Mutagenesis of the Phosphatidylinositol 4,5-Bisphosphate (PIP$_2$) Binding Site in the NH$_2$-Terminal Domain of Ezrin Correlates with Its Altered Cellular Distribution

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Abstract. The cytoskeleton-membrane linker protein ezrin has been shown to associate with phosphatidylinositol 4,5-bisphosphate (PIP$_2$)-containing liposomes via its NH$_2$-terminal domain. Using internal deletions and COOH-terminal truncations, determinants of PIP$_2$ binding were located to amino acids 12–115 and 233–310. Both regions contain a KK(X)$_2$K/RK motif conserved in the ezrin/radixin/moesin family. K/N mutations of residues 253 and 254 or 262 and 263 did not affect cosedimentation of ezrin 1-333 with PIP$_2$-containing liposomes, but their combination almost completely abolished the capacity for interaction. Similarly, double mutation of Lys 63, 64 to Asn only partially reduced lipid interaction, but combined with the double mutation K253N, K254N, the interaction of PIP$_2$ with ezrin 1-333 was strongly inhibited. Similar data were obtained with full-length ezrin. When residues 253, 254, 262, and 263 were mutated in full-length ezrin, the in vitro interaction with the cytoplasmic tail of CD44 was not impaired but was no longer PIP$_2$ dependent. This construct was also expressed in COS1 and A431 cells. Unlike wild-type ezrin, it was not any more localized to dorsal actin-rich structures, but redistributed to the cytoplasm without strongly affecting the actin-rich structures. We have thus identified determinants of the PIP$_2$ binding site in ezrin whose mutagenesis correlates with an altered cellular localization.

Key words: cytoskeleton • actin • CD44 • A431 cells • COS1 cells

Introduction

Members of the ezrin/radixin/moesin (ERM) family and the related tumor suppressor merlin are part of the protein 4.1 superfamily. They are defined as cytoskeleton-membrane linkers (for reviews, see Bretscher, 1999; Mangeat et al., 1999). The anchorage of the ERM family to the plasma membrane occurs in different ways. Indirect binding of ERM to different proteins with several transmembranous domains, such as the cystic fibrosis transmembrane conductance regulator (Short et al., 1998) or the Na$^+$/H$^+$ antiporter (Murthy et al., 1998; Yun et al., 1998), involves an adaptor protein such as EBP 50 (Reczek et al., 1997). ERM can also be linked to the cytoplasmic tail of membrane proteins with a single transmembranous domain such as CD43, CD44, I-CAM 1, I-CAM 2, and I-CAM 3. These interactions appear to be facilitated by the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (Hiraо et al., 1996; Serrador et al., 1997; Yonemura et al., 1998). It is not clear whether this dependency is due to a requirement for PIP$_2$, of the transmembranous protein or of the ERM itself (Hiraо et al., 1996; Heiska et al., 1998). Some studies suggest that PIP$_2$ could help unmask cryptic binding sites in ERM. PIP$_2$ could thus be involved in the activation process of the ERM, switching the inactive “dormant” molecule to an activated state (Matsui et al., 1999; Nakamura et al., 1999). ERM could also directly interact with PIP$_2$-containing phospholipid bilayers (Niggli et al., 1995; Hiraо et al., 1996; Heiska et al., 1998). Thus, the direct association of ezrin with PIP$_2$ and also with the p85 subunit of the phosphatidylinositol 3-kinase may provide a mechanism to anchor the enzyme in proximity to its substrate (Gautreau et al., 1999).

Talin, another member of the 4.1 family, has been shown to insert into bilayers of liposomes containing acidic phospholipids using hydrophobic photolabeling. Lipid interaction was attributed to a 47 kD NH$_2$-terminal domain (Niggli et al., 1994). Similarly, we have demonstrated that the NH$_2$-terminal domain of ezrin (amino acids 1–309) was...
necessary and sufficient for interaction with PIP2-containing liposomes. We showed that ezrin binds preferentially to liposomes containing PIP2 as compared with other phospholipids such as phosphatidylerine, and that this interaction occurs at physiological ionic strength. Under these conditions, ezrin discriminates between PIP2, phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-bisphosphate (Niggli et al., 1995).

In this study, we further characterized the interaction of the NH2-terminal domain of ezrin with PIP2-containing liposomes by truncation and site-directed mutagenesis. Amino acids that are involved in binding to PIP2-containing liposomes have been identified. They are located in two distinct regions of this domain. Similarly, PIP2 interaction was abolished when the same critical mutations were introduced in the full-length ezrin molecule. Although the PIP2-independent interaction of ezrin with proteins was not affected, its intracellular localization was drastically changed. Expression of the mutated first 310 amino acids of ezrin led to a loss of the ability of the NH2-terminal domain of ezrin to promote cell extensions (Martin et al., 1997; Amieva et al., 1999).

Materials and Methods

Site-directed Mutagenesis

The preparation of constructs with internal deletions as well as COOH-terminal truncations has already been described (Roy et al., 1997). The numbering of the amino acid sequence of ezrin includes the first methionine, corresponding to the protein expressed in bacteria. The quick change site-directed mutagenesis kit from Stratagene was used. The changed bases are underlined. The oligonucleotides (sense) (5’–3’) used for introducing the K63N mutation in pGEX-2T ezrin 1-333 were GGCTGAAGCTGGATAAT and TCTGTGTTGCTCAGG. The corresponding oligonucleotide for introducing the K253N mutation in pGEX-2T ezrin 1-586 was GAGGTCAGG (S66A). For this later series, 14 cycles were performed (95°C, 1 min; 68°C, 1 min)

Determination of Protein Concentration

The UV spectra were recorded and the protein concentration was calculated from the absorption coefficients at 220 and 280 nm. The Bradford reagent from Bio-Rad was used for protein determination. The Bradford assay (Bradford, 1976) did not allow a quantitative estimation of the protein content because of the peculiar amino acid composition of the NH2-terminal constructs; this assay was used only for comparing relative amounts of the protein.

Phospholipids

Phosphatidylcholine (PC) and PIP2 were obtained from Lipid Products. For some experiments, PIP2 was also obtained from Sigma-Aldrich. No difference was detected between the two products.

Cosedimentation of Ezrin and Ezrin Constructs with Lipid Vesicles

Analysis of protein-lipid interactions by cosedimentation of proteins with large multimolecular liposomes has been documented in detail elsewhere (Niggli et al., 1994). Large, multimolecular liposomes were prepared from PC and PIP2; in a buffer containing 20 mM Hepes, pH 7.4, 0.2 mM EDTA, 15 mM mercaptoethanol, and 50 mM NaCl. In this case, EGTA was added to the protein solution to neutralize Ca2+, which interferes with the liposome cosedimentation assay (see Results). This resulted in a protein solution containing (mM): 50 Tris/HCl, pH 7.4, 100 NaCl, 1.25 CaCl2, 2.8 EGTA, and 0.05% NaN3. No difference in lipid interaction of the same preparation of wild-type ezrin domains could be detected when proteins were added to the lipids before or after dialysis provided that Ca2+ was neutralized by EGTA. Proteins were kept under nitrogen during incubation with lipids. Final concentrations of protein were 27.5–55 μg/ml and of
lipid 0.5 mg/ml. The fraction of wild-type ezrin (1-333) cosedimenting with PIP2-containing liposomes was similar for both protein concentrations. The mixtures were subsequently centrifuged for 30 min at 100,000 g, 4°C. The pellets were solubilized in 50–100 μl sample buffer (Niggli et al., 1994). The supernatants were mixed with the corresponding amount of the threefold concentrated sample buffer. After heating the samples for 5–10 min at 95°C, they were applied to SDS-polyacrylamide gradient gels (Niggli et al., 1995). The amount of protein present in pellets and supernatants was quantified by scanning the bands of the Coomassie blue-stained gels. The amount of ezrin sedimented in the absence of liposomes was always subtracted from that sedimenting in the presence of lipid (= specific cosedimentation). In a series of representative experiments, 22 ± 8% (n = 10) of wild-type ezrin 1-333 sedimented in the absence of lipid. Comparable data were obtained for mutated proteins. The amount of wild-type ezrin 1-333 recovered in the pellet was increased in the presence of liposomes containing 20% PIP2 to 77 ± 9% (n = 10) of total protein. The specific cosedimentation of wild-type ezrin 1-333 corresponds thus to 300 ± 160% (n = 10) of the amount of protein sedimenting in the absence of lipid. Some variations were observed in the extent of cosedimentation with liposomes when comparing different protein preparations, possibly due to variable denaturation during purification and/or storage. The data are given as mean ± SD of n experiments. Differences between data were analyzed with the Student’s t test for paired data, with a P < 0.05 considered significant.

**Interaction of Ezrin with the Cytosolic Tail of CD44**

GST or GST-CD44 (5 μg, 10 μl) bound to glutathione agarose beads were equilibrated in the reaction buffer (25 mM Tris-HCl, pH 7.4, 150 mM KCl, 1 mM MgCl2, and 1 mM DTT). PIP2 was added in micellar form (Hirao et al., 1996). Full-length wild-type ezrin or ezrin K253N, K254N, K262N, and K263N (3.5 μg, 1 μM) were incubated in a final volume of 50 μl with beads for 1 h at room temperature. Beads were then washed three times with 500 μl buffer at 4°C. After the final wash, 20 mM glutathione was added for 1 h. Aliquots of the supernatant were then analyzed by SDS-PAGE and Western blotting for the presence of ezrin using procedures described in Roy et al. (1997).

**Homotypic Interaction Assay**

Wild-type ezrin 1-586 was coated in wells of a microtiter plate and residual beads saturated with 2% bovine serum albumin (Roy et al., 1997). Ezrin 1-333 constructs (0.5 μM each) were then added in buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 100 mM KCl, and 0.2 mM dithiothreitol). After 1 h of incubation, wells were rinsed with the same buffer and samples were processed for SDS-PAGE analysis and Western blotting.

**Intrinsic Tryptophane Fluorescence Measurements**

Fluorescence spectra were recorded with an Amino-Bowman Series 2 luminescence spectrometer at 30°C. Protein samples were incubated in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl. The tryptophane emission fluorescence spectrum was recorded by excitation at 292 nm. Both excitation and emission beams were set at 2-nm bandwidth.

**Cell Culture and Transient Transfection**

COS1 and A431 cells were maintained in DMEM containing 10% FBS. Cells were seeded 24 h before transfection at a density of 200,000 cells per 35-mm dish. Transfection of plasmids into COS1 cells was carried out by using Effectene (Qiagen) as recommended by the manufacturer (0.4 μg plasmid in 100 μl EC buffer and 3.2 μl enhancer for 5 min at room temperature, then addition of 10 μl Effectene followed by contact with cells 10 μl later). Superfact (QIAGEN) was used for transfection of A431 cells: 2 μg DNA was mixed with 100 μl DMEM and 10 μl Superfact for 5 min at room temperature before addition to cells. The medium was changed 3 h later. Immunofluorescence and cell fractionation were carried out 30 and 42 h after transfection, respectively.

**Cell Fractionation**

The cells were washed twice with PBS with 0.5 mM MgCl2 and 2 mM CaCl2. Lysis buffer (mM) 10 Hepes, pH 7.4, 2.5 MgCl2, 1 EGTA, 1 EDTA, 1 vanadate, 10 NaF, 0.1 PMSF, 100 μg/ml CHAPS, 10% glycerol, and a cocktail of antiproteases) was added (300 μl per 35-mm dish). Cells were scraped off with a rubber policeman and homogenized by 20 strikes in a Dounce homogenizer (pestle B). The homogenates were centrifuged at 25,000 g for 30 min at room temperature. The supernatant corresponds to the cytosolic fraction. The pellet was resuspended in 300 μl buffer A [(mM) 50 MES, pH 6.4, 2.5 MgCl2, 1 EGTA, 1 vanadate, 10 NaF, 10% glycerol, 0.5% Triton X-100, antiprotease cocktail] and centrifuged at 18,000 g for 15 min at 4°C. The pellet (Triton X-100 nonextractable material) was resuspended in 300 μl buffer A and sonicated. Fractions (30 μl) were applied to SDS-polyacrylamide gels and immunoblotted with PSD4 anti–VSV-G mAb or anti-ezrin antibodies (Andréoli et al., 1994; Roy et al., 1997). Anti-ezrin antibodies generated either against the full-length protein or the first 310 residues were defined as anti-C and -N antibodies, respectively (Andréoli et al., 1994).

**Immunofluorescence Analysis**

Cells were fixed with 3.7% formaldehyde in PBS for 20 min and treated with TBS containing 0.2% Triton X-100 for 4 min. Cells were incubated with anti-VSV-G mAb (clone PSD4; Kreis, 1986) for 1 h at room temperature, washed, and incubated with FITC-conjugated phalloidin and Texas red-conjugated anti–mouse Ig Ab (Sigma-Aldrich). After final washes and mounting in Mowiol, cells were examined using a laser scanning confocal microscope (Leica or Bio-Rad Laboratories) using a 63× 1.4 oil immersion objective.

**Results**

**Mapping of the PIP2 Binding Site**

Association of ezrin with the lipid bilayer was first studied with fusion proteins corresponding to the NH2-terminal domain of ezrin, a region which has been previously shown to mediate the interaction of ezrin with PIP2-containing liposomes (Niggli et al., 1995). As a measure for bilayer association, cosedimentation of proteins with large liposomes containing 20% PIP2 and 80% PC was analyzed. Control values of protein sedimenting in the absence of lipid were always subtracted. Lipid interactions were measured under physiological ionic strength conditions. We have found previously that addition of 1 mM MgCl2 (in the presence of 60 μM EGTA and 50 μM EDTA) markedly reduced cosedimentation of wild-type full-length ezrin with PIP2-containing liposomes by 40–70% (data not shown). According to Flanagan et al. (1997), divalent cations promote fusion of PIP2 micelles into insoluble aggregates and may thus also adversely affect PIP2-containing liposomes. All the experiments were therefore carried out in the absence of added divalent cations.
Progressive deletions from the COOH terminus of ezrin 1-333 illustrated the importance of amino acids 233–310 in the assay of cosedimentation with liposomes (95 ± 8% inhibition of cosedimentation for ezrin 1-233, n = 4; Fig. 1). The internal deletion of residues 13–114 also led to a dramatic loss of the resulting construct to interact with liposomes (89 ± 18% inhibition, n = 3). GST alone did not cosediment with the liposomes and elimination of the GST moiety did not affect association with PIP2-containing liposomes (Fig. 1). This finding shows that unspecific trapping of protein in the lipid pellet is negligible under our experimental conditions. The results shown in Fig. 1 also demonstrate that ezrin 1-333 is capable of recruiting a protein incapable of membrane interaction (GST) to the bilayer. The PIP2 binding domain of ezrin therefore appears to consist of nonlinear determinants and involves distinct portions of the molecule.

By analogy with several PIP2-binding proteins and pleckstrin homology (PH) domains, it was inferred that a number of basic residues could be involved in such an interaction. Typical PIP2-binding motifs consist of clusters of basic amino acids; e.g., (K/R)XXXXXX(K/R)/(K/R) in gelsolin or (K/R)XXXXX(K/R)/(K/R) in villin (Jannney et al., 1992; Yu et al., 1992). Two similar motifs are conserved in the entire ERM family at identical positions and also at comparable amino acids; e.g., (K/R)XXXKX(K/R)(K/R) in gelsolin or (K/R)XXXXX(K/R)/(K/R) in villin (Jannney et al., 1992; Yu et al., 1992). Two similar motifs are conserved in the entire ERM family at positions 273–279 and 293–296, respectively. However, we have shown previously that this domain does not significantly interact with PIP2-containing liposomes. (Niggli et al., 1995). These motifs thus are not functionally relevant for lipid interaction.

**Table I. Effects of K to N Mutations on Cosedimentation of Ezrin 1-133 with PIP2-containing Liposomes**

| Mutations K > N introduced in positions: | Percent protein in pellet | n |
|----------------------------------------|--------------------------|---|
| 63 and 64 | 253 and 254 | 262 and 263 | 6 |
| – | – | – | 59 ± 13 | 11 |
| + | – | – | 27 ± 4 | 3 |
| + | + | – | 9 ± 2 | 3 |
| – | + | – | 61 ± 12 | 6 |
| – | – | + | 48 ± 18 | 6 |
| – | + | + | 6 ± 4 | 6 |
| + | + | + | 10 ± 5 | 3 |

Wild-type and mutant ezrin 1-333 domains (27.5–55 μg protein/ml) were incubated in the presence of 100 mM NaCl and in the absence or presence of large liposomes containing 20% PIP2 and 80% PC (0.5 mg lipid/ml), followed by determination of the fraction of liposome-associated protein, as described in Materials and Methods. Mean ± SD of n experiments.

Several double mutations of the lysine residues located in the presumed motifs were introduced in the ezrin 1-333 molecule. As shown in Table I, double mutations of either lysines 253 and 254, or lysines 262 and 263 to asparagine did not significantly impair the ability of the mutated construct to cosediment with PIP2-containing liposomes. The double substitution K63N, K64N by contrast led to a significant (P < 0.025) partial reduction in the amount of mutated ezrin 1-333 that cosedimented suggesting a particularly important role of these residues (Table I). The combination of the K253N, K254N and the K262N, K263N mutations led to an almost complete loss of interaction with PIP2 (84 ± 11% inhibition, n = 6). The combination of K63N, K64N and K253N, K254N mutations resulted similarly in a protein with strongly reduced PIP2-binding capacity (78 ± 11% inhibition, n = 3), far exceeding the effect of the double mutation of K63N, K64N alone. The introduction of the K262N, K263N mutations into this mutant did not further reduce binding. For optimal binding, the presence of either
Figure 4. The mutations introduced specifically target ezrin-PIP₂ interactions. (A) Introduction of four K > N mutations in ezrin did not change the chymotrypsin digestion pattern. Wild-type (WT) and mutated ezrin 1-586 (K253N, K254N, K262N, and K263N) (285 μg/ml) were incubated in the presence or absence of 3.3 μg/ml chymotrypsin at room temperature for the indicated times. After SDSPAGE, membranes were immunoblotted with a polyclonal antibody targeted against the first 310 amino acids of ezrin. No major difference in the digestion patterns was observed when wild-type or mutated ezrin were compared. (B) Introduction of four K > N mutations in ezrin (K253N, K254N, K262N, and K263N) did not alter F-actin binding. F-actin binding was measured using a solid phase assay as described by Roy et al. (1997). Actin concentrations were chosen so that they were below, around, and above the dissociation constant (500 nM) for actin binding to ezrin. For each actin concentration, binding was measured in the presence or absence of PIP₂. Bound actin was detected using a monoclonal anti-actin antibody. The top two blots illustrate the binding of actin to the NH₂-terminal domain of wild-type and mutant ezrin (residues 1–333). The bottom two blots correspond to actin binding to full-length ezrin with or without the four K > N mutations. No obvious difference in F-actin binding capacity was observed when comparing mutated ezrin constructs with their parent wild-type counterparts. (C) Mutagenesis of the PIP₂ binding site in ezrin 1-333 did not impair the interaction of the NH₂-terminal domain of ezrin with the cytoplasmic tail of EBP50. The binding of wild-type ezrin 1-333 (WT and *) or mutated ezrin 1-333 (mutated and **) (1 μM) to GST-EBP 50 or GST was assessed as described for binding to CD44 in Materials and Methods. (Top) Coo- masie staining, (bottom) Western blotting. The experiment had been done in triplicate for each construct. Western blotting showed that there was no major difference between WT ezrin 1-333 and the mutated form concerning binding to GST-EBP50. The right two lanes demonstrate the lack of binding of ezrin 1-333 WT (*) or mutated (***) when assayed with the control GST free of fusion protein. (D) Introduction of up to six K > N mutations in ezrin did not affect homotypic interactions. Wild-type ezrin 1-586 was coated in wells of a microtiter plate (Roy et al., 1997). Ezrin 1-333 constructs (0.5 μM each) were then added in F-actin buffer, except for the first lane, where no construct was added (*). Similar amounts of ezrin 1-586 were coated in each well as judged by the Coomassie stain of the top blot. The same polyclonal anti-ezrin antibody as in A was used to detect the construct overlaid and, irrespective of the mutations introduced, no major difference in the amount of ezrin 1-333 construct bound to ezrin 1-586 was observed (bottom). When no ezrin 1-586 was coated, no ezrin 1-333 was detectable (not shown). (E) Intrinsic tryptophane fluorescence was not modified upon introduction of four K > N mutations in full-length ezrin. (Top curve) Full-length mutated ezrin K253N, K254N, K262N, and K263N; (bottom curve) wild-type ezrin 1-586. Note that neither the maximum intensity of tryptophane fluorescence nor the wavelength for maximum emission were significantly affected upon introduction of the four mutations.
loss of PIP2 requirement of the interaction of ezrin with the cyto-
coupled agarose beads independently of the addition of PIP2 mi-
tuations in ezrin induced constitutive binding to GST-CD44–
left), K253N, K254N, K262N, and K263N (K253,254,262,263N)
in all lanes (top, incubation with GST-CD44 beads; bottom, incu-
bation with GST beads). In contrast to wild-type (WT) ezrin (top
were then analyzed by SDS-PAGE and immunoblotted for the
presence of ezrin. (A) Coomassie blue staining of the blots shows
that comparable amounts of GST-CD44 (or GST) were detected
on the gel. (B) The amount of wild-type or mutated ezrin bound to
GST-CD44 or to GST alone was quantified by densitometry of the
immunoblots shown in A. Black and hatched columns represent
ezrin bound to GST and GST-CD44, respectively. The results
shown are representative of three independent experiments.

lysines 63, 64, 253, and 254 or lysines 253, 254, 262, and 263
is thus required. The presence of lysines 63 and 64 alone is
not sufficient to maintain binding, correlating with the ex-
periments on the truncated NH2 terminus (Fig. 1).

The finding that some double mutations (K253N, K254N or K262N, K263N) did not significantly alter PIP2
binding of ezrin 1-333 could be due to the use of saturating
amounts of PIP2 in the liposomes, which may not allow us
to detect subtle changes in lipid binding. According to our
previous findings, the interaction of full-length wild-type
ezrin with liposomes is maximal at 20% PIP2 and 80% PC
(Niggli et al., 1995). We therefore compared interactions
of mutated and wild-type proteins with liposomes contain-
ing lower amounts of PIP2. As shown in Fig. 3, at 10 and
5% PIP2, no statistically significant difference was detect-
ble between cosedimentation of mutants ezrin 1-333
K253N, K254N, ezrin 1-333 K262N, K263N, and the wild-
type form of ezrin 1-333.

Since the serine 66 residue corresponds to a potential
protein kinase A phosphorylation site, we performed a
S66D mutation in ezrin 1-333 to analyze the effects of in-
roducing a negative charge next to the neighboring lysine
residues involved in PIP2 binding. However, the S66D mu-
ant behaved as the wild-type domain (not shown). Intro-
duction of a negative charge next to lysines 63 and 64 thus
does not disturb PIP2 binding.

On the basis of the findings summarized in Fig. 1 and Table
I, the full-length ezrin molecule was mutated in positions 253,
254, 262, and 263 and in combination with positions 63 and
64. The amount of mutated construct recovered in the lipos-
osome-containing pellet corresponded to 2% of total protein
for K253N, K254N, K262N, and K263N and to 3% for K63N,
K64N, K253N, K254N, K262N, and K263N, as compared
with 24% of wild-type ezrin cosedimenting with liposomes
(\(\alpha = 2\)). PIP2 interaction of these two mutants was thus
almost completely abolished, corresponding to the findings
with the 1-333 constructs.

Conformation and Functional Properties of
Mutated Ezrin

To exclude that our findings were due to an unstable folding
and/or overall denaturation of the mutated protein, we com-
pared the sensitivity of wild-type ezrin and ezrin K253N,
K254N, K262N, and K263N to chymotrypsin (Fig. 4 A).
Chymotrypsin has been previously used to partially proteo-
yze ezrin and identify a chymotryptic-resistant NH2-termi-
nal domain (Franck et al., 1993; Niggli et al., 1995). No ma-
jor differences in the degradation patterns were observed
when ezrin was mutated: the 35- and 24-kD bands detected
with an antibody directed against the first 310 amino acids of
ezrin were the major degradation products for both the
wild-type and the mutated ezrin, indicating therefore that
both molecules were similarly folded (Fig. 4 A). However,
the 24-kD degradation product appeared earlier with ezrin
K253N, K254N, K262N, and K263N, suggesting that its fold-
ing was less stable than that of wild-type ezrin.

The same mutations also did not alter the ability of the full-
length ezrin molecule nor that of the ezrin 1-333 construct to
bind to F-actin at relatively high concentrations (0.5–2.5 \(\mu M\)
F-actin) as measured in the solid phase assay (Fig. 4 B) (Roy
et al., 1997). Under more stringent conditions (0.1 \(\mu M\)
F-actin), the binding capacity of mutated molecules may be
lowered. The addition of PIP2 was without effect on the F-actin
binding of both constructs whether they were mutated or not,
emphasizing therefore the lack of requirement for PIP2 to
bind to F-actin under our assay conditions. The K253N,
K254N, K262N, and K263N ezrin 1-333 also interacted with
the cytoplasmic tail (amino acids 329–358) of EBP 50 in fusi-
on with GST to the same extent as wild-type ezrin 1-333
(Fig. 4 C). No interaction with control GST was detectable.
Figure 6. Mutated ezrin loses its cell membrane localization in transfected A431 and COS1 cells. Human adenocarcinoma A431 epithelioid cells (a–f) and monkey kidney COS1 fibroblasts (g–l) were transfected with either VSV-G–tagged wild-type ezrin (a–c and g–i) or mutated ezrin (K253N, K254N, K262N, and K263N) (d–f and j–l) DNA and treated for indirect Texas red localization of ezrin with anti-VSV antibody (left) and F-actin with FITC-coupled phalloidin (middle). Pictures represent the projected maximum intensities of several focal planes ranging from substrate to apical level, except for d and j. Vertical xz sections at the level of the dotted lines are under each figure. Insets are enlargements of the squared areas in a–c. Dual localizations of ezrin and actin are merged in color, colocalization appearing in yellow. Transfected wild-type ezrin is located in actin-rich cell surface structures such as microspikes, dorsal microvilli (a–c, circles) or ruffles (g–i, arrows), and in lateral cell membranes (a and c, intercellular contacts). Note that focal adhesion plaques (b, c, e, and f, insets, arrowheads) and stress fibers (k) are devoid of any ezrin. Transfected mutated ezrin is essentially located as a cytosolic network (d, f, j, and l; note that single focal planes are used only in d and j for clearer visualization of ezrin), but is also faintly detectable on the cell membranes in COS1 cells (j, arrow). Localization of wild-type ezrin in actin-rich dorsal structures and mutated ezrin in cytoplasm is evident in xz sections. Bars, 10 μm.
The bottom blot was performed with anti–VSV-G mAb. Detectable in the nonextractable material (N). Immunodetection of the full-length ezrin. Note that some degradation of ezrin was detected with this antibody is lower than that presented in A due to the poor accessibility of the anti–N antibody to the epitopes in the cytosol (C), Triton X-100 extractable (E), or nonextractable (N) material, as revealed after immunoblotting with anti–VSV-G mAb. Anti-ezrin antibody, which recognizes both endogenous and VSV-G–tagged ezrin, was used as a control (top). *Internal control with recombinant wild-type ezrin (100 ng load). Results shown are representative of three independent experiments. (B) Transfection of ezrin 1-310. Detection of the top blot was performed with an antibody against the NH2-terminal portion of ezrin. The proportion of cytosolic full-length ezrin (#) detected with this antibody is lower than that presented in A due to the poor accessibility of the anti–N antibody to the epitopes in the full-length ezrin. Note that some degradation of ezrin was detectable in the nonextractable material (N). Immunodetection of the bottom blot was performed with anti–VSV-G mAb.

Using the solid phase assay, we demonstrated that the three pair mutations (K > N; residues 63 and 64, 253 and 254, and 262 and 263) introduced in ezrin 1-333, either alone or in combination, did not impair its homotypic association with the full-length molecule (Fig. 4 D).

In addition, the intrinsic tryptophane fluorescence spectra of both wild-type ezrin and mutated molecule were similar upon normalization and exhibited identical maximum wavelength emission (Fig. 4 E). The 10% difference observed in the maximum intensity may be due to an overestimation of the protein concentration of wild-type ezrin, but in no case did the tryptophane fluorescence of the mutated ezrin appear to be significantly quenched upon exposure of tryptophane(s) to solvent due to an inappropriate folding of the molecule.

Overall, the differences in the properties of wild-type and mutated ezrin documented in Fig. 4 are all very small, whereas the effects of the mutations on PIP2 binding were drastic. The overall conformation of the ezrin molecule as well as some of its major functional binding properties appeared to be essentially preserved after introducing these mutations, except for its ability to interact with PIP2-containing liposomes.

Alteration of the PIP2-dependent Ezrin-CD44 Interaction

The interaction of ezrin with CD44 has been demonstrated to be PIP2-dependent under physiological ionic strength conditions (Hirao et al., 1996). We therefore tested whether mutations in the residues involved in PIP2 binding affected ezrin interaction with the cytoplasmic tail of CD44 in vitro. Indeed in the presence of PIP2 micelles, the amount of wild-type ezrin binding to GST-CD44 immobilized on glutathione agarose beads was significantly increased (Fig. 5). In contrast, the binding of ezrin K253N, K254N, K262N, and K263N to the same beads was unaffected by PIP2 addition, and was already comparable with the level obtained with wild-type ezrin in the presence of PIP2. As a control, both the wild-type and mutated forms of ezrin did not bind to GST-glutathione agarose beads, irrespective of the presence of PIP2 (Fig. 5).

Abnormal Localization of Ezrin Deficient in PIP2 Binding

A431 epithelial cells and COS1 fibroblasts were transfected with ezrin K253N, K254N, K262N, and K263N or with wild-type ezrin, both constructs being tagged with VSV-G epitope. In A431 cells, wild-type ezrin VSV-G colocalized with F-actin at apical ruffles and microvilli, plasma membranes, and microspikes, as already described for endogenous ezrin (Gould et al., 1986; Algrain et al., 1993). Stress fibers and adhesive plaques were decorated with actin but not with ezrin (Fig. 6, a–c and g–i). On the contrary, in A431 and COS1 cells, mutated ezrin VSV-G was found to be located as a sponge-like network within the cytoplasm, clearly visible on single focal planes (Fig. 6, d and j) and xz sections. Mutated ezrin was only faintly detectable at the leading edge of plasma membranes or microspikes (Fig. 6 j). Clearly, mutated ezrin VSV-G labeling was excluded from vesicular structures. Expression of this mutated form of ezrin, however, did not alter cell size and morphology or actin-rich structures.

The subcellular distribution of wild-type and K253N, K254N, K262N, and K263N VSV-G–tagged ezrin was compared upon cell fractionation of COS1 cells. No marked difference was observed in partition of these proteins between cytosol and total membrane fractions (Fig. 7 A). However, the amount of Triton X-100 insoluble ezrin associated with the cytoskeleton was significantly lower in the case of mutated ezrin (37% of total cellular ezrin VSV-G vs. 27% of K253N, K254N, K262N, and K263N ezrin VSV-G; Fig. 7 A). As pointed out for actin, the distribution of endogenous ezrin was not significantly affected by expression of either form of ezrin VSV-G.

Loss of the Cell Extension Activity of the NH2-Terminal Domain of Ezrin Upon Mutation of Its PIP2 Binding Domain

COS1 fibroblasts were also transfected with the VSV-G–tagged NH2-terminal domain of either wild-type or mutated ezrin. As previously described (Martin et al., 1997),
Figure 8. The mutated NH₂-terminal domain of ezrin partly loses its interactions with membranes. Monkey COS1 fibroblasts were transfected with either VSV-G–tagged wild-type (a–c) or mutated (K253N, K254N, K262N, and K263N) (d–f) ezrin 1-310 and treated for immunolocalization of actin (b and e) and VSV (a and d) as in Fig. 6. Transfected wild-type NH₂-terminal ezrin was mainly localized in actin-rich structures such as dorsal microvilli and ruffles (b and c, arrows; xz sections, circles). It induces formation of long filopodia containing both actin (b and c, arrowheads) and transfected ezrin (a and c, arrowheads). Note the presence of nodes along filipodia, particularly rich in NH₂-terminal ezrin (open arrows). The transfected mutated form of NH₂-terminal ezrin was predominantly localized in the cytoplasm (d and f), although some mutant ezrin localized in the same actin-rich structures (e and f) as wild-type NH₂-terminal ezrin. Staining of nuclei was observed only in cells transfected with mutated ezrin 1-310 (d and f). Bars, 10 μm.

Figure 9. Location of the targeted lysines in moesin. The crystallographic data of moesin were treated with the molecular visualization program RASMOL (R. Sayle, Glaxo Wellcome, Greenford, UK). (A) The different domains of the molecule defined by Pearson et al. (2000) were colored as follows: residues 4–94, violet (domain FI); residues 95–201, blue (domain F2); residues 202–297, green (domain F3); and residues 488–577, red (COOH-terminal domain). The lysines of interest have been highlighted in yellow. The distances between the epsilon NH₂ of lysines 63–262, 63–254, and 254–263 are equal to 38.1, 27.6, and 19 Å, respectively. Note that all these lysines are accessible to solvent and therefore to PIP₂. They are all located on the same side of the molecule. (B) Moesin residues that may be in direct contact with the bilayer. Using RASMOL, residues exposed on the surface of moesin and enclosed by the triangle formed by lysines 63, 64, 253, 254, 262, and 263 (bold and underlined) have been identified (bold).
the transfection of the 1–310 fragment of ezrin induced formation of long filopodia, which contain both tagged ezrin 1-310 and actin (Fig. 8, a–c). This cell extension activity of the ezrin NH2-terminal domain is dependent on the cell type used and was not observed in the case of CV1 cells (Algrain et al., 1993). Ezrin 1-310 and actin colocalized to a variable extent along these structures. Transfected ezrin 1-310 was also localized in actin-containing structures such as surface microvilli, dorsal ruffles, and lateral microspikes, comparable with the wild-type full-length molecule. Possibly due to its overexpression, tagged ezrin 1-310 was regularly observed in the cytoplasm and was excluded from vesicles. Strikingly, upon transfection of the cells with mutated ezrin 1-310, almost no cell extensions developed and the cells were extremely flattened. The localization of the mutated form of NH2-terminal domain of ezrin appeared essentially cytoplasmic even if colabeling of some extensions containing actin persisted (Fig. 8, d–f). Note that with this construct some staining of the nucleoli was apparent (see Discussion). Upon cell fractionation, wild-type ezrin and mutated ezrin 1-310 were recovered in the particulate fraction (Fig. 7 B). At variance with the microscopic observations, no ezrin 1-310 was recovered in the cytosolic fraction. This discrepancy can be explained with the relative insolubility of the NH2-terminal domains of ezrin, especially in low salt buffers such as those used for cell lysis.

Discussion

PIP2, a lipid whose turnover is modified upon cell activation, has been implicated in the regulation of the functions of a number of cytoskeletal proteins (Janmey, 1995; Isenberg and Niggli, 1998). Depending on the protein studied, PIP2 interactions may concentrate proteins at the plasma membrane in a manner affected by cell activation, possibly within PIP2-enriched domains (Martin, 1998). PIP2 may also modulate the function of the protein to which it binds (Gascard et al., 1993; Huttelmaier et al., 1998; Steinle et al., 1999). Knowledge of the molecular determinants involved in lipid–protein interactions is a prerequisite for studying their physiological role in intact cells. We have therefore analyzed the nature of PIP2-binding determinants in ezrin by truncations, internal deletion, and site-directed mutagenesis. PIP2 binding was studied using large liposomes containing 20% PIP2 and 80% PC, in physiological salt concentrations. Presenting PIP2 in mixed bilayers is much closer to the cellular environmental conditions than presentation as micelles of pure lipid. The advantage of the cosedimentation assays is also that free ezrin is always exposed to the bilayer and never separated during the assay. A disadvantage is that it cannot be completely excluded, that ezrin extracts some PIP2 molecules to form a small complex that does not sediment. As previously shown, binding of full-length ezrin is maximal at ~20% PIP2, and 5–10% of PIP2 still induced significant association (Niggli et al., 1995). This is similarly the case for wild-type ezrin 1-333 (Fig. 3).

We first showed, by progressive truncations and internal deletions, that the NH2 terminus of ezrin contains two domains essential for PIP2 binding, located in regions 12–115 and 233–310. The loss of one of the sites resulted in almost complete loss of binding. Interestingly, recent findings for gelsolin similarly suggest that this protein binds to PIP2-containing bilayers via a site formed by the juxtaposition of the NH2- and COOH-terminal domains of gelsolin (Tuominen et al., 1999). To our knowledge, these are so far the only two proteins known with a pair of PIP2-binding sites.

Inspection of the sequence of the NH2-terminal domain of ezrin revealed two potential PIP2 binding motifs, which are similar to the motifs identified in gelsolin and villin (Fig. 2). We have analyzed the role of these motifs in ezrin-PIP2 binding by mutation of selected lysines to asparagines. These experiments revealed an important role of lysines 63 and 64, which are required for optimal binding. In contrast, two independent double mutations of lysines 253 and 254 or 262 and 263 to asparagines were of no consequence for the interaction with PIP2. However, this does not mean that they are not involved in binding since mutation of all four lysines 253, 254, 262, and 263 resulted in an almost complete loss of binding similar to that observed after mutation of lysines 63, 64, 253, and 254. The presence of the lysines in the 63–72 motif is thus clearly necessary, but not sufficient for interaction. This conclusion is supported also by the inability of fragment 1–233 to bind to PIP2. For optimal binding, the combined presence of K63, K64, K253, and K254 or K253, K254, K262, and K263 is thus required. Our results suggest that the two domains located in regions 63–72 and 253–263 do cooperate in PIP2 binding. The site at amino acids 63–72 corresponds to a KXXXXXXX(K/R)K motif, which is similar to, but not identical with, the consensus sequence in villin, (K/R)XMKXX(K/R)(K/R) (Yu et al., 1992). The side at amino acids 253–263, a KXXXXXXXXKK motif resembles a consensus sequence for gelsolin, (K/R)XXX(K/R)(K/R) (Yu et al., 1992). Typically, the motifs involved in ezrin-PIP2 interaction start and end with two basic amino acids, whereas the sites in gelsolin and villin start with only one lysine or arginine. In addition, the numbers of interspersed amino acids are different in ezrin. Another motif has been detected in the cytoskeletal protein α-actinin, and also in spectrin and the PH domain of phospholipase C-81 and Grb7: RXXXXXXX(R/K)(R/K)(K/R) (Fukami et al., 1996). Again, this motif is not identical with those detected in ezrin.

During the completion of this manuscript, the crystal structure of a complex of the NH2-terminal domain of moesin (residues 1–297) with the tail (residues 467–577) has been published (Pearson et al., 2000). According to this work, residues 63, 64, 253, 254, 262, and 263 are all located in loops connecting β sheets. In a space-filling model of moesin, the residues implicated in PIP2 binding are all located on the surface of the molecule (Fig. 9 A). These residues form a triangle (Fig. 9 A). The surface enclosed by this triangle is relatively planar, so that a simultaneous contact of all residues with several PIP2 molecules in a bilayer appears to be feasible. The residues enclosed by this triangle and exposed on the surface of moesin have been highlighted in the moesin sequence (Fig. 9 B). These residues, which could form a zone of contact with a PIP2-containing bilayer, are identical in moesin and ezrin, with the exception of asparagine 62 in moesin, which corresponds to aspartic acid 62 in ezrin. Of these residues, including the lysines identified in this study, 36% are positively charged,
16% are negatively charged, 24% are uncharged but polar, and 24% are hydrophobic. Interestingly, Pearson et al. (2000) detected a hitherto unrecognized PH-like domain in the structural module F3 of moesin, where residues 253, 254, 262, and 263 are located. Such PH domains are involved in phosphoinositide binding and feature an antiparallel β sheet consisting of seven strands and a COOH-terminal α-helix (Harlan et al., 1995), comparable with the structure of F3 of moesin. Using the web-based tool SMART (Simple Molecular Architecture Research Tool; Schultz et al., 1998), we searched for a PH domain in the NH2-terminal domain of ezrin, but were unsuccessful. If F3 indeed corresponds to a functional PH domain, then the distribution of charged amino acids is not typical for such domains. According to Pearson et al. (2000), the highly positively charged surface of lobe F3 of moesin may be suitable for binding of negatively charged phospholipids, in agreement with our results. Based on the crystallization results, the PIP2 binding domain in ezrin is thus also different from the lipid-binding domain identified in vinculin, which has been shown to form an amphipathic helical hairpin (Johnson et al., 1998). Ezrin appears to contain two structural elements cooperating in PIP2 binding and one of these elements may be part of a PH domain. These two domains do not appear to be directly involved in F-actin binding, nor do these mutations affect a distal F-actin binding site, as F-actin interaction of ezrin K253N, K254N, K262N, and K263N was not altered. A similar conclusion can be drawn for the interaction of ezrin with EBP 50.

It is unlikely that the effects reported in this paper on ezrin–PIP2 interaction could be simply due to an overall change of the positive charges of the protein. For example, the double mutation K63N, K64N significantly decreased PIP2 binding, whereas the two double mutations of lysines to asparagines at positions 253 and 254 or 262 and 263 had no significant effects. At pH 7.4, the calculated net global charge for the wild-type protein is +6.26 (ezrin 1-333) and −6.34 (ezrin 1-586). Introducing six K > N mutations yielded global charges that were +0.27 and −14.34. In spite of the net charge difference between ezrin 1-333 and full-length ezrin, the very same PIP2-binding determinants were found to be important in the two molecules. Moreover, experiments were carried out under physiological ionic strength conditions. Under these conditions, the range of distances where electrostatics overcome thermal energies is relatively small, and the precise positioning of specific basic amino acids, rather than the overall charge of the NH2-terminal domain, is likely to be important for ezrin–PIP2 interaction. For example, the PH domain of pleckstrin is electrically polarized (Blomberg and Nilges, 1997), and mutation of three conserved lysine residues in this domain has been shown to result in 10-fold decrease in lipid-binding affinity (Harlan et al., 1995). As outlined for the PH domain, other determinants for PIP2 interaction, based, for example, on interactions with acyl chains of the lipid, exist and may account for a pronounced, albeit incomplete, loss in affinity as described in the case of the mutated PH domain of pleckstrin (Harlan et al., 1995).

Interestingly, the introduction of two double mutations (K253N, K254N and K262N, K263N) results in the loss of PIP2 requirement for optimal binding of ezrin to the cytoplasmic tail of CD44. As shown in Fig. 5, the binding between CD44 and the mutated form of ezrin is not impaired but becomes PIP2 independent. This portion of the CD44 molecule contains clusters of basic amino acids involved in the CD44/ezrin interaction (Legg and Isacke, 1998). PIP2 may neutralize basic residues in ezrin, resulting in elimination of repulsive forces between the two molecules. Introducing the mutations may generate the same effect. However, using truncation experiments, Yonemura et al. (1998) demonstrated that the first 19 residues of the cytoplasmic tail of CD44, containing a cluster of basic residues, were sufficient, at physiological ionic strength, to allow ERM-CD44 interactions, whereas binding of the full-length cytoplasmic tail was markedly reduced under these conditions. These interactions were studied in the absence of PIP2. This finding cannot be reconciled with the hypothesis that charge neutralization by lipid is required for such a protein interaction to occur.

Wild-type VSV-G–tagged ezrin is massively recruited to dorsal actin-rich structures. The K253N, K254N, K262N, and K263N mutated form of ezrin was introduced into both epithelioid and fibroblastic cell lines. Immunofluorescence and fluorescence localization showed that the VSV-G–tagged mutant was now mainly located in the cytoplasm, without colocalization with F-actin and only weak binding to plasma membranes. Based on our in vitro findings, one would expect that mutated ezrin would bind to plasma membrane proteins independently of PIP2. In fact, Legg and Isacke (1998) concluded, using CD44-negative cells, that the localization of ezrin to microvilli was not mediated through an interaction with CD44, but rather with other sites at which the actin network closely associated with the plasma membrane. In our study, mutated ezrin expressed in cells no longer localizes to microvilli and ruffles, suggesting that a direct PIP2–ezrin interaction may be one of the necessary events to address ezrin to these specific membrane structures. Since ERM proteins are involved in the formation of the very same structures, the cytoplasmic localization of the mutated ezrin protein might simply result from an upstream defect (PIP2 binding) absolutely required for the formation of a novel ezrin-containing membrane structure. Cell fractionation studies showed indeed that recovery of the mutated ezrin in the cytoskeleton-associated membrane fraction was significantly reduced as compared with the wild-type protein, supporting the immunofluorescence studies. However, total recovery in the membrane fraction appeared not to be altered. This may be due to redistribution occurring during the isolation procedure using detergent.

It has been suggested that multimeric forms of ezrin are involved in the generation of actin-rich structures (Gary and Bretscher, 1993). Interactions between NH2-terminal domains of ERM proteins and full-length proteins resulting in multimer formation have been shown to interfere with the normal functions of endogenous proteins in the NIH3T3 cell line (Henry et al., 1995; Amieva et al., 1999). Using a solid phase assay, we found that wild type as well as mutated ezrin 1-333 and full-length ezrin engaged in homotypic interactions (Fig. 4 D). However, by immunolocalization, no mutated ezrin was found associated with actin-rich structures. We can thus conclude that the formation of heterodimers of wild-type and mutated ezrin is not sufficient for the association with actin-containing domains.
A number of reasons led us to transfact cells with the whole NH$_2$-terminal domain of ezrin (residues 1–310) rather than the portion of the protein containing the PIP$_2$ binding determinants (residues 63–263). Amino acids beyond 296 are necessary for engaging in homotypic as well as heterotypic interactions (Gary and Bretscher, 1993). Deletion of amino acids 13–30 in ezrin (Martin et al., 1997) or of the first 11 residues of moesin (Amieva et al., 1999) led to drastic changes of the localization of the NH$_2$-terminal portion of these proteins. Ezrin 1-320 still localizes to the membrane, whereas fragment 1-276 is cytosolic (Amieva et al., 1999). Thus, deleting any of these portions of the molecule may be dominant over the mutations of the PIP$_2$ binding determinants. In fact, introduction of the latter mutations led to an expression pattern similar to the one observed for any of the deletions described above. These observations suggest that any change in the NH$_2$-terminal domain of the ERM can drastically affect the protein structure and/or function. Of interest is the observation that when the PIP$_2$ binding determinants were mutated, the NH$_2$-terminal domain of ezrin localized within the nucleoli. Although the possibility of an artifact has to be considered, a 55-kD ezrin-related protein that cross-reacts with antibodies against the NH$_2$-terminal domain of ezrin has been detected in the nucleus of many human transformed cells (Kaul et al., 1999). Evidence has also been presented for the nuclear localization of isoforms of protein 4.1, the first member of the ERM family (Correas, 1991). In this latter case, at variance with our observations, a cluster of basic residues was necessary for such a localization to occur (Luque et al., 1998).

In conclusion, two PIP$_2$ binding determinants exist in ezrin in regions 12-115 and 233-310 of the protein, with these mutated residues are likely candidates involved in the targeting of ezrin to, and presumably in the formation of, F-actin–rich membrane structures.

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