A Dual Involvement of the Amino-terminal Domain of Ezrin in F- and G-actin Binding*

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Human recombinant ezrin, or truncated forms, were coated in microtiter plate and their capacity to bind actin determined. F-actin bound ezrin with a $K_d$ of 504 ± 230 nM and a molecular stoichiometry of 10.6 actin per ezrin. Ezrin bound both α- and βγ-actin essentially as F-form. F-actin binding was totally prevented or drastically reduced when residues 534–586 or 13–30 were deleted, respectively. An actin binding activity was detected in amino-terminal constructs (ezrin 1–310 and 1–333) provided the glutathione S-transferase moiety of the fusion protein was removed. Series of carboxy-terminal truncations confirmed the presence of this actin-binding site which bound both F- and G-actin. The F- and G-actin-binding sites were differently sensitive to various chemical effectors and distinct specific ezrin antibodies. The internal actin-binding site was mapped between residues 281 and 333. The association of ezrin amino-terminal fragment to full-length ezrin blocked F-actin binding to ezrin. It is proposed that, in full-length ezrin, the F-actin-binding site required the juxtaposition of the distal-most amino- and carboxyl-terminal residues of the ezrin molecule.

Proteins, located at the interface between the plasma membrane and the cytoskeleton, are essential elements involved in cell plasticity, and are expected to possess association properties tuned by both intra- and extracellular regulators. Ezrin is a protein linker between the cortical skeleton and the plasma membrane (1, 2), and, in polarized epithelial cells, colocalizes with actin predominantly in apical microvilli (3, 10). With talin, ezrin is part of the superfamily of protein 4.1-like proteins sharing a homologous NH2-terminal domain (11–14). With radixin (15, 16) and moesin (17, 18), which share ~70% homology, ezrin define the ERM 1 family to which merlin (19, 20) is also related. Ezrin NH2-terminal domain is reported to interact with the plasma membrane, while the COOH-terminal domain would link the actin cytoskeleton (21). In some cell types, ERMs associate with CD44, a transmembrane receptor for hyaluronan (22) through regulation by the rho GTPase pathway (23).

In multicellular organisms, the relative level of ERM expression is tissue specific (4, 16, 24–28). However, ERMs are coexpressed and play a redundant role in most cell lines since major phenotypic alterations were only observed when the expression of all three ERMs was down-regulated (29). The subcellular redistribution of ERMs upon cell has been best studied in gastric parietal cells (2, 8). The elongation of ezrin-enriched secretory microvilli is linked to ezrin phosphorylation on serine and threonine residues (30), and ezrin acts as a protein kinase A anchoring protein in these cells (31). Ezrin is also tyrosine-phosphorylated (32–35) on two major sites (36) and a differential sensitivity to various growth factors exists between ERMs (37).

Ezrin can self-associate and, through interactions between two domains, the N- and C-ERMADs, form dimers or oligomers, a property shared by other ERMs, with which ezrin can form heterotypic associations (38–43). A COOH-terminal epitope is normally inaccessible in the molecule. Since this epitope is part of the C-ERMAD (42) and is involved in F-actin binding (44), the masking of this site by self-association, between monomers or within dimers, has been emphasized. Up to now little, and, sometimes, contradictory information were reported on the nature of interaction existing between actin and ERMs. Radixin was characterized as a barbed-end capping protein (45). Shuster and Herman (46) claimed that ezrin-actin interaction could not be direct. On the contrary, using a column-affinity assay, Turunen et al. (44) identified a F-actin-binding site within ezrin 35 COOH-terminal amino acids. This conclusion was enlarged to moesin (47). Finally, gastric parietal cell ezrin was found to colocalize and to preferentially interact with βγ-actin (48, 49).

Our attempts to demonstrate a direct ezrin-actin interaction using experimental procedures similar or derived from those described above were unsuccessful. We therefore developed a new assay. Human recombinant ezrin purified from Escherichia coli was coated in wells of a microtiter plate. After incubation with actin, the specifically bound proteins were recovered and analyzed by SDS-PAGE and Western blotting. Ezrin, and a few ezrin recombinant constructs, bound F- and G-actin and the existence of a new actin-binding site in the NH2-terminal domain of ezrin was identified.

MATERIALS AND METHODS

Chemicals, glutathione-s-garose beads, and thrombin were from Sigma, and antiprotease tablets from Boehringer Mannheim. Anti-actin monoclonal antibody was a gift of Dr. N. Lamb (Montpellier). Anti β-actin antibody and all secondary antibodies were from Sigma. A monoclonal antibody directed against ezrin residues 576–586 was from Transduction Laboratories and was equivalent to the Z036 antibody used in Ref. 42.

α-Actin Preparation—Rabbit muscle acetone powder was prepared and actin purified from as in Ref. 50. Actin was isolated as CaATP-G-actin after cycling through 2–4 rounds of polymerization-depolymerization prior to gel permeation chromatography on Sephacryl S-300 in G buffer (2 mM Tris-HCl, pH 8.0, 0.2 mM ATPNa2, 0.2 mM CaCl2, 0.005%
Actin-binding Sites in Ezrin Amino Terminus

Na$_2$CO$_3$ and 0.5 mM β-mercaptoethanol. Occasionally, actin purified by chromatography on DEAE-Trisacryl was used and behaved similarly for ezrin-actin interaction (not shown). A single 43-kDa band was stained by Coomassie Blue when 30 μg of actin was loaded on SDS-PAGE. Actin was stored either as G- or F-actin at 4 °C. After 3 weeks of storing in liquid nitrogen, G-actin depolymerized into 5–10 kDa and chromatographed again on Sephacryl S-300. Binding studies involving G-actin were always performed with freshly gel-salted G-actin.

βγ-Aktin Preparation—Outdated platelets were obtained from the CTRS (Montpellier, France). βγ-Aktin was purified on polyproline-Sepharose (51) and eluted with 0.5 M KI (52), dialyzed, polymerized, and stored as a 10% stock solution. A single 45-kDa band was observed after Coomassie Blue staining of 7 μg of protein analyzed by SDS-PAGE.

Construction of Plasmids—The subcloning of the full-length human ezrin cDNA into the pGEX-2T vector (Pharmacia Biotech Inc.) was described (38) (Fig. 1, A and B). Carboxy-terminal deletions were obtained using the double stranded nested deletion kit from Pharmacia. The ezrin-pGEX-2T vector was cleaved with EcoRI and blunt with thi-dNTPs. The plasmid was cleaved with NcoI and digested with the Klenow DNA polymerase fragment and overnight ligation, TG1 bacteria were transformed with DNA. Randomly picked clones were grown overnight in 3 ml of LB medium, induced for 1 h with 0.5 mM isopropyl-β-D-thiogalactopyranoside, sonicated, and recombinant fusion proteins adsorbed on glutathione-agarose columns with 5 IU thrombin in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM CaCl$_2$, 1 mM dithiothreitol. After thrombin cleavage HI was added and the reaction stopped every 30 s for 10 min. After nuclease S1 action, filling-in with the Klenow DNA polymerase fragment and overnight ligation, TG1 bacteria were transformed with DNA. These gels were processed similarly as the samples. When series of truncated molecules coated was found a saturable and reproducible process. Half-maximum coating was achieved with 0.5–1.5 μg of ezrin. The maximum amount of ezrin coated was 50–100 ng (i.e. 0.7–1.4 pmol), a value similar to what announced by the manufacturer for IgGs. Therefore, the average ezrin density was estimated in the range of 2.5–5 pmol/cm$^2$ (1 molecule/3000–6000 Å$^2$). Thus, ezrin molecules could be evenly spaced on plastic and coating was not formerly dependent upon a self-association process although this cannot be definitively ruled out. 50 ng of coated ezrin led to a final ezrin concentration of 15 nm during the incubation step with actin. When different constructs had to be compared, 0.01 OD 280 nm of each construct was used for coating. Although all constructs had different molar extinction coefficient, this corresponded to an amount of protein larger than the one for which saturation of coating had been demonstrated for full-length ezrin. Thus the number of truncated molecules coated was found very similar. For the shortest constructs, at most a 2-fold increase in the number of molecules coated was measured when compared with full-length ezrin. Therefore, for these constructs, the actin binding ability would then be overestimated if no correction was introduced. This is of no consequence since they were actually found unable to bind actin (see “Results”).

Quantification Procedures—Ezrin or F-actin membranes were stained with Coomassie Blue to check the efficiency of the transfer (5–10 ng of ezrin or actin could be detected). Western blotting was used to identify the bands, using either rabbit anti-ezrin or mouse anti-actin antibodies and appropriate peroxidase-labeled secondary antibodies. The ECL (Amersham) detection kit was used for the revelation. In some experiments, the Vistra ECF amplification module (Amersham) was used instead in conjunction with second antibodies labeled with alkaline phosphatase and blots scanned with a STORM PhosphorImager (Molecular Dynamics). Autoradiograms (ECL) and Coomassie Blue-stained membranes were scanned using a 8-bit CCD camera and the data digitized. In all cases, the ImageQuant software (Molecular Dynamics) was used to quantify the data. Calibrations were performed by running on separate gels serial dilutions of known amounts of ezrin and actin. These gels were processed similarly as the samples. When series of NH$_2$- or COOH-terminal deletions had to be compared, their molar amounts were determined. It was assumed that the epitopes recognized by a mixture of antibodies recognizing either full-length ezrin or its NH$_2$-terminal domain were evenly localized along the ezrin molecule. Thus the detected signal was corrected for the number of amino acids of each construct.

Anti-ezrin Antibodies—Anti-ezrin rabbit antibodies against full-length ezrin or its NH$_2$-terminal domain (amino acids 1–310) were characterized by Andréolé et al. (38) and will be referred to as anti-C and anti-N antibodies, respectively. Antisera were used as such for Western blotting. For competition binding assays, both antisera were affinity purified on resins covalently coupled with the proteins used for immunization.

RESULTS

Expression of Truncated Forms of Ezrin—A summary of the constructs used in this study is presented in Fig. 1. Constructs were produced as GST fusion proteins (Fig. 1, A–D), purified, cleaved from the GST moiety, and characterized by Western blotting with anti-ezrin antibodies (Fig. 2).

Ezrin Is a F-actin-binding Protein—A new procedure to assay actin-ezrin interaction was developed by analogy with enzyme-linked immunosorbent assay tests (see “Materials and Methods”). A 96-well plate was coated with purified full-length or truncated forms of ezrin or the respective GST fusion proteins (Fig. 3). After incubation with βγ-(left panel) or α-(middle panel) F-actin and subsequent washings, bound proteins were analyzed by Western blotting. When GST was

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2 Martin, M. (1997) Mol. Biol. Cell. In press.
coated as a control, no actin was ever found bound. However, a significant binding was reproducibly detected with some specific ezrin constructs. Full-length ezrin (ez1–586) bound actin. Interestingly, ez1–310 also bound actin, whereas the respective GST fusion protein never did. GST as a fusion protein with NH2-terminal forms of ezrin did lower or even prevent actin binding to chimeric ezrin molecules, and this effect was also observed when \( \alpha \)-actin was used (not shown). Therefore, for all subsequent studies, only recombinant proteins, from which the GST moiety was cleaved, were used.

\( \alpha \)-Actin also bound full-length ezrin and other ezrin constructs (Fig. 3, middle panel). Western blots with anti-ezrin and anti-actin antibodies indicated the relative levels of construct coating and actin binding in each well, respectively. In Fig. 3 (right graph), the same data were normalized to allow a quantitative comparison and shown for two actin concentrations. Ez1–586 bound \( \alpha \)-actin. Deleting the 53 COOH-terminal amino acids (ez1–533) severely impaired actin binding. This was consistent with previous results (44, 47). However, no binding of actin to ez219–536, i.e. the COOH-terminal part of ezrin containing the previously identified actin-binding site was detected under these assay conditions, whereas ez1–310 did significantly bind actin. When amino acids 13–30 were deleted (ezD13–30), actin binding was drastically reduced. These residues were deleted because they were found important to regulate the cell extension phenotype observed in insect cells. Overall, the presence of a binding site in the NH2 terminus domain of ezrin was therefore consistently detected whatever the actin source.

**Actin Binding to Ezrin Is Saturable**—In this assay, actin nonspecific binding was undetectable and binding to holo-ezrin was saturable (Fig. 4, A and B). Heating ezrin to 65 °C for 30 min before the coating step led to a large decrease in actin binding capacity (Fig. 4B), although heat denaturation drastically enhanced ezrin coating (see “Discussion”). Ezrin storage at −20 °C led to a complete loss in actin binding (not shown).

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**Fig. 1.** Plasmids used to generate internal NH2- and COOH-terminal deletions in ezrin: relative actin binding capacity of the truncated proteins. Upper part: the map (A) localizes the unique sites used to generate unidirectional deletions from the plasmid described in Ref. 38. 3′-UTR refers to the 3′-untranslated region of ezrin cDNA. B, full-length GST-ez1–586 fusion protein produced by ezrin pGEX-2T. C, constructions expressing ezrin COOH-terminal domains in fusion with residues 1–11. D, constructions expressing ezrin NH2-terminal domains. Once expressed, all fusion proteins were cleaved with thrombin to eliminate the GST moiety. Lower part, linear representation of ezrin constructs. Plain lines, portions of ezrin expressed. Dotted lines, internal deletions. On the right of the figure the relative actin binding capacity of each construct was compared. The amount of each construct coated was quantified (right most column) and these values converted to molar amounts (see “Materials and Methods”). The amount of actin bound at the two concentrations used (1 or 3 μg) was quantified and divided by this latter value. 100% binding was taken as the binding detected with ez1–586 in the presence of 3 μg of actin. Mutation of amino acids 293–296 from RRRK to LTGN in ez219–310 yielded values of 1 and 3% for actin binding at 1 and 3 μg of actin, respectively. These values have to be compared with those obtained with wild type ez1–310, namely, 9 and 16%.
The threshold concentration of α-actin required to achieve a significant binding was 50 nM (2 μg/ml). Half-saturation was reached at 20 μg/ml α-actin (0.5 μM, 1000 ng of actin added). Scatchard analysis (Fig. 4A, inset) demonstrated that actin interacted reversibly with a homogenous population of ezrin molecules, with a K_d value of 504 ± 230 nM (n = 13) and a stoichiometry of 10.6 actin monomers bound per ezrin molecule (range 6–18.4).

Mapping of Actin-binding Sites—Actin binding was analyzed with all constructs described in Figs. 1 and 2. The results were compared for two actin concentrations after quantification and normalization (Fig. 1). The data were fully consistent with those described in Figs. 3 and 4. The short deletion of amino acids 13–30 drastically reduced actin binding (Figs. 1, 3 and 4B). Extending the deletion to residue 115, and beyond up to position 507, completely abolished actin binding. On the other hand, the truncation of ezrin COOH-terminal last 53 amino acids totally impaired actin binding while shorter constructs, ez1–310 and ez1–333, did bind actin, ez1–333 being more efficient that ez1–310 (Fig. 1). A further increase in size to position 366 led to a sudden loss in actin binding. This suggested that a small number of amino acids beyond position 333 were sufficient to negatively control the binding observed with shorter NH2-terminal ezrin fragments. COOH-terminal truncations of ez1–310 led also to a loss in actin binding as shown with ez1–280 and shorter constructs. As already noted above, ezrin COOH-terminal domains containing the previously published F-actin-binding site were incapable of binding (ez310–586 and ez280–586).

Finally, the mutation of amino acids 293–296 from RRRK to LTGN in ez1–310 led to a significant drop in actin binding which was consistent with the demonstration that the same mutations in ez1–310 impaired actin-based cell extension in SF9-infected cells.

Full-length Ezrin and Ezrin NH2-terminal Domain Discriminate between F- and G-actin—The data suggested that ezrin might contain two distinct actin-binding sites with distinct properties. For instance, ez1–356 and ez1–333 bound F-actin with different stoichiometry (Fig. 4B). The K_d of ez1–586 for G-actin

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**FIG. 2. Characterization of ezrin constructs.** Almost all constructs used in this study are present on this figure after SDS-PAGE and transfer onto Immobilon P membranes. *Left panel,* Coomassie Blue staining of the membrane. The band under the 29-kDa marker corresponded to the GST moiety of the cleaved fusion proteins. *Right panel,* anti-ezrin Western blot of the same membrane: the other bands of the left panel are ezrin products. The slowest migrating band in the ez280–586 lane is a residual fusion protein. Ez1–310* is ez1–310 from which amino acids 293–296 were mutated. The abnormal migration of some of the constructs observed had already been documented (42).

**FIG. 3. Major determinants in ezrin for actin binding.** *Left,* anti-actin Western blot. The interactions between β/γ-actin and various ezrin constructs were determined at 40 and 200 μg/ml actin. *Middle,* the indicated proteins were coated and incubated with 40 or 200 μg/ml α-actin. After incubation and washings, eluted proteins were analyzed by Western blotting. The amount of the coated constructs was detected using a mixture of anti-ezrin antibodies (*upper part*). Bound actin was detected on the same blots (*lower parts*). *Right,* the data shown in the middle of the figure (40 μg/ml α-actin, gray bars; 200 μg/ml, black bars) were quantified and normalized to the molar amount of construct coated (see “Materials and Methods”). The variation of the data obtained from 4 different blots is indicated.
The binding capacity of different constructs was compared: Δ, ez1–586; ez1–333, ez1–333; ez310–586; GST; and A, ez1–586 heated to 65 °C for 30 min prior to coating. C, binding of G-actin to ez1–586 (with the ordinate axis expanded 10 times). In D (ez1–333) and E (ez1–566), G-actin binding was analyzed according to Scatchard. For ez1–566, \( K_d = 920 \text{ nm} \) with 0.75 actin molecule bound per ezrin molecule. For ez1–333, \( K_d = 280 \text{ nm} \) with a stoichiometry of 0.27 actin per ez1–333.

(710 nm, \( n = 4 \)) was similar to the value determined for F-actin (see above and Fig. 4E). However, the binding capacity was markedly different since the ratio of actin monomer bound per ez1–586 was 0.9 (range 0.75–0.94). With ez1–333, large variations markedly different since the ratio of actin monomer bound per E

Fig. 4. Actin binding properties of ezrin. A, the actin binding on ez1–586 is shown as a function of the dose of actin (nanograms added or micromolar). In the inset, the data were plotted according to Scatchard (\( K_d = 504 \text{ nm} \) with 14.2 actin molecules bound per ezrin molecule). In C (left), calibration blots for both actin and ezrin are shown. They were used to determine the amounts of ezrin coated and actin bound in the corresponding wells (C, right) at each dose of F-actin. These data yielded the results presented in A. ECF detection kit was used for this experiment.

To better distinguish these different sites, the actions of various effectors were tested. NaCl addition did not alter F-actin binding to ez1–586 or ez1–333 (Fig. 5B). Using 40 μg/ml G-actin, increasing NaCl concentration resulted in enhanced actin binding, consistent with actin polymerization triggered by salt addition (Fig. 5B, right) and recovery of pelletable actin from the wells. At 4 μg/ml G-actin, a value below the critical G-actin concentration required for polymerization (55), the binding was independent of ionic strength (Fig. 5B, left). Therefore, the G-actin binding appeared to be initially due to the G form of actin. Whether this initial binding led to nucleation sites remains to be determined.

N-Ethylmaleimide treatment of ez1–566 or ez1–333 had no effect on either F- or G-actin binding (Fig. 5C). Succinimidyl-6-biotinylhexanoate addition reduced F-actin binding to ez1–333 only, while 3,3′-dithio-bis(propionic acid N-hydroxysuccinimide ester) treatment abolished F-actin binding of both constructs. None of these compounds significantly altered G-actin binding to either protein (Fig. 5C). Therefore, the regions for both ezrin constructs involved in F-actin binding shared similar sensitivities to these effectors, and were clearly distinct from those involved in G-actin binding.

Next, the effects of different affinity purified antibodies were tested on the binding of F- or G-actin to ez1–586 or ez1–333 (Fig. 5D). The anti-C antibody did not recognize the NH2-terminal ezrin domain (positions 1–310) (21, 38). Anti-N and anti-C antibodies bound both ez1–586 and ez1–333. Anti-C antibody bound much better onto holo-ezrin than onto ez1–333, and subsequent addition of either F- or G-actin did not interfere with antibody binding (not shown). No major effect of anti-N and -C antibodies was detected on F-actin binding to ez1–586. However, anti-N antibody interfered slightly with F-actin binding to ez1–333. At high concentrations, anti-N antibody reduced G-actin binding to ez1–566, while both anti-N and -C antibodies, alone or in combination, blocked G-actin binding to ez1–333. A commercial monoclonal antibody directed against amino acids 576–586 failed to bind coated ez1–586 and to block actin-binding (data not shown). Therefore, it was concluded that: 1) the F-actin-binding sites on either ez1–566 or ez1–333 was distinct from the G-actin-binding site; and 2) the G-actin-binding site detected in ez1–333 should involve residues 281–310, the only stretch of residues accessible to anti-N antibodies and residues 311–333 accessible to C blocking antibodies.

NH2-terminal Fragments of Ezrin Associate with Ezrin and Block Actin Binding—At variance with the results of Turunen et al. (44), no F-actin binding was detected with ezrin COOH terminus constructs (Figs. 1 and 3). To test if this failure resulted from the coating procedure, ez1–310 was added in the assay as a competitor of F-actin binding to coated ez1–586. The competitive action of ez1–310 was also compared (Fig. 6, left panel). Whether F-actin was added alone or in combination with ez310–586, no differences were observed for actin binding to ez1–586. On the contrary, when ez1–310 was added together with

3 C. Roy, unpublished data.
F-actin, actin binding to ez1–586 was drastically reduced. Ez1–310 appeared as a strong competitor for actin binding to ez1–586. However, ez1–310 was recovered from wells suggesting that an association between ez1–310 and ez1–586 took place during the assay. The possibility that such interaction was responsible for the observed loss of actin binding was next tested using ez1–333 which behaved similarly (Fig. 6, right graph). When ez1–333 was added after F-actin had bound to ez1–586, it still associated with ezrin (not shown) but did not affect much actin binding. Simultaneous addition of actin and ez1–333 led to a dramatic drop in actin binding. Incubation of ez1–333 prior to actin addition resulted in an almost complete block in actin binding. Ez1–333 binding to ez1–586 was independent of the presence of bound actin, whereas the presence of ez1–333 did prevent actin binding to full-length ezrin.

**DISCUSSION**

The major result of this paper is the description of the actin-binding properties of recombinant ezrin using a solid phase assay and the characterization of important determinants within ezrin NH2-terminal domain involved in actin binding.

**Validity of the Assay**—The assay was specific on different criteria. Actin never bound to proteins such as bovine serum albumin or GST. Ezrin and actin interacted within a reasonable range of concentration: in the nanomolar range for ezrin; saturation was achieved with micromolar actin concentrations.
Among 24 ezrin constructs, only four, including ezrin, bound actin. One of them was deleted of a few amino acids (ez1-310) and showed very reduced binding. The two others are very similar constructs and only differ by a 23-amino acid extension (ez1-310 and ez1-333). The effects of various compounds (Fig. 5) and competitors (Fig. 6) strongly argue for the selectivity and specificity of binding events.

The conformation of the coated proteins is an important issue. Some change(s) did occur in ezrin since the soluble form was unable to bind actin in standard biochemical assays. Did coating promote ezrin denaturation or self-association? Heat denaturation increased coating by a factor of 10 (not shown), whereas the resulting normalized actin binding was low (Fig. 4B), and presumably represented binding to ezrin molecules that had either escaped denaturation or renatured during overnight coating. The enhancement of coating reflected aggregation of ezrin molecules after heat treatment. Therefore, when native ezrin was coated, if self-association took place, it should have remained minimal. The antibody directed against residues 576–586 failed to interact with ezrin coated on plastic, a result consistent with the C-ERMAD being not accessible in full-length ezrin unless the protein is SDS-treated (42). Altogether the data supported that ezrin was coated as a majority of monomers, in a configuration distinct from soluble ezrin, but also distinct from heat-denatured, thawed, or blotted ezrin. Since the presence of intact both C- and N-ERMAdas (42) is necessary to achieve oligomerization, all other constructs tested could not self-associate.

Discrepancies with Other Studies—The ezrin-actin interactions are not yet fully understood. Conflicting results were reported, probably because different sources of ezrin, with various post-translational states, and different assays were used. Soluble gastric ezrin discriminated between β/γ- and α-actin isoforms (48, 49). On overlay assay, blotted ERMs bound F-actin but not G-actin (47). The truncation of the 22 COOH-terminal residues in GST-moesin abolished actin binding. Turunen et al. (44) used an immobilized GST-erm COOH-terminal domain to demonstrate that the last 35 amino acids of the molecule are required for F-actin binding. Using GST-fusion proteins, no binding was detected in ezrin (44) and moesin (47) NH2-terminal domains. We used recombinant proteins from which the GST moiety was cleaved. A free NH2-terminal extremity is of critical importance for unmasking ezrin NH2-terminal actin-binding properties. In this respect, Henry et al. (56) stressed the importance of proper radixin tagging for correct cellular addressing. SDS treatment is a prerequisite of any overlay assay (47) and Turunen et al. (44) mentioned that a SDS step was necessary to solubilize NH2-terminal constructs. Since SDS treatment inactivated the association properties of ezrin N-ERMAD (42), it might have also affected actin binding in previous studies.

The deletion of amino acids 13–30, a sequence most probably hindered by the GST moiety in fusion proteins, considerably reduced actin binding, and this result further strengthens the importance of ezrin NH2-terminal residues in actin binding. Actually, ez1-310 and ez1-333 were much more efficient than any COOH-terminal ezrin construct to bind actin. One should note, however, that some actin binding was detected in COOH-terminal constructs, but this required that, at the ECL reaction step, films were exposed much longer (15 min instead of 30 s) (not shown). It is possible that, when C-domains were coated, the F-actin-binding site stayed masked or was involved in interaction with plastic. In both cases it would be inaccessible to actin. However, the soluble form of the C-domain did not act as a competitor either (Fig. 6). Then it is possible that this site is of much lower affinity than was earlier anticipated and that it cannot be detected in this type of assay. Actually, no $K_d$ value was previously measured for ezrin C-domain.

Albeit, apparently 10-fold higher, the $K_d$ (500 nM) for F-actin determined in this study agrees with the value measured by Yao et al. (48). Their analysis was performed in the reciprocal way keeping actin concentration constant and varying that of ezrin. Therefore, the slope of the Scatchard was expressed in nM$^{-1}$ ezrin ($K_d = 50$ nM). In both studies, one ezrin molecule bound every 10 actin monomers. Either due to steric hindrance or because of actin conformational change due to ezrin binding, the actual actin binding motif is an actin decamer. Thus, the affinity of actin for ezrin was either 500 nM relatively to the full population of actin molecules or 50 nM when the decameric actin binding motif is considered.

The low stoichiometry (<1.0) between G-actin and ez1-586 or, especially, ez1-333 suggested that either some actin was lost during the final washing steps due to a high dissociation rate or some uncontrolled aggregation occurred during coating, especially with NH2-terminal constructs of low solubility. If resulting aggregates were unable to bind actin, the measured stoichiometry would be lowered accordingly. Alternatively, the G-actin-binding site might be a nucleation center. Such a process to be effective would then concern only those of ez1-333 molecules which were close enough to bring three actin molecules together.

**Implications for Ezrin Function**—Our attempts to demonstrate ezrin-actin interactions using conventional procedures failed except when ezrin was immobilized on a solid phase. It is tempting to speculate that adsorption to plastic mimics anchoring to the plasma membrane. This is consistent with a recent model describing soluble ezrin in a dormant state (39). Similarly, the re-association of ezrin COOH- and NH2-terminal domains was never possible in solution, whereas, it is very efficient using either overlay assays (38, 41–42) or surface plasmon resonance technology, i.e. after immobilization of one partner.

The observation that the NH2-terminal domain possesses actin-binding properties justifies to reconsider previous models of ezrin function. Deletions of ezrin NH2-terminal residues (1–30 or 13–30) or truncation of the COOH-terminal last ones (566–586) promoted a cell extension phenotype with concomitant actin mobilization in Sf9 cells. For ez13–30 or COOH-terminal truncated forms, actin binding was either reduced or abolished. Thus, the F-actin-binding site could involve both the very NH2- and COOH-terminal ends of the molecule implying a folding process or the involvement of NH2- and COOH-terminal ends of two distinct molecules to constitute the actin-binding site. This interpretation is consistent with the specificity of anti-ezrin antibodies (38, 21, 42), and their lack of effect on F-actin binding (Fig. 5D). Whether ezrin molecules are positioned head-to-tail or folded, there exists an additional site within amino acids 281–333 as previously suggested (21). It exhibits distinct properties toward F- and G-actin (see Figs. 4 and 5), and we propose that it actually represents a G-actin-binding site. Amino acids 334–366 prevent the accessibility of this site and this further suggests that a folding event occurs within full-length ezrin masking this G-actin site (there is no evidence that upon F-actin addition this internal G-actin site is still accessible). Such a head-to-tail interaction exists in vinculin and prevents actin binding. Addition of phosphatidylinositol 4,5-bisphosphate released this conformation and allows actin and talin binding (57). Such a mechanism, controlled by intracellular signaling, may well be involved in ezrin for which a phosphatidylinositol 4,5-bisphosphate-binding site is located in

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4 C. Roy, unpublished results.
The capacity for some forms of ezrin to bind G-actin is of physiological interest. It is important to determine if this site can be turned on/off in native ezrin, upon (de)phosphorylation, and whether it can act to regulate microfilament assembly/disassembly. The F-actin binding capacity of radixin and ezrin were similar (data not shown). Radixin acts as a barbed-end capping protein (45) which implies that one actin filament would bind one radixin molecule. Other studies concluded that ERM proteins bind along the sides of actin filaments (47, 49), a proposal consistent with the comparable amount of both ezrin and actin found in purified microvilli (2, 39), and with the stoichiometry determined in vitro (this paper and Ref. 49).

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