

The Calpactin Light Chain Is Tightly Linked to the Cytoskeletal Form of Calpactin I: Studies Using Monoclonal Antibodies to Calpactin Subunits

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Abstract. Calpactins are a family of related Ca^{++} -regulated cytoskeletal proteins. To analyze the expression and cytoskeletal association of calpactins we raised monoclonal antibodies with specificity for the heavy or light chains of calpactin I or to calpactin II. Comparison of the tissue distribution of calpactin I heavy and light chains by Western blots revealed that these subunits are coordinately expressed. Both soluble and cytoskeletal forms of the heavy chain of calpactin I were detected in human fibroblasts whereas only a soluble pool of calpactin II was found. These two forms of the calpactin I heavy chain differed both in their state of association with the light chain and in their rate of turnover. Both the soluble pool of the calpactin I heavy chain and calpactin II turned over three to four times faster than the cytoskeletal pool of heavy and light chains. Immunofluorescence microscopy re-

vealed that the calpactin I light chain was present exclusively in the cytoskeleton whereas the calpactin I heavy chain distribution was more diffuse. No difference in the amount of light chain or the cytoskeletal attachment of phosphorylated calpactin I heavy chain was found in Rous sarcoma virus-transformed chick embryo fibroblasts compared with their normal counterpart. The antibody to the light chain of calpactin I was microinjected into cultured fibroblasts and kidney epithelial cells. In many cases antibody clustering was observed with the concomitant aggregation of the associated calpactin I heavy chain. The distribution of fodrin and calpactin II in injected cells remained unchanged. These results are consistent with the existence of two functionally distinct pools of calpactin I which differ in their association with the cytoskeleton.

CALPACTINS comprise a family of Ca^{++} -binding proteins which interact with phospholipids and the cytoskeletal proteins actin and spectrin (15, 16, 21, 22, 24, 50). Calpactin I (also termed p34, p36, or p39) is a major substrate of tyrosine protein kinases (11, 12, 34, 46) and is found in cells as either a 38-kD monomer or a 90,000 molecular mass tetramer of heavy (38 kD) and light (11 kD) chain subunits (13, 16). The 11-kD light chain was found to share amino acid sequence homology with the Ca^{++} -binding proteins termed S-100 (18, 25, 32). In addition, recent reports have documented homology between the calpactin light chain and the predicted sequence of a growth factor-inducible gene termed 2A9 (3) as well as the cystic fibrosis antigen (9). The function of the calpactin light chain is unclear and during evolution it has apparently lost the Ca^{++} -binding function expressed in S-100 (18, 22). It has been suggested that the calpactin light chain may serve a regulatory role since it binds to the same region of the calpactin heavy chain that is phosphorylated by the tyrosine kinases (26, 36). Indeed, recent evidence has shown that some serine phosphorylations in the amino terminus of the heavy chain will prevent light chain binding (35).

A second 39-kD substrate of the epidermal growth factor

receptor tyrosine kinase has been identified (14, 43). Initially termed p35, we refer to this protein as calpactin II, since it is known to be a Ca^{++} -, phospholipid-, and actin-binding protein and related to calpactin I (7, 21, 27).

The cDNAs encoding both the heavy chains of calpactins I and II have been cloned and sequenced (33, 38, 49, 51). The calpactins were first thought to be lipocortins, anti-inflammatory, secreted proteins that bind to and inhibit phospholipase A_2 (51). More recent investigations have revealed that the inhibition of phospholipase A_2 is the result of nonspecific shielding of phospholipids by calpactins (6). In addition, calpactins are clearly abundant intracellular proteins and numerous studies have shown that calpactin I is present under the membrane in a cytoskeletal meshwork partially overlapping the distribution of spectrin in the same cells (see below).

The cytoskeleton is a term used to refer to the cellular framework that includes microtubules, intermediate filaments, and microfilaments. Operationally, investigators generally refer to those proteins insoluble in nonionic detergent solution under a defined set of buffer conditions to be cytoskeletal, although clearly the nucleus and other organelles are found in this detergent-insoluble residue. The

heavy chain of calpactin I has been found in the detergent-insoluble residue in fibroblasts (5, 30, 40, 45). Furthermore in cells fixed before extraction, immunofluorescence microscopy revealed that calpactin I heavy chain was found just under the membrane, although with a notable lack of staining in those regions of the cells where actin bundles (stress fibers) were also present (45). In intestinal epithelial cells the heavy chain of calpactin I is found in the terminal web regions of the brush border (16, 28, 31), a specialized microfilament-rich region of the cell from which other membrane systems and organelles are excluded. Consistent with this distribution calpactin is known to bind to the cytoskeletal proteins actin and spectrin *in vitro* (16, 24), albeit at high Ca^{++} levels (17). The Ca^{++} sensitivity of calpactin can be shifted from the millimolar to the micromolar level by anionic phospholipids (20, 22). It is not known whether these *in vitro* associations reflect the targets in the cell, yet clearly the available data point to association with some cellular structure(s) in close proximity to the plasma membrane.

The functional significance of two forms of calpactin I (monomer vs. complex) is unknown. To study the distribution of individual subunits of the calpactin family we have raised monoclonal antibodies to the heavy and light chains of calpactin I and the 39-kD calpactin II. The antibodies detect calpactin subunits in Western blots, immunoprecipitation, and immunofluorescence microscopy. We focused on the synthesis and distribution of calpactin in normal human fibroblasts and the results suggest that the light chain of calpactin I is tightly associated with the membrane skeletal form of calpactin I.

Materials and Methods

Protein and Antibodies

Calpactins I and II were isolated from bovine lung essentially as described (27). Polyclonal antibodies to calpactin were as in Glenney et al. (27), and to fodrin were as in Glenney and Glenney (23). Rabbit antibodies to phosphotyrosine were a generous gift from Dr. Mark Kamps (Salk Institute). For monoclonal antibodies, the calpactin I complex of subunits was used as immunogen in BALB/c mice (37) to the calpactin I heavy and light chain. Calpactin II, which has been shown to be identical by sequence to p35 and lipocortin (27), was injected separately in other mice.

The protocol for injections and screening of hybridoma will be provided in detail elsewhere. Briefly, hybridomas were screened first by an ELISA and second by Western blots using total bovine lung extracts. Hybridomas positive by these two assays were cloned twice (or until upon rescreeing all of the subclones were positive) by limiting dilution.

Western Blots

Cells and tissues were used for Western blotting by a modification of a previous method (19). Normal and Rous sarcoma virus-transformed chick embryo fibroblasts were provided by Dr. Bart Sefton (Salk Institute). Bovine tissues were frozen on dry ice at the slaughter house and subsequently homogenized in a blender (Waring Products Div., Dynamic Corp. of America, New Hartford, CT) for 1–2 min in 10 vol of H_2O . Aliquots were either used directly for protein determination (BCA assay; Pierce Chemical Co., Rockford, IL) or added to an equal volume of two times concentrated sample buffer and boiled for 5 min. For Western blots, 25 μg of total cell protein was loaded per lane and resolved on a 13% acrylamide SDS gel. Since we found that the light chain adsorbs very poorly to nitrocellulose, we modified the transfer conditions to include 40% methanol and then readjusted the pH to 8.6. Transfer to nitrocellulose was performed at 1 A for 1 h (using an electrophoretic transfer apparatus from Hoefer Scientific Instruments, San Francisco, CA). Blots were dried in a 65°C vacuum oven for 15 min, stained with India ink for 4–5 h, and after blocking, incubated in hybridoma culture supernatant diluted 1:2 with MTA buffer (19). Blots were incubated with first antibody overnight, washed, and incubated with ^{125}I -

conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) for 1–2 h, washed 30 min, and exposed to Kodak AR-5 film for 2–24 h.

Metabolic Labeling and Immunoprecipitation

Pulse-chase experiments followed by selective extraction and immunoprecipitation were performed as follows. Human fibroblasts (21) were plated into 6-well tissue culture plates and grown in DME supplemented with 10% FBS and the following morning were preincubated 30 min with DME without methionine and substituting dialyzed fetal bovine serum. Cells were then pulse-labeled with the same media containing 200 μCi [^{35}S]methionine (ICN Biomedicals Inc., Irvine, CA) for 2 h. The media was removed and after rinsing with PBS was replaced with DME supplemented with 1 mM unlabeled methionine. Cells were harvested at the specified chase times. The media was removed and cells were extracted with sequential 250 μl aliquots of 10 mM imidazole (pH 7.3), 75 mM KCl, 2 mM MgCl_2 , 0.5 mM CaCl_2 , 1 mM NaN_3 , 0.5% Triton X-100 (buffer 1) for 2 min at 4°C. The residual cytoskeleton was then extracted with 500 μl of 0.5% SDS, 2 mM EDTA (buffer 2) for 5 min at room temperature. The two extractions were then adjusted with 500 μl of the opposite buffer, and frozen at -70°C until all time points were collected. Samples were thawed and centrifuged at 10,000 rpm for 15 min at 4°C. Each sample was divided into three equal parts, adjusted to 1 ml with RIPA buffer (20 mM NaPO_4 , pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS). First and second extractions were combined for immunoprecipitation with anti-light chain and anti-calpactin II antibodies. Hybridoma culture supernatants were added and the samples were incubated at 4°C for 4 h. Goat anti-mouse second antibody was then added (10 μl of 1 mg antibody/ml; Sigma Chemical Co.) and further incubated 1 h at 4°C. Washed Pansorbin (25 μl of 10% solution; Calbiochem-Behring Corp., La Jolla, CA) was then added and the tubes rotated end over end for 30 min. The Pansorbin was collected by centrifugation, washed three times in RIPA buffer, and the antigen was eluted with SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and gels were treated with Enlightning (New England Nuclear, Cambridge, MA), dried, and exposed to preflashed x-ray film. The relative amount of radioactivity remaining at different chase times was determined by scanning the autoradiograms. Each experiment was repeated four times and the results were averaged.

Immunofluorescence Microscopy

Human fibroblasts, grown on glass coverslips, were either directly fixed and permeabilized with methanol/acetone (1:1) at -20°C or extracted 5 min at 4°C with the cytoskeletal-stabilizing buffer (30) before methanol/acetone fixation. Coverslips were air dried and incubated with a mixture of culture supernatant plus affinity-purified antibodies to calpactin or fodrin (20 $\mu\text{g}/\text{ml}$). Controls, omitting one of the first antibodies, were always included in parallel. After incubating 45 min at 37°C, coverslips were washed and incubated with affinity-purified second antibodies (fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit). After incubation with secondary antibody for 1 h at 37°C, coverslips were washed and viewed in a Nikon optiphot microscope equipped with epi-illumination. Photographs were taken on Ilford XP-1 or Kodak tmax 400 film using an ASA of 400 for fluorescein and 800 for rhodamine.

Antibody to the calpactin I light chain (II48) was microinjected into live fibroblasts or kidney cells (Madin-Darby bovine kidney, [MDBK]; American Type Culture Collection, Rockville, MD) essentially as described in Mangeat and Burridge (42). Monoclonal IgG was purified by ammonium sulfate fractionation (50%), gel filtration on sepharose 4BCL, and ion exchange chromatography (FPLC, mono Q), and the purity checked by SDS-PAGE and Coomassie staining. The monoclonal IgG at 5 mg/ml was injected into randomly selected cells that were then returned to culture for various times, rinsed in PBS, fixed, and treated for immunofluorescence microscopy as above. The injected antibody was visualized with a fluorescein-conjugated goat anti-mouse second antibody, and cells were counterstained with rabbit polyclonal antibodies, and rhodamine-conjugated goat anti-rabbit second antibody.

Results

Homogeneous bovine calpactin I (as a complex of heavy and light chains) and calpactin II were used as immunogens for

1. *Abbreviation used in this paper:* MDBK, Madin-Darby bovine kidney.

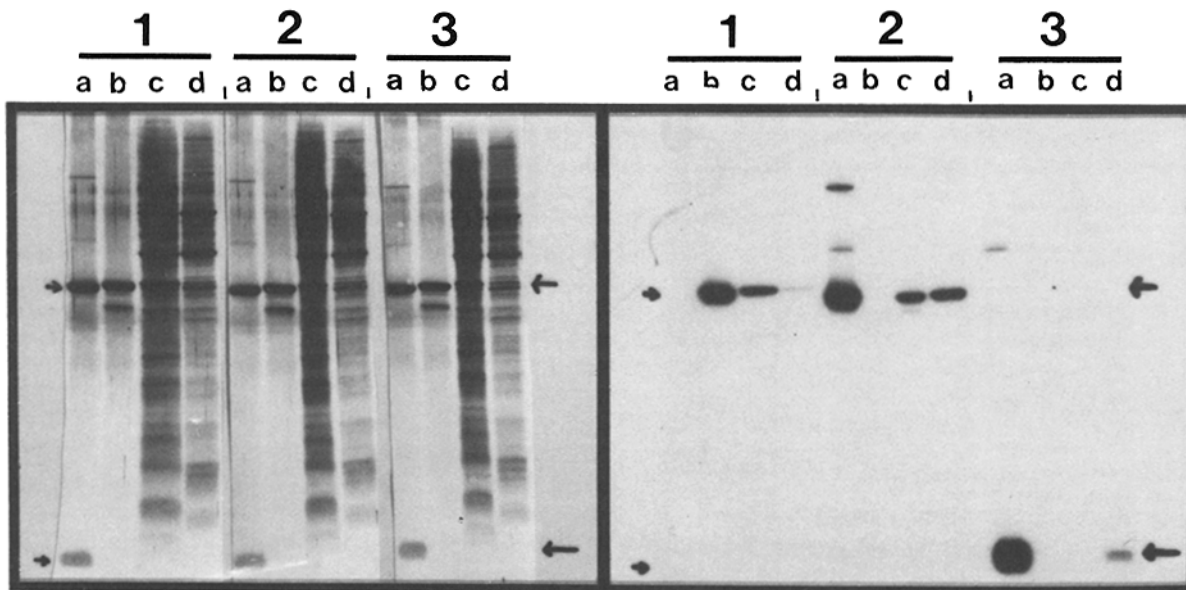


Figure 1. Reactivity of monoclonal antibodies to calpactin I heavy and light chains and calpactin II in Western blots of isolated protein and fractionated human fibroblasts. Calpactin I complex of heavy and light chains (a), calpactin II (b), or human fibroblasts that had been separated into soluble (c) and cytoskeletal (d) fractions were resolved on 13% acrylamide gels, and the protein transferred to nitrocellulose. The nitrocellulose membranes were stained for protein with India ink (left) and further incubated with monoclonal antibodies (panel 1) I1p29 (to calpactin II), (panel 2) I186, or (panel 3) I148 (to the heavy and light chains, respectively, of calpactin I). Reactivity was then assessed using ^{125}I -labeled second antibody followed by autoradiography (right). Arrows on the sides of the stained blots and autoradiographs were made with radioactive ink to allow precise alignment of the two images.

the production of monoclonal antibodies in BALB/c mice. The primary assays for detection of anti-calpactin antibodies involved ELISA assays followed by screening on Western blots. Those hybridomas that were strongly positive by both assays were selected and cloned twice by limiting dilution. In the present report, we will restrict ourselves to the analysis of one antibody reactive against each subunit. All three antibodies reacted with the isolated proteins displayed on Western blots (Figs. 1 and 2). The immunoreactive band could unambiguously be assigned to the major India ink-stained protein bands in blots that were stained with India ink for protein before their use in Western blots (Fig. 1). Antibodies to calpactin I did not cross react with calpactin II and vice versa. Two higher molecular mass bands were reactive with the anti-calpactin I antibodies in samples of purified protein (Fig. 1 left), which probably represent aggregates of calpactin subunits. Since these were never observed in samples of total cell protein (Figs. 1 and 2) we did not further pursue the identity of these bands. Strongly reactive bands corresponding to the 38-kD calpactin I, the 39-kD calpactin II, or the 11-kD calpactin I light chain were detected in total lysates of both human cells and bovine tissues (Figs. 1 and 2).

To analyze the distribution of the calpactin I heavy and light chains in various tissues, the antibodies were used on duplicate Western blots of total bovine tissues and compared with standards of known amounts of protein. As shown in Fig. 2, this assay was quite sensitive since both heavy and light chains could be easily detected in a sample of 4 ng of the unfractionated calpactin I complex. The tissue distribution of both the heavy and light chains were essentially identical. The highest level of both was found in lung and intestine (Fig. 2), two sources that have been used as starting material to purify the calpactin I complex (16, 27). Neither

calpactin subunit was detectable in red blood cells and both were present at very low levels in liver and brain tissue. This would also indicate that antibodies to the calpactin light chain do not recognize the S-100 proteins, since these proteins are expressed at high levels in brain (1, 10).

Antibodies to the calpactin I heavy and light chains and calpactin II all recognized the homologous proteins in human cells (Figs. 1 and 2 k). This allowed us to analyze the subcellular distribution and turnover of calpactins in normal human diploid fibroblasts.

The cytoskeletal association of proteins can be detected biochemically by their relative insolubility in nonionic detergent (see Introduction). To determine the cytoskeletal association of calpactin subunits human fibroblasts were extracted briefly with a mild detergent solution, fractionated into detergent soluble and insoluble pools, and analyzed by Western blots (Fig. 1). A marked difference in the behavior was detected by the three antibodies. Whereas >80% of calpactin II is soluble upon brief extraction, only 50% of the heavy chain and <10% of the light chain of calpactin I was extractible under these conditions. This suggested that two pools of calpactin, which differ in their association with the cytoskeleton, exist in human fibroblasts.

We examined the turnover rate of soluble and detergent-extractable calpactin I heavy chain and compared it with the turnover rate of the light chain and calpactin II. As shown in Fig. 3, a dramatic difference was observed in the half-life of soluble and cytoskeletal forms of the calpactin I heavy chain. The soluble pool turned over with a half-life of 15 h, whereas the insoluble pool lagged far behind with a half-life of ~40–50 h. By comparison, the calpactin I light chain had a relatively long half-life (50–60 h) and calpactin II had a shorter half-life (15 h).

Anti-light chain antibodies were used in immunofluores-

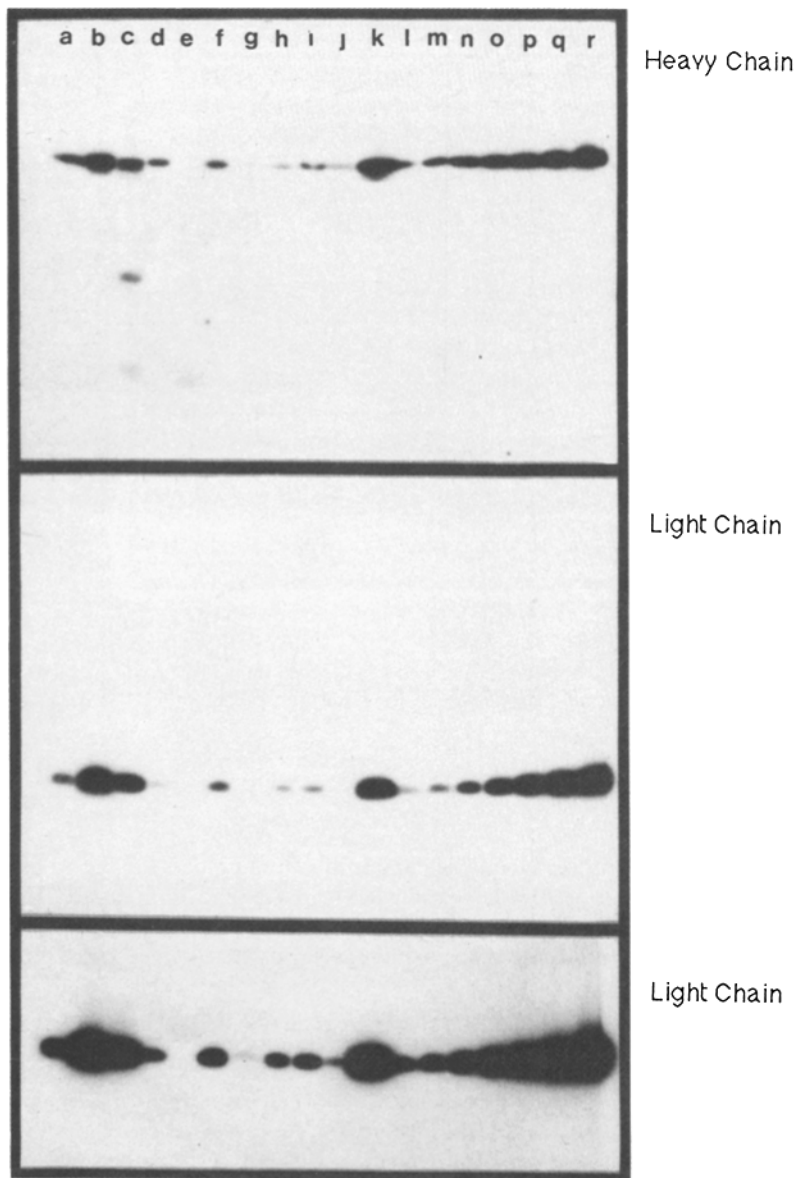


Figure 2. Comparison of the tissue levels of the calpactin I heavy chain and light chain by Western blotting with monoclonal antibodies to the individual subunits. 25 μ g of total protein from bovine spleen (a), lung (b), intestine (c), adrenal gland (d), red blood cells (e), kidney (f), brain (g), skeletal muscle (h), cardiac muscle (i), and liver (j) as well as human A431 (k), and 4 (l), 8 (m), 16 (n), 32 (o), 64 (p), 128 (q), and 256 (r) ng of the pure calpactin I complex were resolved by SDS-PAGE and transferred to nitrocellulose. Duplicate blots were treated with antibodies to the calpactin I heavy chain (top) or light chain (center and bottom) followed by 125 I-conjugated second antibody. (Bottom) An overexposed autoradiogram of the middle panel to detect very low level expression. Note that the distribution of the light chain closely parallels the heavy chain.

cence microscopy on fixed and permeabilized human fibroblasts (Fig. 4). When cells were fixed directly in organic solvents, a staining pattern similar to that shown previously with polyclonal antibodies to the calpactin I complex was observed (30, 40, 45). The dominant feature of this distribution was the absence of staining in certain areas of the cell previously identified as actin-containing stress fibers (45). This staining pattern was found with methanol/acetone fixation (Fig. 4), 4% formaldehyde fixation (1 h), followed by Triton permeabilization (not shown), or formaldehyde fixation followed by methanol/acetone treatment (not shown). This lack of stress fiber staining was faintly detectible with polyclonal antibodies to the calpactin heavy chain, but this appeared to be overlaid by a more diffuse cytoplasmic staining. A similar more diffuse distribution was found when the monoclonal antibody to the heavy chain was used (data not shown). Since the subcellular distribution of calpactin I has been shown to partially overlap that of fodrin, double staining was performed with the mouse monoclonal antibody to the calpactin

I light chain and polyclonal rabbit antibodies to fodrin. As shown in Fig. 4, the calpactin staining extends further out to the edge of the cells by comparison to fodrin, but where the two are in the same area a similar submembrane reticular network is stained.

Previous studies have shown that when fibroblasts are extracted with a cytoskeletal stabilizing buffer, a characteristic reticular network is stained by antibodies to calpactin I and fodrin (30). When this extraction procedure was applied to human fibroblasts, a reticular network was visualized using antibodies to the calpactin I heavy and light chain but not to calpactin II (Fig. 5).

Antibodies to the calpactin I light chain were microinjected into living cells grown on coverslips. The cells were returned to culture for various times and then fixed and permeabilized with methanol/acetone. The injected cells could be easily located using a fluorescently tagged anti-mouse antibody. Preliminary experiments showed that the injected anti-light chain antibody was initially localized in a distribu-

tion indistinguishable from the pattern when used in conventional immunofluorescence microscopy. Upon longer times after injection in some (10%) fibroblasts or most (>60%) MDBK cells, the antibody was clustered into large patches (Fig. 6). We had the impression that the patches of anti-light chain antibody were confined to the plane of the membrane. When the antibody to calpactin II was injected in duplicate experiments, aggregates were never observed. No abnormalities in cell shape were apparent in injected cells. Counterstaining with a rabbit antibody to the calpactin I heavy chain revealed that these antibody patches contained both subunits. The patches were not stained with anti-calpactin II antibodies (not shown), consistent with the observation that calpactin II is not associated with the light chain *in vitro*. The anti-light chain antibody aggregates were also not stained with antibodies to fodrin (Fig. 6).

Calpactin I phosphorylation has been suggested to play an important functional role in transformation by retroviruses containing tyrosine-specific protein kinases (see Discussion). To assess whether there may be differences in association of calpactin I with either the cytoskeleton or the light chain, normal and transformed chick fibroblasts were separated into soluble and cytoskeletal fractions and probed with antibodies to calpactin I and to phosphotyrosine. As shown in Fig. 7 the monoclonal antibodies react strongly with the chicken calpactin I subunits. In chick fibroblasts (unlike human) the majority of the calpactin I heavy chain is associated with the cytoskeleton. This association is Ca^{++} dependent and is similar in both normal and transformed cells. The extent of cytoskeletal association of calpactin I is mirrored by the relative amount of phosphorylated calpactin in the Triton-insoluble pool detected with an antibody to phosphotyrosine (Fig. 7). It should be noted that the gel system and transfer conditions have been optimized for the detection of calpactin subunits and hence the antiphosphotyrosine blot appears simpler than expected. Under other conditions this same antibody will detect many other phosphoproteins (Kamps, M., and B. Sefton, manuscript submitted for publication).

Discussion

The first exogenous substrate identified for the transforming tyrosine kinase pp60^{src} was a protein that has been variously termed p34, p36, or p39 (11, 12, 34, 44, 46). We have been referring to this and related proteins more recently as calpactin to denote their calcium-, phospholipid-, and actin-binding properties. What was initially thought to be a single polypeptide is now known to be more complex. In a pioneering biochemical study on the isolation and characterization of calpactin I (p34) from chick fibroblasts, Erikson and co-workers (13) noted that two forms of this protein were observed, with the difference in the two forms being the presence of a low molecular weight peptide (estimated then to be 6,000 but now known to be 11,000). Shortly thereafter Gerke and Weber (16) isolated a Ca^{++} -binding cytoskeletal protein from intestinal epithelium with subunits of 36 and 11 kD, which they referred to as protein I. Gerke and Weber went on to show that the 36-kD subunit of protein I is identical with the 36-kD substrate of the retroviral tyrosine kinases. Further studies by three independent groups (18, 25, 32) demonstrated that the light chain is strikingly related by

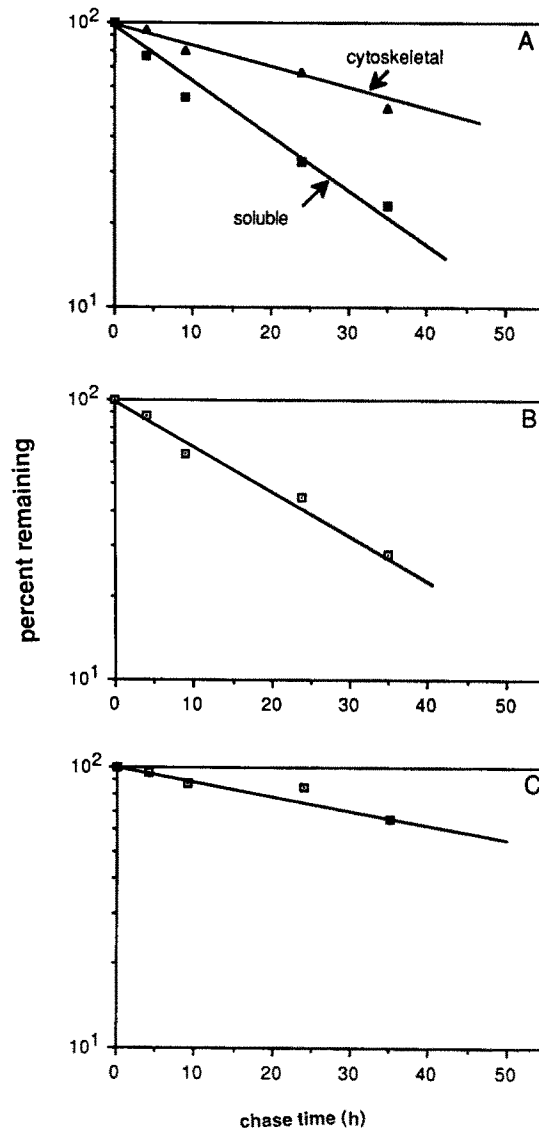


Figure 3. Pulse-chase analysis of the calpactin subunits. Human fibroblasts were labeled with [^{35}S]methionine for 2 h and chased for the indicated times. The amount of radioactivity in soluble and cytoskeleton (insoluble) associated pools of the heavy chain of calpactin I (A), calpactin II (B), or the calpactin I light chain (C) was determined by immunoprecipitation with monoclonal antibodies followed by autoradiography.

amino acid sequence to the S-100 proteins of brain. Although investigators have been studying S-100 for some 20 yr, no consensus has emerged as to the role of S-100 in the cell. Similarly, the role of the calpactin light chain has been the subject of considerable speculation with little data to support it. Studies have shown that the calpactin light chain binds to that region of the heavy chain (amino terminus; 26, 35), which is phosphorylated by pp60^{src} (25) and serine/threonine kinases (29, 35), suggesting that this region of the heavy chain may be involved in the regulation of calpactin functions. The biological property being regulated, however, has been elusive. The present study suggests that one regulatory event potentially mediated by the light chain is the association of calpactin I with the membrane skeleton.

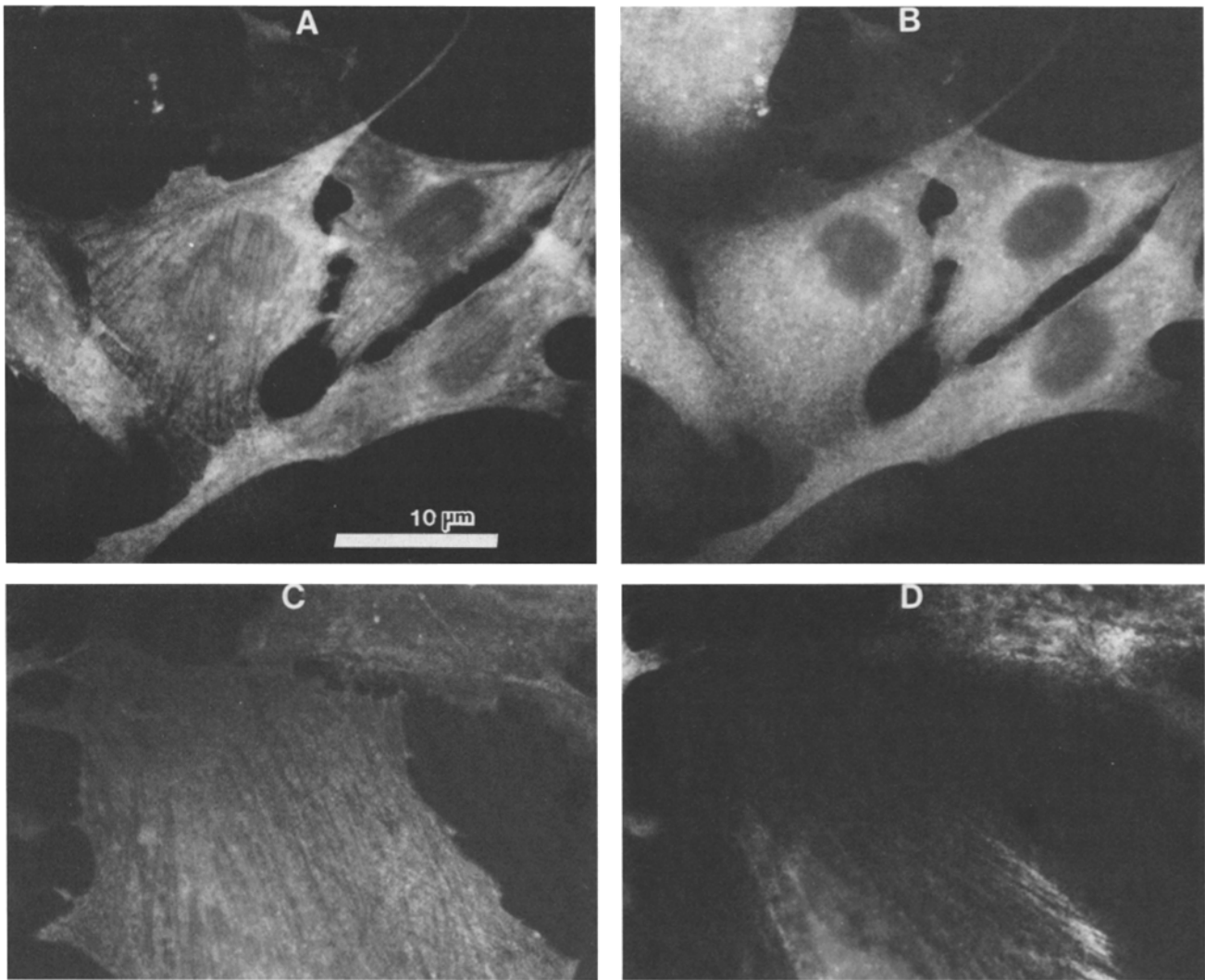


Figure 4. The subcellular distribution of calpactin I light chain compared with the heavy chain and fodrin in prefixed human fibroblasts as revealed by double-label immunofluorescence microscopy. Cells were fixed and permeabilized with -20°C methanol/acetone followed by treatment with the monoclonal antibody to the calpactin I light chain (A and C) in combination with polyclonal rabbit antibodies to the calpactin I heavy chain (B), or fodrin (D). Reactivity was detected with fluorescein anti-mouse (A and C) and rhodamine anti-rabbit (B and D) immunoglobulin.

Previous studies using antibodies to calpactin I isolated from chick fibroblasts revealed that calpactin is present in a cytoskeletal meshwork under the plasma membrane, partially overlapping the distribution of spectrin in these same cells (5, 30, 40, 45). Calpactin has also been found in close association with the membrane in tissues (28, 31) and in one location, the intestinal epithelial cell, calpactin was identified in the highly organized brush border region (16, 28), a location rich in actin and actin-binding proteins. Many of these localization studies relied on antibodies to the calpactin I complex and most of these antibody preparations are known to contain significant reactivity with the light chain as well as the heavy chain (16, 25). Thus, these studies could not distinguish between the location of the complex of heavy and light chains, for instance, from the location of either monomer alone. In the present study we used monoclonal antibodies that recognize individual subunits. The results suggest that two functionally distinct populations of calpac-

tin exist in fibroblasts, each with a distinctive location and inherent stability.

Several lines of evidence suggest that distinct soluble and cytoskeletal forms of calpactin exist. First, calpactin II (which is similar in structure and function to calpactin I but lacks a light chain [27]) was present exclusively in the detergent-soluble fraction, whereas the calpactin I light chain (presumably reflecting the distribution of the calpactin I complex) was only found in the cytoskeletal fraction (Fig. 1). The calpactin I heavy chain partitioned equally between detergent-soluble and particulate fractions, indicating that only about half is associated with the cytoskeleton. Previous studies have also noted a heterogeneity in the extractability of the calpactin heavy chain (45).

Immunofluorescence microscopy using antibodies to the heavy and light chains revealed that the light chain is present exclusively in the membrane structural network. Particularly apparent in prefixed cells is the absence of staining of stress

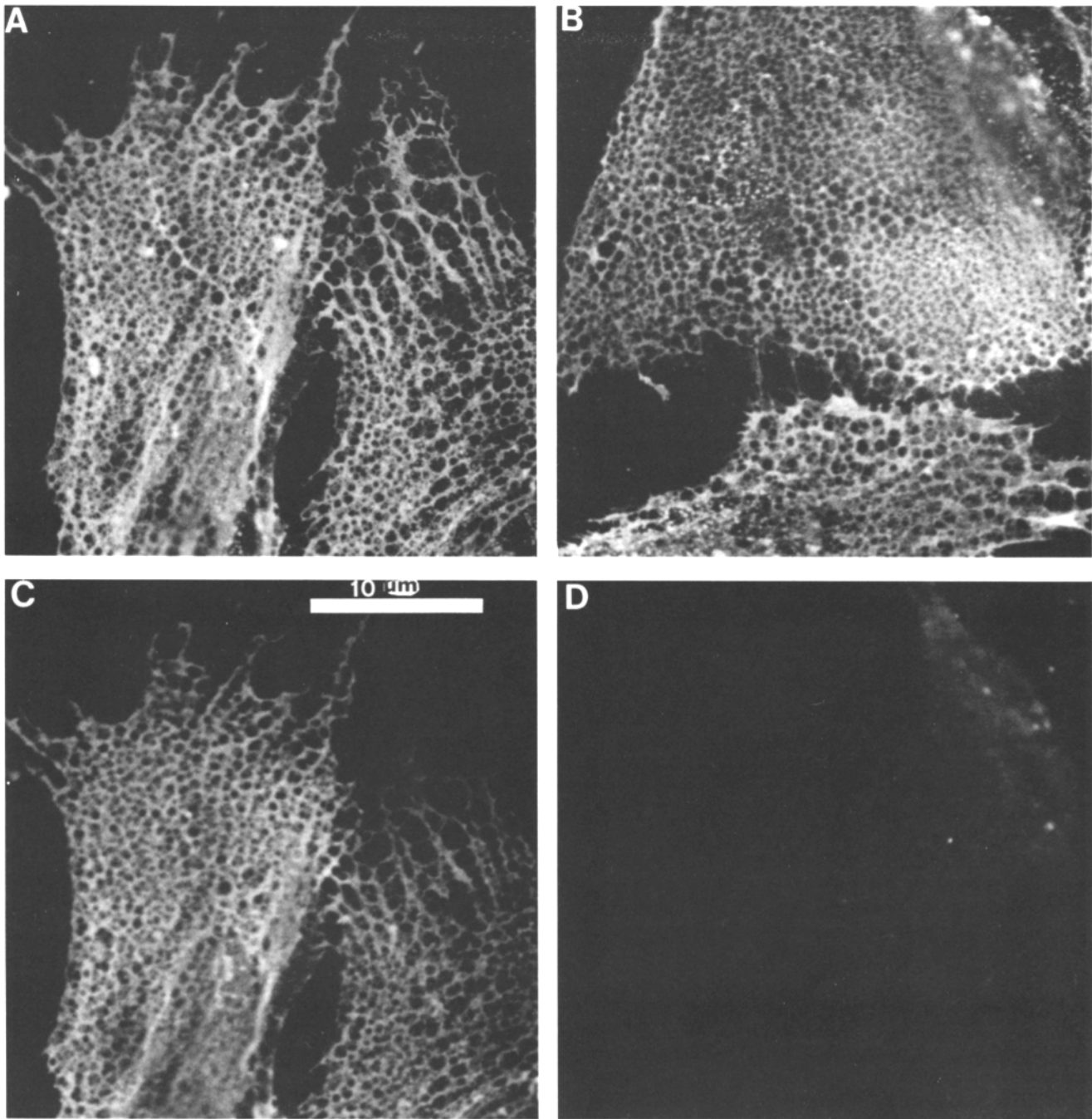
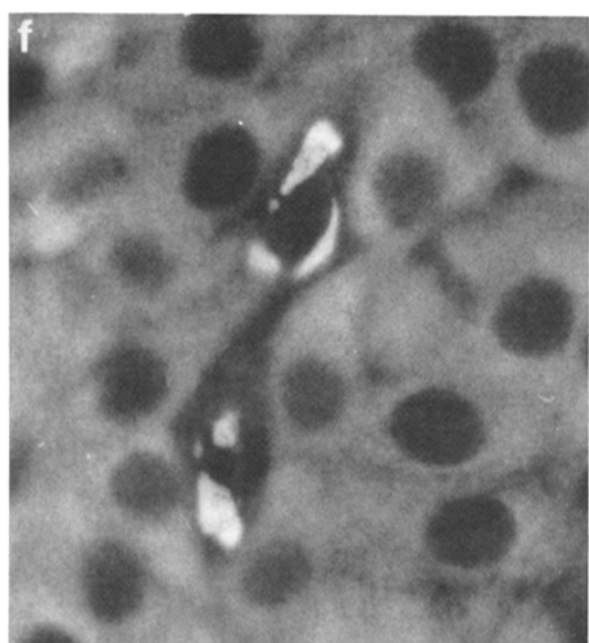
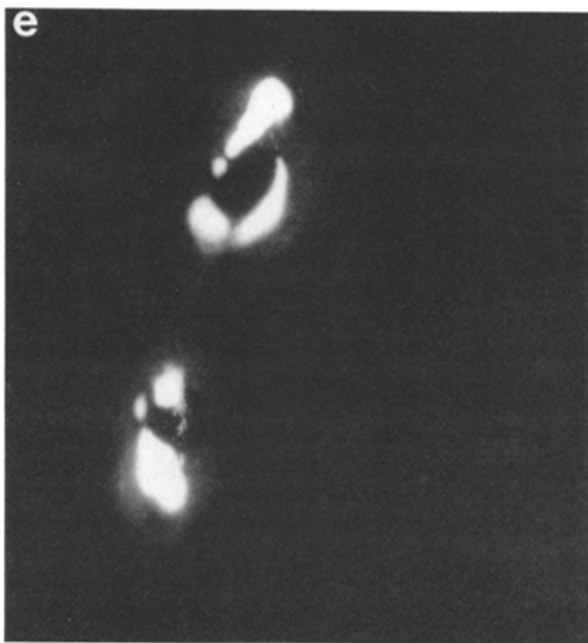
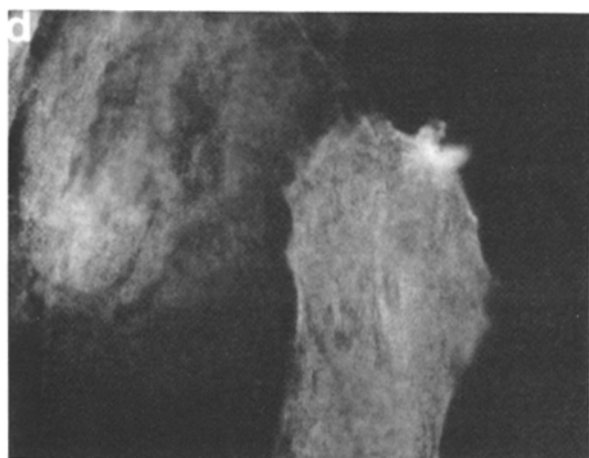
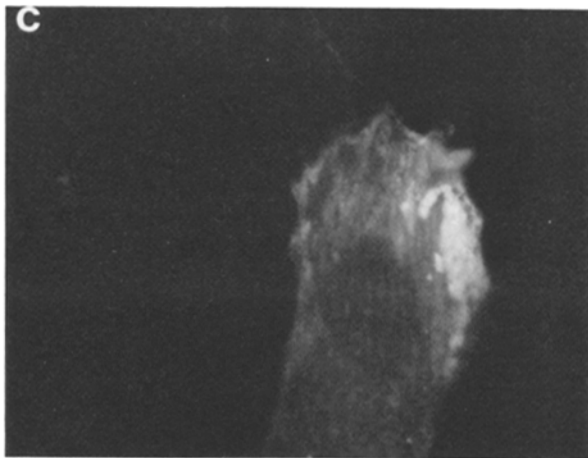
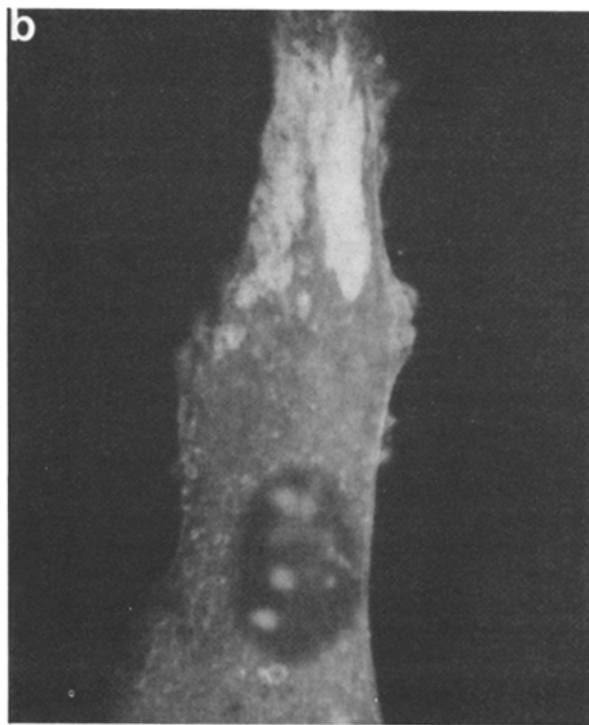


Figure 5. Localization of calpactin subunits in human fibroblasts extracted with a cytoskeletal stabilizing buffer. Cells were extracted in a detergent containing solution before fixation. Cells were then treated with rabbit antibodies to the calpactin I heavy chain (*A* and *B*) together with monoclonal antibody reactive with the calpactin I light chain (*C*) or calpactin II (*D*). Reactivity was detected with fluorescein anti-mouse (*C* and *D*) and rhodamine anti-rabbit (*A* and *B*) immunoglobulin.

fibers (Fig. 4), a characteristic noted previously and shared with the membrane-skeletal protein fodrin (see above). The distribution of the heavy chain, by contrast, was more diffuse. In cells that were extracted with a cytoskeleton-stabilizing buffer, the light chain was found in the reticular network whereas calpactin II was not (Fig. 5). Since this reticular network is not observed in prefixed cells, it may not represent an accurate picture of the membrane skeleton organization in living cells. We favor the notion that the delicate

membrane filament network collapses upon itself when the lipid bilayer is disrupted with detergent. It is possible, for instance, that calpactin I is associated with both the lipid bilayer and the cytoskeleton (27). When the lipid bilayer is disrupted, Ca^{++} is needed to stabilize the association with its cytoskeletal target. The majority of actin in these extracted cells is found in stress fibers, detected using fluorescent phalloidin (not shown). Thus it would appear that this is not simply a case of calpactin artifactually binding to actin under



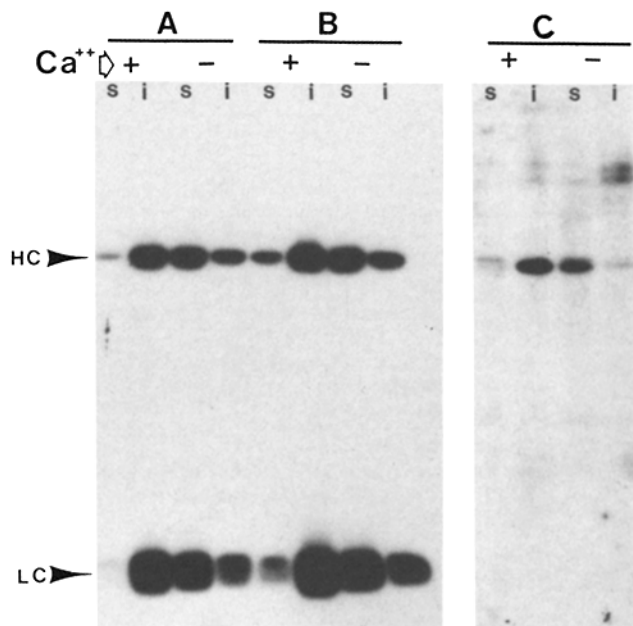


Figure 7. Detection of calpactin I subunits in normal and transformed chick fibroblasts. Normal (A) or transformed (B and C) fibroblasts were extracted in a cytoskeletal stabilizing buffer containing 1 mM Ca^{++} (+) or 5 mM EGTA (-), and separated into soluble (s) and insoluble (i) fractions. Equivalent amounts were subjected to SDS-PAGE, transferred to nitrocellulose, and reacted with a mixture of anti-calpactin I heavy and light chain monoclonal antibodies (A and B) or polyclonal rabbit anti-phosphotyrosine antibodies (C). Blots were further treated with ^{125}I -anti-mouse IgG (A and B), or ^{125}I -protein A (C), followed by autoradiography.

extraction conditions containing high Ca^{++} . Consistent with this we have found that when MDBK cells are lysed in low Ca^{++} -buffer in the absence of detergent, most of the calpactin remains with the particulate fraction. The exact targets *in vivo*, however, remain to be determined.

The immunofluorescence images of heavy and light chain subunits in fixed cells appears quite different (Fig. 4). The calpactin light chain is firmly associated with a population of the heavy chain as shown clearly by microinjection experiments (Fig. 6). The antibody to the light chain injected into fibroblasts resulted in many cases in the patching of antibody within the cells with the concomitant patching of the heavy chain of calpactin I (but not calpactin II). We have found no cells in which the injected anti-light chain clusters were not stained with anti-heavy chain. Interestingly, fodrin was not enriched in these patches (Fig. 6), suggesting that the membrane location of these two structural proteins is not tightly linked. A similar result was found with microinjected antibodies to fodrin (42). This aggregation of the cytoskeletal form of calpactin I should allow us to evaluate the possible associations in the cell.

Figure 6. Microinjection of antibody to the calpactin light chain. Human fibroblasts (a-d), or MDBK cells (e and f) were injected with antibody II48 and returned to culture for 18 h. Cells were then fixed and permeabilized with methanol/acetone and stained with rabbit antibodies to the calpactin I heavy chain (b and f) or fodrin (d). The injected antibody was localized with fluorescein anti-mouse (a, c, and e) and the rabbit antibody with rhodamine anti-rabbit (b, d and f). Note the patching of injected antibody (a and e) with the copatching of the calpactin heavy chain (b and f) in the same cells.

The third line of evidence for two discrete populations of calpactin I comes from experiments on their stability. The soluble fraction of the calpactin I heavy chain has a similar turnover rate (~ 15 h) as calpactin II, which is also soluble, and the cytoskeletal pool of the heavy and light chains of calpactin I have a similar turnover rate (~ 50 h). This difference between the turnover rate of the two pools would appear to rule out the possibility that calpactin is released after lysing the cells, and artifactually rebinding to the cytoskeleton. If this were the case, the turnover rate of the two populations would be the same. Clearly, two pools of nonexchangeable calpactin I are indicated. Studies with another membrane skeletal protein, spectrin, have resulted in a similar conclusion, e.g., the soluble fraction of spectrin turns over much more quickly than the cytoskeleton-associated fraction (2, 39). In this example, however, the soluble subunits of spectrin turned over with a half-life of 50 min (as compared with 15 h for soluble calpactins). A second analogy to this previous work is the regulation of the amount of one subunit in a protein complex by a second subunit. In the case of calpactin, then, the amount of the light chain is probably limiting in cells, similar to the limiting amounts of beta-subunit in the spectrin complex (2). By increasing the amount of light chain, then, the heavy chain would be stabilized and the steady state amount of the heavy chain would increase without the necessity of changes in the messenger RNA level or protein synthesis rate. This may partially explain the apparent induction of the calpactin heavy chain when chick embryo fibroblasts are placed in culture. In that study the accumulation of the heavy chain of calpactin I could be correlated with cell adhesion or spreading (4). Similarly, the spreading behavior of mammary cells has been correlated with the expression of calcium-binding proteins (47), one of which is known to be calpactin I. The expression of the light chain may well be the controlling (or stabilizing) element in such experiments. Alternatively, other elements in the cell such as membrane or cytoskeletal targets may be the stabilizing element for the complex of both subunits. The use of the antibodies described in this report should help address these questions.

The amount of the calpactin light chain appears tightly coupled to the amount of heavy chain in every tissue examined (Fig. 2). This includes tissues with high level expression of both subunits (lung, intestine), intermediate levels (spleen, muscle), or very low levels (liver, brain). It should be noted that the relative level of expression of the calpactin I heavy chain detected with a monoclonal antibody used in the present study is in substantial agreement with the distribution reported previously using polyclonal antibodies to the chick fibroblast protein (28). In no tissue did we find the expression of one subunit in the absence of the other; nor did we find vastly different ratios. This finding is interesting in light of the recent observation of moderate levels of mRNA for the calpactin I light chain in brain tissue (48), whereas we find almost no light chain protein in brain (Fig. 2). It is

likely that the calpactin light chain protein is unstable in the absence of the heavy chain and future experiments should address this possibility. We know that in lung and intestine calpactin I is found predominantly as a complex of subunits (16, 27). Although we have isolated the native monomer from lung, the yields are <10% of the amount of the complex isolated (27). The precision of the Western-blotting method is not high enough to conclude that the ratio of heavy and light chains is identical in every tissue, and certainly the tissues represent heterogeneous populations of cells that may be expressing varying ratios of monomer and complex. Despite these limitations, we suggest that no cell type contains substantial amounts of the calpactin light chain in the absence of heavy chain. In this regard, the situation is quite different than with the brain homolog of the calpactin light chain, S-100. S-100 is isolated as a dimer of alpha and beta subunits (1, 41). Although a fraction of S-100 has been detected associated with membranes (8) and S-100 can bind to and activate the enzyme aldolase (52), it is clear that most is not complexed with an additional subunit. The significance of the 50% amino acid sequence homology between the calpactin light chain and S-100 (alpha subunit) remains unclear.

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