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Antibodies to the N-Terminus of Calpactin II (p35) Affect Ca^{2+} Binding and Phosphorylation by the Epidermal Growth Factor Receptor in Vitro†

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ABSTRACT: Calpactins I and II are related 39-kilodalton (kDa) proteins that interact with phospholipids and actin in a calcium-dependent manner and are substrates of tyrosine protein kinases. They contain a short amino-terminal tail attached to a 36-kDa core domain. Monoclonal antibodies (Mabs) were raised to bovine calpactin II and used as site-specific probes of its structure and function. All of the antibodies reacted with native calpactin II and gave rise to a single band of 39 kDa among total cell protein displayed on Western blots. Most of the antibodies (9/14) reacted with determinants on the tail as shown by Western blots and competition with a synthetic tail peptide. Four antibodies reacted with determinants on the core and a 10-kDa tryptic fragment. Antibody–calpactin II complexes were tested for their ability to interact with lipid, actin, and Ca^{2+} and to serve as substrates of the epidermal growth factor (EGF) receptor tyrosine protein kinase. Whereas none of the antibodies had a detectable effect on actin binding, two anticore antibodies reduced calpactin's affinity for phospholipid. Ca^{2+}-binding sites are known to reside within the core region, yet most antitail antibodies markedly increased the affinity of calpactin II for Ca^{2+}, with four Ca^{2+}-binding sites observed. Antitail antibodies either (i) abolished or (ii) greatly stimulated (10-fold) the phosphorylation of calpactin II by the EGF receptor. These results suggest that the interactions between calpactin II and Ca^{2+}, phospholipid, or the EGF receptor are more complex than previously thought and can be modulated by interactions occurring in the tail.

Calpactins are Ca^{2+}-binding proteins which interact with phospholipid and actin in vitro and have been suggested to be components of the cytoskeletal matrix under the plasma membrane in cells (Gerke & Weber, 1984; Greenberg & Edelman, 1983; Radke et al., 1983; Nigg et al., 1983; Glenney, 1986b; Glenney et al., 1987). Considerable interest has focused on these proteins because they are substrates of the oncogene and growth factor receptor tyrosine kinases (Erikson & Erikson, 1980; Sawyer & Cohen, 1985; Fava & Cohen, 1984; Pepinsky & Sinclair, 1986; Radke & Martin, 1979). Although originally thought to be a single 39-kilodalton (kDa) protein, more recent evidence has demonstrated the existence of at least three forms of calpactin (Glenney, 1986b; Glenney et al., 1987). Calpactin I is found in cells as either a 38-kDa monomer or a tetramer with an associated 11-kDa light chain (Gerke & Weber, 1984; Erikson et al., 1984; Zokas & Glenney, 1987). Calpactin II, on the other hand, is found only as a monomer (Glenney, 1986b; Fava & Cohen, 1984).

The cDNAs encoding calpactins I and II have been molecularly cloned and sequenced (Saris et al., 1986; Kristensen et al., 1986; Wallner et al., 1986; Huang et al., 1986), which allows correlation between structure and function. Calpactins had been suggested to be lipocortins (Pepinsky & Sinclair, 1986; Huang et al., 1986), proteins thought to be potent and specific inhibitors of phospholipase A_2. Subsequent investigations, however, revealed that this apparent A_2 inhibition by calpactin was not due to direct interaction between calpactin and phospholipase but was due to the shielding of the phospholipid substrate (Davidson et al., 1987; Haigler et al., 1987), in accordance with the known lipid-binding properties of calpactin (Glenney, 1985, 1986a,b; Davidson et al., 1987; Geisow et al., 1986; Johnsson et al., 1986; Haigler et al., 1987; Schlaepfer & Haigler, 1987). Thus, recent studies on frag-

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ments of lipocortin (calpactin II) have probably identified the lipid-binding site on the molecule (Huang et al., 1987). Our interpretation is that calpactin is not a true lipocortin and other functions of the protein should be considered.

Calpactin II is known to bind to and bundle actin filaments (Glenney et al., 1987; Schlaepfer & Haigler, 1987). Two Ca^{2+}-binding sites have been detected on calpactin II in the presence of phospholipid by equilibrium dialysis using $^{45}CaCl_2$ (Glenney et al., 1987). The subcellular distribution of calpactin II was shown to be similar to, although not identical with, the distribution of calpactin I (Glenney et al., 1987). Calpactin II is not found in association with a light chain (Glenney, 1986b; Fava & Cohen, 1984; De et al., 1986). This observation is consistent with the striking sequence differences in the amino-terminal tails of the two calpactins (Saris et al., 1986; Kristensen et al., 1986), the region of the molecule known to bind the light chain in calpactin I (Glenney et al., 1986).

In the present study, monoclonal antibodies (Mabs) were raised to bovine calpactin II. The Mabs recognize two major antigenic sites: one on the tail and a second on a 10-kDa tryptic peptide derived from the core. The effects of the Mabs on the known properties of calpactin II reveal a more complex model than has been previously recognized.

Materials and Methods

Purification of Calpactins. The calpactin II used for injection of mice and screening hybridoma supernatants was isolated as described (Glenney et al., 1987). For the larger scale isolation of calpactins, the method was modified as follows: After homogenization, centrifugation, precipitation with a Ca^{2+}-containing solution (pH 6.8), and resolubilization with a Ca^{2+} chelating buffer (pH 8.0) as described (Glenney et al., 1987), the solution was dialyzed overnight against 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8.8), 100 mM NaCl, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 mM dithiothreitol (DTT), and 2 mM $MgCl_2$. The solution was centrifuged at 100000*g*, 90 min, the supernatant adjusted to 1 mM free Ca^{2+} (at pH 6.8), and the precipitate collected by centrifugation (30000*g*, 30 min). The clear supernatant was applied to a 100-mL column of phosphatidylserine–acrylamide (Uchida & Filburn, 1984) at 4 °C, and after the column was washed with buffer alone, calpactin II was eluted with the same buffer containing 5 mM EGTA (pH 8.0). Peak fractions were pooled, dialyzed against 10 mM imidazole, 1 mM EGTA, and 0.5 mM DTT, pH 7.3, and passed through a column of DE-52 (Whatman). Under these conditions, most of the calpactin II bound to the column and was eluted between 10 and 50 mM NaCl. Fractions containing calpactin II were pooled, dialyzed, and chromatographed on a hydroxylapatite column as described previously (Glenney et al., 1987).

Production of Monoclonal Antibodies. Female Balb/C mice were injected with 100–200 μg of calpactin II in Freunds incomplete adjuvant followed by injection 1 month later with the same amount of protein in incomplete adjuvant. One to two months following the second injection, mice were injected intravenously with 50–100 μg of calpactin II in phosphate-buffered saline (PBS) 4 times over the last week before the spleen was harvested. Spleen cells were fused with SP 2/O myeloma cells essentially as described (Stahli et al., 1980). Cells were plated into 96-well tissue culture plates, and the culture supernatant was tested at 10 or 11 days after fusion. The first screening involved an ELISA assay in which calpactin II (0.1 μg/well) was used to coat 96-well Immulon II test plates (Dynatech Laboratories). After the plates were blocked with 5% powdered milk in PBS for 1 h, plates were incubated with hybridoma supernatant 2 h at 37 °C, washed 4 times with PBS, and further incubated with peroxidase-conjugated goat anti-mouse second antibody (Fisher), 1 h at 37 °C. Plates were again washed 4 times with PBS and incubated with 150 μL of a solution containing 0.4 mg of *o*-phenylenediamine (Sigma) and 1 μL of 30% H_2O_2 per milliliter of 75 mM sodium citrate, pH 5.5. The reaction was quenched after 10–30 min by addition of 50 μL of 4 N H_2SO_4, and the absorbance was monitored at 492 nm by using an automatic plate reader (Dynatech). Positive colonies were transferred to 24-well tissue culture plates and the media tested by modifications of the Western blotting method (Glenney, 1986c; Westgeest et al., 1986) on Ca^{2+}-precipitated protein (Glenney et al., 1987) using ^{125}I goat anti-mouse IgG as the detecting reagent. Hybridomas positive by both assays were then cloned twice by limiting dilution and grown in mice primed with pristane (Brodeur et al., 1984) or Freunds adjuvant (Mueller et al., 1986).

Mabs were purified from ascites fluid by using protein A coupled to affigel and the buffer kit purchased from Bio-Rad, used according to manufacturer's instructions. The antibody solution eluted from the protein A column was immediately neutralized with 1 M Tris buffer, and after dialysis, Mabs were further purified by using a fast protein liquid chromatography (FPLC) mono Q column (Pharmacia) followed by precipitation with 50% ammonium sulfate. The Mabs were dialyzed 2 days against a large volume of 10 mM imidazole, 50 mM NaCl, and 2 mM NaN_3, pH 7.3, clarified by centrifugation (10000*g*, 15 min), and stored at 4 °C.

Fab fragments of two of the Mabs (6 and 11) were made by first isolating the IgG using a protein A column as above, dialyzing against 10 mM PO_4 (pH 7.5), 25 mM NaCl, and 1 mM NaN_3, and digesting with 0.5 μg of papain [+20 mM β-mercaptoethanol(β-ME)]/mL at 37 °C. The solution was cooled and applied directly to a FPLC mono Q ion-exchange column equilibrated in the same buffer (without β-ME). The unretarded protein was dialyzed against 20 mM sodium acetate and 20 mM NaCl, pH 5.5, and was subjected to ion-exchange chromatography on an FPLC mono-S column eluting with a 20–100 mM NaCl gradient. The digestion and purification were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining.

Limited Proteolysis. Calpactin II was cleaved with trypsin in two types of experiments. First, calpactin at 200 μg/mL was incubated with 0.5, 5.0, and 25 μg of trypsin/mL in a buffer consisting of 10 mM imidazole, 1 mM EGTA, 50 mM NaCl, and 1 mM DTT, pH 7.3, for 1 h at 20 °C. The digest (as well as undigested calpactin and trypsin alone) was resolved on a 12% acrylamide gel, and the peptides were transferred to nitrocellulose and stained with India ink as in Glenney (1986c). A few of the Mabs were used to stain these blots (Mabs were used at 1 μg/mL). In a second type of experiment, calpactin II at 100 μg/mL was treated as above with 1 μg of trypsin/mL. This digest was then run on a single slot gel and transferred to nitrocellulose, stained with India ink, and each of the Mabs was used to stain strips of the nitrocellulose.

Peptide Inhibition. In some experiments, the ability of a synthetic peptide to inhibit antibody reactivity was tested as follows: A peptide with the sequence Ac-Q-A-W-F-I-E-N-E-E-Q-E-Y-V-Q-T-V-K-S-S-Y-NH_2, where Ac represents an acetyl blocking group at the amino terminus, was provided by the Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, as in Glenney et al. (1986). This corresponds

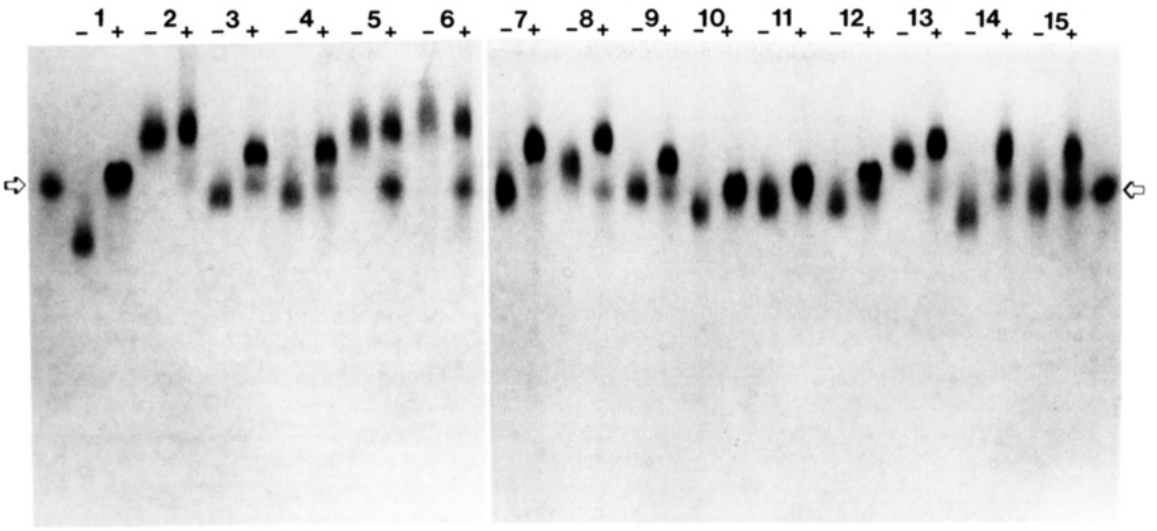

FIGURE 1: Electrophoresis of Mabs and calpactin–antibody complexes under nondenaturing conditions. Mabs 1–15, either alone (–) or with calpactin (+), were adjusted to 60 mM Tris, pH 6.8, and 5% glycerol at 250 μg of calpactin and 500 μg of antibody per milliliter. The proteins were then subjected to electrophoresis at 70 V (constant voltage) on a 5% acrylamide gel. After electrophoresis for 7 h at room temperature (with cooling), the gel was stained with Coomassie blue. Calpactin II alone at the same concentrations was included in the outside lanes (and noted with arrows) for comparison. Note the shift in electrophoretic mobility of most Mabs when calpactin II was present [compare (–) lanes to (+) lanes].

to amino acids 9–27 of the intact human calpactin II (Wallner et al., 1986; termed lipocoptin), with the bovine protein differing at positions 21 and 22 (Glenney et al., 1987). The Mabs at 50 ng/mL were incubated with 2 μg of peptide or intact protein/mL in PBS + 2% bovine serum albumin (BSA) for 1 h at 37 °C. The solution was then added to a 96-well plate and tested by our standard ELISA assay as above. All assays were performed in duplicate and the results averaged.

Phosphorylation of Calpactin. Phosphorylation assays were performed by using a preparation of EGF receptor provided by Dr. Gordon Gill (University of California at San Diego), prepared as in Weber et al. (1984). Calpactin II at 200 μg/mL, with or without bound antibody, was incubated in a solution containing 50 mM Tris, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 10 μM ATP, and 5 μCi of [^{32}P]ATP, pH 6.8, with 5 nM EGF receptor [with bound EGF as in Weber et al. (1984)]. After reaction for 10 min at room temperature, SDS sample buffer was added, and the samples were boiled and analyzed by SDS–PAGE. Gels were stained for protein with Coomassie blue and subjected to autoradiography. The calpactin bands were excised from the gel and Cherenkov counted.

Other Methods. Calcium ion binding, lipid-binding, and actin-binding assays were performed as described previously (Glenney, 1986a; Glenney & Glenney, 1985). In all experiments, calpactin was first incubated with antibody for 30 min at room temperature. Western blots were performed essentially as described previously (Glenney, 1986c). In some experiments (Figure 2), a special slot apparatus was used (Westgeest et al., 1986), and in other experiments (Figure 3), strips were cut from a single slot blot as in Glenney (1986c). All protein concentrations were determined by the Pierce Chemical Co. BCA assay. Subtyping of Mabs was performed by the ELISA assay described above but substituting peroxidase-conjugated antiisotype Mabs (Fisher). Nondenaturing gels were performed by using the standard buffer systems but omitting SDS from all buffers and also omitting β-ME from the sample buffer.

RESULTS

Calpactin II was used as immunogen for the production of monoclonal antibodies (Mabs) in Balb/C mice (Kohler & Milstein, 1975). Although 15 Mabs from 3 fusions were used in this study, the majority (2–13) were derived from a single fusion. To rule out the possibility that many of these represent coisolates of the same antibody-producing cells, Mabs were resolved by electrophoresis in nondenaturing gels in the presence or absence of calpactin II. As can be seen in Figure 1, the Mabs can be easily distinguished from each other by this method. In all cases, a single Coomassie-stained band was observed for the antibody alone. Most importantly, the presence of calpactin II changed the electrophoretic mobility of the antibody, indicating that all of the antibody is binding to calpactin II under nondenaturing conditions. Many Mabs have been shown to react with the denatured protein yet react only weakly with the native molecule (Smith & Wilson, 1986; Brennand et al., 1986). In many cases, the entire antibody band was shifted to a new position which stained more heavily with Coomassie blue. In some lanes, unbound calpactin was also observed, but this could be due to the inaccuracy of mixing the antigen and antibody at exactly equivalent molar amounts.

As shown in Figure 2, all 15 Mabs reacted with a single band on Western blots of total bovine lung protein. In some blots, a calpactin II standard was run in a lane adjacent to the cell lysate, and it was observed that the immunoreactive band comigrated with calpactin II (not shown). Since calpactin II is known to be expressed at high levels in human A431 cells (Glenney, 1986b), we tested our Mabs on total A431 cell protein. Seven of the Mabs (2, 4, 6, 8, 9, 14, and 15) resulted in a strong reactivity, three Mabs gave rise to weak reactivity (3, 5, and 7), and five Mabs were unreactive (1, 10, 11, 12, and 13). These differences in reactivity between bovine lung and human A431 cell protein probably represent differences between bovine and human calpactin II. Alternatively, some type of posttranslational modification of calpactin II may occur in A431 cells, causing the antigenic determinant to be masked.

Calpactins I and II are known to consist of two domains: a short amino-terminal tail and a protease-resistant core (Glenney, 1986a; Johnsson et al., 1986). In order to map the approximate location of the antigenic determinants, we digested calpactin II with trypsin and tested the reactivity of the fragments by Western blots. All Mabs reacted with the 39K undigested calpactin II (see Figure 3b, for instance), and

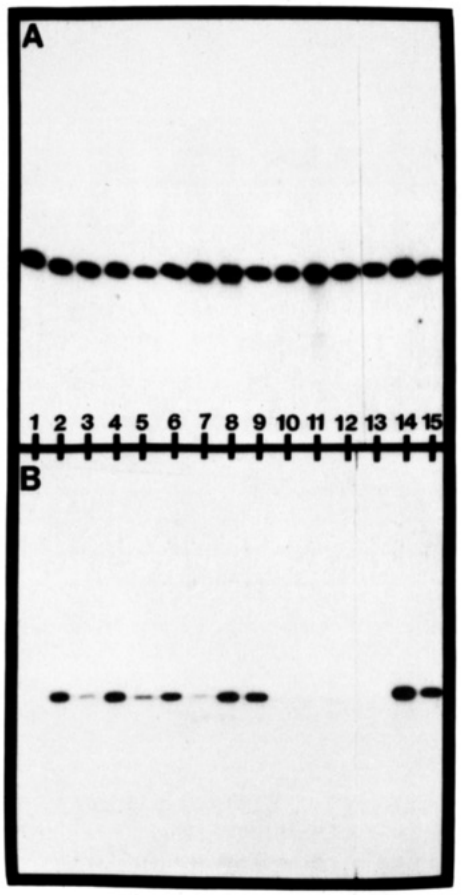

FIGURE 2: Western blot analysis of anti-calpactin II Mabs. Mabs were used to stain Western blots of total bovine lung (A) or human A431 cell protein (B). Protein was resolved on 10% acrylamide–SDS gels and electrophoretically transferred to nitrocellulose filters which were then stained with India ink and blocked with 5% powdered milk. After the nitrocellulose was incubated with 1 μg of antibody/mL (indicated in center; Mabs 1–15), reactivity was detected by further incubating with ^{125}I-labeled goat IgG against mouse immunoglobulin followed by autoradiography.

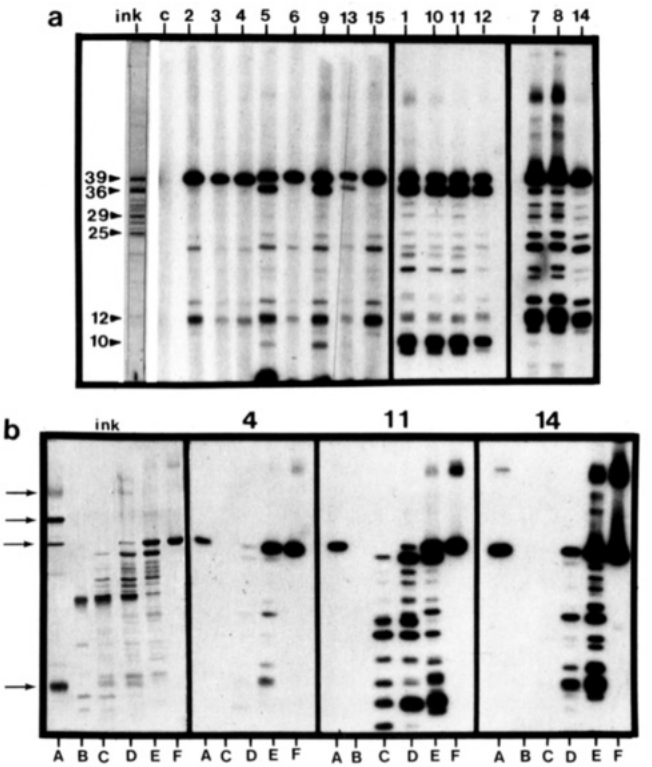

FIGURE 3: Western blots using the Mabs on trypsin-digested calpactin II. (a) Calpactin II at 100 μg/mL was digested with trypsin for 1 h at 1 μg of trypsin/mL. The digestion was quenched with SDS sample buffer, and the fragments were resolved on a 12% gel. The protein was transferred to nitrocellulose and stained with India ink, and strips of nitrocellulose were probed with antibody as described in the legend to Figure 2. A control in which the first antibody was omitted (c) and one India ink stained strip (ink) are shown for comparison. The molecular weights of some of the major fragments are listed, and some of the Mabs with similar reactivity are grouped together to allow direct comparison. (b) Calpactin II, either undigested (lanes F) or digested with 0.5 (E), 5.0 (D), or 25 (C) μg of trypsin/mL, was resolved by SDS–PAGE along with trypsin alone (B), or the protein standards BSA, actin, calpactin II, and cytochrome *c* (lanes A; also indicated by arrows). Protein was transferred to nitrocellulose, stained with India ink, and probed with the Mabs listed above each panel as described in the legend to Figure 2.

many reacted with discrete fragments in trypsin-digested samples (Figure 3a). Higher molecular weight bands were observed for undigested calpactin II stained with some of the Mabs in samples of purified protein (see Figure 3b, Mab14), which probably represent aggregates of calpactin. Since these were never observed in samples of total cell protein (Figure 1), we did not pursue the identity of these bands further. Trypsin digestion of calpactin II resulted in fragments of 36, 29, 25, 12, and 10 kDa as detected by India ink staining. A likely interpretation is that the 36-kDa fragment represents the core domain [the tail is known to be hypersensitive to proteolysis (Glenney et al., 1987; De et al., 1986; Huang et al., 1987; Haigler et al., 1987)] which is further cleaved into lower molecular weight fragments. Surprisingly, the reactivity of many of the Mabs was lost in conversion of intact calpactin to the 36-kDa core (Figure 3, Mabs 2, 3, 4, 6, 7, 8, 13, 14, and 15). By contrast, Mabs 1, 10, 11, and 12 reacted strongly both with the core and with a 10-kDa fragment. The 29-kDa tryptic peptide was only weakly reactive with any of the Mabs. These results suggest that two major antigenic regions exist: one in the 3-kDa tail and a second residing in a 10-kDa fragment.

To further define the binding site for Mabs reactive with the tail, a synthetic peptide which represents part of the human calpactin II tail was assayed for its ability to inhibit antibody reactivity using a solid-phase ELISA assay. To simplify analysis, only those Mabs which react with human calpactin

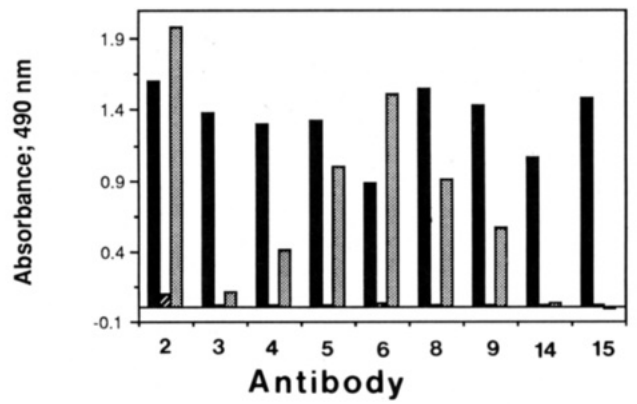

FIGURE 4: Inhibition of anti-calpactin II Mabs by soluble calpactin or a tail peptide. Calpactin II was used to coat the bottom of 96-well test plates. Mabs which react with human calpactin II (see Figure 2B) were then either added alone (solid bars; left) or added after preincubation with calpactin II (cross-hatched; middle) or a synthetic peptide corresponding to amino acids 9–27 (stippled; right). After a further incubation for 1 h, wells were incubated with peroxidase-conjugated second antibody followed by *o*-phenylenediamine and H_2O_2. Reactivity was monitored by the absorbance at 490 nm.

II were tested (see Figure 2B). As shown in Figure 4, the reactivity of all of the Mabs was completely inhibited by

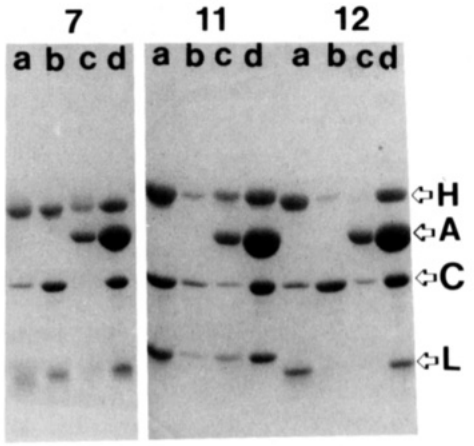

FIGURE 5: Effect of Mabs on the binding of calpactin II to actin filaments or phospholipid liposomes. Calpactin II was incubated 30 min with the antibody listed above each panel. The antibody–calpactin II complex was then added to an assay mixture containing 0.5 mg of phospholipid liposomes (a, b) per milliliter or 0.6 mg of actin/mL (c, d) in 60 mM Tris (pH 6.8) containing 1 mM $CaCl_2$. After further incubation for 1–1.5 h at room temperature, the liposomes and actin filaments were harvested by centrifugation at 100000*g* for 30 min in an airfuge. Supernatant (a, c) and pellet (b, d) fractions were adjusted to equal volumes of SDS sample buffer and resolved on a 10% acrylamide–SDS gel which was then stained with Coomassie blue. The identity of the polypeptides in the gel is listed on the right (H and L, IgG heavy and light chains, respectively; A, actin; C, calpactin II). Although only three examples are shown, Mabs 1–9 and 13–15 gave the same results as Mab 7; Mab 10 had the same effect as Mab 11, and Mab 12 was the only one observed in which the antibody polypeptides were not found in the liposome pellet together with calpactin II.

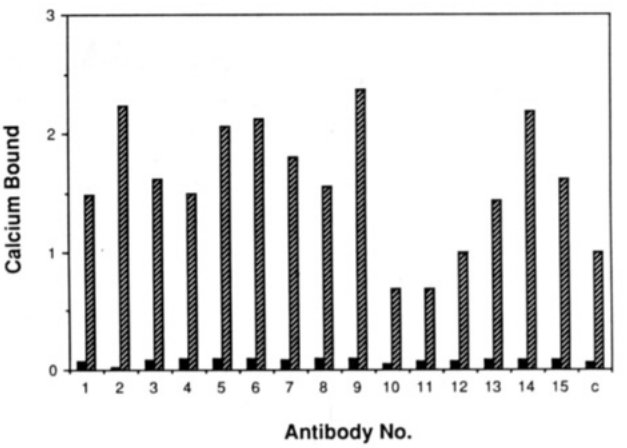

FIGURE 6: Effect of Mabs on Ca^{2+} binding by calpactin II in the presence or absence of phospholipid. Calpactin II was incubated with each of the 15 Mabs and subjected to equilibrium dialysis with $^{45}CaCl_2$ in the absence (solid bars) or presence (hatched bars) of 0.5 mg of phosphatidylserine liposomes/mL. After dialysis for 20 h at 22 °C, aliquots inside the dialysis membrane were tested for protein content as well as radioactivity, and the amount of radioactivity was measured in an equal aliquot of the dialysis buffer. The amounts of Ca^{2+} bound in two separate assays (for +PS) were averaged and compared to that of calpactin alone (listed as c).

preincubation with soluble native calpactin II. In addition, three of the Mabs were completely inhibited by the peptide (Figure 4; Mabs 3, 14, and 15). Interestingly, two of the Mabs (Mabs 2 and 6) which give the same reactivity on trypsin-digested calpactin II (Figure 3) were not inhibited by peptide. Other peptides will be necessary to more completely define the antibody-binding sites.

To test whether the Mabs had an effect on actin or lipid binding, a simple high-speed sedimentation assay was employed. The assay relies on the observation that actin filaments (or liposomes) will sediment upon high-speed centrifugation together with bound protein whereas calpactin II will not sediment alone under these conditions. Bound calpactin, in the presence or absence of antibody, was determined by comparing the soluble and pelleted proteins on a polyacrylamide gel in the presence of SDS. None of the Mabs had any detectable effect on the binding of calpactin to actin (see Figure 5 for examples). Indeed, the antibody heavy and light chains could be detected in the actin–calpactin pellet (Figure 5), confirming the results in Figure 3 that the two proteins interact under native conditions. In controls in which the actin or phospholipids were omitted, calpactin alone or with bound antibody was never observed in the pellet.

Most of the Mabs had no effect on the association of calpactin II with phosphatidylserine liposomes (Figure 5). Two of the Mabs (10 and 11) strongly inhibited the binding of calpactin II to phospholipids under these conditions, as detected by free calpactin in the supernatant. One antibody (12) did not inhibit the binding of calpactin to liposomes, but the antibody was not found in the phospholipid pellet, indicating that phospholipid would displace the binding of antibody 12 to calpactin (compare, for instance, the actin-binding component of this experiment).

We tested antibody–calpactin complexes for their ability to bind Ca^{2+} in the presence and absence of phospholipid using equilibrium dialysis with $^{45}CaCl_2$. As shown previously, at micromolar free Ca^{2+}, calpactin II binds very little Ca^{2+} in the absence of phospholipid and stoichiometric amounts in its presence (Glenney et al., 1987; Schlaepfer & Haigler, 1987). In the absence of phospholipid, none of the Mabs had any stimulatory effect on Ca^{2+} binding (Figure 6). By contrast, in the presence of phospholipid, most of the antibody–calpactin complexes actually bound more Ca^{2+} than calpactin + phospholipid alone. In control experiments, Ca^{2+} binding of antibody + phospholipid alone was negligible. Two Mabs (10 and 11) caused a slight decrease in the amount of Ca^{2+} bound (Figure 6). In order to determine whether this inhibition or enhancement was an effect on Ca^{2+} affinity or stoichiometry, we selected two Mabs and tested their effects over a wide range of Ca^{2+} concentration levels. To rule out the effect of simple cross-linking by the bivalent antibody, the effects of the monovalent Fabs are reported here although similar effects were observed for the intact Mabs. Antibody 6 caused an increase (and Mab 11 caused a decrease) in bound Ca^{2+}. Scatchard analysis (Figure 7) revealed that this was due to an effect on the affinity of calpactin for Ca^{2+}. Scatchard plots of all three conditions (without calpactin or with the two Mabs) extrapolated to four Ca^{2+}-binding sites per calpactin II molecule, and only the slope of the lines (affinity) is appreciably different.

Calpactin II is known to serve as a substrate of the EGF receptor tyrosine kinase (Sawyer & Cohen, 1985; Pepinsky & Sinclair, 1986), with the site of phosphorylation assigned to tyrosine-20 (De et al., 1986). Since many of the anti-calpactin II Mabs bind to this region of the molecule, we tested them for their ability to affect this phosphorylation in vitro. As expected, none of the anticore Mabs (Mabs 1, 10, 11, and 12) had a significant effect on the phosphorylation of calpactin II by the EGF receptor (Figure 8). Again not surprisingly, many of the antitail Mabs (Mabs 3, 7, 8, 13, 14, and 15) abolished this phosphorylation. This inhibition was specific since these Mabs had no effect on the autophosphorylation of the receptor itself (Figure 8). Quite unexpectedly, many of the antitail Mabs actually enhanced the phosphorylation of calpactin II up to 10-fold (Figure 8). In other experiments, it was found that this enhancement was due to an effect on the V_{max} of the reaction with no effect on the K_m (not shown).

Table I: Summary of the Reactivity and Effect of Mabs to Calpactin II[a]

	designation	subclass	bovine lung	human A431	36-kDa core	10-kDa tryptic peptide	Ca^{2+} binding (+ps)	Ca^{2+} binding (−ps)	lipid binding	actin binding	phosphor-ylation
1	w47	G1	+	−	+	+	+	−	−	−	+
2	II2	G1	+	+	−	−	+	−	−	−	++
3	II29	G1	+	w	−	−	+	−	−	−	−
4	II38	G1	+	+	−	−	+	−	−	−	+
5	II39	G1	+	w	+	−	+	−	−	−	++
6	II44	G1	+	+	−	−	+	−	−	−	++
7	II48	G1	+	w	−		+	−	−	−	−
8	II54	G1	+	+	−	−	+	−	−	−	−
9	II58	G1	+	+	+	−	+	−	−	−	++
10	II65	G1	+	−	+	+	−	−	+	−	+
11	II77	G1	+	−	+	+	−	−	+	−	+
12	II85	G1	+	−	+	+	+	−	•	−	+
13	II103	G1	+	−	−	−	+	−	−	−	−
14	IIp29	G1	+	+	−	−	+	−	−	−	−
15	IIp40	G1	+	+	−	−	+	−	−	−	−

[a] The second column is the designation which was given to the Mabs initially (and which we refer to them in the laboratory). The reactivity of the antibodies on Western blots is listed in columns 4–7 (see Figure 2 and 3), where (+) indicates strong reactivity, (−) indicates no detectable reactivity, and (w) denotes weak staining. The effect of Mabs on the Ca^{2+} binding is taken from Figure 6, where (+) indicates no effect or an increase in bound Ca^{2+} and (−) indicates a decrease. The effects of Mabs on actin and lipid binding are taken from Figure 5, where (−) indicates no effect and (+) points out those Mabs which inhibit lipid binding. The asterisk for Mab 12 denotes the observation that antibody was not bound to the lipid–calpactin complex. The effect of Mabs on phosphorylation is taken from Figure 8, where (+) indicates no change in the phosphorylation, (++) indicates those Mabs which enhance phosphorylation, and (−) shows those Mabs which abolish phosphorylation of tyrosine-20 by the EGF receptor.

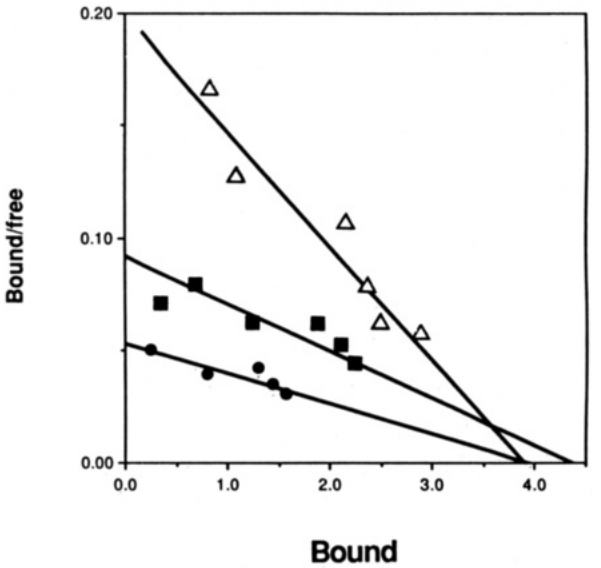

FIGURE 7: Effect of Fab fragments on Ca^{2+} binding by calpactin II. Monovalent Fab fragments were prepared from Mabs 6 (●) and 11 (△) and incubated with calpactin II at a weight ratio of 2:1 (Fab:calpactins). The Fab–calpactin complex or calpactin alone (■) was then added to liposomes as in Figure 6 and subjected to dialysis (in duplicate) at the specified Ca^{2+} concentration with trace levels of $^{45}CaCl_2$. The data were analyzed by the method of Scatchard as described previously (Glenney et al., 1987).

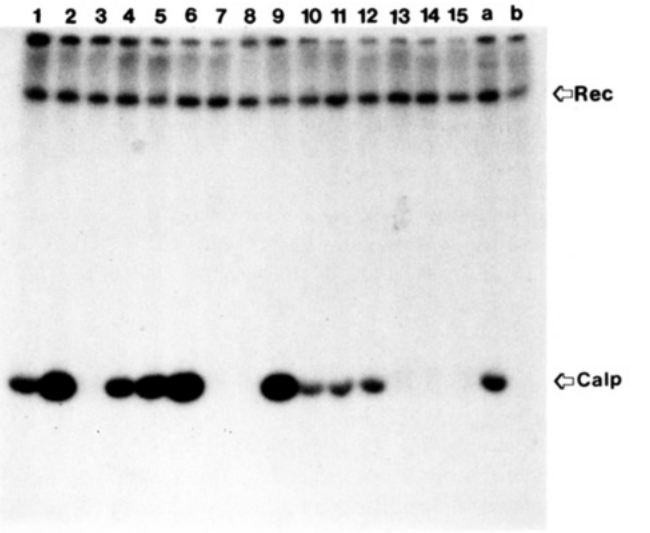

FIGURE 8: Effect of Mabs on the phosphorylation of calpactin II by the EGF receptor. Phosphorylation reactions were carried out with [^{32}P]ATP and the EGF receptor alone (b), with added calpactin II without antibody (a), or with calpactin II incubated with the specified Mabs (1–15). The reaction was stopped by the addition of SDS sample buffer and run on a 10% acrylamide–SDS gel which was then stained with Coomassie blue and exposed to X-ray film; the autoradiogram is shown.

Discussion

The 38-kDa subunit of calpactin I comprises two domains: a tightly packed core domain connected to a small (3-kDa) protease-sensitive tail (Glenney et al., 1987; Johnsson et al., 1986). The core has been shown to contain the Ca^{2+}-, phospholipid-, and actin-binding sites (Glenney, 1986a; Glenney et al., 1987), while the tails of both calpactins I (Glenney & Tack, 1985) and II (De et al., 1986; Haigler et al., 1987) contain the site of phosphorylation as well as the site of association of the light chain with the heavy chain in calpactin I (Johnsson et al., 1986; Glenney et al., 1986). It has been postulated that the tail represents a regulatory site of calpactin, and for calpactin I, only the fraction which contains the light chain is associated with the cytoskeleton (Zokas & Glenney, 1987). Clearly, monoclonal Mabs recognizing different structural features of the calpactins would be useful in testing the functional role of different regions of the molecule. In the present investigation, we examined the effect of 15 Mabs directed against bovine calpactin II on the in vitro properties of the protein (summarized in Table I). At least nine of the Mabs are directed against the tail domain, and four react strongly with the core. The immunodominance of the tail could reflect its relative accessibility in the molecule, or, alternatively, Mabs to the core may have been selected against because they were not reactive in Western blots (used in the primary screening method). Certainly, one would expect a more elaborate tertiary structure in the 36-kDa core than in the smaller 3-kDa amino terminus.

The Mabs described in the present study react with native protein as well as denatured calpactin II displayed on Western blots, in contrast to many Mabs which react only with dena-

tured protein (Smith & Wilson, 1986; Brennand et al., 1986). Thus, a complex of antibody with calpactin II is observed by electrophoresis under native conditions (Figure 1), and Mabs are also found in the calpactin–actin and calpactin–phospholipid pellets (see Figure 5 for representative examples). The ability of the Mabs to react strongly with both native and denatured protein, then, makes them ideally suited for use in probing the structure and function of the molecule.

Both calpactin I and calpactin II are known to interact with anionic phospholipids (Glenney, 1985, 1986a,b; Glenney et al., 1987; Geisow et al., 1986; Johnsson et al., 1986; Schleapfer & Haigler, 1987). One effect of this interaction is to shift the Ca^{2+} sensitivity of calpactins from the millimolar to the micromolar level in a manner analogous to the effects of phospholipid on protein kinase C (Glenney, 1985, 1986a; Glenney et al., 1987; Schlapfer & Haigler, 1987). Surprisingly, almost all of the Mabs tested have an effect on either lipid binding or Ca^{2+} binding. Mabs 10 and 11, for instance, result in reduced calpactin II binding to phospholipid liposomes (Figure 5). Significantly, both of these Mabs react with the core region, a region in calpactin I which is known to contain the Ca^{2+}- and phospholipid-binding sites. Of the other two anticore Mabs, liposomes apparently displace the binding of antibody 12 to calpactin. Thus, three different types of effects are observed with Mabs which react with the same 10-kDa fragment. It will be interesting to determine the exact antibody-binding sites of these anticore Mabs in the 4-fold repeat structure of calpactin II.

Most of the Mabs described here have an effect on Ca^{2+} binding by calpactin II. The most consistent feature was that Mabs which recognize the tail increase the affinity of calpactin for Ca^{2+} in the presence of phospholipid. It was found previously that calpactin II binds stoichiometric amounts of Ca^{2+} in the presence of phospholipid (Glenney et al., 1987). Scatchard analysis of that data was interpreted as curvilinear, indicative of two cooperative binding sites. Since the core region has a 4-fold rather than a 2-fold repeat, it was suggested that two repeats may be necessary for complexing Ca^{2+} ions. The results using Mabs suggest that this interpretation was incorrect. Mabs which increase calpactins affinity for Ca^{2+} are particularly illuminating in this regard. As shown in Figure 6, most of the antitail Mabs result in three to four Ca^{2+} ions bound under conditions of this assay. To ensure that this was not due to a problem of cross-bridging of calpactin by the bivalent IgG, Fab fragments were used for an entire Ca^{2+} saturation curve (Figure 7). Scatchard analysis of this binding data reveals four Ca^{2+}-binding sites with a K_d of approximately 20 μM. The previous results (essentially repeated in Figure 7) can now be reinterpreted as four Ca^{2+}-binding sites with a significantly lower affinity. Consistent with this, another antibody which lowers the affinity of calpactin for Ca^{2+} (11) also allows the detection of four Ca^{2+}-binding sites (Figure 8). These results then are in substantial agreement with the studies of Schlaepfer and Haigler (1987) in which they were able to detect four Ca^{2+}-binding sites on calpactin II (lipocortin I).

Previous models have depicted the tail and core to be two noninteracting domains (Glenney et al., 1987). This model is clearly oversimplified since Mabs to the tail dramatically affect Ca^{2+} binding in the core. It has been shown for both calpactin I and calpactin II that Ca^{2+} binding by the core markedly affects phosphorylation of the tail by tyrosine kinases (Fava & Cohen, 1984; Glenney, 1985; Haigler et al., 1987). It would appear then that there is significant communication between these two domains. This observation also raises the possibility that other effects on the tail such as phosphorylation or interaction with other proteins in vivo could increase the affinity of calpactin II for Ca^{2+}. Certainly, the affinity for Ca^{2+} observed in the absence of antibody is too low as to be of significance in resting or even activated cells. Consistent with this hypothesis, Schleapfer and Haigler (1987) have found that phosphorylated calpactin II binds to phosphatidylserine liposomes at a lower Ca^{2+} level than the nonphosphorylated form.

The Mabs described here should be particularly useful in analyzing the effects of phosphorylation of calpactin II. Calpactin II is known to be phosphorylated on tyrosine in EGF-treated cells (Sawyer & Cohen, 1985; Pepinsky & Sinclair, 1986). More recent studies have shown that calpactin II is also phosphorylated in vitro by the EGF receptor on tyrosine-20 in the amino-terminal tail (De et al., 1986; Haigler et al., 1987). This is a position similar to the phosphorylation site of calpactin I by $pp60^{src}$ (Glenney & Tack, 1985). Mabs to the tail of calpactin II have one of two effects on the phosphorylation by the EGF receptor: (i) Mabs abolish the phosphorylation while having little effect on autophosphorylation by the receptor itself (Figure 8). These include Mabs which react with a synthetic peptide (amino acids 9–27) which includes tyrosine-20 (Figure 4; antibodies 3, 14, and 15). (ii) Mabs enhance the phosphorylation of calpactin II (up to 10-fold) by the EGF receptor (Figure 8). These include Mabs which clearly react with the tail (see Figure 3; antibodies 2 and 6) but which are not inhibited by the synthetic peptide. It is possible that this indicates a region of the tail in which interactions, such as might occur by association with another protein, lead to the phosphorylation of calpactin II. This possibility should be kept in mind when analyzing the effect of calpactin II phosphorylation in vivo. These Mabs, by either enhancing or inhibiting the phosphorylation of calpactin II in vitro, should be ideally suited for microinjection studies in which the phosphorylation of this substrate can be modulated independently of the effect on other substrates of the tyrosine kinases.

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