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Identification of a phosphatidylinositol-4,5-bisphosphate-binding domain in the N-terminal region of ezrin

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Abstract Purified human recombinant ezrin cosediments with large liposomes containing phosphatidylserine (PS). This interaction is optimal at low ionic strength. At physiological ionic strength (130 mM KCl) ezrin interacts strongly with liposomes containing ≥5% phosphatidylinositol-4,5-bisphosphate (PIP_2), the residual being phosphatidylcholine (PC). When PIP_2 is replaced by phosphatidylinositol-4-monophosphate (PIP), phosphatidylinositol (PI) or PS, the interaction is markedly reduced. Furthermore we show, that a purified N-terminal glutathione *S*-transferase (GST) fusion protein of ezrin (1–309) still has retained the capacity to interact with PIP_2-containing liposomes, whereas a C-terminal fusion protein (310–586) has lost this ability.

Key words: Ezrin; Phosphatidylinositol-4,5-bisphosphate; Acidic phospholipid; Lipid interaction

1. Introduction

Ezrin is a member of the protein 4.1-superfamily which is involved in the interaction of the cell cytoskeleton with the plasma membrane [1,2,3]. Sequence analysis shows, that ezrin contains an N-terminal domain with homology to protein 4.1 and talin, followed by a long α-helical region and a charged C-terminal domain [4]. The N-terminal domain of ezrin, when expressed in mammalian cells, locates to the plasma membrane, whereas the C-terminal domain is enriched in the actin cytoskeleton [5], possibly via a recently identified actin binding site in that region [6]. Overexpressed intact ezrin accumulates at the plasma membrane of insect cells [7], and overexpression of the C-terminal site in these cells induces formation of cell extensions [8]. Interestingly, the N-terminal domain inhibits the latter effect [8]. Recently, the presence of two self-associating domains in ezrin, termed N- and C-ERMADs (ezrin-radixin-moesin association domains) has been demonstrated [9]. A putative membrane anchor of ezrin and the closely related proteins moesin and radixin may be the cell surface glycoprotein CD44 [10]. The membrane association of the tyrosine kinase substrate ezrin may be regulated by phosphorylation [3].

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Abbreviations: GST, glutathione *S*-transferase; PC, phosphatidylcholine; PIP_2, phospatidyl-inositol-4,5-bisphosphate; PIP, phosphatidylinositol-4-monophosphate; PI, phosphatidyl-inositol; PS, phosphatidylserine.

We have now studied the interaction of human recombinant ezrin with phospholipids, important constituents of cell membranes, and we report on a selective interaction of ezrin with PIP_2 via its N-terminal domain.

2. Materials and methods

2.1. Purification of recombinant human ezrin and domains

Domains of ezrin were produced in *E. coli* as a fusion protein with GST and purified as described [7]. Two constructs were isolated: an N-terminal GST fusion protein (amino acids 1–309) and a C-terminal GST fusion protein (amino acids 310–586) [7].

2.2. Phospholipids

PC and PS were obtained from Lipid products, South Nutfield, Surrey, England. PIP_2, PIP and PI were obtained from Sigma, Buchs, Switzerland.

2.3. Cosedimentation of ezrin and ezrin domains with lipid vesicles

Analysis of protein–lipid interactions by cosedimentation of proteins with large multilamellar liposomes has been documented in detail elsewhere [11]. Large, multilamellar liposomes were prepared from PC, PS, PI as described [11]. PIP_2 or PIP were added as stock solutions in 20 mM Hepes pH 7.4, 0.2 mM EGTA to the dried lipids. Ezrin, and ezrin domains (stock solutions in 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 15 mM mercaptoethanol) were centrifuged prior to the experiments for 20 min at 20,000 × *g*, 4°C, followed by protein determination [12]. Proteins were subsequently incubated for 15 min at 22°C in the absence of liposomes, followed by a further incubation in the absence or presence of liposomes for 15 min. In part of the experiments, 130 mM KCl was included in the buffer. Proteins were kept under nitrogen during incubation with lipids. Final concentrations of protein were 0.1–0.15 mg/ml and of lipid 0.5 mg/ml. The mixtures were subsequently centrifuged for 20 min at 20,000 × *g*, 4°C. The pellets were solubilized in 50–100 μl sample buffer [11]. The supernatants were mixed with the corresponding amount of the three-fold concentrated sample buffer. After heating the samples for 5–10 min at 95°C, they were applied to SDS-polyacrylamide gradient gels [13]. 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. The data are given as mean ± S.D. of *n* experiments. Differences between data were analyzed with the Student's *t* test for paired data, with a *P* value of < 0.05 considered significant.

2.4. Cosedimentation of chymotryptic fragments of ezrin with lipid vesicles

Chymotryptic fragments of ezrin were generated essentially as described previously [14], with the following modifications. Ezrin (10 μg) was incubated with 0.1 μg chymotrypsin (Boehringer, Mannheim, Germany) in a buffer containing 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 15 mM mercaptoethanol (final volume 30 μl) for 80 min at 22°C. The reaction was stopped by addition of 1 mM phenyl methyl sulfonyl fluoride. The fragments were then incubated and centrifuged in the absence or presence of liposomes (0.5 mg/ml) as described in section 2.3. Pellets and supernatants were applied to 5–15% gradient gels, followed by transfer to nitrocellulose and immunodetection [15] with a polyclonal antibody against intact human recombinant ezrin [7]. Mixtures of chymotryptic fragments were analyzed by FPLC in the absence of lipid

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as follows: 200 μl of ezrin, digested as described above and adjusted to 130 mM KCl, was applied to a Pharmacia Superdex 75 (10/30) column equilibrated in 20 mM Tris pH 7.4, 0.1 mM EDTA, 15 mM mercaptoethanol, 130 mM KCl (flow rate 0.5 ml/min; 0.5 ml per fraction). Fractions were analyzed by gel electrophoresis, blotting and reaction with a polyclonal antibody against intact ezrin, and in addition with a second antibody raised against the purified recombinant N-terminal

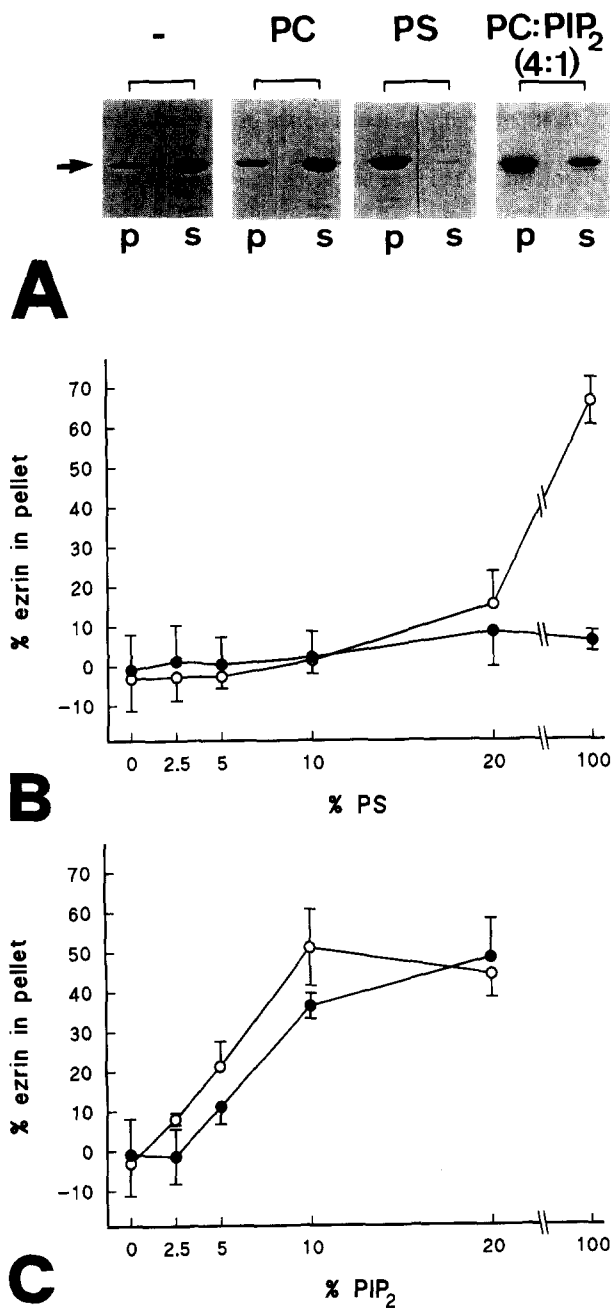


Fig. 1. Cosedimentation of ezrin with large liposomes of different composition. (A) 7.5 μg ezrin (0.15 mg/ml) was incubated for 15 min at 22 °C in the absence of added KCl, and in the absence or presence of large liposomes (0.5 mg/ml) containing different lipids, as indicated. Pellets (p) and supernatants (s) were analyzed on 5–15% gradient gels. A Coomassie Blue-stained gel is shown. The arrow indicates ezrin. (B,C) Quantitative evaluation of the amount of ezrin sedimenting in the absence (open circles) or presence (closed circles) of 130 mM KCl and with large liposomes containing PC and increasing amounts of PS (B) or PIP_2 (C), as indicated (0.5 mg/ml total lipid). 0% PS or PIP_2 correspond to 100% PC. Data correspond to the mean ± S.D. of 3 experiments.

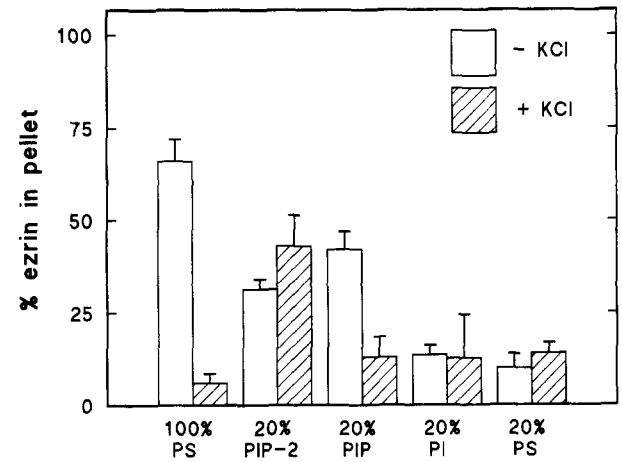


Fig. 2. Cosedimentation of ezrin with large liposomes containing different types of phospholipids. Ezrin (0.11 mg/ml) was incubated for 15 min at 22°C in the absence or presence of 130 mM KCl, and in the presence of large liposomes containig 100% PS, or 80% PC and 20% PIP_2, or 20% PIP, 20% PI, 20% PS, as indicated. After centrifugation at 20,000 × g pellets and supernatants were analyzed by separation on 5–15% gradient gels and scanning of the Coomassie Blue-stained bands (mean ± S.D., $n = 3$).

domain of ezrin as described [7]. Dilution of both antibodies was 1:2000.

Microsequencing of the chymotryptic fragments was carried out as described [16], at the CNRS facility in Vernaison, France.

3. Results

3.1. *Interaction of purified intact human recombinant ezrin with liposomes containing different types of phospholipids*

Purified ezrin was incubated in the absence or presence of large multilamellar liposomes of different composition, followed by centrifugation at 20,000 × g. Ezrin content of pellets and supernatants was analyzed by SDS-PAGE and scanning of the Coomassie blue-stained bands. A gel of a representative experiment is shown (Fig. 1A). 10–30% of ezrin sedimented on its own in the absence of liposomes, possibly due to self-association [7,9]. In the presence of pure PC-liposomes, the amount of sedimenting ezrin was not significantly increased. In contrast, in the presence of vesicles containing 100% PS, 60–85% of ezrin (after subtraction of ezrin sedimenting in the absence of lipid) was recovered in the pellet (Fig. 1A). This interaction was almost abolished by addition of 130 mM KCl, and by decreasing the% of PS in the liposomes to 20% or less. Quantitative evaluation of data is shown in Fig. 1B,C. Liposomes containing 20% PIP_2 and 80% PC were significantly more efficient in inducing cosedimentation of ezrin than liposomes containing the same amount of PS, and, in contrast to PS, cosedimentation with these liposomes was not abolished by 130 mM KCl (Fig. 1B,C). Significantly increased association of ezrin could be measured for liposomes containing PC and as little as 5–10% PIP_2 in the presence of 130 mM KCl ($P<0.05$; Fig. 1C). In contrast, 10% or less of PS did not induce a significant association of ezrin (Fig. 1B). The interactions of ezrin with PS-and PIP_2-containing vesicles differ thus both qualitatively and quantitatively. The latter interaction is already optimal at low (≥10%) concentrations of the lipid and is not very sensitive to an increase of ionic strength.

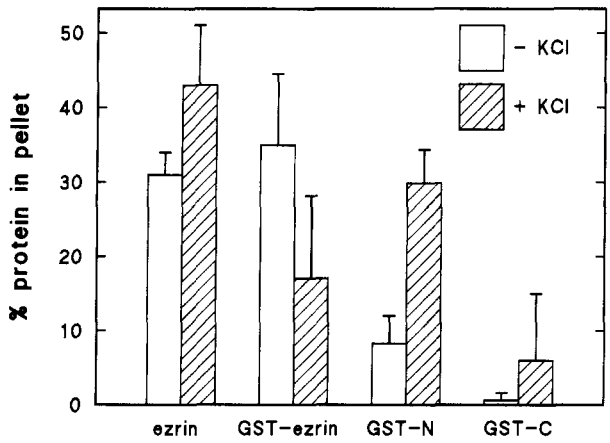


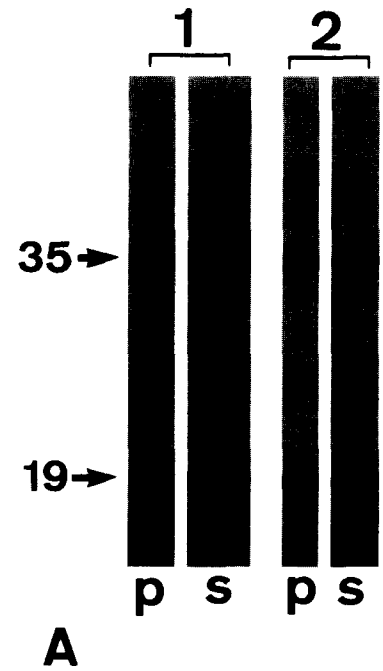
Fig. 3. Cosedimentation of GST-ezrin, purified GST-C- and GST-N-terminal domains of ezrin with large liposomes containing 20% PIP_2 and 80% PC, as compared with ezrin. GST-ezrin (GST-E), GST-N-terminal domain (GST-N) and GST-C-terminal domain (GST-C) (0.15 mg protein/ml) were incubated in the absence or presence of 130 mM KCl, and in the presence of large liposomes containing 20% PIP_2 and 80% PC (0.5 mg lipid/ml). Incubation with lipids, centrifugation and analysis of pellets and supernatants was carried out as described in the legend of Fig. 1. Data for ezrin from Fig. 2 are included in the Figure for comparison (mean ± S.D. of 3–6 experiments).

A control protein, bovine serum albumin (BSA), did not cosediment significantly with PS- or PIP_2-containing liposomes. 5% of total BSA sedimented in the presence of PS-liposomes compared to 2% in its absence. Moreover, preincubation of ezrin with BSA (0.15 mg/ml of each protein) did not affect cosedimentation of ezrin with liposomes containing 20% PIP_2. 46% of ezrin pelleted in the absence of BSA, versus 50% in its presence.

As shown in Fig. 2, ezrin was able to discriminate between PIP_2, PIP and PI, especially at physiological salt concentrations. Under these conditions it interacted most strongly with PIP_2-containing liposomes. Binding to PIP-containing liposomes occurred at low ionic strength, and was decreased by increasing ionic strength (Fig. 2). For the experiments shown in Figs. 1 and 2, purified human recombinant ezrin had been used. GST-ezrin also interacted more strongly with PIP_2, as compared to the other lipids (not shown). The extent of interaction of GST-ezrin with liposomes containing 20% PIP_2 is shown in Fig. 3. Interaction under low salt conditions was comparable for GST-ezrin and ezrin (35 ± 9% and 31 ± 3%, respectively; Fig. 3). In the presence of 130 mM KCl, the extent of interaction of GST-ezrin (17 ± 11%), albeit somewhat lower than that of ezrin (43 ± 8%), was still significantly increased above background ($P < 0.005$). The GST-moiety thus does not appear to modify substantially the lipid-binding properties of ezrin.

3.2. *Interaction of different purified domains of ezrin with PIP_2-containing liposomes*

Interaction of the N- and C-terminal domains with liposomes consisting of 20% PIP_2/80% PC was compared in the same experiment with that of GST-ezrin. As shown in Fig. 3, the



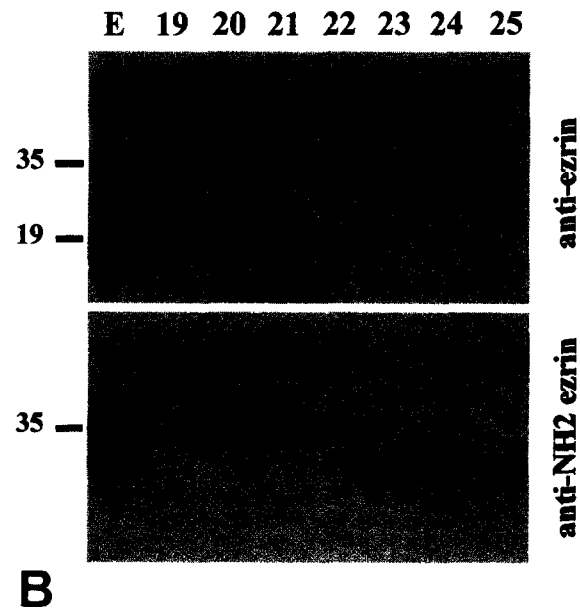


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Fig. 4. (A) Cosedimentation of chymotrypsin digestion products of ezrin with large lipsomes containing 10% PIP_2 and 90% PC. Recombinant ezrin (10 μg) was partially digested with chymotrypsin, incubated and sedimented in the absence (1) or presence (2) of liposomes as described in section 2.4. Pellets (p) and supernatants (s) were analyzed by separation on 5–15% gradient gels and immunoblotting. A blot incubated with a polyclonal antibody against human recombinant ezrin is shown. Numbers on the left indicate molecular masses of two major fragments in kDa. (B) The 34 and 19 kDa chymotrypsin digestion products of ezrin coelute from a Superdex 75 column. Recombinant ezrin was digested as described in section 2.4. Aliquots of the digestion products (lane E) and of fractions 19–25 eluted from the Superdex 75 column were analyzed by gel electrophoresis and immunoblotting. Blots were incubated either with antibodies recognizing both N-terminal and C-terminal domains of ezrin (upper blot), or with an antibody recognizing only the N-terminal domain (lower blot). The indicated fragments of 34 and 19 kDa coeluted as a complex (peak fraction 21). For comparison, note that a degradation product of the N-terminal domain eluted later in fractions 23–24, although its apparent molecular mass is higher than that of the 19 kDa peptide.

C-terminal domain did not interact significantly with PIP_2-liposomes, neither in the presence nor in the absence of added KCl. The N-terminal domain interacted at least as strongly as GST-ezrin with PIP_2-liposomes in the presence of 130 mM KCl, but much less in the absence of added salt, in contrast to the intact protein (Fig. 3).

3.3. Interaction of chymotryptic fragments of ezrin with PIP_2-containing liposomes

Limited chymotryptic digestion of ezrin yielded two major fragments of 35 and 19 kDa. Both bands reacted with the anti-ezrin antibody (Fig. 4A,B), but only the 35 kDa band reacted with the antibody directed against the N-terminal domain of ezrin (Fig. 4B), indicating that the 19 kDa fragment is derived from the C-terminal domain. This result was confirmed by microsequencing of the two fragments, showing that the 35 kDa fragment corresponded to the entire N-terminal domain (1–291), whereas the 19 kDa fragment corresponded to the last part of the C-terminal domain (484–585). Both domains did not significantly pellet in the absence of lipid (Fig. 4A,1), but approximately 60% of both the 35 kDa and the 19 kDa fragment cosedimented with liposomes containing 10% PIP_2/90% PC (Fig. 4A,2). These experiments were repeated with liposomes containing 20% PIP_2/80% PC and evaluated by scanning of the Coomassie Blue-stained gels. In the absence of added KCl, 58–59% of the 35 kDa band and 48–58% of the 19 kDa fragment cosedimented with the liposomes. In the presence of 130 mM KCl, cosedimentation of the 35 kDa fragment was reduced to 32–47%, and that of the 19 kDa fragment to 18–20% ($n = 2$).

Gel filtration chromatography of a mixture of the chymotryptic fragments in the absence of lipid using a Pharmacia Superdex 75 column showed, that the 35 and the 19 kDa fragments coelute (Fig. 4B, fraction 21). On this column, ovalbumin (45 kDa) elutes well before ribonuclease (13.5 kDa) suggesting that the two ezrin fragments derived from the N- and the C-terminus form a complex.

4. Discussion

We have demonstrated an extensive interaction of purified human recombinant ezrin with bilayers containing acidic phospholipids, and a selectivity for PIP_2 revealed at physiological ionic strength. Interaction of ezrin with PIP_2, in contrast to that with other related lipids such as PIP, PI, or to PS, was not sensitive to increasing ionic strength to 130 mM KCl, and occurred significantly at relatively low (5–20%) levels of PIP_2 in the bilayer and at low (μM) concentrations of ezrin (Figs. 1 and 2). 20% of PIP_2 in mixed liposomes are required to functionally regulate gelsolin, a well-known PIP_2-binding protein [17]. Incorporation of PIP_2 into liposomes containing other lipids decreases, but does not abolish, the actin-severing activity of gelsolin, compared to the effect of pure PIP_2 micelles. It has been proposed, that gelsolin and also profilin bind to clusters of ca. five molecules on the bilayer, explaining why incorporation of PIP_2 into bilayers decreases interaction [17,18]. The total concentration in our experiments of PIP_2 was, for liposomes containing 20% of this lipid, ca 100 μM. This corresponds to about 50 μM exposed on the outer surface of monolamellar liposomes, and certainly less than 50 μM in multilamellar liposomes. In platelets, the concentration of PIP_2 may be as high as 140–240 μM [18]. The interaction of ezrin with PIP_2 occurs thus under physiological ionic strength and concentrations of PIP_2. Moreover, not the whole protein, but a specific domain, the N-terminal GST-construct of ezrin interacts selectively with the PIP_2-containing liposomes at physiological ionic strength (Fig. 3), another indication for a relevant interaction. Both the C-terminal GST-construct of ezrin, and a control protein, BSA, did not significantly interact with PIP_2-containing liposomes. Interestingly, in mixtures of chymotryptic fragments of ezrin, a 19 kDa fragment derived from the C-terminus of ezrin also cosediments with PIP_2-containing liposomes (Fig. 4A), in contrast to the data obtained with the purified C-terminal GST-construct of ezrin. This may be explained by the recently described self-associating domains in the N- and C-terminal regions of ezrin; N-ERMAD and C-ERMAD [9]. These domains have been mapped to amino acids 1–296 and 479–585, respectively [9]. The chymotryptic fragment of 35 kDa (1–291) in our experiments thus contains the N-ERMAD, and the 19 kDa fragment (484–585) the C-ERMAD. We also could demonstrate interaction of the two fragments by FPLC (Fig. 4B). Our results shown in Fig. 4A may therefore be explained by the N-ERMAD indirectly linking the C-ERMAD to the liposomes. The interaction of the two domains appears not to be disturbed by liposome interaction of the N-terminal domain of ezrin.

The ability of ezrin to interact directly with phospholipid bilayers is comparable to that of the related proteins talin and protein 4.1, which have also been demonstrated to interact in vitro via their N-terminal domain with acidic phospholipid bilayers [11,19]. Moreover, the interaction of protein 4.1 with the transmembrane protein glycophorin is enhanced by PIP_2 [20]. It will be interesting to see whether closely related amino acid sequences mediate lipid-interaction for all three proteins, and whether the interaction of ezrin with other proteins (e.g. actin and/or CD44) is modulated by PIP_2, as described for different proteins regulating actin filament length or membrane attachment such as protein 4.1, gelsolin, profilin, α-actinin [21]. Direct interaction with phospholipids has been demonstrated for a number of actin-associated proteins [22] and may be relevant also in intact cells [23].

In conclusion, ezrin contains in its N-terminal domain a phospholipid binding site which interacts in vitro with high affinity and under physiological conditions with bilayers containing PIP_2, a molecule involved in signalling processes. This binding site may be involved in recruiting ezrin and the N-terminal domain to plasma membranes in intact cells. Interaction of the N-terminal part of ezrin with liposomes does not disturb N-ERMAD/C-ERMAD interaction.

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