

Ezrin Has a COOH-Terminal Actin-binding Site That Is Conserved in the Ezrin Protein Family

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Abstract. Ezrin, previously also known as cytovillin, p81, and 80K, is a cytoplasmic protein enriched in microvilli and other cell surface structures. Ezrin is postulated to have a membrane-cytoskeleton linker role. Recent findings have also revealed that the NH₂-terminal domain of ezrin is associated with the plasma membrane and the COOH-terminal domain with the cytoskeleton (Algrain, M., O. Turunen, A. Vaheri, D. Louvard, and M. Arpin. 1993. *J. Cell Biol.* 120: 129–139). Using bacterially expressed fragments of ezrin we now demonstrate that ezrin has an actin-binding capability. We used glutathione-S-transferase fusion proteins of truncated ezrin in affinity chromatography to bind actin from the cell extract or purified rabbit muscle actin. We detected a binding site for filamentous actin that was localized to the COOH-

terminal 34 amino acids of ezrin. No binding of monomeric actin was detected in the assay. The region corresponding to the COOH-terminal actin-binding site in ezrin is highly conserved in moesin, actin-capping protein radixin and EM10 protein of *E. multilocularis*, but not in merlin/schwannomin. Consequently, this site is a potential actin-binding site also in the other members of the protein family. Furthermore, the actin-binding site in ezrin shows sequence homology to the actin-binding site in the COOH terminus of the β subunit of the actin-capping protein CapZ and one of the potential actin-binding sites in myosin heavy chain. The actin-binding capability of ezrin supports its proposed role as a membrane-cytoskeleton linker.

THE ezrin protein family (also called ERM family; Sato et al., 1992) has potentially an important role at the cortical cytoskeleton and may participate in the regulation of several cellular functions (see a recent review by Arpin et al., 1994). Indirect evidence shows that members of the ezrin protein family interact with membranes and cytoskeleton, but more data are needed on the components binding directly to ezrin and other proteins of the family and how these associations are regulated. Here our focus is on the cytoskeletal interactions of ezrin, the prototype protein of the family.

Ezrin is a widely distributed protein located in microvilli and other cell surface structures (Bretscher, 1983; Gould et al., 1986; Pakkanen et al., 1987; 1988; Pakkanen, 1988; Hanzel et al., 1989; Berryman et al. 1993; Franck et al., 1993). Based on the homologies in amino acid sequence (Turunen et al., 1989; Gould et al., 1989) with other cytoskeletal proteins, it has been suggested that ezrin links the cytoskeleton to the plasma membrane. The NH₂-terminal domain of ezrin (200–300 amino acids; see Fig. 1, domain EzN) shows 37% identity to erythrocyte protein band 4.1 (Conboy et al., 1986) and 23% identity to talin (Rees et

al., 1990). Both protein 4.1 and talin are linker proteins at the cortical cytoskeleton. The NH₂-terminal domain of protein 4.1 has been shown to be responsible for the binding to membrane proteins and the central 10-kD domain to spectrin (for reviews see Bennett, 1989; Bretscher, 1991; Luna and Hitt, 1992). A similar functional structure of ezrin has appeared when the domains of ezrin have been expressed in cells. Accordingly, in ezrin the NH₂-terminal domain is localized to membranes and the COOH-terminal part containing the α -helical domain colocalizes with actin filaments (Algrain et al., 1993). Further evidence for the association of ezrin with cytoskeleton comes from several reports that ezrin is associated with the cytoskeleton in cell fractionation experiments, in which ezrin has been found in the detergent-insoluble fraction containing the cytoskeleton (Bretscher, 1983; Hanzel et al., 1991; Egerton et al., 1992; Algrain et al., 1993). No specific binding ligand in the membranes or in the cytoskeleton has been reported thus far for ezrin.

Recently, several proteins have been identified that share a strikingly high identity to ezrin. Radixin (Funayama et al., 1991), moesin (Lankes and Furthmayr, 1991), merlin/schwannomin (Rouleau et al., 1993; Trofatter et al., 1993), and EM10 protein of *Echinococcus multilocularis* (Frosch et al., 1991) are 75, 73, 48, and 43% identical to ezrin, respectively. Although it is possible that the whole ezrin protein family functions as a membrane-cytoskeleton linker, only radixin

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has been reported to be an actin-binding protein (Tsukita et al., 1989). Extensive attempts using sedimentation, affinity-chromatographic and overlay assays to show direct interaction of ezrin with actin or other proteins have been unsuccessful (Bretscher, 1983; 1991; Krieg and Hunter, 1992; Fazioli et al., 1993). A weak interaction of ezrin with actin was detected in sedimentation assays, but the binding was not seen in physiological salt concentrations (Bretscher, 1983, 1991). A direct binding of ezrin to the closely related moesin has been detected recently by immunoprecipitation and overlay assays (Gary and Bretscher, 1993), but the physiological meaning of this association is not yet known.

Using bacterially expressed truncated ezrin constructs we now demonstrate that actin in cell extracts and purified muscle actin bind to ezrin. In our procedure we expressed overlapping parts of ezrin as fusion proteins with glutathione-S-transferase (GST)¹. Ezrin fusion proteins were used in affinity chromatography to identify proteins binding to ezrin. Actin was the only major protein that could be shown to bind to ezrin.

Materials and Methods

Antisera

Polyclonal antibody I: a polyclonal rabbit antiserum raised against human ezrin (cytovillin) was described by Pakkanen (1988). We also raised monoclonal antibodies against human ezrin using the COOH-terminal part of ezrin in immunizing mice (Turunen, O., T. Wahlström, O. Carpen, E.-M. Salonen, and A. Vaheri, manuscript in preparation). The amino acid region 362-585 was cloned as a Pst I fragment pCV1 clone (Turunen et al., 1989) to pEX1 vector for the expression in bacteria. One of the resulting monoclonal antibodies (CVPI) was used in characterization of the GST-ezrin fusion proteins expressed from pGEX vectors. Polyclonal antibody II: rabbit antiserum raised against human ezrin was a kind gift from Dr. A. Bretscher (Cornell University, Ithaca, NY). The monoclonal mouse anti-ezrin antibody Z036, raised against a synthetic peptide comprising the region 575-585 of human ezrin, was purchased from Zymed Labs. (San Francisco, CA), the monoclonal antibodies to actin from Sigma Chem. Co. (St. Louis, MO), and the monoclonal antibodies to β -galactosidase from Promega (Madison, WI).

Creation and Expression of Truncated Fusion Proteins

A series of truncated ezrin-fusion proteins (see Fig. 1) were expressed as GST fusion proteins from pGEX vectors (Pharmacia, Uppsala, Sweden). Subclones of the COOH-terminal part of ezrin were produced by PCR using ezrin cDNA clone pCV6 (Turunen et al., 1989) as the template. Bam HI sites were constructed at both ends of the PCR clones for the subcloning in the pGEX-3X expression vector. The NH₂-terminal fusion protein FP 1-308 was created by subcloning the pCV6 insert to the pGEX-1AT vector and removing the DNA fragment Sma I-Nru I (cuts at pCV6 bases 1003 and 2049, respectively). The starting site for the protein coding of ezrin was regarded as the same as that reported by Gould et al. (1989) and Franck et al. (1993). The FP 1-308 fusion protein contained 26 additional amino acids coming from the 5'-noncoding region of the pCV6 clone. FP 243-585 was created by expressing the ezrin pCV1 clone (containing amino acids 243-585) with EcoRI sites at the ends of the clone (Turunen et al., 1989) in the pGEX-1AT vector. The clones ending in the ezrin residue 585 used the stop codon of ezrin cDNA. The stop codon for the clone FP 1-308 was formed from the non-coding region of ezrin cDNA, thus adding 15 amino acids to the carboxy terminus of FP 1-308 protein: AIIIESPPVLCWEQD. The clones FP 279-485, FP 279-531, and FP 477-579 used the stop codons of the pGEX vector; consequently they all contained 6-15 COOH-terminal amino acids expressed from the vector (the additional amino acids at the COOH terminus of ezrin were: FP 279-485, DPREFIVTD; FP 279-531 and FP 477-531, RIPGNSS; FP 477-579, GSPGIHRD). The correct sequence of the clone FP 552-585 was confirmed by sequencing with Sequenase 2.0

1. Abbreviations used in this paper: GST, glutathione-S-transferase.

(Un. States Biochemicals, Cleveland, OH). The correct expression of all clones except FP 1-308 was confirmed by a positive reaction with antibodies specific to ezrin.

The fusion proteins were produced and purified using basically the method of Smith and Johnson (1988). *Escherichia coli* DH5 cells were used as the host in protein expression. The synthesis of the fusion proteins was induced by incubating the bacteria with 1.0 mM isopropyl β -D-thiogalactopyranoside (IPTG) for 1-5 h at 30°C. The cells were sedimented by centrifugation and the cell pellet was then solubilized in buffer A (10 mM phosphate buffer pH 7.4/0.165 M NaCl/1.0 mM MgCl₂/0.2 mM ATP/0.2 mM DTT/1% Triton X-100/1.0 mM PMSF). After sonication, the cell debris was removed by centrifugation (10,000 g, 10 min) and the supernatant was filtered through a Millipore 0.45 μ m filter. The supernatant was applied to Glutathione-Sepharose 4B beads (Pharmacia) to purify the fusion proteins.

Glutathione-Sepharose 4B beads were washed with buffer A before incubation with bacterial lysate. The fusion proteins were bound from bacterial lysate to Glutathione-Sepharose beads by incubating for a minimum of 10 min with end-over shaking at room temperature. The Glutathione-Sepharose beads containing the fusion protein were washed with buffer A to remove unbound bacterial proteins.

Since the fusion protein FP 1-308 was insoluble in *E. coli* cells, it was purified by preparative SDS-PAGE. After staining with ice-cold 0.9 M potassium acetate, the corresponding gel slice was cut out from the gel and the protein was electroeluted from the gel slice in a buffer containing 100 mM glycine and 30 mM Tris/HCl pH 8.0. After dialysis against buffer A, the FP 1-308 protein was purified by Glutathione-Sepharose 4B beads.

Preparation of [³⁵S]Methionine-labeled Cell Lysate and Incubation with GST-Ezrin-Sepharose Beads

JEG-3 choriocarcinoma cells (Amer. Type Culture Collection, Rockville, MD; HTB 36) were labeled overnight with [³⁵S]methionine (30 μ Ci/ml) in methionine-free MEM supplemented with 2% FCS. The cells were washed with PBS and collected on ice in buffer A. After sonication, the cell debris was removed by centrifugation at 10,000 g (10 min) and the cell lysate was filtered through a Millipore 0.45 μ m filter.

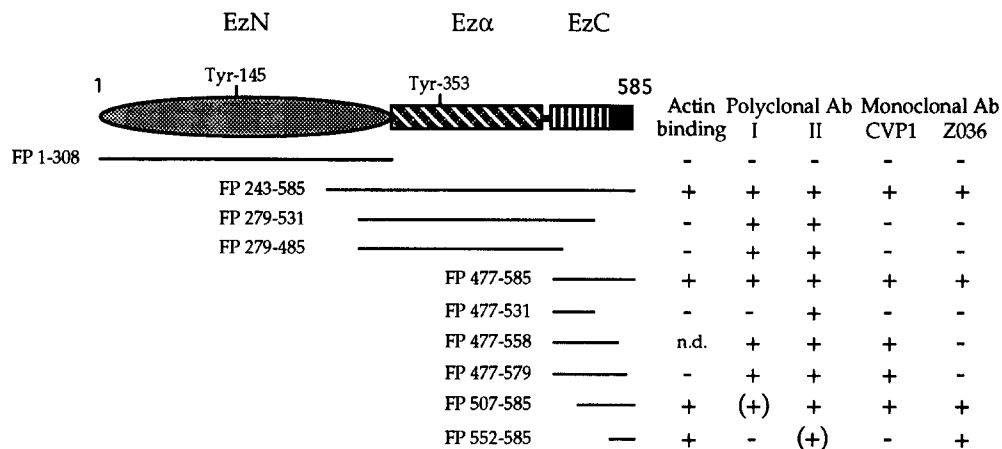
The cell lysate was incubated for a minimum of 15 min with GST-ezrin-Sepharose beads using shaking at room temperature. In the binding experiments ~5-50 μ l of 50% Sepharose slurry containing ~1-20 μ g of fusion protein was incubated with cell extract obtained from a third to fifth part of a confluent 100-mm Petri dish. The total volume in the binding experiment was 0.5-1.0 ml. Before analyzing the proteins bound to GST-ezrin-Sepharose beads, unbound proteins were removed by washing several times with buffer A, more than ten times the bed volume. The GST-ezrin-Sepharose beads containing the bound cellular material and the control GST-Sepharose beads were applied directly in SDS-PAGE.

Effect of NaCl and SDS on the Binding of Actin to GST-Ezrin

The effect of NaCl on binding of [³⁵S]methionine-labeled cellular actin to ezrin is shown for FP 477-585. Concentrations of NaCl were varied in buffer A: 0.15, 0.5, 0.8, and 1.0 M. The binding of actin to GST-ezrin-Sepharose beads and the washing of the beads after binding were accomplished in the corresponding NaCl concentration. We also tested the binding of actin to GST-ezrin fusion proteins in the low salt concentration of buffer B (10 mM phosphate buffer pH 7.4/0.2 mM CaCl₂/0.2 mM ATP/0.2 mM DTT/1% Triton X-100/1.0 mM PMSF). The sodium concentration in buffer B was 10 mM. For the monomer binding assay, [³⁵S]methionine-labeled JEG-3 cells were washed with PBS, and then rapidly once with buffer B without detergent. The cells were directly suspended in buffer B and prepared for the binding assay as described above. The binding of actin to GST-ezrin was also tested with 0.01% and 0.1% SDS added to binding experiment in buffer A.

Binding of Purified Muscle Actin to GST-Ezrin

Rabbit skeletal muscle actin was prepared according to the method of Spudich and Watt (1971). Purified actin (2 μ g as a single band in SDS-PAGE) was stored on ice in G-buffer (2 mM Tris-HCl pH 7.5/0.2 mM CaCl₂/0.2 mM ATP/0.5 mM β -mercaptoethanol). Actin was polymerized during 30 min in buffer A (without PMSF) and incubated at the concentration of 1.0 mg/ml for 30 min in buffer A (without PMSF) with FP 243-585 Sepharose 4B beads or GST-Sepharose 4B beads in a total volume of 200 μ l. The concentration of FP 243-585 was ~40 μ g/ml. As a negative control



region is marked in black. The heptaproline sequence is indicated by a black bar. The tyrosines known to be phosphorylated are shown. GST was located on the NH₂-terminal side of the fusion proteins.

Figure 1. Summary of the localization of the actin-binding site in ezrin and the regions of the protein detected by different antibodies raised against human ezrin. Abbreviations: domains of ezrin are indicated by EzN, Ez α and EzC; —, regions of ezrin expressed as GST-fusion proteins; polyclonal Ab I denotes the antibody raised against ezrin in our laboratory; polyclonal Ab II denotes the antibody raised by Dr. A. Bretscher; *nd*, not determined. The actin-binding COOH-terminal

we used the GST-Sepharose 4B beads at the GST concentration of ~ 200 $\mu\text{g/ml}$. After washing several times by the binding buffer the beads were analyzed in SDS-PAGE. It was important to polymerize the actin before adding to GST-ezrin-Sepharose beads or control GST-Sepharose beads. Otherwise actin was polymerized inside the porous structures of Sepharose beads and consequently remained bound to the beads.

Binding of purified muscle actin was tested also in monomer actin buffer conditions (buffer B) in actin concentration of 1.0 mg/ml. We used both filament free G-actin and a G-actin preparation containing actin filaments: after polymerization with 150 mM KCl and 1.0 mM MgCl₂, the actin was dialyzed two days against G-buffer and was not ultracentrifuged to remove the remaining filaments.

Analysis of Proteins Bound to GST-Ezrin-Sepharose Beads

SDS-PAGE was used to identify the proteins bound to the GST-ezrin-Sepharose beads. The proteins in the gel were detected by autoradiography or by immunoblotting with monoclonal actin antibody in a dilution of 1:2,500. An estimation of the amounts of fusion proteins in the binding experiments was obtained by staining the immunoblot filter with 0.2% Ponceau red in 3% TCA or by Coomassie blue staining of the gels. The binding of purified muscle actin to FP 243-585 Sepharose beads was detected by Coomassie blue staining of the SDS-PAGE gel or by immunoblotting with actin antibody.

Results

General Strategy to Detect Proteins Interacting with Ezrin

We have here used affinity chromatography to identify proteins binding to ezrin. The truncated forms of ezrin were expressed as fusion proteins with GST in pGEX expression system (Smith and Johnson, 1988). GST fusion proteins were purified by using the affinity of GST to glutathione coupled to Sepharose 4B beads. We used GST-ezrin fusion proteins immobilized on Glutathione-Sepharose 4B beads in affinity chromatography to detect which proteins in cell lysate bind to GST-ezrin ("GST-ezrin" is used to refer to all the different truncated ezrin clones created as fusion proteins with GST). The advantages of this method are that no chemical coupling reagents are used and the orientation of the GST-ezrin on the Sepharose beads is known. While the fusion protein is bound to the Sepharose beads by the GST-part, the COOH terminus of ezrin is uniformly oriented away from the matrix and is free to interact with other proteins.

Analysis of the Bacterial Expression Products

A series of truncated GST-ezrin fusion proteins (Fig. 1) covering the whole coding sequence of ezrin was created from ezrin cDNA. Full-length GST-ezrin could not, however, be purified for the actin-binding assay since the expression level was very low and the fusion protein was insoluble. To see whether the fusion proteins were fully expressed, the observed sizes in SDS-PAGE were compared to the sizes calculated on the basis of sequence data. The theoretical sizes of the truncated expression products of ezrin were added to the size of GST protein (27 kD). The observed sizes in SDS-PAGE compared to expected values (in parenthesis) were as follows: FP 1-308, 67 kD (65 kD); FP 243-585, 80 kD (68 kD); FP 279-531, 71 kD (58 kD); FP 279-485, 59 kD (53 kD); FP 477-585, 42 kD (40 kD); FP 477-531, 37 kD (34 kD); FP 477-579, 42 kD (40 kD); FP 507-585, 35 kD (37 kD); FP 552-585, 31 kD (31 kD). The comparison of the observed and estimated sizes indicated that all fusion proteins were expressed in full length. All the GST-ezrin fusion proteins were expressed close to the expected sizes, except the clones containing the heptaproline region (FP 243-585, FP 279-485, FP 279-531; Fig. 1). Their observed sizes were ~ 6 –13 kD higher than the calculated values.

Several antibodies raised against ezrin were used in immunoblotting to confirm the correct expression of the truncated fusion proteins (Fig. 2). All the GST-ezrin fusion proteins, except FP 1-308, were detected by one or more of the ezrin antibodies. The immunoblotting analyses also identified the location of the sites detected by different antibodies. The polyclonal antibody I (PAb I) raised against the full-length cellular ezrin reacted predominantly with the region containing the predicted α -helical domain Ez α and the heptaproline sequence (Fig. 2, lanes 3 and 4). The reaction was weaker against the COOH-terminal domain EzC (Fig. 2, lanes 6–8) and PAb I did not react at all with the NH₂-terminal domain EzN (Fig. 2, Lane 1). The polyclonal ezrin antibodies prepared by Dr. A. Bretscher (PAb II) gave a similar staining pattern except that they reacted with the COOH-terminal epitopes stronger than PAb I (Fig. 2, lanes 5–8) and detected a larger COOH-terminal region. As a result, PAb II detected the region 477-531 and possibly also the region 552-585, while PAb I did not detect the regions 477-531 and 552-585

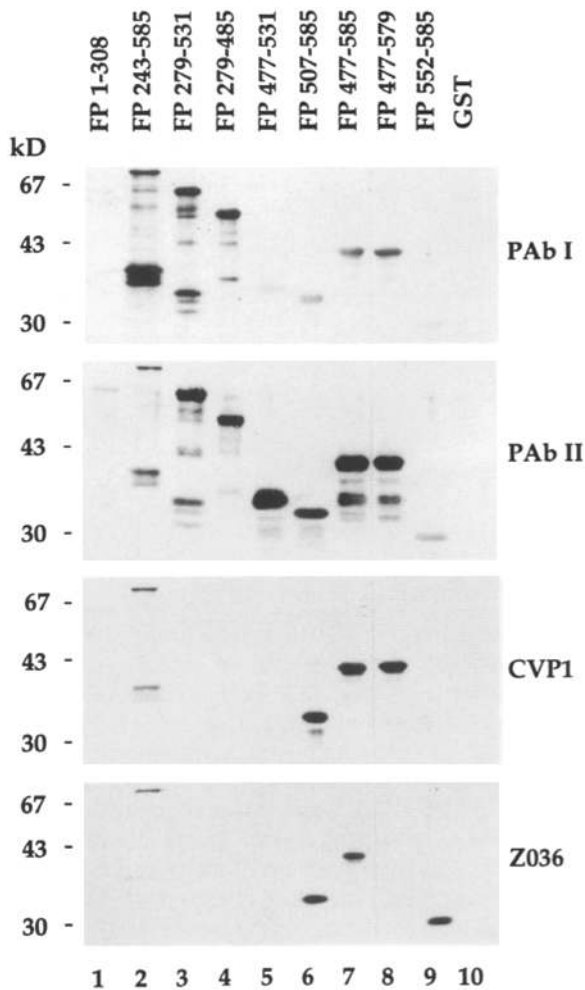


Figure 2. Immunoblotting of the GST-ezrin fusion proteins. The *E. coli* bacterial pellet was solubilized in Laemmli's sample buffer and immunoblotted using polyclonal antibody I (*PAb I*) in a dilution of 1:2,000, polyclonal antibody II (*PAb II*) in a dilution of 1:2,000, monoclonal antibody CVP1 in a dilution of 1:2,000, and monoclonal antibody Z036 in a dilution of 1:5,000.

(Fig. 2, lanes 5 and 9, respectively). The epitope detected by monoclonal antibody CVP1 is located at the site 532-558, since it detected FP 477-579 (Fig. 2, lane 8) and FP 477-558 (not shown), but not FP 279-531 and FP 477-531 (Fig. 2, lanes 3 and 5, respectively). CVP1 is specific to human ezrin (our unpublished data). Since the only differences between mouse and human ezrins in the region 532-558 or close to it are at the sites Val-531 (Leu-532 in mouse ezrin) and Ser-535 (Asn-536 in mouse ezrin) (see Fig. 8 A), the epitope recognized by CVP1 is concluded to include residue 531 and/or residue 535. The monoclonal antibody Z036 is raised by Dr. A. Bretscher against a synthetic peptide comprising the region 575-585. Immunoblotting with Z036 showed that all the fusion proteins ending in the COOH terminus (FP 243-585, FP 477-585, FP 507-585, and FP 552-585) contained an intact epitope detected by the Z036 monoclonal antibody.

Binding of Actin to GST-Ezrin-Sepharose 4B Beads

The major protein binding to GST-ezrin-Sepharose 4B beads from [³⁵S]methionine-labeled JEG-3 choriocarcinoma cell

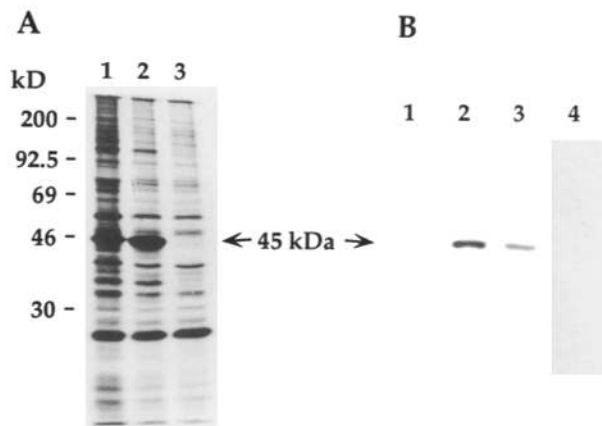


Figure 3. Binding of cellular actin to GST-ezrin-Sepharose beads. (A) A 45-kD protein was bound to the FP 243-585 fusion protein (lane 1) and FP 477-585 fusion protein (lane 2) immobilized on Glutathione-Sepharose 4B beads when cell lysate from JEG-3 choriocarcinoma cells labeled with [³⁵S]methionine was incubated with GST-ezrin-Sepharose beads. The 45-kD protein did not bind to FP 477-579 Sepharose beads (lane 3). In this experiment the Triton-X-100 was omitted from the buffer A. The proteins bound to GST-ezrin-Sepharose beads were detected using autoradiography. (B) The 45-kD protein bound to GST-ezrin-Sepharose beads was identified in immunoblotting using a monoclonal antibody to actin in a dilution of 1:2,500. The immunoblot shows the actin-binding experiments using GST-protein (lane 1), FP 243-585 fusion protein (lane 2), and FP 477-585 fusion protein (lane 3) immobilized on Glutathione-Sepharose 4B beads. As antibody control the material bound to FP 243-585 Sepharose beads was stained with monoclonal antibody to β -galactosidase in a dilution of 1:2,500 (lane 4).

lysate in buffer A had the size of 45 kD in SDS-PAGE (Fig. 3 A, lanes 1 and 2). There were also some unidentified proteins (molecular mass region 70-200 kD) binding in very low amounts to the Ez α domain (FP 243-585; Fig. 3 A, lane 1) but not to the EzC domain (FP 477-585; Fig. 3 A, lane 2). In immunoblotting, the 45-kD protein binding to FP 243-585 and FP 477-585 Sepharose beads was found to be actin (Fig. 3 B, lanes 2 and 3, respectively) and had the same size as cellular actin (not shown). As antibody control, the cellular material bound to FP 243-585 Sepharose beads (Fig. 3 B, lane 4) was stained with monoclonal antibody to β -galactosidase. Actin did not bind to the GST-Sepharose control beads (Fig. 3 B, lane 1). Occasionally very weak binding to control beads was observed, but the amount of binding was insignificant (an example is seen in Fig. 4, lane 8) when compared to the specific actin binding.

In our actin-binding assay we normally used 1% Triton X-100. However, actin was bound to truncated ezrin similarly in the absence (Fig. 3 A, lanes 1 and 2) or presence (Fig. 4, lane 4) of the nonionic detergent. The independence of the use of the nonionic detergent Triton X-100 indicates that hydrophobic interactions do not have a major role in the binding of actin to GST-ezrin.

To see whether ezrin binds also to monomeric actin, we tested the binding of actin to GST-ezrin in the low salt buffer (buffer B) that keeps the actin in monomeric form. In this experiment the actin in [³⁵S]methionine-labeled cell lysate did not bind to GST-ezrin (FP 243-585 and FP 477-585) in

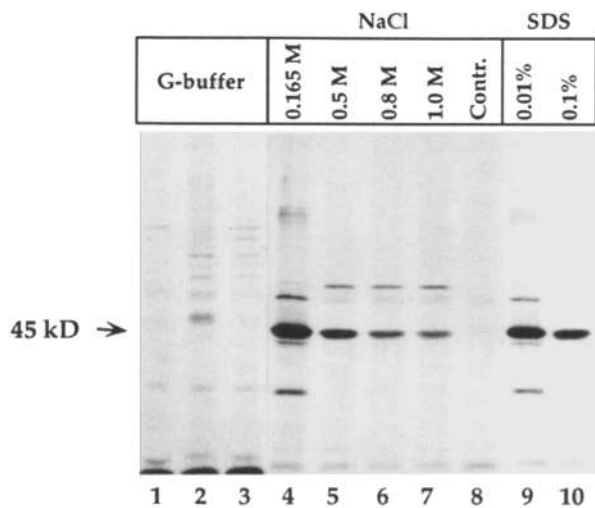


Figure 4. The influence of NaCl and SDS on the actin-binding of the COOH-terminal domain of ezrin (FP 477-585). [³⁵S]Methionine-labeled JEG-3 cell extract was used as the source for actin. When JEG-3 cells were solubilized in buffer B (G-buffer), actin did not bind to FP 243-585 Sepharose beads (lane 2) or FP 477-585 Sepharose beads (lane 3). Lane 1 shows the GST control experiment in buffer B. In lanes 4–7 the influence of higher concentrations of NaCl were tested on the actin binding of FP 477-585. The [³⁵S]methionine-labeled JEG-3 cell lysate was prepared in buffer A containing 0.165 M NaCl (lane 4), 0.5 M NaCl (lane 5), 0.8 M NaCl (lane 6), and 1.0 M NaCl (lane 7). Lane 8 shows the experiment with GST-Sepharose control beads in 0.165 M NaCl. The influence of SDS on the actin binding of FP 477-585 was tested in buffer A with 0.01% SDS (lane 9) or 0.1% SDS (lane 10).

10 mM sodium phosphate buffer (Fig. 4, lanes 2 and 3, respectively). This shows that monomeric actin did not bind to GST-ezrin (region 243-585). The negative result in the low salt buffer was not due to the absence of actin in the cell lysate made in buffer B, as confirmed by immunoblotting (not shown).

The GST-ezrin fusion proteins could be easily eluted by 15 mM glutathione from the Glutathione-Sepharose beads except the fusion protein FP 243-585. However, we could not use the glutathione elution to test whether actin binds specifically to GST-ezrin, since the actin was partially washed away from the beads also with the buffer only (not shown). Probably the washings broke the actin filaments on the beads, but also a potentially low affinity binding of actin to GST-ezrin could explain the elution of actin by binding buffer (buffer A).

In the experiments with [³⁵S]methionine-labeled cell lysate, a few proteins were binding in small amounts (compared to actin) to GST-ezrin-Sepharose beads, but not to the GST-Sepharose control beads. An example is a 36-kD protein that bound to the GST-ezrin-Sepharose beads (Fig. 3 A, lanes 1 and 2; Fig. 4, lane 4) but not to the control beads (Fig. 3 A, lane 3; Fig. 4, lane 8). However, a concentration of 0.5–1.0 M NaCl in the binding buffer (Fig. 4, lanes 5–7) or 0.1% SDS (Fig. 4, lane 10) removed the minor proteins; an upper minor protein appearing in high salt experiments (Fig. 4, lanes 5–7) was occasionally detected. Concentrations of 0.5–1.0 M NaCl or 0.1% SDS did not prevent the binding of GST-ezrin fusion protein to Glutathione-Sepharose beads as assayed by Coomassie blue staining of

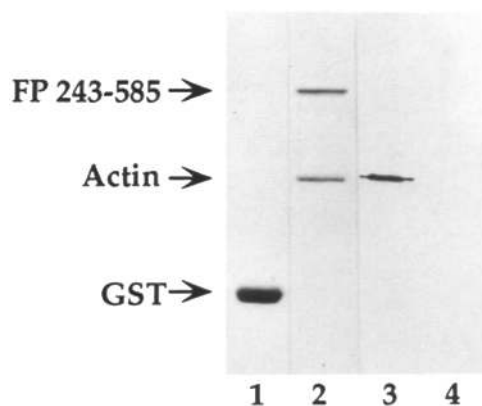


Figure 5. Binding of purified rabbit muscle actin to GST-ezrin Sepharose beads. F-actin was incubated in buffer A with GST-Sepharose beads (lane 1) and FP 243-585 Sepharose beads (lane 2). Lanes 1 and 2 shows Coomassie blue staining of the Sepharose beads after the actin-binding experiment. Lane 3 shows the binding of actin filaments in buffer B to FP 243-585. Lane 4 shows the binding experiment of filament-free monomeric actin in buffer B with FP 243-585 Sepharose beads. The actin-binding in lanes 3 and 4 was detected by immunoblotting with an actin monoclonal antibody in a dilution of 1:2,500.

SDS-PAGE gels (not shown). These results show that the minor binding proteins do not mediate the binding of filament actin to GST-ezrin. They also show that the EzC domain binds only actin.

With increasing salt less actin bound to FP 477-585 Sepharose beads (Fig. 4, lanes 4–7). This can be explained by the short filaments which do not bind in the lower NaCl concentrations (0.5 M) while long filaments can bind to the GST-ezrin-Sepharose beads even in 1.0 M NaCl. Most of the binding was lost in 0.5 M NaCl indicating that the actin binding of ezrin is mediated mainly by electrostatic interactions. Also the GST-ezrin fusion protein FP 243-585 had the same binding profile (not shown).

The GST-ezrin fusion proteins containing the COOH-terminal region of ezrin were partially degraded during the bacterial growth or purification of the fusion proteins. The use of proteinase inhibitors did not eliminate the degradation. The short incubation and centrifugation times during the purification were essential to reduce the degradation. In Fig. 2 (lanes 2–8), the lower minor bands are probably degradation products, since they were stained by antibodies to ezrin. The negative staining of the degradation products with the monoclonal antibody Z036 (detecting the region 575-585) shows that the degradation products of the fusion proteins FP 243-585, FP 507-585, FP 477-585, and FP 552-585 (Fig. 2, lanes 2, 6, 7, and 9, respectively) seen in immunoblotting lack the COOH terminus of ezrin. The negative actin-binding result with the COOH-terminal domain where six amino acids were omitted from the COOH terminus (Fig. 3 A, lane 3 and FP 477-579 in Fig. 6) shows that the possible degradation products are not responsible for the actin binding.

We also used purified rabbit muscle actin to demonstrate the direct binding of actin to ezrin. While the GST-Sepharose control beads did not bind purified rabbit muscle F-actin (Fig. 5, lane 1), the F-actin was bound to FP 243-585

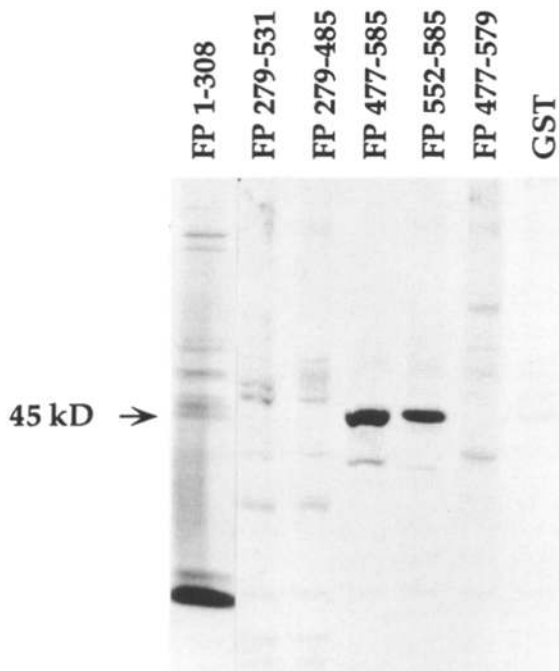


Figure 6. Location of the actin-binding site in ezrin. [³⁵S]Methionine-labeled JEG-3 cell lysate was used in the actin-binding experiments done in buffer A. The actin binding with different GST-ezrin-Sephadex beads and GST-Sephadex beads was detected by autoradiography. The strong low molecular mass band in lane FP 1-308 is background binding.

Sephadex beads in the same way as the actin from cell lysates (Fig. 5, lane 2). Lanes 1 and 2 in Fig. 5 show Coomassie blue staining of the binding results. Although the concentration of purified actin in the binding experiments was in general 1.0 mg/ml, the binding of F-actin to FP 243-585 Sephadex beads could be detected easily in actin concentrations less than 0.1 mg/ml (not shown). The results with the highly purified proteins confirm that filamentous actin bound directly to GST-ezrin and that for the binding no additional protein factors were needed.

Furthermore, we obtained evidence that the F-actin bound to GST-ezrin also in low salt concentration (buffer B). When the G-actin solution was not free from the actin filaments we could see a clear binding to GST-ezrin (FP 243-585; Fig. 5, lane 3). No binding of actin occurred in G-buffer when the filaments were removed by centrifugation (180,000 g for 2 h; Fig. 5, lane 4). Lanes 3 and 4 in Fig. 5 show immunoblotting of the binding results with monoclonal actin antibodies. As a conclusion, physiological salt is needed only for the formation of actin filaments but not for the binding of ezrin. This supports the possibility that ezrin could bind also monomeric actin but the affinity may be too low for the binding to be detected in affinity chromatography.

Identification of the Actin-binding Site in Ezrin

To identify the actin-binding site in ezrin we produced a series of GST-ezrin fusion proteins containing overlapping parts of ezrin. A summary of the actin-binding results is shown in Fig. 1. We observed that all the GST-ezrin fusion proteins binding actin contained an intact COOH terminus

of ezrin. The NH₂-terminal domain (FP 1-308; Fig. 6) as well as the predicted α -helical domain (FP 279-531 and FP 279-485; Fig. 6) did not bind actin in physiological salt concentration. Already the omission of six COOH-terminal amino acids of ezrin fully prevented any actin binding (Fig. 3 A, lane 3; FP 477-579 in Fig. 6). This shows that if other parts in ezrin were binding actin the intact COOH terminus was needed for the binding to occur. The negative actin binding of the fusion proteins FP 279-485 and FP 477-579 was confirmed also with the purified muscle F-actin (not shown). Since SDS in the purification of fusion protein FP 1-308 might affect the binding properties of FP 1-308, it is not fully ruled out that EzN could not bind actin in different conditions.

The actin-binding capability was confined to a stretch of 34 amino acids at the COOH terminus of ezrin (FP 552-585; Fig. 6). Both actin in cell lysate (Fig. 6) and purified muscle actin (not shown) bound to FP 552-585. The inability of different GST-ezrin fusion proteins missing the COOH terminus of ezrin (FP 1-308, FP 279-531, FP 279-485, and FP 477-579) to bind actin shows that possible bacterial impurities or unspecific binding to any fusion protein are not responsible for the observed actin binding of GST-ezrin. The actin binding was specific to a short COOH-terminal region in ezrin. Since the COOH terminus of FP 477-579 contains a few additional amino acids (GSPGIHRD) expressed from the vector, theoretically these amino acids could also prevent the actin binding of a site located nearby. Consequently, we cannot conclude that the short acidic COOH-terminal region (579-585) would be the site binding to actin.

Discussion

The actin binding of ezrin detected in our affinity-chromatographic assay confirms the earlier suggestions that ezrin has a structural role in microvilli and other cell surface structures linking the cytoskeleton to the plasma membrane. Our results showing that the actin-binding site is located within the 34 COOH-terminal amino acids of ezrin confirm the earlier models of the domain structure of ezrin. The expression of truncated ezrins in CV-1 monkey kidney cells had already suggested that the actin-binding site is located in the COOH-terminal region comprising Ez α and EzC domains (Algrain et al., 1993). Since the spectrin/actin-binding site in protein 4.1 is located in the middle of the protein it has been proposed that the predicted α -helical domain in ezrin would be responsible for the actin binding (Krieg and Hunter, 1992). The limited homology in this region to several cytoskeletal proteins gives additional support to this hypothesis (Turunen et al., 1989; Gould et al., 1989).

However, in our experiments we could not find any direct actin binding in the Ez α domain. A contribution of the Ez α domain to the actin-binding of ezrin is not ruled out, since the Ez α domain has 23–28% identity with tropomyosin or ~40% similarity when the identical positions of hydrophobic and charged amino acids are counted in addition. The similarity without any gaps in the sequence (about 100 amino acids) covers two pairs out of seven α and β quasi-equivalent actin-binding sites in tropomyosin (Parry, 1975; McLachlan and Stewart, 1976; Hitchcock-DeGregori and Varnell, 1990). In addition, the Ez α domain is partially amphipathic with hydrophobic amino acids concentrated at po-

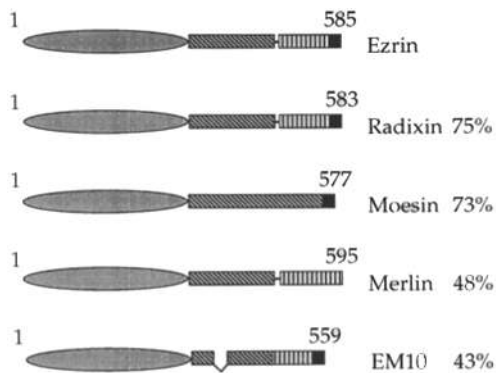


Figure 7. The domain structures of ezrin, radixin, moesin, merlin/schwannomin, and EM10. , NH₂-terminal domain (amino acids 1-300); , predicted α -helical domain; , proline-rich amino acid stretch; , COOH-terminal domain (the actin-binding site in ezrin and the potential actin-binding sites in other proteins are shown in black). Percentage sequence identities are shown in comparison to ezrin.

sitions *a* and *d* in the repeat of seven amino acids (unpublished data). Consequently, the Ez α domain could form a tropomyosin-like actin-binding surface. The homologies of the Ez α domain to different cytoskeletal proteins, like tropomyosin, myosin, caldesmon, troponin T, and keratins (Turunen et al., 1989; Gould et al., 1989) may also indicate interactions with several proteins in the cortical cytoskeleton.

The sequence identities between ezrin, radixin, moesin, merlin (also called schwannomin), and EM10 protein are strongest in the NH₂-terminal domain. The identity between ezrin, radixin, and moesin in this region is over 80%, while the average identity of the whole proteins is 75%. The identity between merlin and other members of the protein family is 62% in the NH₂-terminal domain and 48% for the whole proteins. EM10 protein shows 43% overall identity

with ezrin and 55% identity in the NH₂-terminal domain. The differences appear mostly in the predicted α -helical and COOH-terminal domains (see Fig. 7). For example, a long proline-rich region at the end of the predicted α -helical domain is found in ezrin, radixin and merlin, but not in moesin and EM10 protein. In addition, the predicted α -helical domain is longer in moesin than in the other proteins.

The strong similarities in the amino acid sequences and similar cellular locations suggest a common role for ezrin protein family as membrane-cytoskeleton linkers. The members of the protein family—ezrin, moesin, and radixin—are located in various cell surface structures where they colocalize with actin (Bretscher, 1983; Gould et al., 1986; Pakkanen et al., 1987; Pakkanen, 1988; Hanzel et al., 1989; Tsukita et al., 1989; Sato et al., 1991, 1992; Furthmayr et al., 1992; Berryman et al., 1993; Franck et al., 1993). Also EM10 protein has a cell surface location (Frosch et al., 1991). The different phosphorylation patterns of ezrin, moesin, and radixin indicate different ways to regulate each protein (Fazioli et al., 1993; Franck et al., 1993).

The COOH-terminal region of 25 amino acids in ezrin, moesin, radixin, and EM10 protein forms the most conserved region in the whole COOH-terminal domain (Fig. 8 A), as also noted by Lankes et al. (1993). Especially, the comparison with EM10 sequence shows that the homology is centered around the conserved tyrosine (Tyr-564 in ezrin). The high degree of sequence homology in this region could be indicative of a common actin-binding site. Only radixin has been reported to bind actin (Tsukita et al., 1989), but no data on a specific actin-binding site are yet available.

The COOH-terminal sequence of merlin/schwannomin does not show any remarkable homology to the actin-binding site of ezrin and does not have the conserved tyrosine (see Fig. 7 A). The cellular location of merlin/schwannomin, the tumor suppressor for neurofibromatosis 2 (Rouleau et al., 1993; Trofatter et al., 1993), has not yet been reported. It will be interesting to see whether merlin/schwannomin can

A

h-ezrin	478	VVEPVSYHVQESLQDQEGAEPIGY-SAEISSSEGIRDDRNEEKRI TEAEKNERVQRQLVTLSSSELSQARDENKRTHNDI IHNENM-RQGRDKYKTLRQIRQNTKQRIDEFEAL	585
m-ezrin	477N.....G.....L.....L.....N.....M	586
m-radixin	477	PVI-PT----NEH..QD.NSAEA.....VMNH.S..EWV..TQ....KK..QA....A....T.K.Q..VL.A..V-KA.....HA.....M	583
h-moesin	473	.A..A----.NE..QD.NGAEA..D.RADAMAK..S..E.T.....KH.KA.T...AN....S.K.A..M..A...-L.....SM	577
E.mull-EM10	450	RREKHDKS.VNGSGDA.SQDDESE.KELEVIENVR.T..S.V.AVS...TL.TK.AN.KM...ST..QS.MRDI.RR.EY.V-.E.N.....N..K...MC.VEQ..SM	559
h-merlin	486	PIPAIPLPDIP.FNLI.DLSLSPD-FKDTDMKRLSME-I..KEKVEYM...SKHL.E..NE.KT.IEALKKERE.AL..L...SD.G.SS.HN.IKKLTLQSA.S.VAF...E.	595

B

h-ezrin	502	LSSEGIRDDRNEEK-RITEAEKNERVQRQLVTLSSSELSQARDENKRTHNDI IHNENMRQGRDKYKTLRQIRQNTKQRIDEFEAL	585
c-capZ	190	LTRQMEKDEFTVSDSS-PHIANIGRLVEDMENKIRSTLNEIYFGKTRDIVNGLRSIDAI PDNQYKQLQRELSQVLTRQRIYIQPDN	277
h-ca-mhc	697	GVLEGGIRICRKGFPPNRILYGF -----RQRYRILNPA-AIPEGQFIDSRKGA	742
h-sk-mhc	698	GVLEGGIRICRKGFPPNRILYGF -----KQRYRVLNAS-AILEGQFIDSKKAC	743
c-BBMI	571	GLMENVVRRAGYAFRQLYQPF -----LERYKMLSRK-TWPRWTGGDREGAE	616
h-nm-mhc	696	GVLEGGIRICRKGFPPNRVVFQEF -----RQRYEILTPN-SIPKGFMDGKQACV	741

Figure 8. (A) Comparison of the COOH-terminal domains in the ezrin family of proteins. The actin-binding site in human ezrin is underlined. Identical residues and gaps are indicated by dots and dashes, respectively. Databank sources for the different proteins: human ezrin (P15311 or P23714), mouse ezrin (S66825), mouse radixin (S66820), human moesin (M69066), human merlin (L11353), and *Echinococcus multilocularis* protein EM10 (M61186). (B) Actin-binding regions in chicken CapZ β subunit (P14315) and myosin heavy chain (*mhc*) sequences are compared to similar sequence motifs in human ezrin (P15311 or P23714). The aligned myosin heavy chains were selected to represent different grades of identity to the KYKXL motif in ezrin. The sequences of the myosin heavy chains were obtained by BLAST network service of NCBI. Abbreviations and databank sources for the myosin heavy chains: *h-ca-mhc*, human cardiac muscle (P12883); *h-sk-mhc*, human embryonic fast skeletal muscle (P11055); *c-BBMI*, chicken brush border myosin I (A33620); *h-nm-mhc*, human nonmuscle (M81105). Identities and identical charges in comparison to human ezrin are indicated in bold. Dashes indicate gaps.

bind to actin, since it is not ruled out that it has at its COOH terminus some amino acids essential for the actin-binding. A new feature of merlin/schwannomin is the finding of alternative splicing transcripts affecting the COOH terminus of the protein (Arakawa et al., 1994; Haase et al., 1994; Hara et al., 1994; Pykett et al., 1994). The alternative splicing could change or even regulate the binding properties of merlin/schwannomin.

The region 558-578 in ezrin, (see Fig. 8 B) moesin, radixin, and EM10 protein shows high sequence homology also with a potential actin-binding site in the myosin heavy chain (Keane et al., 1990; Vandekerckhove and Vancompernelle, 1992) and the actin-binding site of CapZ β subunit (Hug et al., 1992). Especially, the KYKXL motif ("K" refers here to both K and R) is found in skeletal and cardiac myosin heavy chains, brush border myosin I (a microvillus protein), CapZ β subunit and the ezrin protein family, except merlin/schwannomin. In myosin heavy chains the KYKXL motif is located in the middle of the protein in the S1 head domain, but does not belong to the major actin-binding sites observed in the crystallographic studies (Rayment et al., 1993; Schröder et al., 1993). Furthermore, the KYKXL motif is not fully conserved in all myosins (see Fig. 8 B). Although the peptide made from this site binds actin (Keane et al., 1990), the importance of the site for the actin binding in myosins is not clear. In CapZ β subunit the actin-binding site is localized in a short COOH-terminal region like in ezrin. In addition, the distance of the KYKXL motif from the COOH terminus differs in CapZ β subunit only by one amino acid from that in ezrin.

CapZ and radixin are reported to be barbed end-capping proteins (Casella et al., 1987; Tsukita et al., 1989) and belongs to class II actin-capping proteins that do not sever actin (for review see Weeds and Maciver, 1993). Although the actin-capping activity is not fully ruled out, the immunoelectron microscopical observations (Pakkanen et al., 1987; Berryman et al., 1993) implicate lateral binding of ezrin to actin filaments to be a major form of interaction of ezrin with actin in microvilli. In this respect ezrin has a similar location in microvilli as brush border myosin I (for reviews see Louvard, 1989; Bretscher, 1991).

The actin-binding site at the COOH terminus of ezrin is a strongly basic region (amino acids 558-578) ending in a short stretch of acidic and hydrophobic amino acids (579-585). Several actin-binding sites in other actin-binding proteins are positively charged interacting with acidic amino acids in actin (for reviews see Kabsch and Vandekerckhove, 1992; Vandekerckhove and Vancompernelle, 1992). More data are required to localize exactly the amino acids in ezrin responsible for the actin binding. To understand the role of ezrin protein family in the dynamics of the cell surface structures it will be important to determine the influence of ezrin and the other members of the protein family on the behavior of actin.

Since experimental evidence for a direct interaction of ezrin with actin has been difficult to obtain (Bretscher, 1983, 1991; Krieg and Hunter, 1992), it has been proposed that phosphorylation would be crucial for the interaction of ezrin with actin (Krieg and Hunter, 1992). Consequently, purified cellular ezrin would occur in a form where the actin-binding site is masked. The phosphorylation of tyrosine or serine/threonine (Bretscher, 1989; Hanzel et al., 1991; Fazioli

et al., 1993) could be a signal opening the actin-binding site in ezrin. Furthermore, it is possible that the affinity of the actin-binding site in ezrin is so weak that only the binding of filament actin to immobilized ezrin (and possibly not the binding of free ezrin to actin) can be seen in the actin-binding assay. Stronger binding occurs presumably in the affinity chromatography when F-actin is bound to the GST-ezrin-Sepharose in several points of the filament. Monomeric actin could be lost during the washings in the case that it binds ezrin weakly. In principle, several factors may contribute to the loss of actin binding of cellular ezrin during the purification.

Evidence for a masked actin-binding site comes from the expression of ezrin cDNA in cultured cells. Expression studies with full-length ezrin and COOH-terminal part of ezrin in cultured cells have indicated that the binding of ezrin to the cytoskeleton is dependent on the unmasking of the binding site in ezrin (Algrain et al., 1993). Both full-length ezrin and the COOH-terminal part of ezrin expressed from cDNA colocalized to microvilli and other cell surface structures. However, the full-length ezrin did not bind to stress fibers, whereas the COOH-terminal part of ezrin expressed in cells is seen to be colocalized with stress fibers in immunofluorescence analysis (Algrain et al., 1993). This shows that the truncated ezrin missing the NH₂-terminal domain has lost its physiological regulation of the binding to cytoskeleton. The inability of full-length ezrin to bind to stress fibers indicates that the actin-binding site of ezrin must be unmasked by specific signals for the binding to actin to occur.

The cloning of phosphotyrosine phosphatases sharing over 30% identity with the EzN domain gives indirect support for the phosphorylation as an essential regulatory signal in the function of ezrin protein family (Gu et al., 1991; Yang and Tonks, 1991). This is indicated also by experimental data. In A-431 human epidermoid carcinoma cells stimulated by EGF, the tyrosine and serine are transiently phosphorylated, concomitantly with the redistribution of ezrin to microvilli (Bretscher, 1989). In gastric parietal cells the phosphorylation of serine is increased when ezrin is induced to redistribute to microvilli (Urushidani et al., 1989). More data on the structure-function relationship are required to understand how the phosphorylation regulates ezrin. Tyr-145 and Tyr-353 have been identified as the major tyrosine phosphorylation sites of ezrin in the EGF stimulated A-431 cells (Krieg and Hunter, 1992). Another potential site for the regulation by tyrosine phosphorylation is the conserved Tyr-564 in KYKXL motif of the actin-binding site in ezrin. One theoretical mechanism for tyrosine or serine/threonine phosphorylation to regulate actin-binding would be by an influence on the dimerization of ezrin (Ullrich et al., 1986; Pakkanen and Vaheri, 1989; Gary and Bretscher, 1993).

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