecular weight peak resolved on AcA 44, which contains lipocortin I and lipocortin II, did not appear to bundle F-actin as determined by either light scattering (Table III) or low-speed centrifugation (data not shown). Therefore, although lipocortin I and lipocortin II (monomer) bind to F-actin, they do not appear to induce bundle formation under these experimental conditions.

The ability of purified CAPs to induce Ca^{2+}-dependent F-actin bundling was measured by light scattering (Table III) at an equimolar ratio of CAP to actin. Of all the CAPs tested, only lipocortin-85 and CAP-III were capable of inducing Ca^{2+}-dependent increases in light scattering. Since CAP-III was found to generate an increase in light scattering in the absence of F-actin, the values for light scattering in the absence of F-actin have been subtracted. This result provides further evidence that only lipocortin-85 is capable of the Ca^{2+}-dependent formation of F-actin bundles. In contrast to the report by Glenney et al. (1987), we are unable to demonstrate F-actin bundling activity for lipocortin I or lipocortin II under our

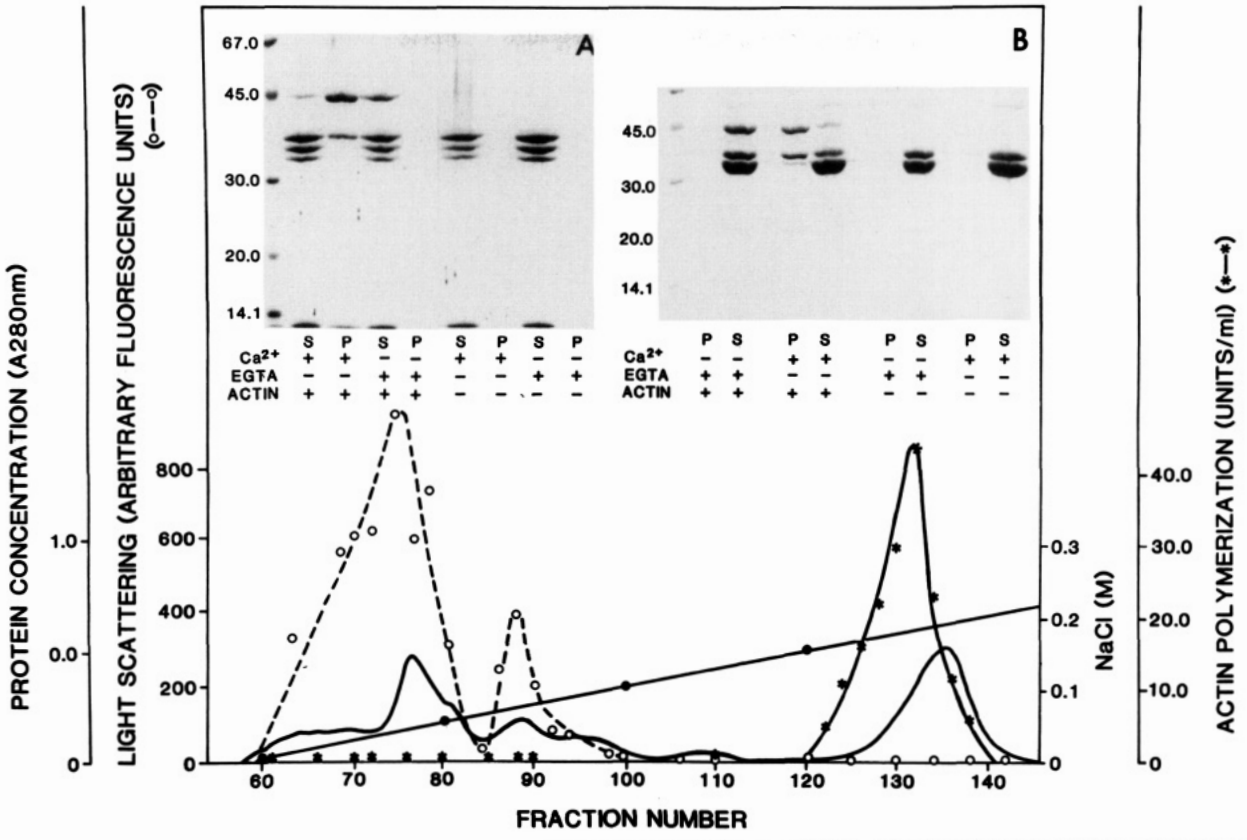

FIGURE 10: Analysis of the interaction of HPLC–DEAE-resolved CAPs with actin. The EGTA-eluted pool (Figure 8, lane b) containing the complete complement of CAPs was chromatographed on HPLC–DEAE as outlined under Experimental Procedures. Fractions were assayed for Ca^{2+}-dependent F-actin bundling activity (O---O) by light scattering or for Ca^{2+}-dependent changes in the initial rate of actin polymerization (*—*) by measuring the ability of test samples to influence the initial rate of conversion of G-actin to F-actin (Experimental Procedures). F-Actin bundling was measured in the presence of 1.16 μM F-actin by light scattering at 400 nm in 2 mM Tris (pH 7.5), 0.33 mM ATP, 0.01% NaN_3, 0.5 mM DTT, 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM EGTA, and 1.2 mM Ca^{2+}. The value for light scattering in the absence of added Ca^{2+} has been subtracted. Actin polymerization was measured with pyrene-labeled G-actin (3.97 μM) in buffer containing 2 mM Tris (pH 7.5), 0.5 mM DTT, 0.33 mM ATP, 0.5 mM Ca^{2+}, and 0.01% NaN_3 at 22 °C, in the presence of test samples, by the addition of KCl and $MgCl_2$ to 100 and 1 mM, respectively. The excitation wavelength was 365 nm, and the emission wavelength was 407 nm. Values for actin polymerization in the absence of Ca^{2+} have been subtracted. Protein concentration is represented by the A_{280nm} (●—●). Inset: Fractions demonstrating Ca^{2+}-dependent increases in light scattering were pooled, dialyzed vs 40 mM Tris (pH 7.5)/0.5 mM DTT, and tested for actin bundling activity by centrifugation at low speed (Experimental Procedures). Test fractions were incubated with F-actin (1.16 μM) in 2 mM Tris (7.5), 0.33 mM ATP, 0.01% NaN_3, 0.5 mM DTT, 100 mM KCl, 1 mM $MgCl_2$, and 0.2 mM EGTA in the presence or absence of 1.2 mM Ca^{2+} for 1 h, at 22 °C, and centrifuged at 15600g for 10 min. The pellet was adjusted to the identical volume as the supernatant with buffer, and the superantants (S) and pellets (P) were lyophilized and analyzed by SDS–PAGE. (A) Fractions 68–78; (B) fractions 87–91, concentrated about 10-fold by pressure filtration prior to incubation with F-actin.

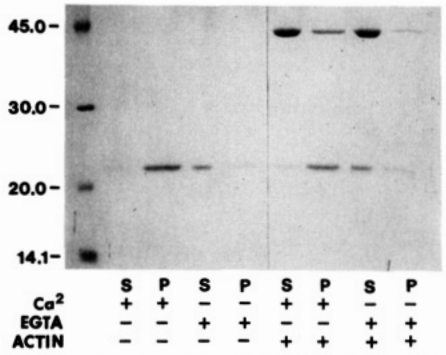

FIGURE 11: Interaction of purified CAP-III with F-actin. CAP-III (3.6 μM) was incubated with F-actin (2.4 μM) and subjected to low-speed centrifugation as detailed in Figure 10.

assay conditions. Although F-actin bundling activity measured at 1.0 mM Ca^{2+} is of doubtful physiological significance, we have recently measured a $K_d(Ca^{2+})$ of 1–2 μM for F-actin bundling by equimolar complexes of lipocortin-85 and F-actin and a $K_d(Ca^{2+})$ of 40 μM for F-actin binding by equimolar complexes of CAP-III and F-actin (Ikebuchi and Waisman, unpublished results).

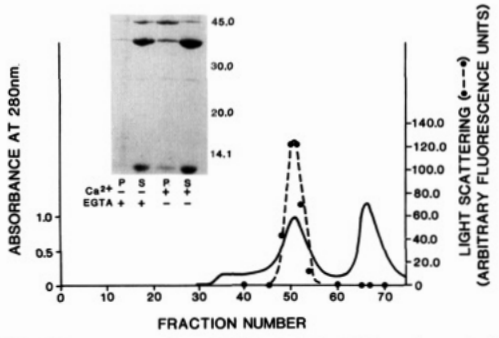

FIGURE 12: Chromatography of HPLC–DEAE flow-through fractions on AcA 44 and analysis for F-actin bundling activity. The flow-through fraction from HPLC–DEAE was concentrated by pressure filtration and applied to a 2.6 cm × 90 cm column of AcA 44. Fractions were collected and assayed for F-actin bundling activity as detailed in the legend to Figure 10. Inset: Fractions 48–53 were analyzed for F-actin bundling activity by low-speed centrifugation as detailed in the legend to Figure 10.

Effects of CAPs on Actin Polymerization. We have also tested the ability of CAPs, resolved by HPLC–DEAE, to influence the initial rate of actin polymerization. Actin binding proteins which interact either with G-actin or with the actin

Table III: Effect of Calcium-Activated Proteins on Actin Bundling[a]

addition	light scattering (arbitrary fluorescence units)	
	Ca^{2+}	EGTA
none	23.0	23.0
L-I	22.0	19.0
L-II	22.0	24.0
L-85	755.0	20.0
CAP-I	22.0	22.0
CAP-II	25.0	24.0
CAP-III	127.0	21.0
CAP-IV	20.0	23.0
CAP-V	23.0	22.0
CAP-VI	24.0	23.0

[a] Actin bundling was measured in the presence of equimolar actin and CAP by light scattering at 400 nm. The reaction mixture contained 2 mM Tris (pH 7.5), 0.3 mM ATP, 0.01% NaN_3, 0.5 mM DTT, 100 mM KCl, 1 mM $MgCl_2$, 1.16 μM F-actin, 0.2 mM EGTA, and, where indicated, 1.2 mM $CaCl_2$. The value for light scattering in the presence of CAP but in the absence of actin has been subtracted.

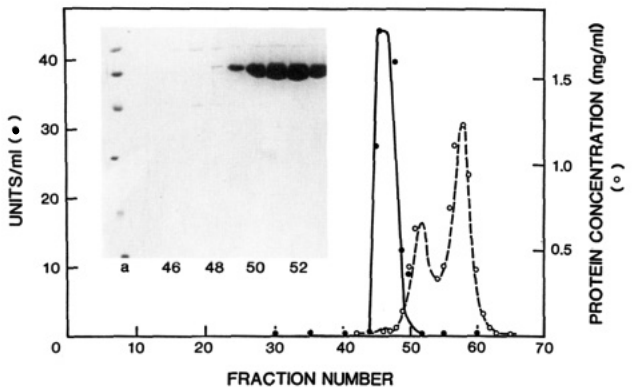

FIGURE 13: Gel permeation chromatography of actin nucleation activity. The fractions from HPLC–DEAE chromatography of EGTA-eluted proteins (Figure 10) which stimulated actin polymerization (fractions 128–135) were pooled, concentrated by pressure filtration, and applied to a 2.6 cm × 90 cm column of AcA 44. Fractions were pooled, dialyzed against 40 mM Tris (pH 7.5)/0.5 mM DTT, and analyzed for potential effects on actin polymerization (O---O) as detailed in Figure 10.

nucleation complex have been shown to have either stimulatory or inhibitory effects on the initial rate of conversion of G-actin to F-actin. Therefore, this assay is a useful tool in establishing a functional correlate for proteins suspected of interacting with actin. As shown in Figure 10, chromatography of EGTA-eluted proteins on HPLC–DEAE resolved a peak of G-actin stimulatory activity. Stimulation of the initial rate of actin polymerization by this activity peak was totally Ca^{2+} dependent. The activity peak appeared to coincide with CAP-V (data not shown). When the peak of activity was pooled and chromatographed on ACA 44, the peak of stimulatory activity was clearly resolved from the CAP-V protein peak (Figure 13). The protein(s) responsible for the stimulation of actin polymerization was (were) detected by SDS–PAGE analysis and consisted of a 90-kDa band and a 42-kDa band. Preliminary analysis based on antibody cross-reactivity has suggested the identity of this protein as a 1 to 1 molar complex of gelsolin and actin.

In order to confirm our observation that the partially purified CAPs do not affect the initial rate of actin polymerization, we have reexamined this possibility with purified CAPs. As shown in Table IV, of all the CAPs tested, only CAP-III appears to affect the initial rate of actin polymerization. The inhibition of actin polymerization by CAP-III was not observed when the EGTA-eluted proteins, containing CAP-III, were chromatographed on HPLC–DEAE (Figure 10) because of

Table IV: Effect of Calcium-Activated Proteins on the Rate of Actin Polymerization[a]

protein	protein:actin ratio (mol/mol)	rate of actin assembly ($\times 10^{-10}$ M s^{-1})
CAP-I	0.25	6.86 ± 0.75
CAP-II	0.56	6.90 ± 0.48
CAP-III	0.06	2.74 ± 0.26
CAP-IV	0.73	6.92 ± 0.97
CAP-V	0.63	6.71 ± 0.54
CAP-VI	0.70	6.92 ± 0.42
L-I	0.20	6.74 ± 0.41
L-II	0.20	6.81 ± 0.28
L-85	0.25	6.72 ± 0.56
none		6.86 ± 0.28

[a] Pyrene-labeled G-actin at 3.97 μM was incubated with calcium-activated proteins in 0.5 mM $CaCl_2$ immediately prior to addition of 100 mM KCl and 1 mM $MgCl_2$ to initiate polymerization.

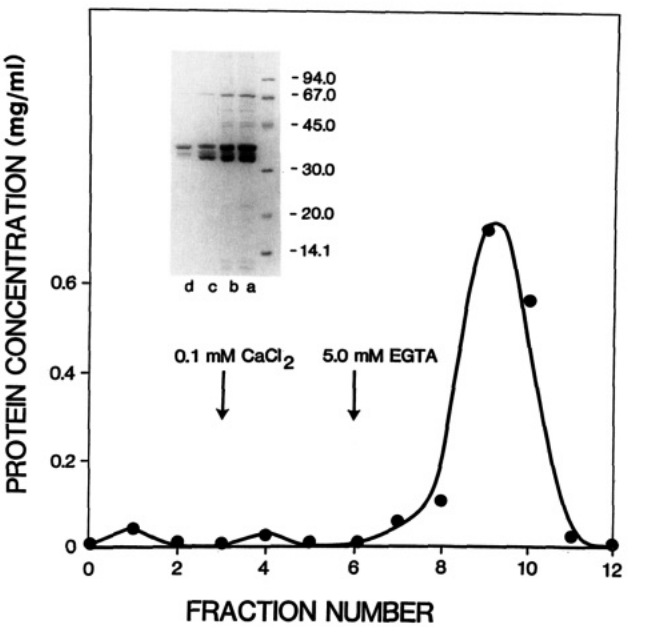

FIGURE 14: Interaction of CAPs with calmodulin. The EGTA-eluted proteins (10 μg) were applied to a calmodulin affinity column previously equilibrated with 40 mM Tris (pH 7.5), 1 mM $MgCl_2$, 0.25 M NaCl, 0.5 mM DTT, and 0.5 mM Ca^{2+}. After being washed, the calmodulin column was eluted with buffer containing 0.1 mM Ca^{2+} or 5.0 mM EGTA. Inset: SDS–PAGE analysis of proteins eluted by 5.0 mM EGTA: (a) fraction 9; (b) fraction 10; (c) fraction 8; (d) fraction 7.

the very low concentration of the protein. The inhibition of actin polymerization by CAP-III suggests that this protein may interact directly with G-actin and prevent incorporation of the G-actin into the actin polymers. Alternatively, CAP-III may bind to, and destabilize, the actin nucleation complex, thereby inhibiting further growth of actin on the nucleation complex.

Interaction of CAPs with Calmodulin. Martin et al. (1988) have suggested, based on experiments with a calmodulin affinity column, that lipocortin-85 is a calmodulin binding protein. Considering the sequence homology between lipocortin-85 and the other annexin proteins, it was reasonable to suspect that the annexins might bind to calmodulin. We therefore applied the entire fraction of proteins eluted from lung membranes with EGTA to a calmodulin affinity column previously equilibrated with Ca^{2+}-containing buffer and washed the column extensively with the Ca^{2+}-containing buffer (Figure 14). All the proteins were retained on the column and were not eluted by washing the column with 0.1 mM Ca^{2+}. Proteins were eluted from the calmodulin affinity column with 5.0 mM EGTA. In control experiments in which bovine serum

Table V: Effect of Calcium-Activated Protein on Calmodulin-Stimulated Cyclic Nucleotide Phosphodiesterase Activity[a]

protein	% inhibition	protein	% inhibition
L-I	<1	CAP-IV	<1
L-II	<1	CAP-V	<1
L-85	<1	CAP-VI	<1
CAP-I	<1	calcineurin	93.5
CAP-II	<1	none	0
CAP-III	<1		

[a] Cyclic nucleotide phosphodiesterase was assayed according to Teo et al. (1973) in the presence of 20 ng/mL calmodulin and in the absence or presence of 10 μg/mL added CAP or 2 μg/mL calcineurin.

albumin was conjugated to the affinity resin, binding in the presence of Ca^{2+} was not observed.

Since our results suggested that our CAPs might form a complex with calmodulin, we expected these proteins to inhibit calmodulin-activated cyclic nucleotide phosphodiesterase by competing with the enzyme for calmodulin. Therefore, cyclic nucleotide phosphodiesterase was titrated with enough calmodulin to assure 70% of the total activation of the enzyme, and the enzyme was assayed in the presence of 1000-fold molar excess of purified CAPs. As shown in Table V, the CAPs did not inhibit calmodulin-activated cyclic nucleotide phosphodiesterase under these conditions. In contrast, calcineurin, at a 25-fold molar excess over calmodulin, completely inhibited the calmodulin-stimulated cyclic nucleotide phosphodiesterase activity.

We have also attempted to demonstrate the formation of a complex between purified CAPs and calmodulin by chromatography of purified CAPs and calmodulin, preincubated in the presence of Ca^{2+}, on gel permeation columns (see Experimental Procedures). However, under our experimental conditions, we have been unable to detect any complexes between these proteins. These results suggest that although CAPs bind to immobilized calmodulin, they do not bind to the native protein.

Discussion

One of the major difficulties in the characterization of the Ca^{2+} and phospholipid binding proteins, known generally as the annexins, has been the confusing nomenclature which has resulted from the purification of some of these proteins from a variety of tissues by many different laboratories. In the present paper, we report a simple and rapid procedure for the purification of several proteins which exhibit Ca^{2+}-dependent binding to the detergent-extracted 29000*g* pellet of bovine lung. These proteins, referred to as Ca^{2+}-activated proteins (CAPs), were characterized by peptide mapping and shown not to be proteolytic fragments. Comparison of the amino acid sequence of the CAPs has identified seven of these proteins as annexin proteins. Of the two novel CAPs, one of these proteins, CAP-IV, demonstrated Ca^{2+}-dependent binding to phosphatidylserine vesicles and a high degree of homology to CAP-VI which suggested that this protein might be a novel annexin protein. Further sequence information is necessary to resolve this question. The limited sequence information obtained for CAP-III suggests that this protein was not a proteolytic fragment of other annexin proteins nor was it related to any other sequenced protein. Unfortunately, the limited concentration of this protein in bovine lung precluded extensive characterization.

We have characterized the interaction of the CAPs with phospholipid, actin, and calmodulin. Although the CAPs (CAP-III was not tested) showed Ca^{2+}-dependent phospholipid binding, only lipocortin I, lipocortin II, lipocortin-85, CAP-III, CAP-IV, and CAP-VI bound F-actin in the presence of Ca^{2+}. The Ca^{2+}-dependent binding of F-actin by lipocortin-85 resulted in the formation of F-actin bundles. Only CAP-III affected the initial rate of actin polymerization. Taken collectively, our studies suggest that lipocortin-85, lipocortin II, lipocortin I, CAP-III, CAP-IV, and CAP-VI bind to F-actin in a Ca^{2+}-dependent manner and that only CAP-III influences the initial rate of actin polymerization. Of all the CAPs tested, only lipocortin-85 is capable of Ca^{2+}-dependent F-actin bundling.

Although all CAPs bound to calmodulin immobilized on an affinity resin, interaction of these proteins with native calmodulin could not be demonstrated. Several laboratories have documented the cosedimentation of the lipocortins with F-actin, and the interaction of the lipocortins with calmodulin. Considering the extensive sequence homology between the various annexins, these results have presented the possibility that the annexins might in general represent actin and calmodulin binding proteins. However, our results suggest that calmodulin binding is probably an artifact. Furthermore, the interaction of the annexins with F-actin is not a general property of these proteins, and the functional changes in F-actin that occur as a consequence of annexin/F-actin interaction seem unique to each protein. The Ca^{2+}-dependent formation of F-actin bundles by lipocortin-85 is particularly interesting. The demonstration that lipocortin-85 is immunocytochemically colocalized with the actin binding proteins that form a reticular network underneath the plasma membrane (Gerke & Weber, 1984; Osborn et al., 1988) is consistent with the proposed function of the protein as an F-actin bundling protein. The report that lipocortin-85, but not the other lipocortins, is capable of aggregation of chromaffin granules at micromolar concentrations of Ca^{2+} is also consistent with the proposed function of the protein (Drust & Creutz, 1988). Current work in our laboratory is aimed at examining, quantitatively, the interaction between lipocortin-85 and F-actin. Preliminary results have suggested that micromolar concentrations of Ca^{2+} are required for F-actin bundling activity.

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