Calpactins: Two Distinct Ca++-regulated Phospholipid- and Actin-binding Proteins Isolated from Lung and Placenta

John R. Glenney, Jr.,* Brian Tack,‡ and Mark A. Powell*

* Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138; and
‡ Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037

Abstract. Three forms of calpactin, the 36,000 M_r Ca++-binding cytoskeletal protein, were isolated in large amounts from bovine lung and human placenta using cycles of calcium-dependent precipitation followed by solubilization with EGTA-containing buffers. Calpactin-I as a tetramer of heavy (36 kD) and light (11 kD) chains was the predominant form of calpactin isolated, however milligram amounts of the calpactin-I heavy chain monomer and calpactin-II, a related but distinct molecule, were also isolated by this method. Calpactin-II was characterized in some detail and found to bind two Ca++ ions with K_d's of 10 μM in the presence of phosphatidylserine. Both calpactin-I and -II were found to aggregate liposomes at micromolar Ca++ concentrations, suggesting that at least two phospholipid-binding sites are present on these molecules. Both calpactin monomers bind to and bundle actin filament at high (1 mM) but not low (<1 μM) Ca++ concentrations. Amino-terminal sequence analysis of a lower molecular mass variant of calpactin-II revealed that this protein was the previously identified human "lipocortin" molecule. Antibodies were elicited to calpactin-I and -II and the cell and subcellular distribution of each was compared. Calpactin-II was only present at high levels in tissues (lung, placenta) which contained high levels of calpactin-I. Other tissues (intestine) contained high calpactin-I and undetectable levels of calpactin-II. Double-label immunofluorescence microscopy on human fibroblasts revealed that, like calpactin-I, calpactin-II is present in a submembraneous reticular network, although the distribution of the two calpactins is not identical.

T HE molecules which participate in connecting the plasma membrane to the microfilament system have remained rather elusive due to the complexity of the cortical cytoplasm in nucleated cells (16, 41). One protein which has been suggested to be involved in membrane-to-filament linkage is spectrin, the major membrane–skeletal protein of the erythrocyte (3, 33). A variant of spectrin has also been found in non-erythroid cells where it has been localized to a reticular network just under the membrane (2, 30). Although additional erythrocyte membrane skeletal proteins have been identified in other cell types (1, 5, 9, 34, 35, 50), the mechanism and components involved in such a subcortical network need not be identical in all cells.

The various functions of the microfilament systems appear to be governed by the actin-binding proteins in a given cell or subcellular compartment (51, 54). The observation that actin-binding proteins are present in only a limited region of the total cellular actin distribution is consistent with microfilaments being involved in a variety of functions as governed by the associated proteins. Antibodies to non-erythroid spectrin have allowed a glimpse of a distinct actin organization in the cortical cytoplasm (2, 26, 30, 47). More recently, another actin-binding protein, 36,000 M_r, has been

identified which also appears to be localized to the cytoskeleton (4) in a similar distribution as spectrin (6, 36, 44). This protein, termed "calpactin" (also referred to as p36, p34, p39, or protein I) is a major substrate of the oncogene or growth factor receptor–associated protein tyrosine kinases (12, 13, 39). Calpactin isolated from mammalian intestine (18, 28), lymphocytes (8), or chick fibroblasts (14) is a complex of two distinct subunits with molecular masses of 38,500 (heavy chain) and 11,000 (light chain) as estimated by amino acid sequence. The light chain is related by amino acid sequence to the Ca++-binding S-100 proteins of brain (20, 28, 37). The calpactin light chain has apparently lost the ability to bind Ca++ (20, 23), and instead the Ca++-binding sites of calpactin-I have been assigned to the heavy chain subunit (19, 23). The affinity of calpactin for Ca++ can be modulated by phospholipids (21, 23) with two Ca++-binding sites detected for each calpactin heavy chain ($K_d = 5$ μM) in the presence of phosphatidylserine (23).

The domains of calpactin have been analyzed in some detail. Chymotrypsin cleaves the calpactin-I heavy chain (36 kD) into COOH-terminal core (33 kD) and NH2-terminal tail (3 kD) domains (28, 42). The core has been shown to contain all of the Ca++- and phospholipid-binding activity

(23) whereas the tail has been shown to contain the site of interaction with the calpactin light chain (29, 42) in addition to the sites of phosphorylation by pp60[src] (31) and protein kinase C (32).

Recently, a 36-kD substrate of the epidermal growth factor receptor tyrosine kinase has been detected in human A431 cells (15, 49). This molecule, which has been termed p35 or calpactin-II, shares a number of structural features with calpactin-I and, using an assay based on the ability to interact with actin and phospholipid, appears to share these activities as well (22). Unlike calpactin-I, however, calpactin-II is only found as a monomer (15, 22), whereas calpactin-I can be detected as a monomer or complex with light chain (14, 19, 22).

We now report the isolation of the calpactin-I complex, calpactin-I monomer, and calpactin-II from bovine lung and human placenta. We show that (a) calpactin-II is identical to lipocortin I; (b) calpactin-II binds 2 mol of Ca^{++} per mol protein, phospholipid dependently; (c) both calpactins bind to and bundle actin filaments at high Ca^{++} concentrations; and (d) there are significant differences in the tissue and subcellular distributions of calpactin-I and -II.

Materials and Methods

Purification of Calpactins

Frozen bovine lung (or fresh human placenta) is homogenized with a Waring blender in small aliquots (100 g) with an equal volume of 40 mM Tris, 1% Triton X-100, 10 mM EGTA, 2 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM benzamidine, pH 8.8. Homogenates were centrifuged 30 min at 15,000 g, and the supernatant further centrifuged at 100,000 g for 1 h. The soluble protein was adjusted to 1 mM free Ca^{++} (monitored with a Ca^{++}-selective electrode), while maintaining the pH at 7.3–7.5 with 1 M Tris base. After stirring 20 min at 4°C, precipitated protein was collected by centrifugation (100,000 g, 1 h), resuspended in 100 ml 10 mM imidazole, 2 mM $MgCl_2$, 0.5 mM DTT, 2 mM $CaCl_2$, pH 7.3, layered over 400 ml of 5% sucrose in the same solution, and centrifuged as above. The pellets were resuspended in 200 ml of 20 mM imidazole, 2 mM $MgCl_2$, 0.5 mM DTT, 25 mM EGTA, 200 mM NaCl, pH 7.3, stirred 30 min at 4°C, and centrifuged at 120,000 g for 1 h. The supernatant was dialyzed overnight against 10 mM imidazole, 25 mM NaCl, 0.5 mM EGTA, 0.5 mM DTT, pH 7.3, and then adjusted to 1 mM free Ca^{++} as described above. Precipitated protein was collected by centrifugation as before, resuspended in 15 ml 20 mM Tris, 1 M NaCl, 50 mM EGTA, 1 mM DTT, 2 mM $MgCl_2$, pH 8.0, and after stirring 30 min was centrifuged at 150,000 g for 1 h. The supernatant was applied to a 2.8- × 110-cm column of Sephacryl S-300 equilibrated and run in 20 mM $NaPO_4$, 0.5 M NaCl, 0.5 mM DTT, 20 mM EGTA, 2 mM $MgCl_2$. Fractions (6 ml) were collected and monitored by absorbance at 280 nm and every other fraction beginning at the void volume was analyzed by SDS PAGE. Peaks corresponding to the complex of calpactin-I with light chain (~90,000 M_r) or the monomer peak (~35–40 kD) were separately pooled, dialyzed against 10 mM imidazole, 1 mM EGTA, 1 mM NaN_3, 0.5 mM DTT, pH 7.3, and applied to a 10-ml DE-52 column equilibrated in the same buffer. Calpactins flow through the DE-52 column unretarded and the other major proteins in the preparation, the 73-kD protein (23) and the 32.5-kD protein (16) bound to the column and could be recovered by elution with salt. The calpactin pools were adjusted to 20 mM Na acetate, the pH adjusted to 5.6, and the solution was applied to a CM52 (Whatman Inc., Clifton, NJ) column and eluted with 1 M NaCl. This was used as the pure calpactin-I complex which was dialyzed against 0.5 mM DTT and stored as aliquots at −70°C. For the monomer fraction, the solution was dialyzed against 10 mM imidazole, 1 mM NaN_3, and applied to a 3-ml hydroxylapatite column (Bio-Rad Laboratories, Richmond, CA) which was developed with a 0–200-mM linear gradient of $NaPO_4$, pH 7.5. Fractions were monitored by absorbance at 280 nM, SDS PAGE, Ca^{++}-binding, and Western blotting. The peak fractions corresponding to calpactin-II were pooled and dialyzed against 20 mM Na acetate, pH 5.6, applied to an FPLC mono-S-column (Pharmacia Fine Chemicals, Piscataway, NJ) which was eluted with a 150–400-mM NaCl gradient. Calpactin-II was then dialyzed against 0.2 mM DTT and stored at 4°C.

Liposome Aggregation

Fluorescently loaded liposomes were prepared by drying 1 mg phosphatidylserine + 1 mg phosphatidylethanolamine together and sonicating into a solution of rhodamine-conjugated IgG (Sigma Chemical Co., St. Louis, MO). The liposomes were separated from free rhodamine IgG using a sucrose flotation gradient. The liposomes were then mixed with either BSA (control), calpactin-I, calpactin-I core, or calpactin-II in a solution containing 10 mM imidazole, 40 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 10 mM EGTA, 8.8 mM $CaCl_2$ (10 mM free Ca^{++}), pH 6.8. After 30 min, the liposome suspension was observed in a Nikon optophot microscope equipped with epi-illumination. The interaction of calpactin with liposomes was also monitored by light scattering as in reference 51 and low speed centrifugation of ^{14}C-PS-containing liposomes as follows. Calpactins were mixed with liposomes prepared as above (without fluorescent loading) in 20 mM imidazole, pH 7.3, 150 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and allowed to sit at room temperature for 1 h. The absorbance at 320 nM was measured, and then the solution was centrifuged for 6 min at 13,000 g. Lipids in supernatant and pellet fractions were determined by scintillation counting of ^{14}C-PS.

Other Methods

Two-dimensional gels of purified calpactins was performed with the nonequilibrium pH gradient electrophoresis (NEPHGE) first dimension (45) as described previously (22). Peptide mapping of iodinated calpactin heavy
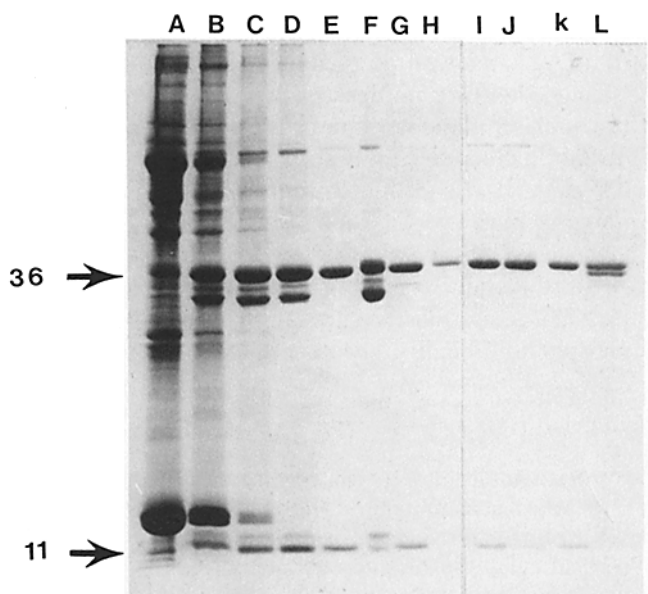

Figure 1. SDS PAGE analysis of fractions at various stages in the purification of calpactin. Frozen bovine lung tissue was homogenized in EGTA-containing buffer and after a high speed centrifugation the extract (lane A) was adjusted to 1 mM free Ca^{++} while maintaining the pH at 7.3. The precipitate (lane B) was collected by centrifugation, resuspended in buffer containing 1 mM free Ca^{++}, and centrifuged through a 5% sucrose cushion. The pelleted material (lane C) was resolubilized in EGTA-containing solution and after dialysis followed by another cycle of Ca^{++}-induced precipitation, the protein soluble in 1 M NaCl, 25 mM EGTA, 50 mM $NaPO_4$ (lane D) was applied to a Sephacryl S-200 column where a 90,000 M_r peak (E) and a 35,000–40,000 M_r peak (lane F) were observed. Peak fractions were pooled, dialyzed against low salt buffer, and passed through a column of DEAE-cellulose. The peak of calpactin-I complex (lane G) was used as the pure protein whereas the monomer fraction (lane H) was further fractionated by hydroxylapatite chromatography (see Fig. 2). Additional samples represent the calpactin-I complex (I and K) and monomer (J and L) fractions from preparations of human placenta (I and J) and bovine adrenal gland (K and L). Coomassie Blue–stained 12% acrylamide SDS gel.

Table I. Summary of the Purification of Calpactin from Bovine Lung and Human Placenta

	Bovine lung		Human placenta	
	Total protein	Calpactin-I	Total protein	Calpactin-I
	mg	*mg*	*mg*	*mg*
Extract	24,600	204	31,000	53
First Ca^{++} pellet	3,240	201	3,080	43
Second Ca^{++} pellet	1,250	232	570	36
EGTA soluble protein	780	230	304	48
Third Ca^{++} pellet	440	195	96	34
S-200 column starting solution	171	108	81	22
Complex (90,000 M_r) peak	83	60	31	11
Monomer (36,000 M_r) peak	27	12	19	7
DEAE flow-through (complex)	45	45	9.6	9.6
DEAE flow-through (monomer)	14	6.1	11	5

The total protein at each step was determined using the BCA protein assay (Pierce Chemical Co.) and the amount of calpactin was determined by Western blots using (*a*) anti–bovine intestinal calpactin-I and (*b*) comparison to known amounts of pure calpactin run in adjacent lanes of the same gel.

and light chains was as described (22). Ca^{++}-binding by equilibrium dialysis of calpactin-II in the presence or absence of phospholipid was performed as described (23). Actin-binding and -bundling was monitored as in reference 31. Sequence determination was performed as in reference 28. Antibodies were elicited in rabbits against bovine calpactin-II (see Fig. 2, Peak *A*) which was further purified by reverse-phase HPLC using a wide pore C-4 column (J. T. Baker Chemical Co., Philipsburg, NJ) and eluting with a linear 40–60% acetonitrile gradient in 0.1% trifluoroacetic acid. Peak fractions were dried under vacuum, sonicated in PBS, emulsified with an equal volume of Freund's complete (first injection) or incomplete (subsequent injections) adjuvant and 400 μg was injected subcutaneously and intramuscularly. Antibodies were purified from the serum by affinity chromatography using Peak A protein (see Fig. 2) coupled to Sepharose and elution from the column with 4 M MgCl$_2$. Western blots were performed as described (25) using a 1:200 dilution of anti–p35 serum (see Fig. 2) (generously provided by Stanley Cohen, Vanderbilt University, Nashville, TN) or 2 μg/ml affinity-purified anti–calpactin-II (see Fig. 9). Double-label immunofluorescence microscopy on human fibroblasts was performed on cells fixed for 2 h in 3.7% formaldehyde and permeabilized with 0.2% saponin in PBS containing 20 mM ethanolamine, pH 8.0. A mouse monoclonal antibody to human calpactin-I (40) was provided by Dr. Claire Isacke (The Salk Institute) and used at a 1:20 dilution of ascites fluid.

The calpactin-I heavy chain, derived from the calpactin-I complex was separated from the light chain by denaturation in 6 M guanidine, gel filtration on Sephacryl s-300, and renaturation essentially as described previously (28). The core was purified from chymotryptic digests of calpactin-I as in reference 29. Protein determinations were made using the BCA assay (Pierce Chemical Co., Rockford, IL).
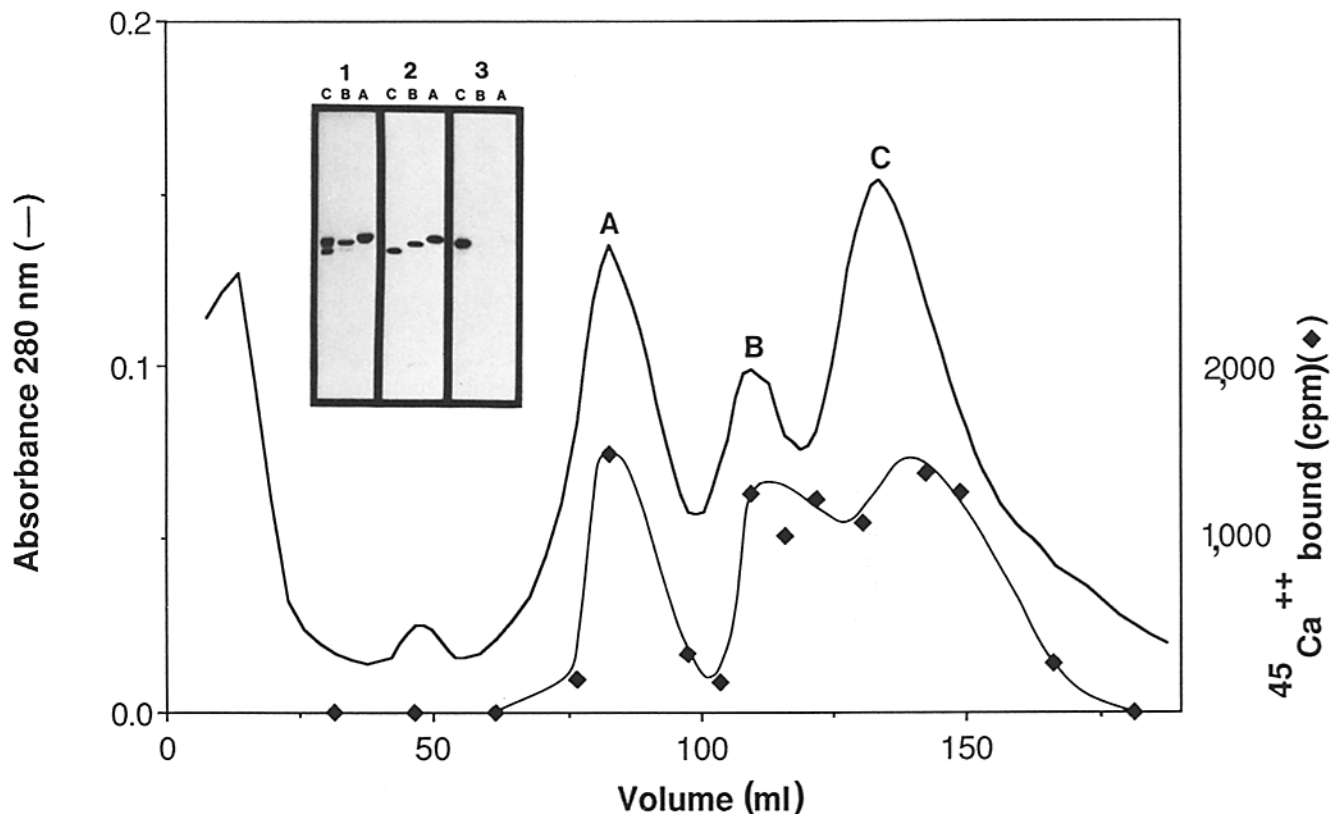

Figure 2. Fractionation of monomeric calpactins on hydroxylapatite. The calpactin monomer peak, after passing through a DEAE column in low salt, was loaded onto a hydroxylapatite column which was developed with a linear 0–200 mM NaPO$_4$ gradient. Fractions were monitored for absorbance at 280 nm (*solid line*) and selected fractions tested for Ca^{++}-binding activity in the presence of 200 μg phosphatidylserine per ml by equilibrium dialysis at 10 μM free Ca^{++} (*solid symbols*). The radioactivity bound represents cpm per 15 μl of the fraction above buffer where the buffer alone gave a value of 6,000 cpm. (*Inset*) SDS PAGE and Western blot analysis of calpactin peaks from the hydroxylapatite column. Lanes *A*, *B*, and *C* represent pooled fractions corresponding to peaks *A*, *B*, and *C*. One set (*1*) was stained with Coomassie Blue while the other two were transferred to nitrocellulose and treated with antibodies to human calpactin-II from A431 cells (*2*) or antibodies to bovine intestinal calpactin (*3*). Blots were then treated with ^{125}I–protein A and subjected to autoradiography.
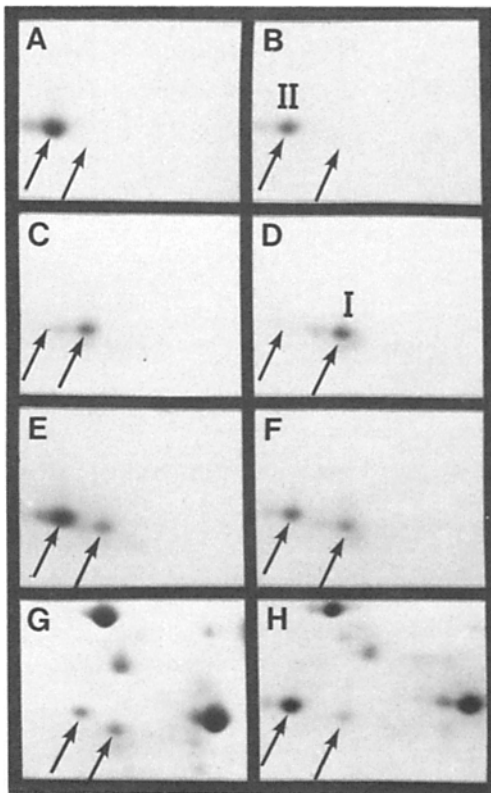
Figure 3. Two-dimensional gel electrophoresis of calpactin monomer fractions. Bovine lung (*A, C,* and *E*) or human placenta (*B, D,* and *F*) calpactin-I (*C* and *D*), calpactin-II (*A* and *B*), or a mixture of the two (*E* and *F*) were run on a non-equilibrium pH gradient electrophoretic (NEPHGE) separation system for separation by charge in the first dimension (migration from left to right, with the more basic component to the right) and size (SDS PAGE) in the second dimension (migration from top to bottom). For comparison, an extract of A431 cells either alone (*G*) or mixed with placental calpactin-II (*H*) were analyzed. Only the relevant portions of the Coomassie Blue–stained gels are shown.

Results

Purification and Characterization of Calpactins

Previous methods for the purification of calpactin-I as a complex of heavy and light chain subunits from mammalian tissue involved the preparation of microvillus vesicles in Ca^{++} and the elution of calpactin with EGTA (18, 27). With the finding that a high degree of purity can be achieved by reprecipitating with Ca^{++} (24), we sought to exploit this observation using other tissue sources. As shown in Fig. 1, cycles of Ca^{++}-induced precipitation followed by EGTA resolubilization from an extract of lung resulted in the en-

richment of polypeptides with molecular masses of 73,000, 36,000, and 32,000. Gel filtration of this material in high salt, low Ca^{++}-containing buffers allowed the separation of a high molecular mass material, which eluted at the void volume, from the other polypeptides. Of particular interest were two peaks containing 36-kD proteins, one peak eluting at a position corresponding to a native molecular mass of 95,000 and the second at the 35,000–40,000-mol-wt position. We used Western blotting to quantitate the amount of calpactin-I present at various stages in this procedure using an antibody to intestinal calpactin-I (23). As shown in Table I, this procedure is quite selective for calpactin. Starting with 750 g of tissue, we could isolate 45 mg of calpactin-I as a complex with light chain from bovine lung or 10 mg from human placenta (Table I). When the amount of total protein is compared to the amount of calpactin-I as determined with a Western blot, it is clear that the peak of calpactin eluting from the gel filtration column in the monomer position consisted of more than one component, even after ion exchange chromatography (Table I). Fractionation of this protein on a hydroxylapetite column revealed that at least three Ca^{++}-binding components (labeled *A, B,* and *C*) were present (Fig. 2). Since previous studies from this laboratory have shown that two distinct calpactins are detectable in cultured cells (22), we tested the three peaks for immunoreactivity with antibodies to calpactin-I and -II (also termed p35). As shown in the inset to Fig. 2, antibodies to calpactin-II react with distinct molecular weight variants in all three peaks whereas anti–calpactin-I reactivity is largely confined to a 36-kD band in peak C. A likely interpretation is that intact calpactin-II is represented in Peak A, and slightly smaller variants of calpactin-II, due to proteolytic nicking, were separated into peaks B and C. A similar profile was observed with protein derived from human placenta (not shown).

Single Coomassie-stained spots were observed for the 36-kD proteins from the hydroxylapatite column (Peaks A and C) of both bovine lung and human placenta. When mixed, the two spots (Peaks A and C) were clearly resolved, and the Peak A component from human placenta was found to exactly co-migrate with the previously identified calpactin-II spot (22) from A431 cells (Fig. 3 *H*). The calpactin-I monomer co-migrated with the heavy chain of the calpactin-I complex in this gel system (not shown).

Amino acid sequence analysis was performed on calpactin-II–related proteins (Fig. 4). Whereas the higher molecular mass form (Peak A, Fig. 2) did not give rise to a sequence, indicating an apparently blocked amino terminus, a unique sequence of 28 residues was observed for the slightly lower molecular mass variant of bovine calpactin-II (Peak B, Fig. 2). Comparison of this sequence to the predicted structure of the amino-terminal region of human lipocortin (53) revealed that 25 of 28 residue positions were identical,

PEAK A   NONE

PEAK B   F I E N E E Q E Y I K T V K G S K G G P G S A V S P Y P ....

HUMAN LIPOCORTIN A M V S E F L K Q A W F I E N E E Q E Y V Q T V K S S K G G P G S A V S P Y P T F N ...

Figure 4. Amino-acid sequence analysis of the bovine calpactin-II derivatives (see Fig. 2). No sequence was observed for the highest molecular mass form of calpactin-II (*peak A*) indicating a blocked amino terminus, whereas a sequence commencing with phenylalanine was observed for *peak B* (all sequences given in the single letter amino acid code). The bottom line represents the predicted amino-terminal sequence of human lipocortin (53) for comparison. Vertical lines indicate positions where the amino acid is identical between lipocortin and peak B protein.
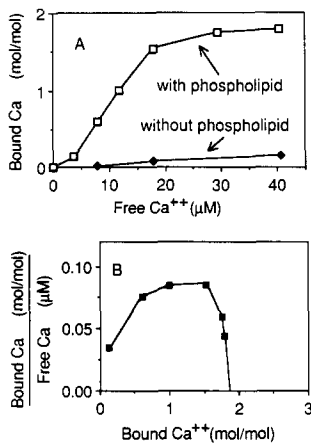
Figure 5. Effect of phospholipid on Ca^{++} binding by calpactin-II. Calpactin-II in the presence (*open squares*) or absence (*solid diamonds*) of phosphatidylserine was subjected to equilibrium dialysis with $^{45}CaCl_2$ at increasing Ca^{++} levels. After dialysis, the amount of Ca^{++} bound was determined by scintillation counting and the amount of protein determined using the Pierce BCA protein assay. *B* is the scatchard plot of Ca^{++} binding by calpactin-II in the presence of phosphatidylserine.
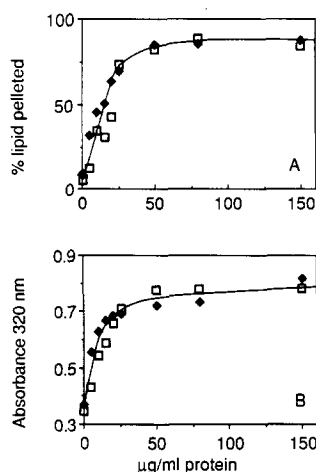

Figure 6. Liposome aggregation induced by calpactin-I and Ca^{++}. Calpactin-I (*solid symbols*) or the calpactin-I core (*open symbols*), which lacks the amino-terminal tail and the light chain, were incubated at the specified concentration with liposomes containing ^{14}C-phosphatidyl-serine/phosphatidylethanolamine/cholesterol (1:1:1). After 30 min, light scattering was measured (absorbance at 320 nm; *b*). The tubes were then capped and centrifuged at 10,000 g for 5 min and the distribution of lipid was determined by scintillation counting of supernatant and pellet fractions (*a*).

thereby indicating that bovine calpactin-II and human lipocortin are equivalent proteins.

Ca^{++} Binding

Calpactin-I and its core have been shown to bind Ca^{++}, with the binding greatly enhanced by phospholipid (23). To determine whether calpactin-II has this activity, equilibrium dialysis was performed with ^{45}Ca in the presence or absence of phospholipid. In the absence of phospholipid, Ca^{++} binding was barely detectable at the micromolar range of Ca^{++} (Fig. 5 *A*). In the presence of phosphatidylserine, by contrast, calpactin bound stochiometric amounts of Ca^{++} with two Ca^{++}-binding sites detected with dissociation constants in the

range of 10 µM. Interestingly, Scatchard analysis revealed a curve rather than a straight line which would be expected if the binding is cooperative (Fig. 5 *B*). Such a phenomenon was not seen with calpactin-I (23).

Phospholipid Aggregation by Calpactin

Calpactins aggregate liposomes as detected by three independent methods. First, at increasing calpactin concentrations, light scattering increased (Fig. 6 *A*). Second, calpactin caused liposomes to form aggregates that were collected by low speed centrifugation (Fig. 6 *B*). Third, when fluores-
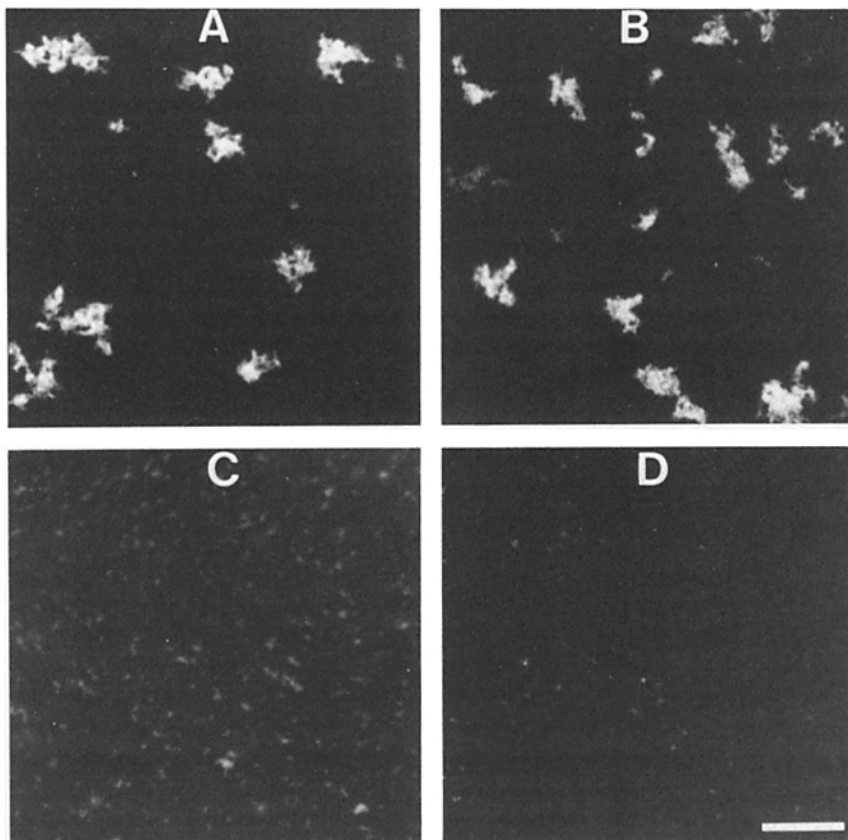

Figure 7. Liposome aggregation as monitored microscopically using rhodamine-loaded liposomes. Liposomes loaded with rhodamine-conjugated IgG were mixed with 125 µg calpactin-I per ml (complex of heavy and light chains) (*A*), with the calpactin-I core (*B*), with calpactin-II (*C*), or with serum albumin (*D*). After 30 min at room temperature, aggregates were visualized in the fluorescence microscope. All micrographs were taken at the same magnification with the same exposure time and printed identically. Bar, 40 µm.
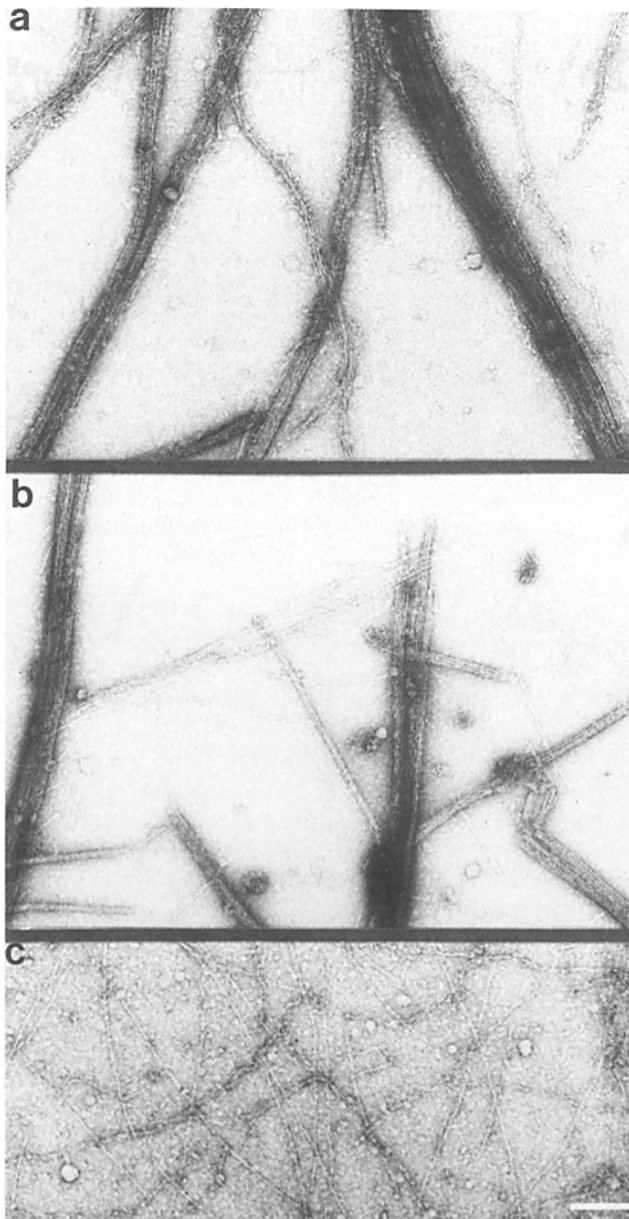
Figure 9. Comparison of the tissue distribution of calpactin-I and -II. Human platelets (*d*), erythrocytes (*e*), or placenta (*f*), and mouse spleen (*g*), kidney (*i*), liver (*j*), lung (*k*), intestine (*l*), cardiac muscle (*m*), skeletal muscle (*n*), and brain (*o*), and rat adrenal gland (*h*) were solubilized directly into SDS sample buffer. Proteins were resolved by SDS PAGE and transferred to nitrocellulose membrane. Membranes were then treated with antibodies to calpactin-I (*upper gel*) or calpactin-II (*lower gel*) followed by ^{125}I-protein A and autoradiography. Standards of calpactin-I (*c*), calpactin-II (see Fig. 2, peak A [*a*] and peak B [*b*]) were included in adjacent lanes of each gel. Only the relevant portion of each autoradiograph is shown.

Figure 8. Actin bundling by calpactins. Calpactin-I core (*a*) or calpactin-II (*b* and *c*) were incubated with actin at high (*a* and *b*) or low (*c*) Ca^{++} concentrations. The solution was applied to a carbon-coated grid, stained with 2% uranyl acetate, and observed in a Zeiss electron microscope operated at 80 kV. Bar, 0.9 μm.

cently loaded liposomes were used, the aggregation could be detected in the fluorescent microscope (Fig. 7). Without calpactin present a fluorescent haze was observed since individual liposomes are below the level of detection in the light microscope. Large fluorescent aggregates were the dominant feature of those liposome preparations to which calpactin was added. Surprisingly, the core (which lacks the amino-terminal tail and light chain) possessed the same lipid-aggregating activity as the calpactin complex as monitored by all three assays (Figs. 6 and 7).

Actin-binding and -bundling by Calpactins

Since the calpactin-I complex has been shown to bind to and bundle actin filaments at high Ca^{++} (18, 27), we tested
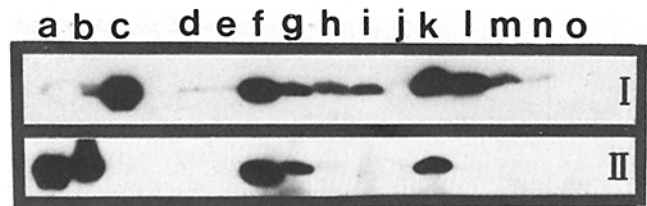
whether calpactin-II and the calpactin-I core have the same effect. Both calpactin-I and -II and the calpactin-I core bind to actin filaments as judged by a cosedimentation assay (data not shown). Controls showed that in the absence of Ca^{++}, calpactins were only found in the supernatant of a high speed centrifugation assay, whereas with actin present they were found in the pellet. Calpactin also bundles actin at high Ca^{++} concentrations. In the absence of calpactin, only single actin filaments were observed, whereas large bundles of actin were found with either calpactin-II, calpactin-I, or the calpactin-I core (Fig. 8).

Both calpactin-I and -II have been found in three different cell lines. Thus, it becomes important to determine whether both calpactins are present together in all situations or whether they are independently expressed. To determine this distribution we used Western blots of total tissue protein solubilized directly in SDS sample buffer, run on a 10% acrylamide gel and probed with anti–intestinal calpactin-I or anti–lung calpactin II. As shown in Fig. 9, in cells where calpactin-I is low or undetectable, such as brain, liver, platelets and red cells, calpactin-II is also negligible. Two tissues stand out as high in calpactin-II; placenta and lung, and both tissues are rich in calpactin-I. In other tissues, such as intestine, a high level of calpactin-I was observed with undetectable amounts of calpactin-II present. Although an extensive tissue survey was not performed, we found no situation where there was high calpactin-II in the absence of calpactin-I.

Since many tissue culture cells are known to contain high levels of calpactin-I and -II, and calpactin-I is known to be just under the membrane, we analyzed the distribution of calpactin-II in human fibroblasts by double-label immunofluorescence microscopy. As shown in Fig. 10, calpactin-II was found in a rather punctate distribution, much more discrete than calpactin-I in the same cells. The pattern of the two calpactins was clearly similar and the distribution was partially overlapping. This indicates that calpactin-II is in the same type of cortical microfilament network.

Discussion

Calpactin was isolated from bovine lung and human placenta. Typical yields of 30–60 mg of the calpactin-I complex
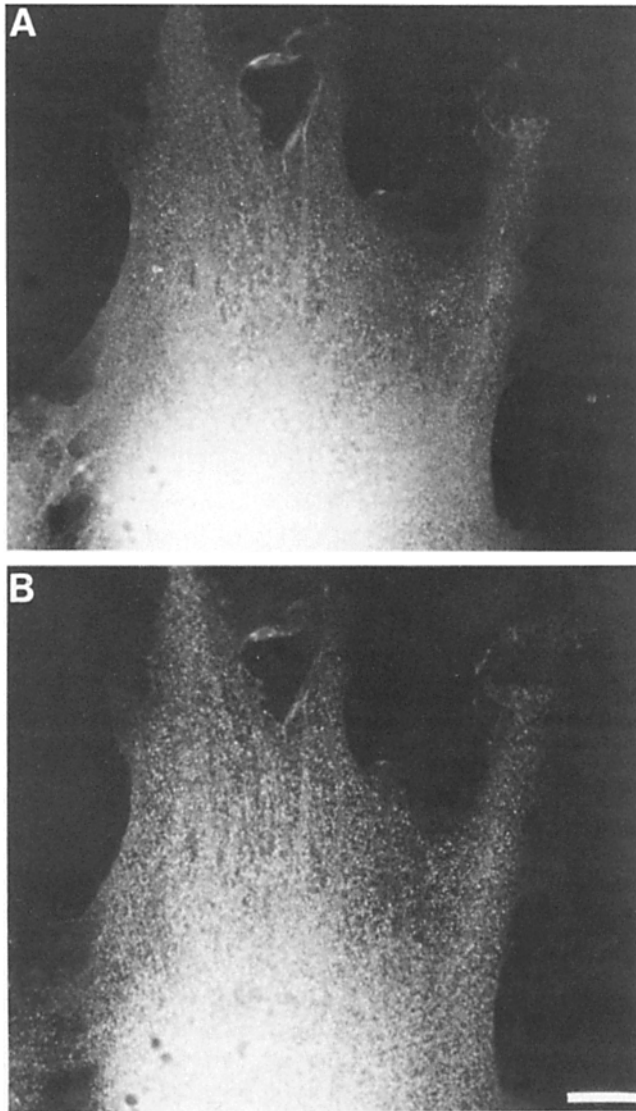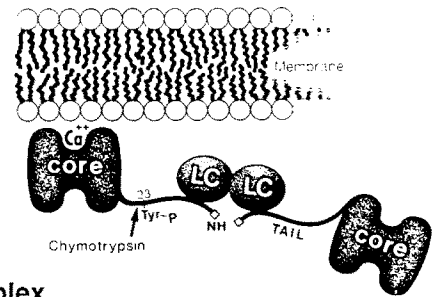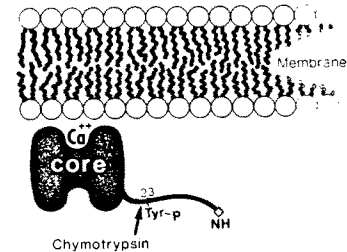
Figure 10. Double-label immunofluorescence microscopy of cal-pactin-I (*A*) and calpactin-II (*B*) in the same human fibroblast cell. Cells grown on coverslips were fixed in formaldehyde, permeabi-lized in saponin, and treated with a mouse monoclonal anti–cal-pactin-I antibody (*A*), plus rabbit anti–calpactin-II affinity-purified antibodies (*B*). Reactivity was visualized with fluorescein-conju-gated sheep anti–mouse IgG plus rhodamine-conjugated goat anti-rabbit IgG antibodies and observed in the fluorescein (*A*) or rhoda-mine (*B*) channels. Bar, 20 μm.

Calpactin-I complex

Calpactin-I monomer

Calpactin-II

Figure 11. Membrane association of three forms of calpactin found in many cells. See Discussion for detail.
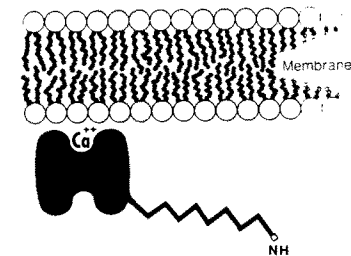
are the highest thus far reported and should allow a more complete analysis of this molecule. In addition, calpactin-II, a related but distinct protein, and the calpactin-I monomer were isolated in lesser amounts as a by-product. We had previously used a similar type of procedure to isolate the calpactin-I complex from bovine intestine (23). In that prep-aration, the calpactin-I core was an abundant component of the monomer peak of the gel filtration separation (23). There was no indication that calpactin-II was present in that fraction and indeed the present results (Fig. 9) indicate that very little calpactin-II is present in intestine. In lung, how-ever, the situation is different. The monomer peak yielded significant amounts of calpactin-II, fragments of calpactin-II

(Fig. 2), and intact calpactin-I monomer, whereas little cal-pactin-I core was found.

Fig. 11 is an attempt to summarize the following features of calpactins. (*a*) Three forms of calpactin have been de-scribed; the calpactin-I complex of heavy and light chains; the calpactin-I monomer, and calpactin-II. (*b*) The calpactin light chain, which is homologous to S-100 (20, 31, 37), is known to exist as a dimer (19). (*c*) The heavy chain of calpac-tin-I can be envisioned as consisting of two distinct domains, a 33-kD core domain and a 3-kD tail which can be dissected by limited chymotryptic cleavage at residues 23 and 29 (28). (*d*) Both calpactins have two Ca^{++}-binding sites, both of which have been localized to the core and are probably on opposite sides of the molecule. (*e*) Calpactins bind to phos-pholipids (21, 23, 42; see also Results), and phospholipids enhance the affinity of calpactin for Ca^{++} (23). (*f*) The light chain interacts with the blocked amino-terminal tail of the calpactin-I heavy chain (29), but not with calpactin-II (22). (*g*) The site of phosphorylation by the oncogene pp60[src] is known to reside in the tail (22). This model does not address the possibility of linkage to the cytoskeleton by actin or spectrin, nor does it incorporate potential phospholi-pase A2 inhibitory activity (43, 46, 48; see below). In addition, the interactions and structure are necessarily oversimplified.

The full sequence of both calpactin-I heavy chain and lipocortin has recently been determined and compared (38, 43, 48). The two proteins display approximately a 50% over-

all sequence homology. Four 70-amino acid repeats were observed for both proteins in the previously recognized "core" region, which is known to bind Ca^{++} and phospholipid and this is connected to a 30-amino acid tail which represents the amino terminus of calpactins. The amino acid sequence homology between lipocortin and the heavy chain of calpactin-I is unequally distributed through the polypeptide with the highest degree found in the repeats within the core (43–70% identity) and the least homology (15%) in the tail (43, 48). A detailed comparison of the functions of these proteins then should help us to understand the implications of this sequence homology.

The core domain of the calpactin-I heavy chain has been shown previously to bind Ca^{++} and phospholipid, with phospholipid able to increase the affinity of calpactin for Ca^{++}. In the presence of phosphatidylserine, each calpactin heavy chain monomer binds two Ca^{++} ions with a K_d of 4.5 μM. Although other proteins such as protein kinase C and the Ca^{++}-activated protease are known to shift their Ca^{++} sensitivity in response to phospholipids, direct Ca^{++}-binding measurements for this class of proteins has only been reported for calpactin-I. We now show that each calpactin-II molecule also binds 2 mol of Ca^{++} per mol, also dependent on phospholipid. Interestingly, the affinity for Ca^{++} is slightly lower (K_d = 10 μM) for calpactin-II than for calpactin-I, and the binding appears cooperative (Fig. 5). These features should be kept in mind when attempting to assign the Ca^{++}-binding site to particular regions of the calpactin-I and -II sequence.

Calpactins bind to and aggregate phospholipids. This was found using a low speed centrifugation assay, light scattering (Fig. 6), and microscopic visualization of fluorochrome trapped in liposomes (Fig. 7). This aggregation phenomenon is probably related to the Ca^{++} binding which is greatly enhanced by phospholipids. Thus, we suggest that a ternary complex of calpactin, Ca^{++}, and phospholipid is formed, with these sites found on opposite sides of each core domain (Fig. 11). If the two Ca^{++} and phospholipid sites were on the same side of the molecule, for instance, one might expect lipid binding but not aggregation or cross-linking. In this respect, calpactin-I and -II display an activity similar to synexin (7) or calelectrin (17, 52). In those cases, however, vesicle aggregation required millimolar concentrations of Ca^{++}, whereas it is found at 10 μM Ca^{++} with calpactins (Fig. 7). Another difference is that aggregation with calpactin was measured using pure lipid rather than membrane vesicles.

Previous studies have shown that the calpactin-I complex binds to, and bundles actin filaments in the presence of Ca^{++} (18, 27). In the present study, we find that the calpactin-I core and calpactin-II also bundle actin filaments. The simplest model of a bundling protein is one which has two actin-binding sites and forms cross-linkages between parallel-oriented actin filaments at regular intervals (11). The fact that the calpactin core has this effect would suggest that two actin-binding sites are found on each heavy chain subunit. Thus, the fourfold repeat structure may also include two or more actin-binding sites in addition to two Ca^{++}/phospholipid sites.

Calpactin-I has been previously shown to have the properties of a cytoskeletal protein (4) and immunofluorescence microscopy has demonstrated that it is present in a submembraneous distribution similar to spectrin. The list of proteins which are present in this network includes erythrocyte cytoskeletal proteins (see above) and a 73-kD protein which co-purifies with calpactin-I through much of the procedure (24) and is probably the 68-kD protein which has been isolated from porcine lymphocytes (8). We can now include calpactin-II in this cytoskeletal network. Immunofluorescence microscopy reveals that the two calpactins have a very similar, although not identical, distribution. The distribution of calpactin-II appears more punctate although the nature of these fluorescent dots is not known. It is possible that this corresponds to some type of small vesicle population in the cell. Future experiments will explore this possibility.

After this work was completed, a report by De et al. appeared concerning the isolation of a 35-kD substrate for the epidermal growth factor receptor kinase from porcine lung (10). According to the amino acid sequence which was presented, they have isolated the truncated form of calpactin-II (peak B of Fig. 2; see Fig. 4) and shown that tyrosine 20 is phosphorylated by the epidermal growth factor receptor. The results on the tissue distribution of p35 in adult tissues is in substantial agreement with the data presented herein (e.g., high in lung and placenta and low in liver and intestine). In that study they suggested that calpactin-II (p35) binds Ca^{++}. This is now confirmed by direct Ca^{++}-binding measurements in the presence or absence of phospholipids (Fig. 5).

Lipocortin has been characterized as an extracellular protein which inhibits pancreatic phospholipase A_2. Although both calpactins have an identical phospholipase inhibitory activity (Davidson, F. F., E. A. Dennis, M. A. Powell, and J. R. Glenney, Jr., J. Biol. Chem., in press) this is due to their effect on phospholipids rather than the enzyme itself. Regardless of the mechanism, the question arises as to whether the major biological function of calpactin is the inhibition of intracellular phospholipase A_2. Given that calpactins are major cellular proteins, and are cytoskeletal proteins that directly interact with the lipid bilayer, we prefer the idea that they are membrane-to-cytoskeleton linkage molecules. The availability of a method for the large-scale purification of these proteins should allow rapid progress in answering these questions.

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