Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4258–4262, June 1986
Biochemistry

Two related but distinct forms of the M_r 36,000 tyrosine kinase substrate (calpactin) that interact with phospholipid and actin in a Ca^{2+}-dependent manner

(cytoskeleton/membrane/growth factor/p36)

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Communicated by Robert W. Holley, March 6, 1986

ABSTRACT **A method was devised that allows the identification of proteins related to the M_r 36,000 tyrosine kinase substrate calpactin based on their ability to interact with actin and phospholipid in a calcium-dependent manner. Two distinct proteins, detected in human A431 cells and fibroblasts, were resolved by two-dimensional gel electrophoresis. One of these proteins (calpactin I) appears identical to the M_r 34,000–39,000 substrate of the pp60^{src} tyrosine kinase and the second (calpactin II) reacts with antibodies to the M_r 35,000 substrate of the epidermal growth factor receptor. Both proteins interact with phospholipid and actin, are rather basic, and share structural and antigenic determinants. A major difference between the two proteins is noted in their state of association with the M_r 10,000 light chain; i.e., calpactin I is associated with the light chain while calpactin II is not.**

A major substrate of the oncogene and growth-factor receptor tyrosine kinases is a protein with an apparent molecular weight estimated at 34,000–39,000 on $NaDodSO_4$/PAGE (1–6). This protein has been characterized as rather basic in net charge with the properties of a cytoskeletal protein (7, 8). Indeed, immunofluorescence microscopy has demonstrated that it is part of the submembrane skeleton and at least partially codistributes with the membrane–skeletal element spectrin (9–12). Although having a somewhat limited tissue distribution (13, 14), p36 (calpactin) is particularly abundant in intestinal epithelial cells from which it can be isolated in large amounts as a M_r 90,000 complex (15). This complex consists of two copies of a M_r 10,000 subunit (15, 16) homologous to the S-100 proteins of brain (17–19), together with two M_r 36,000 subunits.

Consistent with its subcellular distribution, intestinal calpactin has been shown to have the ability to interact with actin and spectrin, requiring millimolar levels of Ca^{2+} to detect this interaction (15, 20). Intestinal calpactin also serves as an excellent substrate for the protein tyrosine kinase pp60^{src} *in vitro* (21), being phosphorylated at tyrosine-23 (17). Although the *in vitro* phosphorylation of calpactin was also greatly enhanced by millimolar levels of Ca^{2+} (21), it was found that the Ca^{2+} sensitivity could be shifted to the micromolar level with phospholipids in a manner similar to the activation of protein kinase C by Ca^{2+} and phospholipid. Consistent with this it was shown that calpactin interacts directly with phospholipid liposomes in the presence of Ca^{2+} but not in its absence (21).

The identification of substrates for the epidermal growth factor receptor tyrosine kinase in human A431 cells has revealed an additional substrate with M_r 35,000 (p35) (22, 23). p35 is thought to be unrelated to p34 (calpactin), even though its properties are rather similar, being a basic protein that interacts with the membrane in a Ca^{2+}-dependent fashion in addition to being Ca^{2+}-dependently phosphorylated by the epidermal growth factor receptor (22). To resolve the relationship among these M_r 36,000 tyrosine kinase substrates and to identify the number of similar proteins in A431 cells and fibroblasts, a two-step affinity procedure was developed based on the Ca^{2+}-dependent interaction with actin and phospholipid. Using this method, two major components can be resolved by two-dimensional gel electrophoresis. Since both proteins are calcium-dependent phospholipid- and actin-binding proteins, they are referred to as calpactins. The more basic and slightly lower molecular weight form (termed calpactin I) appears more related to what has been termed p34, p36, or p39, and the second form (calpactin II) is probably the 35-kDa protein.

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MATERIALS AND METHODS

A431 cells were from the American Type Culture Collection. Human diploid fibroblasts (AG1523), obtained from the National Institute of Aging, Aging Cell Repository, Institute for Medical Research (Camden, NJ), were a gift from Clare Isacke (The Salk Institute), and NIH 3T3 cells were a gift from Inder Verma (The Salk Institute). Cells were cultured in Dulbecco–Vogt-modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum. For [^{35}S]methionine labeling, cells were grown overnight in DMEM without methionine, and supplemented with 200 μCi of [^{35}S]methionine per ml (1 Ci = 37 GBq; New England Nuclear) in addition to 10% (vol/vol) dialyzed fetal bovine serum.

To identify proteins related to calpactin in labeled cells, monolayers were rinsed with Tris-buffered saline (20 mM Tris·HCl, pH 7.5, 0.15 M NaCl), then once with E buffer (10 mM imidazole, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM NaN_3, pH 7.3) followed by extraction in E buffer containing 0.5% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2 mM benzamidine. Typically a 2-cm dish of subconfluent cells was treated with 175 μl of E buffer and 0.5% Triton X-100 for 5 min at room temperature with constant agitation. The extract was clarified by centrifugation (100,000 × *g*, 20 min in a Beckman Airfuge operated at 4°C). An aliquot was removed for two-dimensional gels, and the clarified extract was adjusted to 0.5 mg of chicken skeletal muscle F-actin (24) per ml, 50 mM imidazole (pH 7.3), 2.5 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, and 2 mM $CaCl_2$. After incubation for 30 min at room temperature, actin and associated proteins were pelleted by centrifugation in the Airfuge as before. The actin pellet was resuspended in 60 μl of elution buffer (10 mM imidazole, pH 7.3, 10 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP) and, after 20 min, centrifuged as before. The soluble protein was mixed with 2 vol of 50 mM imidazole (pH 7.3), 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 mM $CaCl_2$, 500 μg of phospholipid liposomes per ml. Phospho-

lipid liposomes consisted of 2 mg of cholesterol, 1 mg of phosphatidylcholine, 1 mg of phosphatidylserine, 1 mg of phosphatidylinositol (all lipids from Sigma), dried under vacuum, and sonicated in H_2O. Labeled protein with liposomes and Ca^{2+} were incubated 0.5 hr at room temperature followed by centrifugation (100,000 × *g*, 30 min). The pellet was dissolved directly in two-dimensional gel sample buffer. Samples at other stages in the procedure were dried under vacuum (Speedvac, Savant) and dissolved in sample buffer. ^{35}S-labeled proteins were mixed with unlabeled bovine intestinal calpactin (17), which was localized by Coomassie blue staining after two-dimensional gel electrophoresis.

For larger scale preparation of proteins, cells were grown in roller bottles and harvested by scraping in Tris-buffered saline followed by low-speed centrifugation. Cell pellets were extracted with 2 vol of E buffer and 0.5% Triton X-100 for 30 min at 0°C, followed by high-speed centrifugation (150,000 × *g*, 1 hr). Extracts were then processed for Ca^{2+}-dependent actin and phospholipid binding as above or dialyzed 3–4 hr against 10 mM imidazole (pH 7.3), 1 mM EGTA, 1 mM NaN_3, and passed through a DE-52 (Whatman) column equilibrated in the same buffer at 4°C. Protein was used directly for two-dimensional electrophoresis followed by either Coomassie staining or immunoblotting. Alternatively, the solution passed through the DE-52 column was concentrated in a dialysis bag over dry Sephadex G-200 and applied to a 1 × 100 cm column of Sephacryl S-200 (Pharmacia) equilibrated and eluted in 100 mM NaCl, 10 mM imidazole (pH 7.3), 1 mM EGTA, 1 mM NaN_3, 0.5 mM dithiothreitol. Fractions beginning with the excluded volume (V_0) were analyzed by $NaDodSO_4$/PAGE followed by immunoblotting using antibodies to bovine intestinal p36. Fractions of highest immunoreactivity were pooled, dialyzed briefly against H_2O, concentrated under vacuum, and analyzed by two-dimensional gels and immunoblotting.

Two-dimensional gels were electrophoresed using the nonequilibrium pH gradient electrophoresis (NEPHGE) system (25) in the first dimension (400 V, 4 hr) followed by the standard $NaDodSO_4$/PAGE (10% acrylamide) in the second dimension. ^{35}S was visualized by fluorography after treating the gel with Amplify (Amersham).

Peptide maps of iodinated proteins from Coomassie blue-stained spots excised from two-dimensional gels were by the method of Edler *et al.* (26) with the modifications described (27). Controls always included iodination and peptide mapping of a region of the gel with no detectable protein. Immunoblots were performed as described (20) with modifications. After electrophoretic transfer to nitrocellulose, protein was stained with india ink, followed by blocking, antibody incubation, and ^{125}I-labeled protein A (28). Anti-chicken fibroblast p36 (10, 14) was provided by Tony Hunter (The Salk Institute) and anti-35-kDa protein (23) was provided by Giugni *et al.* (29). Both were used at a dilution of 1:500 in immunoblots. Calpactin (p36) was purified from bovine intestine and lung as described (17). For antibody production, intestinal calpactin was further purified by preparative $NaDodSO_4$/PAGE and electrophoretic elution. Anti-intestinal calpactin antibodies, raised in rabbits, and purified by antigen affinity chromatography, were used in immunoblots at a concentration of 1–2 μg/ml.

RESULTS

The procedure employed to identify proteins related to calpactin takes advantage of the functional properties of this molecule. Since calpactin (p36) has been shown to interact with both actin (15, 20) and phospholipid (21) in a Ca^{2+}-dependent fashion, a simple two-stage centrifugation procedure based on these properties was devised. As shown in Fig. 1, when this procedure was applied to A431 cells, two components were detected with one variant slightly more acidic and with a slightly higher apparent molecular weight. Using an internal marker of bovine calpactin (which gave rise to only a single Coomassie-stained spot) these same two spots could be detected in the initial extract and were present in about the same ratio in the actin-binding (Fig. 1*B*) and phospholipid-binding (Fig. 1*C*) steps in the procedure. The overall yield of these two components in the phospholipid pellet was always more than 50% compared to the initial extract. Both components were also observed in human (Fig. 1*D*) and mouse (Fig. 1*E*) fibroblasts.

Since both proteins observed in [^{35}S]methionine-labeled cells have the properties one would expect for calpactin (p36), we tested them for reactivity with antibodies to chicken and bovine proteins. These were both major components in A431 cells and human fibroblast and were easily

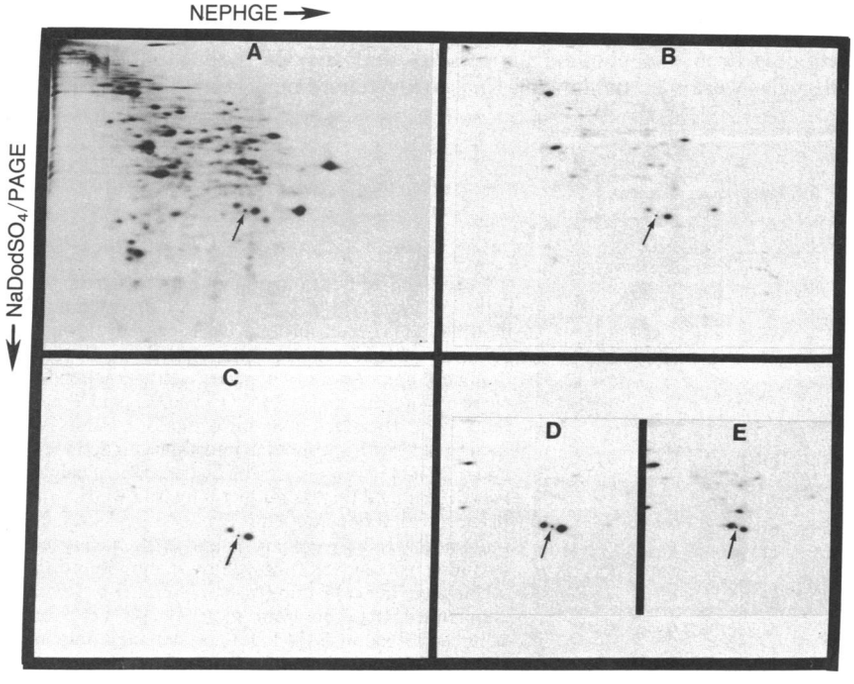

FIG. 1. Identification of calpactins in cells grown in culture. Human A431 cells (*A–C*), human fibroblasts (*D*), or NIH 3T3 cells (*E*) were labeled overnight with [^{35}S]methionine and lysed in detergent and EGTA-containing solution. After centrifugation the clarified extract (*A*) was adjusted to 0.5 mg of actin per ml, 1 mM free Ca^{2+}, and the actin filaments were harvested by centrifugation. The components eluted from actin with low Ca^{2+} (*B*, *D*, and *E*) were then adjusted to 3 mM free Ca^{2+} and 0.5 mg of phospholipid (as liposomes) per ml. Liposomes containing bound protein were collected by centrifugation (*C*). Samples were analyzed by two-dimensional gel electrophoresis based on separation by charge (NEPHGE) in the first dimension (more basic proteins to the right side) and size ($NaDodSO_4$/PAGE) in the second. An internal marker of 1 μg of bovine intestinal calpactin was included and visualized by Coomassie blue staining; the position in each gel denoted an arrow and dot. Only the relevant portions of the gels in *D* and *E* are shown. Note the two components in all three cell lines tested.

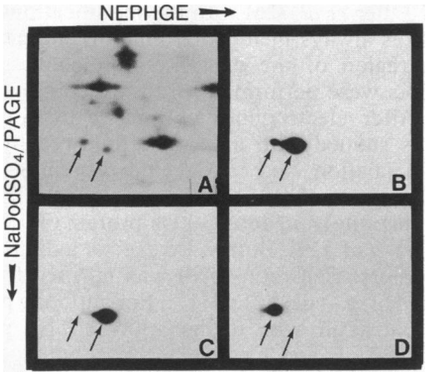

FIG. 2. Reactivity of the two calpactin spots identified in Fig. 1 with antibodies to M_r 34,000–36,000 protein-tyrosine kinase substrates. An unlabeled extract of human A431 cells was subjected to two-dimensional gel electrophoresis as in Fig. 1, and the proteins were transferred to nitrocellulose. After staining protein with india ink (*A*), blots were treated with antibodies to bovine intestinal calpactin (p36) (*B*), chick fibroblast p36 (*C*), or the 35-kDa protein described by Fava and Cohen (22) (*D*). After incubating the blots in ^{125}I-labeled protein A, reactivity was detected by autoradiography (exposure times, 2–4 hr). Arrows indicate the position of the two india ink-stained calpactin spots on the same blots. Only the relevant portions of the blots are shown.

localized in gels stained with Coomassie (not shown) or protein blots stained with india ink (Fig. 2). Since india ink does not interfere with the subsequent antibody detection (not shown), this allowed the direct comparison of antibody reactivity with the stained blot. As shown in Fig. 2, antibodies to both chicken and bovine calpactin displayed a marked preference for the lower, more basic spot (also referred to as calpactin I). Heavily overexposed autoradiograms also demonstrated that the anti-bovine calpactin also reacted weakly with the more acidic higher molecular weight form (referred to as calpactin II). This cross-reactivity was quite specific since no other proteins on the two-dimensional gels were immunoreactive. Since the 35-kDa protein described by Fava and Cohen (22) has similar properties, an antibody to this protein was tested on duplicate immunoblots. As shown (Fig. 2*d*) this antibody recognized only calpactin II with no detectable reactivity with calpactin I.

Calpactin I (p36) has been shown to exist as a M_r 90,000 complex with a light chain (10 kDa), or as a monomer, whereas the A431 p35 protein (calpactin II) has only been detected as a monomer (22). Extracts of A431 cells were size-fractionated on a Sephacryl S-200 column, and fractions were monitored by NaDodSO$_4$/PAGE and immunoblotting using anti-bovine calpactin. As shown in Fig. 3, two peaks of immunoreactivity were observed corresponding to molecular weights of 90,000 (Peak A) and 35–40,000 (Peak B). These two peaks were further analyzed by two-dimensional gels and immunoblotting using anti-bovine calpactin. In the higher molecular weight peak, only a single form (corresponding to calpactin I) was observed whereas both calpactins were detected in the lower molecular weight peak.

To determine the structural relationship between the two forms of calpactins identified in A431 cells and human fibroblasts, peptide maps of iodinated proteins were compared. Both calpactin I and calpactin II gave rise to 15–16 major iodinated chymotryptic peptides. The maps of calpactin I from human A431 cells and fibroblasts were identical to each other and strikingly similar to the bovine protein (Fig. 4), and similarly the maps of calpactin II from the two cell types were identical. Comparison of the peptide maps between calpactin I and calpactin II, however, revealed few comigrating peptides. Mixing experiments confirmed that the majority of the peptides of calpactin I were resolved from those of calpactin II. Two major iodinated peptides derived from calpactin I, however, appeared to comigrate with peptides from calpactin II (indicated by arrows in Fig. 4).

DISCUSSION

Two variants of the M_r 36,000 protein-tyrosine kinase substrate appear to exist in at least three cell lines. I will refer to these as calpactins (for calcium-dependent phospholipid- and actin-binding proteins), with the variant first described in chicken fibroblasts (1, 3) as calpactin I, and the related protein described by Fava and Cohen (22) as calpactin II. These two proteins display a number of common structural and functional features (see Table 1). Calpactin I and II appear to be Ca^{2+}-binding proteins with Ca^{2+} modulating their phosphorylation (21, 22) as well as their ability to interact with phospholipid and actin (Fig. 1). The assignment of actin and phospholipid binding by calpactin II in the present study is based on cosedimentation followed by elution with EGTA (Fig. 1); thus, further experiments with purified calpactin II are necessary to define these interactions more completely. As shown for intestinal calpactin, phospholipid appears to shift the Ca^{2+} sensitivity from high (millimolar) Ca^{2+} to low (micromolar) Ca^{2+} concentrations in

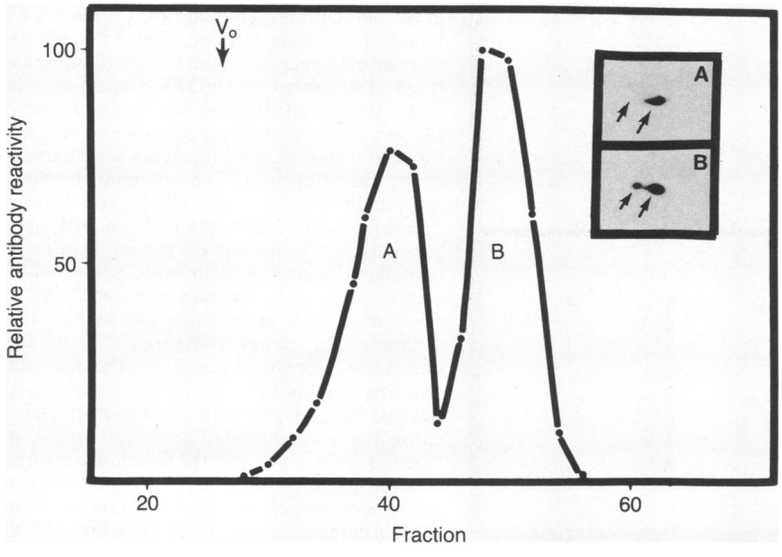

FIG. 3. Size-fractionation of proteins from A431 cells and detection of calpactin variants by immunoblotting. A431 cell protein was passed through a column of DE-52 cellulose and applied to a Sephacryl S-200 column. Aliquots of every other fraction were electrophoresed on NaDodSO$_4$/PAGE, and the proteins were transferred to nitrocellulose. Blots were then treated with anti-bovine intestinal calpactin I (p36) and ^{125}I-labeled protein A. The distribution of calpactin was determined by autoradiography followed by quantitation with a densitometer. The two peaks (A, B) of immunoreactivity were pooled and further analyzed by two-dimensional gels followed by immunoblotting using anti-bovine calpactin (*Inset*). Note the presence of calpactin I (p36) in both molecular weight forms, whereas calpactin II (35 kDa) was detected only in the position of the monomer.

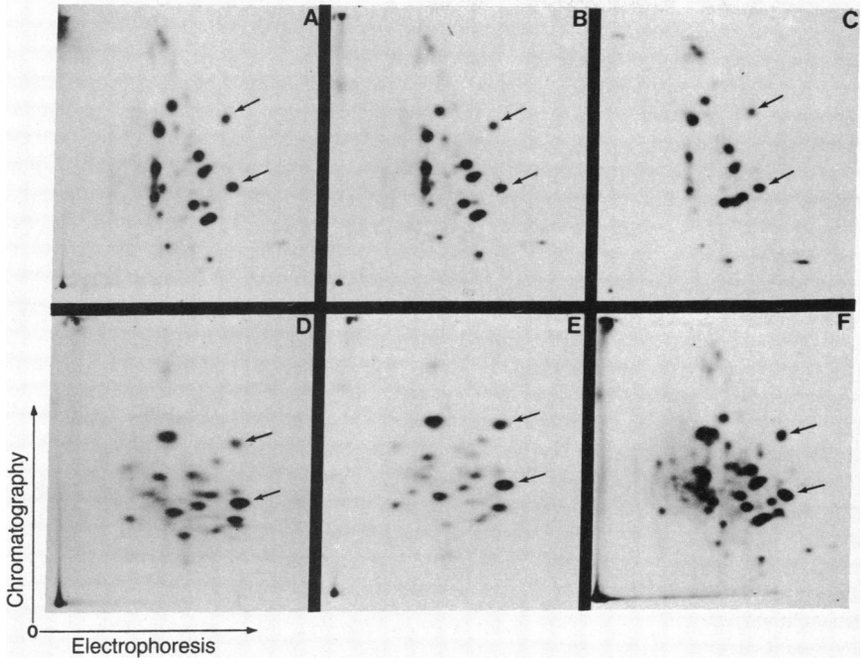

FIG. 4. Iodinated peptide maps of calpactin I and II from A431 cells (*A* and *D*) and human fibroblasts (*B*, *E*, and *F*). Calpactins I (*A–C*) or II (*D* and *E*) were excised from a Coomassie blue-stained two-dimensional gel, iodinated with chloroamine-T in the gel slice, and digested with chymotrypsin. ^{125}I-labeled peptides were resolved by electrophoresis at pH 1.9 followed by chromatography and detected by autoradiography. (*C*) Bovine calpactin I. (*F*) Mixture of human calpactins I and II. Arrows indicate the position of two peptides that are found in both calpactins I and II.

a manner similar to the effects of Ca^{2+} and phospholipid on protein kinase C (see ref. 21). In the presence of phosphatidylserine, calpactin I binds 2 mol of Ca^{2+} per mol ($K_d \approx 5$ μM), with this Ca^{2+} binding assigned to the carboxyl-terminal 33-kDa core (lacking the phosphorylation site and the light chain) (30).

Common structural features (Table 1) shared by calpactins I and II are reflected first by antibody cross-reactivity. Anti-bovine intestinal calpactin appears to recognize predominantly calpactin I from human cells, with weak, but specific, cross-reactivity with calpactin II (Fig. 2). Other antibody preparations (anti-chicken calpactin I and human calpactin II) appear to be more specific for only one of the two variants. This result is reminiscent of the situation with spectrin-related molecules. The two major forms of spectrin are clearly members of the same family yet most antibody preparations to the mammalian proteins recognize only one of the two forms (see ref. 27 for references). Secondly, peptide maps of ^{125}I-labeled calpactins reveal the presence of two apparently comigrating peptides, although the majority of peptides do not seem to be common to the two proteins. Consistent with this, one [^{35}S]methionine peptide of immunoprecipitated p36 (calpactin I) appears to comigrate with a peptide from the 35-kDa protein immunoprecipitated by anti-p35 (calpactin II) (J. Cooper and T. Hunter, personal communication). It is impossible to estimate the extent of amino acid-sequence homology reflected by such data, but it should be pointed out that since a single-amino acid change in a peptide would probably result in an altered mobility in this high resolution system, the mapping procedure may give a false overestimate of the extent of the structural difference of two related proteins. Clearly the limited number of common peptides would point to these proteins being encoded by different genes and not simply different due to the results of posttranslational modifications.

Other proteins with properties similar to the calpactins have been identified. Protein kinase C, for instance, is known to associate with phospholipid in a Ca^{2+}-dependent manner (reviewed in ref. 31). A M_r 36,000 protein from the electric organ of the eel (calelectrin) has also been shown to Ca^{2+}-dependently associate with lipid (32). Still other polypeptides, of similar size termed the chromobindins, which are extracted from adrenal medulla chromaffin cells and bind to chromaffin granules only in the presence of Ca^{2+} (33), may include one or both calpactins. The number and relatedness of calpactins, however, will ultimately come from comparison of the genes and deduced primary amino acid sequence. Full-length cDNA encoding calpactin I has been cloned and

Table 1. Comparison between M_r 34,000 and M_r 39,000 tyrosine kinase substrates

Property	Calpactin I	Calpactin II
M_r on NaDodSO$_4$/PAGE	34–39,000 (1–6)	35,000 (21)
Net charge	Basic (1–6)	Basic (21)
Association state		
Dimer with p10	+ (15, 16, *)	− (21, *)
Monomer	+ (15, 16, *)	+ (21, *)
Phosphorylated on tyrosine *in vivo*	+ (1–6)	+ (22)
Ca^{2+}-dependence of tyrosine phosphorylation *in vitro*	+ (20)	+ (21)
Antibody reactivity		
Anti-chicken p36	+ (*)	− (*)
Anti-bovine p36	+ (*)	+ (*)
Anti-35 kDa	− (*)	+ (*)
Ca^{2+}-dependent actin binding	+ (*)	+ (*)
Ca^{2+}-dependent phospholipid binding	+ (*)	+ (*)
Common ^{125}I-labeled peptides	2/16 (*)	2/16 (*)

The reference is in parentheses. +, Positive result or yes. −, Negative result or no.
*This paper.

sequenced (34). A more complete analysis of this family of proteins should now be possible.

Why does a cell need two calpactins? The two forms of calpactin are not identical, and these differences may reflect subtle differences in its function. One obvious difference resides in the association state of calpactins. Calpactin I has been shown to exist both as a monomer and as a complex with a M_r 10,000 light chain that displays a marked sequence homology with the S-100 proteins from brain (17-19). Since calpactin II is only detected as the monomeric form (ref. 22 and Fig. 3), it would appear that it has lost this high-affinity binding site for the light chain. Alternatively, other factors in the cell may govern the association between calpactin heavy and light chains.

The role of tyrosine phosphorylation of calpactins is also unclear. Calpactin I has been shown to be phosphorylated on tyrosine by both oncogene- (1-3) and growth factor receptor- (4-6) associated protein-tyrosine kinases. The phosphorylation of calpactin II thus far has only been detected in epidermal growth factor-treated A431 cells (23) and human fibroblasts (29). The use of the technique described here (Ca^{2+}-dependent actin and phospholipid binding), being quite selective for these family members, should allow the evaluation of the state of phosphorylation of calpactins in a variety of normal and transformed cells. Preliminary results (not shown) have confirmed that phosphorylated calpactin I from epidermal growth factor-treated A431 cells and Rous sarcoma virus-transformed 3T3 cells can be easily detected by this method.

Note Added in Proof. Bovine calpactin II has now been purified and Ca^{2+} binding was found to be stimulated by phospholipid (J.G. and M. Powell, unpublished data).

I thank Tony Hunter for his interest and encouragement in this project and for donating one of the antibodies used. I also thank Dr. Harry Haigler and Stanley Cohen for the gift of anti-35-kDa antibodies, Clare Isacke for helpful discussions, and Lorna White for typing the manuscript. This work was supported by U.S. Public Health Service Grant GM32866 from the National Institutes of Health.

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