Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin

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Introduction

Ezrin, a membrane–actin cytoskeleton linker, which participates in epithelial cell morphogenesis, is held inactive in the cytoplasm through an intramolecular interaction. Phosphatidylinositol 4,5-bisphosphate (PIP2) binding and the phosphorylation of threonine 567 (T567) are involved in the activation process that unmask both membrane and actin binding sites. Here, we demonstrate that ezrin binding to PIP2 through its NH2-terminal domain, is required for T567 phosphorylation and thus for the conformational activation of ezrin in vivo. Furthermore, we found that the T567D mutation mimicking T567 phosphorylation bypasses the need for PIP2 binding for unmasking both membrane and actin binding sites. However, PIP2 binding and T567 phosphorylation are both necessary for the correct apical localization of ezrin and for its role in epithelial cell morphogenesis. These results establish that PIP2 binding and T567 phosphorylation act sequentially to allow ezrin to exert its cellular functions.

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Abbreviations used in this paper: C-ERMAD, COOH-terminal ERM association domain; ERM, ezrin/radixin/moesin; N-ERMAD, NH2-terminal ERMAD; PH, pleckstrin homology; PIP2, phosphatidylinositol 4,5-bisphosphate; VSV G, vesicular stomatitis virus glycoprotein; wt, wild-type.
proteins. However, although this phosphorylation is required, it is not sufficient in vitro for the association of ERM proteins with F-actin indicating that this phosphorylation event is only one step of the activation process (Nakamura et al., 1999). The binding to PIP$_2$ has also been proposed to play an essential role in the conformational activation of ERM proteins (Nakamura et al., 1999; Yonemura et al., 2002). A PIP$_2$ binding mutant of ezrin is not recruited to the plasma membrane, suggesting that PIP$_2$ binding is essential for the membrane localization of ERM proteins (Barret et al., 2000). Moreover, in vitro, PIP$_2$ regulates the binding of ERM proteins to the cytoplasmic tail of several transmembrane proteins (Hirao et al., 1996; Heiska et al., 1998) and together with the C-ERMAD threonine phosphorylation, the binding to F-actin (Nakamura et al., 1999). The crystal structure of the N-ERMAD of radixin complexed with the polar headgroup of PIP$_2$ shows a slight change of conformation in contrast to N-ERMAD alone (Hamada et al., 2000). These observations indicate that the binding to PIP$_2$ is an additional step required in the conformational activation of ERM proteins.

To analyze the synergy between these two events in the conformational activation of ezrin in vivo, we made use of the mutations abolishing PIP$_2$ binding alone or in combination with the T567D mutation. We demonstrate that PIP$_2$ binding is the primary requirement in the conformational activation of ezrin followed by the threonine phosphorylation. Moreover, we show that this sequence of events is necessary for the apical targeting of ezrin and for the morphogenesis of epithelial cells.

Results and discussion

**PIP$_2$-ezrin is maintained in an inactive conformation in the cytoplasm**

To explore the role of PIP$_2$ in the conformational activation of ezrin, we used the LLC-PK1 epithelial cell line, which is
derived from the kidney proximal tubule and develops microvilli at the apical surface. We derived stable clones producing an ezrin variant in which the binding to PIP$_2$ is abolished (PIP$_2^-$ ezrin; Barret et al., 2000). This ezrin mutant has two lysine doublets mutated to asparagine (K253/254N and K262/263N). Wild-type (wt) ezrin was mainly localized at the apical microvilli, but also observed as a weak diffuse staining corresponding to the cytoplasmic pool and as a faint signal at cell–cell contacts. In contrast, the PIP$_2^-$ ezrin was completely cytoplasmic and absent from microvilli (Fig. 1 A). To assess the ability of PIP$_2^-$ ezrin to associate with the actin cytoskeleton we performed, before immunofluorescence, an extraction with a Triton X-100 buffer preserving the cytoskeleton and cytoskeleton-associated proteins. No staining could be detected in cells expressing PIP$_2^-$ ezrin in contrast to wt ezrin, which remained associated with the microvillus actin cytoskeleton (Fig. 1 A). Quantification after Western blot analysis of soluble and insoluble fractions confirmed that PIP$_2^-$ ezrin was threefold less insoluble than wt ezrin (Fig. 1 B). This indicates that PIP$_2^-$ ezrin cannot bind to the actin cytoskeleton. Therefore, we quantified the amount of ezrin in the membrane and cytosolic pools after cell fractionation. We found a threefold reduction in the amount of PIP$_2^-$ ezrin in the membrane fraction in comparison with wt ezrin showing that PIP$_2^-$ ezrin can no longer be recruited at the membrane (Fig. 1 C). To confirm the role of PIP$_2$ in the recruitment of ezrin to the membrane, we treated cells with ionomycin in presence of Ca$^{2+}$. This pharmacological treatment leads to the hydrolysis of PIP$_2$ (Várnavi and Balla, 1998). After 30 min, wt ezrin was released from the membrane and resided in the cytoplasm where upon it became fully extractable with Triton X-100 buffer (Fig. 1 D). Thus, in these treated cells, wt ezrin behaved similarly to PIP$_2^-$ ezrin. Because the cytoplasmic localization of PIP$_2^-$ ezrin is not due to a folding defect, confirmed by both tryptophan fluorescence spectrum analysis and chymotrypsin digestion (unpublished data), we conclude that the cytoplasmic localization of PIP$_2^-$ ezrin is due to its inability to bind PIP$_2$.

The accumulation of PIP$_2^-$ ezrin in the cytoplasm suggests either that PIP$_2$ is the major membrane binding partner of ezrin or alternatively that PIP$_2$ is required for its conformational activation unmasking other NH$_2$-terminal membrane binding sites. To discriminate between these two possibilities we derived stable LLC-PK1 clones expressing the NH$_2$-terminal domain either in its wt form (Nter ezrin) or carrying the PIP$_2^-$ mutation (NterPIP$_2^-$ ezrin). We showed that both Nter ezrin and NterPIP$_2^-$ ezrin were localized at the membrane in an unpolarized manner and were not found in the cytoplasm (Fig. 2 A). Consistent with these fluorescence data, analysis of membrane and cytosolic pools indicated that both Nter ezrin and NterPIP$_2^-$ ezrin were almost exclusively in the membrane fraction (Fig. 2 B). This result highlights the importance of the N-ERMAD–C-ERMAD interaction in the regulation of the recruitment of ezrin at the membrane because, in comparison, only 10% of full-length ezrin is present at the membrane (Fig. 2 C). Most importantly, it demonstrates that PIP$_2$ is not the major membrane partner that dictates the membrane association of ezrin but is the primary requirement in the conformational activation of the full-length molecule unmasking other membrane binding sites. Altogether, these results indicate that PIP$_2^-$ ezrin is cytoplasmic because it is maintained in an inactive conformation.
Ezrin phosphorylation at T567 requires the interaction of the N-ERMAD with PIP<sub>2</sub>

Because the T567 phosphorylation of ezrin has been shown to play an important role in its conformational activation, we examined the level of T567 phosphorylation of PIP<sub>2</sub><sup>−/−</sup> ezrin using a T567 phosphospecific antibody. Western blots were performed after gel electrophoresis of immunoprecipitated proteins. Although a signal was detected with wt ezrin, which corresponded to the phosphorylated protein because it was abolished after phosphatase treatment, no signal was observed with PIP<sub>2</sub><sup>−/−</sup> ezrin (Fig. 3 A). This result indicates that the binding of ezrin to PIP<sub>2</sub> is required for and precedes T567 phosphorylation in the mechanism of ezrin activation. The interaction of ezrin with PIP<sub>2</sub> could target the protein at the membrane at the vicinity of the kinase. To test this hypothesis, we fused wt and PIP<sub>2</sub><sup>−/−</sup> ezrin to the pleckstrin homology (PH) domain of the phospholipase C-81 (wtPH and PIP<sub>2</sub><sup>−/−</sup> PH ezrin), which interacts with PIP<sub>2</sub> at the plasma membrane, and we analyzed the subcellular distribution and the phosphorylation of these chimeras after transient transfection of LLC-PK1 cells. Both wtPH and PIP<sub>2</sub><sup>−/−</sup> PH ezrin were localized at the plasma membrane and in the microvilli (Fig. 3 B). After cell fractionation, both chimeras were found almost exclusively in the membrane fraction (Fig. 3 C). Although these two chimeras had the same localization in the cells, only wtPH ezrin was phosphorylated at T567 (Fig. 3 D). Altogether, these results show that the targeting of ezrin to the membrane is not sufficient for its phosphorylation at T567. Rather, our results indicate that the interaction of the ezrin N-ERMAD with PIP<sub>2</sub> is necessary for the subsequent phosphorylation of T567. This suggests that the interaction of the N-ERMAD with PIP<sub>2</sub> induces a conformational rearrangement that allows the phosphorylation at T567. Such a conformational change has been observed upon analysis of the crystal structure of the N-ERMAD complexed to the polar headgroup of PIP<sub>2</sub> (Hamada et al., 2000).

The T567D mutation restores the membrane–cytoskeleton linker capacity of PIP<sub>2</sub><sup>−/−</sup> ezrin

Because the binding of ezrin to PIP<sub>2</sub> is required for its subsequent phosphorylation, we determined whether the T567D mutation in the PIP<sub>2</sub><sup>−/−</sup> ezrin mutant (PIP<sub>2</sub><sup>−/−</sup> T/D ezrin)
would be sufficient to restore the apical membrane localization and the binding to the actin cytoskeleton. Whereas PIP2− ezrin was found restricted to the cytoplasm (Fig. 1 A), PIP2− T/D ezrin was also recruited to the plasma membrane, similar to ezrin carrying the T567D mutation alone (T/D ezrin; Fig. 4 A). This suggests that the T567D mutation allows the unmasking of the PIP2-independent membrane binding sites (Fig. 1 C). In addition, both T/D ezrin and PIP2− T/D ezrin remained associated with the cortical actin cytoskeleton after extraction of cells with the Triton X-100 buffer indicating that T567D mutation is sufficient to unmask the actin binding site independently of PIP2 binding (Fig. 1 B and Fig. 4 A). Moreover, ionomycin treatment had no effect on either the membrane localization or the association with the actin cytoskeleton of both T/D and PIP2− T/D ezrin (Fig. 4 B). Altogether, our results show that the T567D mutation restores the capacity of PIP2− ezrin to act as a membrane–actin cytoskeleton linker and demonstrates that binding of ezrin to PIP2 is dispensable for the unmasking of functional binding sites in vivo, in contrast to what was described in vitro (Nakamura et al., 1999).

However, neither PIP2− T/D ezrin, nor T/D ezrin had a restricted apical localization (Fig. 4 A). The factors controlling the apical localization of ezrin are still unknown. But our results indicate that the release of the N-ERMAD–C-ERMAD interaction leading to the unmasking of PIP2 independent membrane binding sites is not sufficient, in itself, for the apical localization of ezrin. This indicates that regulated unmasking of the membrane binding sites in the NH2-terminal domain is required for this apical localization consistent with our observations using the Nter mutants (Fig. 2 A). The sequence of events consisting of binding to PIP2 followed by phosphorylation at T567 is critical for the restricted apical localization of wt ezrin.

Unlike T/D ezrin, PIP2− T/D ezrin does not alter epithelial cell morphology

We have shown previously that production of T/D ezrin in epithelial cells induced the formation of lamellipodia, membrane ruffles, tufts of microvilli, and impaired cell–cell contacts indicating a critical role for ezrin in epithelial cell morphogenesis (Gautreau et al., 2000; Pujuguet et al., 2003). To test whether ezrin binding to PIP2 is necessary for its role in epithelial cell morphogenesis we analyzed the surface of cells expressing PIP2− and PIP2− T/D ezrin by scanning electron microscopy (Fig. 5). In contrast to cells producing T/D ezrin, we observed that cells producing PIP2− T/D ezrin formed a regular monolayer with surface microvilli resembling those of cells overexpressing wt ezrin (Fig. 5). This suggests that despite the ability of PIP2− T/D ezrin to act as a linker between the membrane and the actin cytoskeleton, its expression does not affect epithelial cell morphology as seen with T/D ezrin. This suggests that the conformational activation unmasking functional binding sites alone is not sufficient to allow ezrin to exert its signaling functions. Therefore, correct regulation of ezrin activation is dependent on the binding of ezrin to PIP2.

We observed that although cells expressing PIP2− ezrin formed a well-organized monolayer, they displayed, on their apical surface, fewer and shorter microvilli compared with the abundant and regular microvilli seen on cells expressing wt ezrin. Thus, production of PIP2− ezrin had a strong dominant negative effect on microvillus formation. We did not detect any effect of PIP2− ezrin on the amount and localization of the phosphorylated endogenous ERM proteins (unpublished data). This indicates that the absence of microvilli in cells producing PIP2− ezrin is not due to a dominant negative effect on the activation of endogenous ERM proteins, but rather is due to an effect on a downstream effector of ezrin involved in microvillus formation.

Recently, genetic analysis of Drosophila moesin, the only ERM protein in this organism, has stressed the role of this protein in the control of epithelial cell integrity and polarity (Polesello et al., 2002; Speck et al., 2003). Our experiments have demonstrated that to exert its cellular functions at the apical pole of epithelial cells, ezrin undergoes a conformational activation, which requires primarily PIP2 binding and the subsequent phosphorylation at T567. Future works using these well-characterized mutants of ezrin should further the understanding of how the regulated conformational activation of ezrin controls its signaling functions in the development and maintenance of epithelial cell polarity.
Materials and methods

Cell culture
LLC-PK1 cells (ICL 101; American Type Culture Collection) were grown in DME (GIBCO BRL) and supplemented with 10% FCS, at 37°C in 10% CO₂.

Antibodies
The mouse monoclonal anti-vesicular stomatitis virus glycoprotein (VSV G) antibody (clone P5D4) was described previously (Kreis, 1986). T567 phospho-ezrin pAb was purchased from Cell Signaling Technology.

DNA constructs and stable transfection
The pCB6 vectors containing cDNA coding for VSV G-tagged ezrin carrying the K253N, K254N, K262N, and K263N mutations (PIP2 ezrin) or the T567D mutation (T/D ezrin) were described previously (Barnet et al., 2000; Gautreau et al., 2006). The pCB6 vector containing cDNA coding for VSV G-tagged Nter ezrin (1-309) was described previously (Algrain et al., 1993). The pCB6 vectors containing cDNA coding for wTPH and PIP2 PH ezrin were obtained by an in-frame insertion of a fragment coding for the PH domain of rat phospholipase C-8I (10-139) at the 3’ end of the VSV G–ezrin cDNA. All constructs were obtained by standard techniques and verified by sequencing. Stable LLC-PK1 clones were obtained as described previously (Gautreau et al., 2000). All experiments were performed with three independent clones for each construct and gave similar results.

Immunofluorescence
10² cells were seeded on 1-cm² polyester filters (Transwell; Costar Corp.) and grown for 4 d. Cells were fixed with 3% PFA and permeabilized with 0.5% Triton X-100. Cells were subsequently incubated with anti–VSV G antibody and then with Alexa 488-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories) and with TRITC-labeled phalloidin (Sigma Chemical Co.). The samples were analyzed with an SP2 confocal laser scanning microscope (Leica). When indicated, living cells were incubated with anti–VSV G antibody (Jackson ImmunoResearch Laboratories) and with TRITC-labeled phalloidin (Sigma Chemical Co.). The samples were analyzed with an SP2 confocal laser scanning microscope (Leica). When indicated, living cells were incubated for 30 min with 10 mM CaCl₂ and 30 M ionomycin (Sigma Chemical Co.) in PBS. Extraction was performed before fixation by treating the cells for 1 min with a Triton X-100 buffer (50 mM MES, 3 mM KCl, 150 mM NaCl, pH 7.4) containing a cocktail of protease inhibitors. The homogenates were clarified by centrifugation at 100,000 g.

Scanning electron microscopy
5 × 10⁶ cells were seeded on 4.7 cm² polycarbonate filters and grown for 4 d. Samples were fixed with 2.5% glutaraldehyde, dehydrated in a graded series of ethanol incubation, dried by the critical point method using liquid CO₂, coated with gold palladium, and observed with a microscope (model JSM 840A; JEOL).

Immunoprecipitation
Immunoprecipitations were performed as described previously (Gautreau et al., 2000). When indicated, immunoprecipitated proteins were treated with λ-phosphatase (New England Biolabs, Inc.) according to the manufacturer’s instructions.

Analysis of detergent-soluble and -insoluble fractions
Cellular fractions were obtained from 6-well plates confluent cultures. Total cellular fractions were collected with Laemmli buffer at 100°C. Soluble fractions were prepared by a 1-min extraction with Triton X-100 buffer at 20°C and supplemented with 3 × Laemmli buffer. The insoluble fractions were extracted with Laemmli buffer at 100°C. Samples were analyzed by SDS-PAGE and immunoblotting. Densitometric analysis were performed with the Scion image program (NIH image).

Cytosol/membrane fractionation
Confluent cultures from 10-cm dishes were mechanically disrupted using a cell cracker in 10 mM Hepes, 1 mM EDTA, 150 mM NaCl, pH 7.4, buffer containing a cocktail of protease inhibitors. The homogenates were clarified by centrifugation at 600 g. An aliquot of the supernatant was supplemented with 3 × Laemmli buffer and corresponds to the total fraction. The supernatant was subjected to a 30-min centrifugation at 100,000 g. The resulting supernatant was supplemented with 3 × Laemmli buffer (cytosolic fraction) and the membrane pellets were solubilized in Laemmli buffer (membrane fraction). Samples were analyzed by SDS-PAGE and immunoblotting.

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References
Algrain, M., O. Turunen, A. Valeri, D. Louvard, and M. Arpin. 1993. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane–cytoskeleton linker. J. Cell Biol. 120:129–139.
Barret, C., C. Roy, P. Montcourrier, P. Mangeat, and V. Niggli. 2000. Mutagenesis of the phosphatidylinositol 4,5-bisphosphate (PIP2) binding site in the NH2-terminal domain of ezrin correlates with its altered cellular distribution. J. Cell Biol. 151:1067–1079.
Bretschker, A., K. Edwards, and R.G. Fehon. 2002. ERM proteins and merlin: integrators at the cell cortex. Nat. Rev. Mol. Cell Biol. 3:586–599.
Gary, R., and A. Bretscher. 1995. Ezrin self-association involves binding of an N-terminal domain to a normally masked G-terminal domain that includes the F-actin binding site. Mol. Biol. Cell. 6:1061–1075.
Gautreau, A., D. Louvard, and M. Arpin. 2000. Morphogenic effects of ezrin require a phosphorylation-induced transition from oligomers to monomers at the plasma membrane. J. Cell Biol. 150:193–203.
Gautreau, A., D. Louvard, and M. Arpin. 2002. ERM proteins and NF2 tumor suppressor: the Yin and Yang of cortical actin organization and cell growth signaling. Curr. Opin. Cell Biol. 14:104–109.
Hamada, K., T. Shimizu, T. Matsui, S. Tsukita, S. Tsukita, and T. Hakoshima. 2000. Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. EMBO J. 19:4449–4462.
Hayashi, K., S. Yonemura, T. Matsui, and S. Tsukita. 1999. Immunofluorescence detection of ezrin/radixin/moesin (ERM) proteins with their carboxy-terminal threonine phosphorylated in cultured cells and tissues. J. Cell Sci. 112:1149–1158.
Heiska, L., K. Allfinn, M. Groinholm, P. Vilja, A. Valeri, and O. Carpen. 1998. Association of ezrin with intercellular adhesion molecule-1 and –2 (ICAM-1 and ICAM-2). J. Biol. Chem. 273:21893–21900.
Hirao, M., N. Sato, T. Kondo, S. Yonemura, M. Monden, T. Sasaki, Y. Takai, S. Tsukita, and S. Tsukita. 1996. Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and rho-dependent signaling pathway. J. Cell Biol. 135:37–51.
Kreis, T.E. 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. EMBO J. 5:931–941.
Magendanz, M., M.D. Henry, A. Lander, and F. Solomon. 1995. Interdomain interactions of radixin in vitro. J. Biol. Chem. 270:25324–25327.
Matsui, T., M. Maeda, Y. Doi, S. Yonemura, M. Amano, K. Kainibuchi, S. Tsukita, and S. Tsukita. 1998. Rho kinase phosphorylates COOH-terminal threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J. Cell Biol. 140:647–657.
Nakamura, F., M.R. Amieva, C. Hirota, Y. Mizuno, and H. Furthmayr. 1996. Phosphorylation of T-558 of moesin detected by site-specific antibodies in RAW264.7 macrophages. Biochem. Biophys. Res. Commun. 226:650–656.
Nakamura, F., L. Huang, K. Pestaonjampas, E.J. Luna, and H. Furthmayr. 1999. Regulation of F-actin binding to platelet moesin in vitro by both phosphorylation of threonine 558 and polyphosphoinositides. Mol. Biol. Cell. 10:2669–2685.
Niggli, V., C. Andréoli, C. Roy, and P. Mangeat. 1995. Identification of a phosphatidylinositol-4,5-bisphosphate-binding domain in the N-terminal region of ezrin. FEBS Lett. 376:172–176.
Oshiro, N., Y. Fukata, and K. Kaibuchi. 1998. Phosphorylation of moesin by Rho-associated kinase (Rho-kinase) plays a crucial role in the formation of microvilli-like structures. J. Biol. Chem. 273:34663–34666.
Pearson, M.A., D. Reczek, A. Bretscher, and P.A. Karplus. 2000. Structural basis of the membrane-targeting and unmasking mechanisms of the PH domain of PH-domain of rat phospholipase C-ζ. J. Biol. Chem. 275:34663–34666.
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Pujuguet, P., L. Del Maestro, A. Gautreau, D. Louvard, and M. Arpin. 2003.
Ezrin regulates E-cadherin-dependent adherens junction assembly through Rac1 activation. *Mol. Biol. Cell.* 14:2181–2191.

Simons, P.C., S.F. Pietromonaco, D. Reczek, A. Bretscher, and L. Elias. 1998. C-terminal threonine phosphorylation activates ERM proteins to link the cell’s cortical lipid bilayer to the cytoskeleton. *Biochem. Biophys. Res. Commun.* 253:561–565.

Speck, O., S.C. Hughes, N.K. Noren, R.M. Kulikauskas, and R.G. Fehon. 2003. Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. *Nature.* 421:83–87.

Turunen, O., T. Wahlström, and A. Vaheri. 1994. Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin protein family. *J. Cell Biol.* 126:1445–1453.

Várnai, P., and T. Balla. 1998. Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to Myo-[3H]inositol-labeled phosphoinositide pools. *J. Cell Biol.* 143:501–510.

Yonemura, S., S. Tsukita, and S. Tsukita. 1999. Direct involvement of ezrin/radixin/moesin (ERM)-binding membrane proteins in the organization of microvilli in collaboration with activated proteins. *J. Cell Biol.* 145:1497–1509.

Yonemura, S., T. Matsui, S. Tsukita, and S. Tsukita. 2002. Rho-dependent and -independent activation mechanisms of ezrin/radixin/moesin proteins: an essential role for polyphosphoinositides in vivo. *J. Cell Sci.* 115:2569–2580.
