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Heterotypic and homotypic associations between ezrin and moesin, two putative membrane–cytoskeletal linking proteins

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ABSTRACT **Ezrin and moesin are components of actin-rich cell surface structures that are thought to function as membrane–cytoskeletal linking proteins. Here we show that a stable complex of ezrin and moesin can be isolated from cultured cells by immunoprecipitation with specific antibodies. The capacity of these two proteins to interact directly was confirmed with a blot-overlay procedure in which biotin-tagged proteins in solution were incubated with immobilized binding partners. In addition to the heterotypic association of ezrin and moesin, homotypic binding of ezrin to ezrin and of moesin to moesin was also demonstrated *in vitro*. These results suggest mechanisms by which ezrin and moesin might participate in dynamic aspects of cortical cytoskeletal structure.**

Ezrin is enriched in microvilli and other surface projections in a wide range of cells in culture (1–6) and in tissues (7, 8). It colocalizes with F-actin in these structures and partially cofractionates with the cytoskeleton in extraction experiments (1, 4, 8). These data suggest that ezrin may be directly or indirectly associated with the actin cytoskeleton of cell-surface structures.

The ezrin cDNA sequence (9, 10) shows that ezrin is a member of the band 4.1 superfamily, which includes erythrocyte band 4.1, talin, two protein-tyrosine phosphatases, the neurofibromatosis 2 tumor-suppressor candidate merlin, and the ezrin–radixin–moesin (ERM) family (11–19). Band 4.1 is a membrane–cytoskeletal linking protein that connects the integral membrane protein glycophorin to the subcortical actin–spectrin network (20). Talin links the fibronectin receptor to the actin cytoskeleton via vinculin and α-actinin (21). The subcellular localization of ezrin and its sequence homology to band 4.1 and talin have led to the hypothesis that ezrin is also involved in linking plasma membrane and cytoskeletal components (9). Support for this model has come from recent transfection experiments that show that the amino-terminal half of ezrin, which includes the region homologous to the membrane-binding domain of band 4.1 (22), contains a determinant for plasma membrane localization, whereas the carboxyl-terminal half of the protein displays actin cytoskeletal association (23).

Radixin, the second member of the ERM family, is an F-actin barbed end-capping protein originally isolated from hepatic cell–cell adherens junctions (24). The protein shares 75% amino acid-sequence identity with ezrin over the entire length of the coding region (12). Immunolocalization studies indicate that radixin, but not ezrin, is present in focal contacts and cell–cell adherens junctions of cultured cells (2, 16). The third member of the ERM family, moesin, was isolated independently from bovine uterus (25) and human placenta (26); cloning and sequencing of moesin cDNA revealed that the protein is 74% identical to ezrin in amino acid sequence (14). Moesin, like ezrin, colocalizes with F-actin in surface structures (2, 7).

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The extensive homology between ERM family members suggests that the membrane–cytoskeletal linking model proposed for ezrin may be equally applicable to radixin and moesin. It seems likely that these three proteins have evolved to fulfill distinct, specialized linking roles, perhaps by binding to different or partly overlapping sets of membrane or cytoskeletal partners. Support for this view comes from immunolocalization studies in tissues (7) and the fact that amino acid differences between family members have been highly conserved among species. For example, human and mouse ezrin have 96% sequence identity, and radixin and moesin are each 98% identical between the two species. Thus, the ≈25% divergence between ERM family members presumably specifies unique functional attributes because little substitution is tolerated. The identification of protein–protein associations for these proteins should provide clues to their specific functions. In this report, we show that ezrin binds moesin *in vivo* and *in vitro* and discuss the functional implications of such an association.

MATERIALS AND METHODS

Cell Culture. A431 (human epidermoid carcinoma) cells were cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum/antibiotics. For biosynthetic ^{35}S-labeling, cells were grown in methionine-free medium/10% serum/2 mM L-glutamine/10 μM L-methionine/[^{35}S]methionine at 80–200 μCi/ml (Tran^{35}S-Label; ICN; 1 Ci = 37 GBq) for 10–12 hr before lysis.

Antibodies and Purified Proteins. Human placental ezrin and moesin were purified as described (26). Human ezrin and moesin were used to generate rabbit antisera, and affinity-purified polyclonal antibodies specific for ezrin or moesin were prepared as described (2).

Immunoprecipitation. Cells were lysed with 1% Triton X-100/25 mM Tris·HCl/150 mM NaCl/1 mM EDTA/0.25 mM phenylmethylsulfonyl fluoride/0.5 mM benzamidine, pH 7.4, and centrifuged at 100,000 × *g*. Aliquots of the supernatant were mixed with protein A-Sepharose beads and either 1.1 μg of ezrin antibody or 1.4–2.8 μg of moesin antibody and incubated for 2–3 hr at 4°C. In competition experiments, purified human ezrin or moesin was added to this mixture. In one set of experiments, supernatants were subjected to four freeze–thaw cycles at −70°C and 37°C before immunoprecipitation. Immunoprecipitates were washed with the lysis buffer and then eluted from the beads with SDS/PAGE sample buffer.

Gel Electrophoresis and Immunoblotting. Protein samples were analyzed by SDS/8% PAGE. Silver staining was done as described (27). For immunoblot analysis, a semi-dry electroblotter was used to transfer proteins to poly(vinylidene fluoride) membranes (Immobilon-P; Millipore). The blots were blocked with 10% nonfat dry milk and then incubated with either ezrin antibody at 50 ng/ml, moesin antibody at 80 ng/ml, or 1:10,000 moesin antiserum. Immu-

Abbreviation: ERM, ezrin–radixin–moesin.

noreactive bands were detected using peroxidase-conjugated goat anti-rabbit IgG at 2 μg/ml (Cappel Laboratories) and an enhanced chemiluminescence system (Amersham).

Blot Overlay. To generate biotinylated probes, purified human ezrin and moesin (0.3 mg/ml) in 50 mM $NaHCO_3$/20 mM NaCl, pH 8.5, were incubated with NHS-LC-biotin at 0.1 mg/ml (sulfosuccinimidyl-6-(biotinamido) hexanoate; Pierce) for 1 hr on ice, then 50 mM glycine was added, and the solutions were dialyzed. Cell lysates were prepared as described for immunoprecipitation, except that 1% sodium deoxycholate and 0.1% SDS were included in the lysis buffer, and the 100,000 × *g* supernatant was precleared with streptavidin-conjugated agarose beads (Sigma). Proteins were electrophoresed and transferred to membranes as described above. The blots were blocked with 10% milk, followed by sequential incubations with avidin at 0.2 mg/ml and biotin at 0.1 mg/ml to block avidin- and biotin-binding sites. The avidin/biotin blocking steps were omitted for analysis of purified proteins. Blots were incubated with biotinyl–ezrin or biotinyl–moesin at 0.1 μg/ml in 1% milk for 4–12 hr at 4°C; control blots were treated with 1% milk alone. Probe binding was detected using 1:2000 peroxidase-conjugated avidin (Extr-Avidin; Sigma) and enhanced chemiluminescence. A 0.1% Tween-20/25 mM Tris·HCl/150 mM NaCl, pH 7.4 buffer was used for all washes and incubations.

RESULTS

Association of Ezrin and Moesin *in Vivo*. Affinity-purified ezrin antibody, which recognized only ezrin (81 kDa) in immunoblots of A431 cells (Fig. 1*A*), was used for immunoprecipitations from ^{35}S-labeled cell lysates. In addition to ezrin, a 77-kDa protein coprecipitated (Fig. 1 *B* and *C*). To determine whether the 77-kDa protein was present by virtue of an association with endogenous ezrin, an excess of unlabeled purified human ezrin was added to the immunoprecipitation mixture to compete with cellular ^{35}S-labeled ezrin and ezrin-containing complexes for a limiting amount of antibody. This addition resulted in the near elimination of ^{35}S-labeled ezrin and 77-kDa protein from the immunoprecipitate (Fig. 1*B*). In contrast, no effect was seen on nonspecific bands such as those due to proteins that bind to protein A-Sepharose directly. These experiments indicated that the coprecipitating 77-kDa protein was recovered as a result of its association with ezrin. Consistent with this interpretation, subjecting cell lysate to four freeze–thaw cycles before immunoprecipitation resulted in loss of the 77-kDa protein but not of ezrin (Fig. 1*C*), suggesting that this treatment dissociates the ezrin–77-kDa complex.

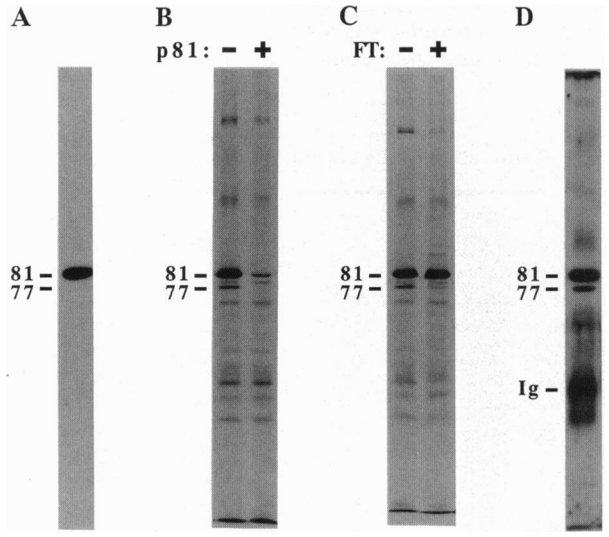

FIG. 1. Coprecipitation of moesin in ezrin immunoprecipitates. (*A*) Immunoblot of total A431 cell lysate probed with ezrin antibody. (*B* and *C*) Ezrin immunoprecipitates from lysates of ^{35}S-labeled A431 cells were electrophoresed and analyzed by autoradiography. (*B*) Excess unlabeled ezrin was omitted (−) or added (+ p81) to the immunoprecipitation mixture to compete with ^{35}S-labeled ezrin and ezrin-containing complexes for antibody. (*C*) Cell lysate was untreated (−) or subjected to four freeze–thaw cycles (+ FT) before immunoprecipitation. (*D*) Immunoblot of ezrin immunoprecipitate from A431 cells probed with moesin antiserum. The immunoglobulin (Ig) band was recognized by the secondary antibody. Molecular sizes are indicated in kDa.

We determined that the coprecipitating 77-kDa protein was moesin, the 77-kDa ERM family protein, using moesin antiserum to probe an immunoblot of the immunoprecipitate (Fig. 1*D*). This antiserum preferentially recognizes moesin but also cross-reacts with ezrin (2).

The ezrin antibody used for immunoprecipitation does not recognize moesin in immunoblots of purified protein (2), cell lysate (Fig. 1*A*), or the ezrin immunoprecipitates (data not shown). However, because ezrin and moesin are closely related, we needed to eliminate the possibility that the ezrin antibody might cross-react with native moesin in our experiments. Purified ezrin or moesin was added to the immunoprecipitation mixture containing protein A-Sepharose, ezrin antibody, and ^{35}S-labeled A431 cell lysate. No displacement of the ^{35}S-labeled ezrin–moesin complex from the immunoprecipitate was observed after moesin addition (Fig. 2). Competition by the same amount of ezrin is shown for comparison. These results prove that the isolation of the ezrin–moesin complex by immunoprecipitation with ezrin antibody depends on the antigenic recognition of ezrin and not of moesin and, in addition, show that the complex is sufficiently stable to prohibit exchange between free unlabeled moesin and the ^{35}S-labeled moesin bound to ezrin.

To confirm that moesin is the 77-kDa binding partner complexed with ezrin, we performed immunoprecipitations with affinity-purified moesin antibody to see whether ezrin could be coprecipitated. This antibody recognized only moesin in immunoblots of total A431 lysate (Fig. 3*A*) and did

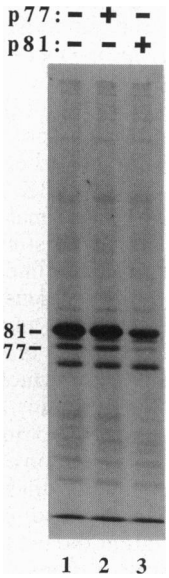

FIG. 2. Ezrin antibody does not recognize moesin in immunoprecipitation experiments. Ezrin immunoprecipitates from ^{35}S-labeled A431 cells were electrophoresed and analyzed by autoradiography. No exogenous protein (lane 1), 12 μg of moesin (+ p77; lane 2), or 12 μg of ezrin (+ p81; lane 3) was added to the immunoprecipitation mixture to compete with ^{35}S-labeled proteins for antibody. Molecular sizes are indicated in kDa.

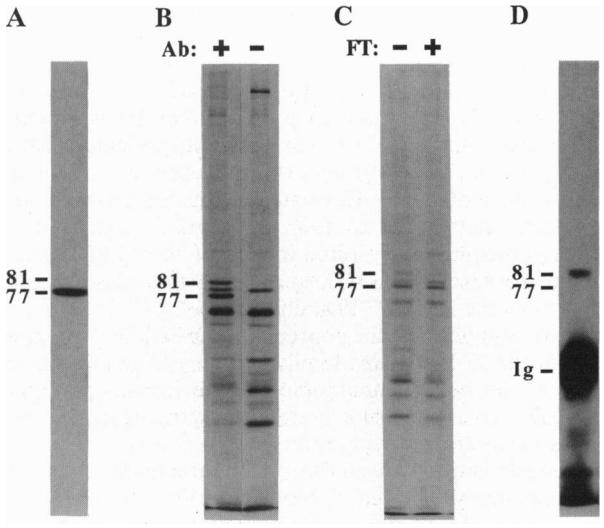

FIG. 3. Coprecipitation of ezrin in moesin immunoprecipitates. (*A*) Immunoblot of total A431 cell lysate probed with moesin antibody. (*B*) Immunoprecipitation from ^{35}S-labeled A431 cells using moesin antibody (+ Ab) or no antibody as a control (−). The immunoprecipitates were electrophoresed and analyzed by autoradiography. (*C*) Moesin immunoprecipitates as described in *B*, except that the cell lysate was untreated (−) or subjected to four freeze–thaw cycles (+ FT) before immunoprecipitation. (*D*) Immunoblot of the moesin immunoprecipitate shown in *B* probed with ezrin antibody. The immunoglobulin (Ig) band was recognized by the secondary antibody. Molecular sizes are indicated in kDa.

not immunoprecipitate purified ezrin in solution (data not shown). In A431 cell lysates, the moesin antibody indeed coprecipitated an 81-kDa protein along with moesin (Fig. 3*B*). A control immunoprecipitation in which the antibody was omitted identified those proteins that bind to protein A-Sepharose directly. As expected, treatment of the cell lysate by freeze–thawing decreased the amount of the coprecipitating 81-kDa protein, but not moesin, in the immunoprecipitate (Fig. 3*C*). Confirmation that the 81-kDa protein in the moesin immunoprecipitate was in fact ezrin was obtained by immunoblotting with the ezrin antibody (Fig. 3*D*). Thus, the ezrin–moesin complex can be isolated by immunoprecipitation with either specific antibody, and disruption of the complex decreases the recovery of the coprecipitating protein but not the antibody-specific protein in each variation of the experiment. We also identified ezrin–moesin complexes by immunoprecipitation in MDBK (Madin–Darby bovine kidney epithelial) and NRK (normal rat kidney, epithelial-like) cells (data not shown), suggesting that these complexes exist in a variety of cultured cell lines.

Hetero- and Homotypic Associations of Ezrin and Moesin *in Vitro*. The capacity of ezrin and moesin to associate was confirmed by using a solid-phase binding assay. We developed a blot-overlay procedure in which biotin-tagged ezrin or moesin was incubated with proteins that had been immobilized on membranes. Biotinyl–ezrin and biotinyl–moesin gave single bands when analyzed on a silver-stained gel (Fig. 4*A*), demonstrating the purity of these reagents.

Binding was assayed under conditions of high stringency. Biotinyl–ezrin (0.1 μg/ml) in the presence of a high concentration of extraneous protein (nonfat dry milk at 10 mg/ml) could readily detect 10 ng of purified ezrin or moesin that had been transferred to a membrane (Fig. 4*C*). No signal was detected with the avidin-peroxidase secondary reagent alone (Fig. 4*B*). These results confirm the conclusion, based on our immunoprecipitation studies, that ezrin and moesin can form a stable complex and also demonstrate that the association is direct. In addition, the experiment revealed that ezrin can

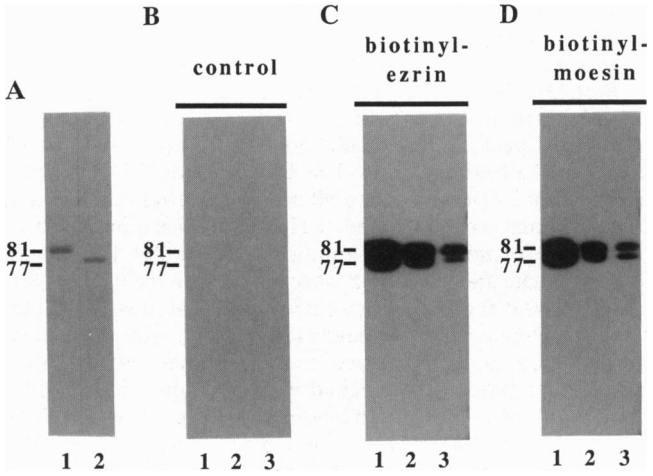

FIG. 4. Associations of ezrin and moesin *in vitro*. (*A*) Silver-stained gel of biotinyl–ezrin (lane 1) and biotinyl–moesin (lane 2). (*B–D*) Unmodified ezrin and moesin (90, 30, and 10 ng of each protein; lanes 1–3) were electrophoresed and transferred to blots. The blots were incubated with buffer alone as a control (*B*) biotinyl–ezrin at 0.1 μg/ml (*C*) or biotinyl–moesin at 0.1 μg/ml (*D*). Peroxidase-conjugated avidin was used as a secondary reagent to detect biotinylated proteins. Molecular sizes are indicated in kDa.

self-associate *in vitro*. In the converse blot-overlay experiment, biotinyl–moesin bound to both proteins (Fig. 4*D*), demonstrating that the ezrin–moesin complex can form regardless of which binding partner has been biotinylated, and establishing that moesin too has the capacity to self-associate.

To further evaluate the specificity of the interaction, we tested whether blot overlays could be used to detect ezrin and moesin in total cell lysates. Aliquots of A431 cell lysate (30 μg of total protein) containing ≈10 ng of ezrin (4) and ≈2 ng of moesin were electrophoresed and analyzed by Coomassie blue staining (Fig. 5*A*) and blot overlay. Biotinyl–ezrin and biotinyl–moesin each specifically bound to bands at 81 and 77 kDa (Fig. 5 *C* and *D*). A control blot showed that nonspecific

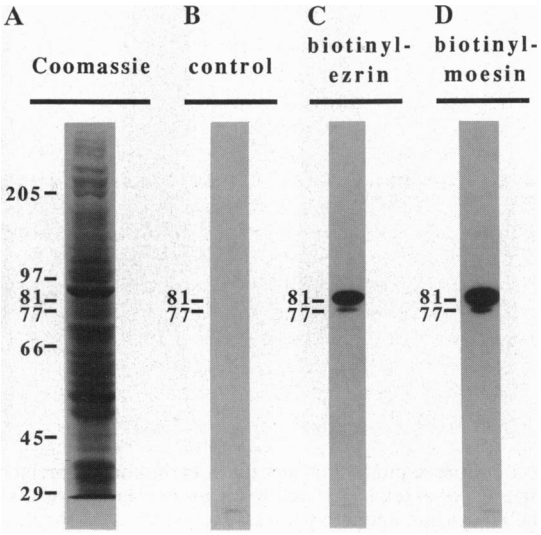

FIG. 5. Ezrin and moesin in total A431 cell lysate can be detected by blot overlay. (*A*) Coomassie blue-stained gel of total cellular protein (30 μg). (*B–D*) Cell lysate was electrophoresed and transferred to a blot. The blots were incubated with buffer alone as a control (*B*), biotinyl–ezrin at 0.1 μg/ml (*C*), or biotinyl–moesin at 0.1 μg/ml (*D*). Peroxidase-conjugated avidin was used as a secondary reagent to detect biotinylated proteins. Molecular sizes are indicated in kDa.

signal was confined to a single faint band at the electrophoretic dye front (Fig. 5B). Partial immunodepletion of ezrin and moesin from the lysate significantly reduced the 81- and 77-kDa signals (data not shown). The specific signals were eliminated by the addition of excess unmodified ezrin to biotinyl–ezrin during the primary incubation step of the blot-overlay procedure, whereas competition with excess biotin had no effect (data not shown). These results show that the hetero- and homotypic interactions of ezrin and moesin are highly specific, allowing even crude or partially purified material to be assayed by blot overlay.

DISCUSSION

The blot-overlay results demonstrate that ezrin and moesin can directly associate in the absence of additional cellular proteins that might mediate the interaction. Furthermore, the coprecipitation of ezrin and moesin in immunoprecipitation experiments indicates that heteromeric ezrin–moesin complexes exist *in vivo*. It seems likely that these complexes are very stable in intact cells because exogenous unlabeled moesin did not exchange with ^{35}S-labeled cellular moesin in complexes isolated by immunoprecipitation. In the three cell lines reported here to contain ezrin–moesin complexes, localization data are consistent with the assertion that these complexes exist *in vivo*. Ezrin and moesin are both concentrated in actin-containing surface structures of A431, MDBK, and NRK cells, as seen by immunofluorescence microscopy (ref. 2; Z. Franck and A.B., unpublished data).

The physiological relevance of the ezrin–moesin complex should be considered in relation to the uncomplexed forms of the proteins. The relative abundance of the complex can be appreciable but appears, nonetheless, to be substoichiometric with respect to each constituent. For A431 cells, we estimate that ≈50% of the total moesin is bound to ezrin, whereas ≈15% of the total ezrin is bound to moesin (A431 cells contain about four times more ezrin than moesin) on the basis of comparisons of total versus coprecipitated proteins from ^{35}S-labeled cells. Knowledge of the distribution of the ezrin–moesin complex among various cell types might provide insight into its role *in vivo*. An obvious prerequisite for formation of the complex within a given cell type is coexpression of the two proteins. Although the localization of ezrin and moesin in developmental systems has not been reported, the majority of fully differentiated cells in adult animal tissues contain predominantly one protein or the other. Coexpression appears restricted to a select subset of cell types in tissues (7). In contrast, demonstrable coexpression of ezrin and moesin is much more prevalent in cultured cells (2, 16). It is not known whether immortalization of cells generally affects the expression of ERM family proteins, but recent studies have found a correlation between ezrin overexpression and tumorigenesis using Meth A cells (28). Further study will be needed to determine the relationship between ezrin and moesin expression levels, complex formation *in vivo*, and cellular phenotype.

An array of different molecular species of ezrin and moesin appears to exist *in vivo*. In addition to the heteromeric ezrin–moesin complex described here, both proteins also occur as monomers in solution (ref. 1; unpublished data). Furthermore, gel-filtration chromatography has been used to identify a homo-oligomeric (dimer or trimer) form of ezrin from JEG-3 human choriocarcinoma cells (29) and from human placenta (unpublished data). The blot-overlay results suggest that the existence in cells of a homomeric form of moesin is likely as well. What factors influence the nature (homo- or heterotypic) and extent of complex formation *in vivo* are unknown, but several observations suggest the involvement of some type of regulation. Heteromeric complexes cannot be formed *in vitro* by simply mixing purified ezrin and moesin together in solution (unpublished data), and such complexes are present at only substoichiometric levels *in vivo*. Self-association also seems to be regulated because the monomeric and oligomeric forms of ezrin seen by gel filtration do not interconvert when rechromatographed (unpublished data). Instead, each fraction re-elutes as a single peak at its original position, suggesting that some stable conformational change or covalent modification determines the capacity to self-associate. In contrast, regulated binding was not apparent in our solid-phase binding assay using immobilized ezrin and moesin. It could be that the SDS/PAGE, electroblotting, and incubation steps of the blot-overlay procedure constitute a denaturation/partial renaturation cycle that facilitates binding by exposing a binding site that would remain conformationally inaccessible in the regulated state *in vivo*. Also, it is possible that only a fraction of the ezrin and moesin used in this assay are competent to undergo association but that signal amplification is adequate to detect even low levels of binding activity.

Several functional consequences of forming homo- and heteromeric complexes of ezrin and moesin can be predicted. These proteins are hypothesized to serve as linkages between the plasma membrane and the cortical actin cytoskeleton. Homotypic or heterotypic association would bring together sites contributed by each subunit to generate a complex that is multivalent with respect to both cytoskeleton and membrane binding sites. Such complexes might therefore be capable of crosslinking cytoskeletal elements to which they bind and of dimerizing or clustering ezrin- and moesin-binding membrane proteins. In addition, the joining of two or more low-affinity-binding sites could create a complex with higher avidity for its ligand, so that certain binding partners might preferentially associate with the oligomeric forms of ezrin and moesin. In summary, complex formation could serve to increase the apparent affinity for membrane or cytoskeletal binding partners or act to bring these partners together in three-dimensional space. Therefore, regulation of the propensity of these proteins to undergo homotypic and heterotypic association may be a mechanism by which dynamic remodeling of the cortical cytoskeletal architecture is achieved.

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