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Interdomain Interactions of Radixin *in Vitro**

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We have assayed the domains of the ERM protein radixin for binding activities *in vitro*. Affinity columns bearing the amino-terminal domain of radixin selectively bound a small subset of the proteins of the chicken erythrocyte cytoskeleton. Two of those proteins were identified as radixin itself and band 4.1. In contrast, the carboxyl-terminal domain of the molecule bound neither protein, and full-length radixin did not bind band 4.1 (binding of full-length radixin to itself was not evaluated). Columns bearing a mixture of the amino- and carboxyl-terminal domains of radixin also failed to bind radixin and band 4.1. These results suggested that the amino- and carboxyl-terminal sequences can interact with one another either *in cis* or *in trans*, and so interfere with radixin's interactions with other ligands. Using affinity co-electrophoresis, we confirmed a direct interaction in solution between the two radixin domains; the data are consistent with the formation of a 1:1 complex with a dissociation constant of $\sim 5 \times 10^{-8}$ M. Competition between intramolecular and intermolecular interactions may help to explain the provocative and dynamic localization of ERM proteins within cells.

The ERM proteins, ezrin, radixin, and moesin, are components of cortical cytoskeleton that are thought to play a role in linking cytoskeletal and membrane elements. They are found in ruffling edges, growth cones, and membrane extensions such as microvilli, filopodia and lamellipodia, regions rich in F-actin, but their precise positions do not coincide with those of F-actin or other major cytoskeletal elements (1–5). For example, in neuronal growth cones, ERM and F-actin staining patterns are overlapping but distinct (3). Moreover, drugs that depolymerize microtubules delocalize ERM proteins from growth cones, but not F-actin (3).

It is not known what molecular interactions are responsible for correct localization of ERM proteins within cells. *In vitro*, ERM proteins do not behave like conventional actin-binding proteins (6). Binding studies, and the isolation of complexes from cell extracts have suggested a number of potential ERM ligands. These include the transmembrane protein CD44 (7) and F-actin (which is reported to interact with the carboxyl-terminal domain of ezrin and moesin (8, 9), although the details of that interaction are controversial (10)). Apparently, ERM proteins may also self-associate into homo- and hetero-oligomeric complexes (11, 12).

Assays in cells demonstrate that separable domains of the ERM proteins contribute information that specifies appropriate localization in the cell (13, 14). For example, at low levels of expression, the carboxyl-terminal domain of radixin localizes to all of the structures in which ERM proteins are normally found, save the cleavage furrow, and associates quite clearly with one cellular element, stress fibers, where ERM proteins are not typically found. The information necessary to target radixin to the cleavage furrow is in the amino-terminal domain of the protein (14).

These cellular assays also reveal evidence for regulatory interactions between the domains of ERM proteins. Expressed at high levels, the carboxyl terminus causes dramatic disruption of normal cell morphology and interferes with cell division, while the amino terminus has neither phenotype (14–16). However, both consequences of high level expression of the carboxyl terminus are suppressed by the presence of the amino terminus, either *in cis* or *in trans* (14, 16). Perhaps, then, the deleterious effects of one domain are prevented by an interaction with the other domain. We have tested this model using *in vitro* methods. Here, we present evidence that the amino- and carboxyl-terminal domains of radixin can bind each other *in vitro*. We also show that this binding event blocks the binding of other ligands.

MATERIALS AND METHODS

We cloned chicken radixin cDNAs by polymerase chain reaction (First Strand cDNA Synthesis Kit; Pharmacia Biotech Inc.) from chicken embryo fibroblast mRNA. The clones representing the full-length, amino-terminal (codons 1–318) and carboxyl-terminal (codons 319–585) sequences were ligated into PQE-70 (Qiagen) using the *Sph*I and *Bgl*II sites, so that the last amino acid of each sequence is followed by arginine, serine, and then 6 histidines and a stop codon. *Escherichia coli* DH5αF′IQ transformants with the correct radixin insert were identified by restriction digests and by DNA sequencing (Sequenase version 2.0 DNA Sequencing Kit; U. S. Biochemical Corp.) at the junction of radixin insert and vector. Expression of the three His_6-tagged radixin polypeptides and His_6-tagged dihydrofolate reductase was induced by growth in 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cell pellets were lysed (50 mM NaH_2PO_4/$NaHPO_4$, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM Pefabloc, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 0.009 TIU/ml aprotinin, and 1 mg/ml lysozyme), sonicated, and clarified by centrifugation, then stored as aliquots at −80 °C. High-speed supernatants containing the His_6-tagged polypeptides were incubated with 0.5 ml of nickel-NTA[1] Sepharose CL-6B resin (Qiagen) in lysis buffer supplemented with 10 mM β-mercaptoethanol. The resin was washed batchwise twice with 15 ml of wash buffer (50 mM NaH_2PO_4/$NaHPO_4$, pH 8.0, 300 mM NaCl, 40 mM imidazole) plus 10% glycerol and once in wash buffer alone.

For affinity adsorption experiments, His_6-tagged proteins were left bound to nickel-NTA matrices and incubated with a chicken erythrocyte cytoskeletal fraction prepared as follows. Freshly washed erythrocytes were extracted with 0.1% Nonidet P-40 in PM2G (100 mM Pipes, 1 mM $MgSO_4$, 2 mM EGTA, pH 6.9) containing 0.009 TIU/ml aprotinin, 1 mM

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[1] The abbreviations used are: NTA, nitrilotriacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; FL, full-length radixin; N-domain, amino-terminal domain; C-domain, carboxyl-terminal domain.

phenylmethylsulfonyl fluoride, and 1 mg/ml leupeptin. After a wash in the same buffer, the pellet was extracted in 8 M urea in phosphate-buffered saline with protease inhibitors. The urea extract was spun at 11,000 × *g* and the supernatant collected, dialyzed in phosphate-buffered saline, and frozen as aliquots. The erythrocyte proteins were incubated with the affinity matrices batchwise for 20 min 4 °C, then repeatedly washed and centrifuged to remove unbound proteins. The matrices were loaded into columns and eluted with two 0.5-ml aliquots of elution buffer (50 mM NaH_2PO_4/$NaHPO_4$, pH 8.0, 300 mM NaCl, and 125 mM imidazole). Fractions were boiled in sample buffer and analyzed by 7.5% SDS-PAGE. Proteins were detected by silver stain or by immunoblotting as described (14), using antibodies that detect epitopes in the amino-terminal domain of ERM proteins (#220), the carboxyl-terminal domain of radixin (#457), and previously characterized antibodies against chicken erythrocyte band 4.1 (17, 18).

For affinity co-electrophoresis (ACE), high-speed supernatants of His_6-tagged radixin amino- and carboxyl-terminal domains were purified on nickel-NTA resin columns. Glutathione *S*-transferase was prepared by expression of pGEX-3X (Pharmacia) in *E. coli* strain HB101. Protein concentrations were determined by the Bradford assay (Bio-Rad). ACE gels were cast as described (19, 20) using 1% low gelling temperature agarose in 125 mM potassium acetate, 50 mM Hepes adjusted to pH 7.5 with NaOH. Gels were run at 60 volts for 4 h and the proteins then transferred to nitrocellulose by capillary action and analyzed by immunoblotting. Retardation coefficients were calculated as described previously, including the application of a correction for "overrunning" (electrophoresis of the detected species beyond the end of the zones containing the retarding species (20)). Dissociation constants were calculated from nonlinear least squares fitting of plots of corrected retardation coefficient *versus* concentration of retarding protein (21). Data were fit to a general form of the binding equation that is appropriate even when the concentration of the detected species is not $\ll K_d$ (20).

RESULTS AND DISCUSSION

Distinct Binding Domains of Radixin in Vitro—To prepare radixin affinity columns, the high speed supernatants of bacterial extracts expressing His_6 versions of full-length radixin (FL) or its amino-terminal domain (N-domain) or carboxyl-terminal domain (C-domain) were applied to nickel-NTA agarose as described above. In each case, the radixin polypeptide encoded by the plasmid is by far the most abundant polypeptide bound, although several bacterial bands are apparent by silver staining (data not shown).

As a source of potential binding partners for radixin, we used the proteins of the cytoskeletal fraction of chicken erythrocytes. All of the cytoskeletal radixin in these cells is in a single structure, the marginal band, from which it can only be extracted by strong chaotropic agents (2). The cells also are available in large quantities. We prepared detergent-extracted cytoskeletons from suspensions of chicken erythrocytes, solubilized these in 8 M urea, removed the urea by dialysis, and applied the extracts to the three radixin affinity columns described above. After extensive washing, columns were eluted with 125 mM imidazole and the eluted proteins analyzed by SDS-PAGE. Several erythrocyte proteins that bind to each of the columns were detected by silver stain, but we have identified by immunoblotting two known proteins that bind either exclusively or preferentially to the NH_2-terminal domain.

A ~80-kDa erythrocyte protein is detected in the eluate from the N-domain column by silver stain. That protein is identified as radixin by immunoblotting with antibody 457, specific for the carboxyl terminus of radixin, and antibody 220, which binds to an epitope present in the amino termini of all ERM proteins (Fig. 1*A*, *AMINO*, both *C* and *N* lanes). This band is not detectable in the eluates from the C-domain column, by either immunoblotting (Fig. 1*A*, *CARBOXY*) or silver stain. These data are consistent with the finding of Andreoli *et al.* (12), who demonstrated that full-length ezrin bound more tightly to its amino terminus than to its carboxyl terminus. We do not detect ezrin or moesin as proteins bound to the N-domain column, perhaps because they are much less abundant

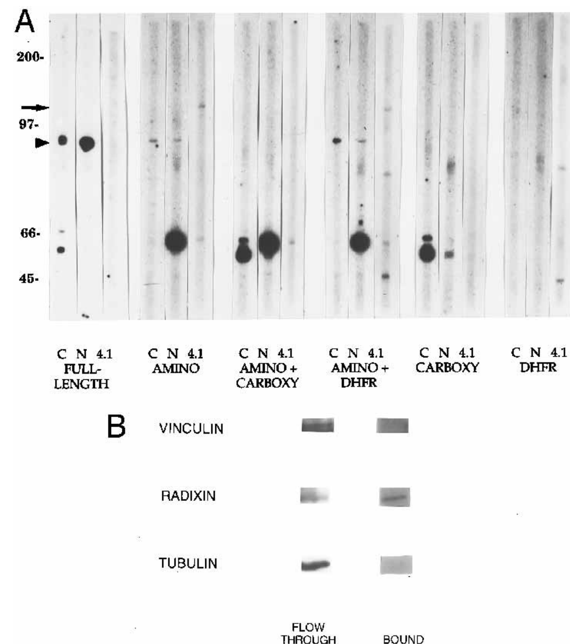

FIG. 1. *A*, Immunoblot analyses of column eluates. High imidazole eluates from columns bearing His-tagged polypeptides (*AFFINITY COLUMN*) plus bound chicken erythrocyte proteins were collected. The His-tagged polypeptides included: full-length radixin (*FULL-LENGTH*), the NH_2- and COOH-terminal domains of radixin, and dihydrofolate reductase. The eluates were separated on 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies recognizing the carboxyl terminus (*C*) or amino terminus (*N*) of radixin, or band 4.1 (*4.1*). The *arrowhead* indicates the position of radixin, the *arrow* indicates the position of band 4.1, and the *numbers* (kilodaltons) indicate mobilities of four molecular weight markers. *B*, partitioning of vinculin, radixin, and tubulin between flow-through and column-bound material, detected by Western blots of fractions loaded to represent equal starting material.

in chicken erythrocytes than radixin.[2] We cannot determine if radixin is bound to the FL column, since the two proteins should co-migrate.

A ~110-kDa polypeptide detected by silver stain binds preferentially to the N-domain column. That protein is identified as band 4.1 in immunoblots, using antibodies against chicken erythrocyte band 4.1 (Fig. 1*A*, *amino*, *4.1 lane*). Granger *et al.* (22) demonstrated that in chicken erythrocytes, band 4.1 occurs in multiple isoforms with a wide range of molecular weights, but that a species of ~115 kDa (in their gel system) is the major one. Our ~110-kDa band co-migrates with the major band 4.1 element in our hands. In some experiments, the antibodies identify a much less intense band in the eluates from the C-domain column and the FL column.

Several properties of the observed protein binding suggest that it is specific. First, the binding is highly selective. Although some erythrocyte proteins appear to bind to all three columns, in fact they and the specific polypeptides named above account only for a small subset of the total complement of proteins in the extract. Second, we can detect both tubulin and vinculin by immunoblotting of the column flow-through, but we detect no signal above background among the proteins bound to the column (Fig. 1*B*). Third, neither band 4.1 nor radixin bind to column bearing an irrelevant His-tagged protein of comparable size, dihydrofolate reductase. In contrast, those erythrocyte proteins that bind to all three radixin columns also bind to the dihydrofolate reductase control (data not shown).

We do not know if band 4.1 and radixin are ligands of radixin

[2] B. Winckler and F. Solomon, unpublished.

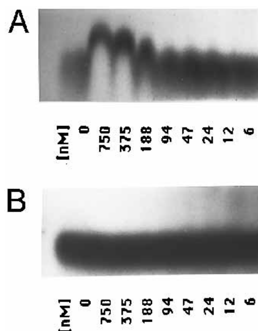

FIG. 2. **Affinity co-electrophoresis of the N- and C-domains of radixin.** *A*, column-purified His_6-N-domain (10^{-7} M) was loaded into a long gel slot perpendicular to the direction of electrophoresis. Column-purified His_6-C-domain was loaded, at the concentrations given, into multiple lanes parallel to the direction of electrophoresis. After electrophoresis, during which time the migrating front of N-domain traversed the zones containing the C-domain, the contents of the gels were transferred to nitrocellulose and visualized by immunoblotting with antibody against the N-domain. *B*, electrophoresis was carried out as in *A*, except that glutathione *S*-transferase was loaded into the slot and was detected using antibodies specific for that protein.

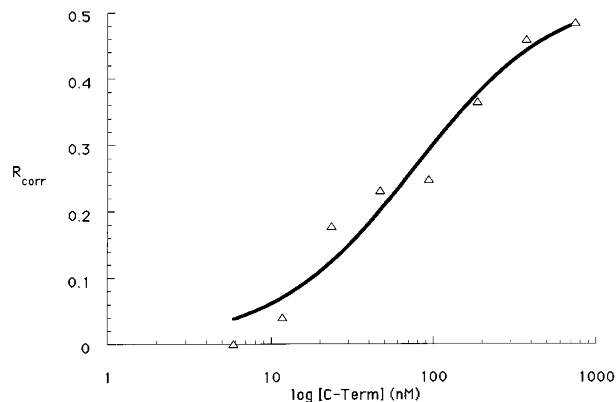

FIG. 3. **Analysis of the binding of the N- and C-domains of radixin as revealed by affinity co-electrophoresis.** R_{corr}, the corrected value of the retardation coefficient, quantifies the electrophoretic retardation of the N-domain. *C-Term* gives the nominal concentration of the C-domain in the lanes. Typically, the concentration of the detected species can be ignored in ACE experiments, because it is $\ll K_d$. Here, that assumption does not apply because detection of the N-domain required relatively high concentrations. The nominal initial concentration of the N-domain was 10^{-7} M, establishing an upper limit. Because the band broadens and diffuses during electrophoresis, the actual concentration of N-domain was likely lower. Varying the assumed concentration of the N-domain in the analysis from its highest possible value (10^{-7} M) to 1.25×10^{-8} M yielded values for K_d that varied about 4-fold range. The data have been fit to an equation derived from the definition of K_d, using the assumption that bound fraction is equal to R_{corr}/R_∞, where R_∞ represents the limiting value of R_{corr} when the concentration of the carboxyl-terminal domain is arbitrarily large. The curve was obtained using nonlinear least squares methods, in which K_d and R_∞ were taken as variables to be fit simultaneously.

in the cell. However, these experiments do suggest that, under the conditions of this assay, specific associations do occur between a small subset of chicken erythrocyte proteins and discrete domains of radixin.

Inhibition of Interactions between the N-domain and in Vitro Ligands by the C-Domain—The observation that columns containing the radixin N-domain retain a protein, band 4.1, that is not retained by FL columns raises the possibility that, in full-length radixin, the carboxyl-terminal domain inhibits the binding properties of the amino-terminal domain. This effect could occur because the presence of the domains as contiguous sequences affects their conformation and therefore their binding properties. Alternatively, the two domains could physically interact with each other in a way that excludes other ligands. To distinguish between these possibilities, we assayed the ability of the C-domain to interfere with the binding properties of the N-domain. As shown in Fig. 1, columns bearing either the N-domain alone, or the N-domain mixed with a control protein, dihydrofolate reductase, both bind radixin and band 4.1. In contrast, columns bearing a mixture of the N-domain and C-domain bind neither radixin nor band 4.1 (Fig. 2, *AMINO* + *CARBOXY*). The results suggest that the C-domain can inhibit the binding of ligands to the N-domain.

Direct Detection of Binding between the Amino- and Carboxyl-terminal Domains of Radixin in Solution—The observation that the C-domain can inhibit the binding properties of the N-domain strongly suggests that these two domains bind to one another. To demonstrate that such direct binding does occur, in solution, and to estimate the strength of binding, we used the technique of affinity co-electrophoresis (ACE, see Refs. 19 and 20)). Briefly, the two His_6-tagged polypeptides were subjected to electrophoresis within a single 1% agarose gel in a physiological buffer (125 mM potassium acetate, 50 mM Hepes, adjusted to pH 7.5 with NaOH). We loaded the N-domain (at 10^{-7} M) into a long transverse slot. We cast (in agarose) the C-domain into nine rectangular wells at concentrations from 0 to 750 nM. The anode was placed so that the faster migrating N-domain passes through the zones containing the C-domain during most of the electrophoresis. The mobility of the N-domain was then detected by transferring the proteins out of the gel to nitrocellulose and probing with an amino-terminal-specific antibody. Fig. 2*A* demonstrates that, where migrating N-domain encountered the C-domain, the migration of the former was retarded in a manner that varied directly with the concentration of the latter. In contrast, the migration of purified glutathione *S*-transferase, a control protein, was not affected by the C-domain over the same range of concentrations (Fig. 2*B*).

From measurements of mobility retardation in Fig. 2*A*, we can estimate the dissociation constant for the interaction of the amino- and carboxyl-terminal polypeptides of radixin (20). Fig. 3 shows the analysis of one such experiment. As described (21), to avoid problems arising from saturation of the film, we used a PhosphorImager to determine the true midpoint of each of the bands. The data have been fit by an equation that assumes 1:1, noncooperative binding, and a concentration of N-domain of 5×10^{-8} M. The curve indicates an apparent value for K_d of 4.5×10^{-8} M. A second experiment, analyzed in the same way, gave a value for K_d of 4.2×10^{-8} M (not shown). Equations that assume higher order or cooperative binding (21) fit the data significantly less well (not shown).

In summary, the data indicate that radixin has separable domains capable of specific binding interactions *in vitro*. Binding partners for radixin's domains include band 4.1 and radixin itself. Furthermore, the N- and C-domains of radixin can bind each other in solution with high affinity. Like the intact full-length protein, the reconstituted complex of the N- and C-domains fails to interact with band 4.1. Since the estimated concentration of the N- and C-domain polypeptides in bacterial extracts is $\sim 2.5 \times 10^{-6}$ M, it is likely that, in the experiment in which both polypeptides were mixed and applied to a single column (Fig. 1), complexes had formed before adsorption to the column (Fig. 1). Taken together with the results of transfection experiments, in which high level expression of the carboxyl-terminal domain of radixin had deleterious consequences that the full-length protein did not have, and that could be suppressed by co-expression of the amino-terminal domain (14, 16), these data strongly suggest that direct interactions between the amino- and carboxyl-terminal domains of the radixin

molecule *in vivo* inhibit the interaction of radixin with other molecules. Presumably, such inhibition is overcome under appropriate circumstances, either because ligands with higher affinity or effective local concentration successfully compete with the interaction between the amino- and carboxyl-terminal domains or because regulatory modifications (*e.g.* phosphorylation) modulate the affinity of that interaction.

It is reasonable to propose that the interaction between amino- and carboxyl-terminal domains of radixin is intramolecular. Such a situation would be strikingly similar to what has been observed in studies of vinculin, in which intramolecular interaction between head and tail domains has been shown to compete with the binding of presumptive ligands (23, 24). Since ERM proteins can self-associate, however, we cannot rule out the possibility that interactions of the amino- and carboxyl-terminal domains of radixin may also be intermolecular. In this regard it is noteworthy that, in the present study, affinity columns of the amino-terminal domain of radixin bound full-length radixin, but columns of the carboxyl-terminal domain did not. Similarly, Andreoli *et al.* (12) reported that full-length ezrin binds its own amino terminus substantially more tightly than its own carboxyl terminus. Although negative results must be interpreted with caution (protein fragments, and proteins immobilized on solid supports, may have artifactually altered binding properties), it is possible that self-association of full-length ERM proteins involves activities other than those demonstrated in the present study. A detailed analysis of binding domains and their activities, both *in vivo* and *in vitro*, will resolve these issues.

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