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Review

Model membranes to shed light on the biochemical and physical properties of ezrin/radixin/moesin

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ABSTRACT

Ezrin, radixin and moesin (ERM) proteins are more and more recognized to play a key role in a large number of important physiological processes such as morphogenesis, cancer metastasis and virus infection. Recent reviews extensively discuss their biological functions [1–4]. In this review, we will first remind the main features of this family of proteins, which are known as linkers and regulators of plasma membrane/cytoskeleton linkage. We will then briefly review their implication in pathological processes such as cancer and viral infection. In a second part, we will focus on biochemical and biophysical approaches to study ERM interaction with lipid membranes and conformational change in well-defined environments. *In vitro* studies using biomimetic lipid membranes, especially large unilamellar vesicles (LUVs), giant unilamellar vesicles (GUVs) and supported lipid bilayers (SLBs) and recombinant proteins help to understand the molecular mechanism of conformational activation of ERM proteins. These tools are aimed to decorticate the different steps of the interaction, to simplify the experiments performed *in vivo* in much more complex biological environments.

1. Biological importance of ezrin, radixin and moesin

1.1. General overview of ERM proteins (ezrin, radixin, moesin)

ERM proteins are localized at the plasma membrane in actin-rich surface structures (Fig. 1) [5], such as microvilli, membrane ruffles, and lamellipodia in gut cells, lymphocytes, hepatocytes, spermatozoids, fibroblasts and in a variety of other cell types [5–9]. They are involved in various physiological processes including establishment of cell polarity, cell motility and cell signaling [10]. They act as linkers between the plasma membrane and the cortical actin cytoskeleton. From a structural point of view, ERMs are constituted of 3 domains: a membrane binding domain, also known as FERM (for band 4.1 protein, ezrin, radixin and moesin), and intermediary region and a actin binding domain called C-ERMAD (for C-terminal ERM-association domain) (Fig. 2A).

The FERM domain, constituted of ~300 amino acids, is characteristic of the proteins of the band 4.1 superfamily. These proteins also present other types of domains, including PDZ, tyrosine phosphatase, SH2-like, tyrosine kinase, kinase-like, myosin head, and PH domains [11]. In ERM proteins, the FERM domain is followed by a long α-helical region, which forms a coiled-coil structure, according to the only structure of full-length moesin available [12] (Fig. 2A). The C-terminal domain, which is constituted of ~80 residues, is known to contain an actin binding site [13](Fig. 2A). ERM function is regulated by head to tail interactions between the FERM domain (Fig. 2B) and the C-terminal (C-ERMAD) domain (Fig. 2C). In the folded conformation, also called closed conformation (or dormant, or inactive), the F-actin binding site is masked. In the unfolded open conformation, also called active, the N-terminus binds the plasma membrane and the C-terminus is accessible for binding actin filaments [14,15].

1.2. Specific roles vs. functional redundancy

ERM proteins play an important role in the organization of the cell cortex [2]. They interact with a large range of proteins, including other cytoplasmic or membrane associated proteins, such as ezrin binding protein 50 (EBP50), also known as Na^+/H^+ exchanger regulating factor 1 (NHERF1) [16] and Na^+/H^+ exchanger type 3 kinase A regulatory protein (E3KARP or NHERF2) [17], as well as transmembrane proteins, among which CD43, CD44 and

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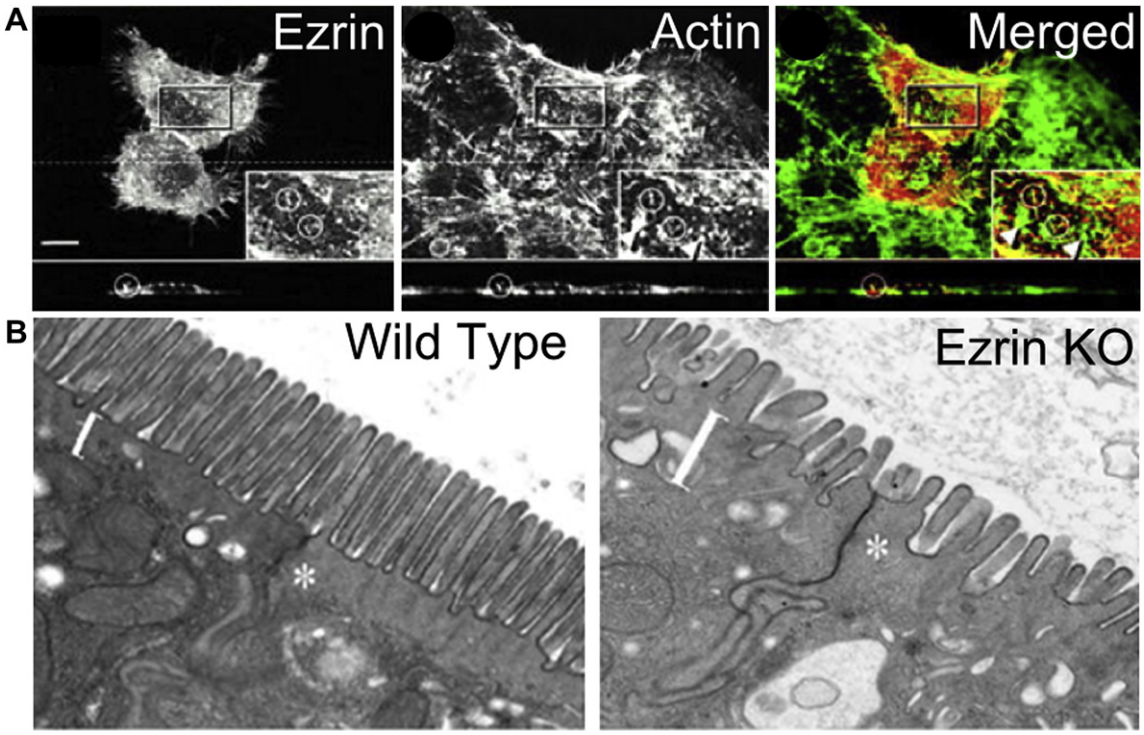

Fig. 1. (A) *In cellulo* localization of ezrin and actin in human adenocarcinoma A431 epitheloid cells. Cells were transfected with either VSV-G-tagged wild-type ezrin and treated for indirect Texas red localization of ezrin with anti-VSV antibody (left) and F-actin with FITC-coupled phalloidin. Colocalization of ezrin with actin filaments in dynamic structures appears in yellow (right) [50] (reproduced with permission from Ref. [50], copyright (2000) Rockefeller University Press 2000). (B) Transmission electron microscopy of intestinal microvilli of WT (left) and ezrin knock-out mouse (right). The wild type intestinal epithelial cells reveal packed and uniform rod-like microvilli across the apical surface whereas the microvilli of knock-out mouse cells show not well oriented, thick and non-uniform [20] (reproduced with permission from Ref. [20], copyright (2004) Elsevier).

intracellular adhesion molecule 2 (ICAM-2) [18]. All these interactions contribute to the formation of functional protein complexes to stabilize the cytoskeleton–plasma membrane linkage. Furthermore, they interact with phospholipids. In vertebrates, the three ERM proteins show tissue specific expression profiles [4]. Ezrin, first isolated in gastric parietal cells, is present mostly in epithelial cells, while moesin is mostly found in endothelial cells. Radixin is rather found in hepatocytes [2]. Bretscher *et al.* first discovered ezrin in 1983 [19] and found that it was present in very large amounts in membrane protrusions, such as intestinal microvilli. However, no clear role for its involvement in the formation of the latter appeared. To investigate more precisely the involvement of ERM in the morphogenesis of epithelial tissues, several studies using mutant mice have emerged. In mutant mice lacking ezrin, intestinal epithelium cells showed malformations: instead of having many microvilli oriented and with defined size, they were thicker, shorter and not well oriented (Fig. 1B). Consequently, the newly born mutant mice did not survive more than a few days as their intestines were unable to incorporate the essential nutrients [20]. Similarly, other reports using the same approach showed that the absence of radixin was not lethal. However, mice lacking radixin showed liver damage after 8 weeks [21]. In addition, radixin appeared to be important for the implementation of the architecture of cochlear stereocilia, but its absence was offset by other ERMs, including ezrin [22]. It seems the lack of radixin was compensated by other ERM. ERM protein functions are redundant and the redundancy was also confirmed by another study [23], which showed that mutant mice deficient in moesin exhibited no functional or structural abnormality of the tissues suggesting that the other ERM proteins take over functions of moesin. Despite their redundancy, ezrin and moesin are differentially distributed in the early steps of melanoma tumor cell invasion [24]. The different distributions of ezrin and moesin were also reported during T cell activation [25,26]. Schaffer *et al.*, reported that ezrin, but not moesin, is transiently present at the immune synapse, before movement to the distal pole complex [26]. However no specific requirements for ezrin vs. moesin in the activation process were evidenced. Ilani *et al.* showed that, upon activation of T-lymphocytes, cell shape changed and a loss of villi and actin polymerization at the interface between the lymphocyte and an antigen-presenting cell was observed. Moesin was absent from the immune synapse, while ezrin was present in this area. In this case, moesin interacted with CD43 whereas ezrin interacted with the signaling kinase ZAP-70. This study pointed out for the first time a non-redundancy in the function of ERM [25]. Recently, Haynes *et al.* [27], showed that increased moesin expression, but not ezrin or radixin, was necessary during epithelial–mesenchymal transition for efficient actin filament remodeling, and for cortical relocalization of adhesion and contractile elements. This relocalization included CD44, α-smooth muscle actinin, and phosphorylated myosin light chain [27].

1.3. ERMs in pathological processes

1.3.1. Cancer

Ezrin was first identified as a crucial molecule in the dissemination of two pediatric tumors, rhabdomyosarcoma [28] and osteosarcoma [29]. Clinical studies have shown that ezrin overexpression also correlated with adult tumor metastasis. Ezrin overexpression increased migration of metastatic melanoma [30], pancreatic cancer cells [31], or hepatocellular carcinoma [32]. Conversely, ezrin silencing or inhibition contributed to decreased cell motility [33–36]. Ezrin implication in cancer has been related to its interactions with a plethora of molecules related to metastatic functions. We will not cite all of them but a few starting with CD44,

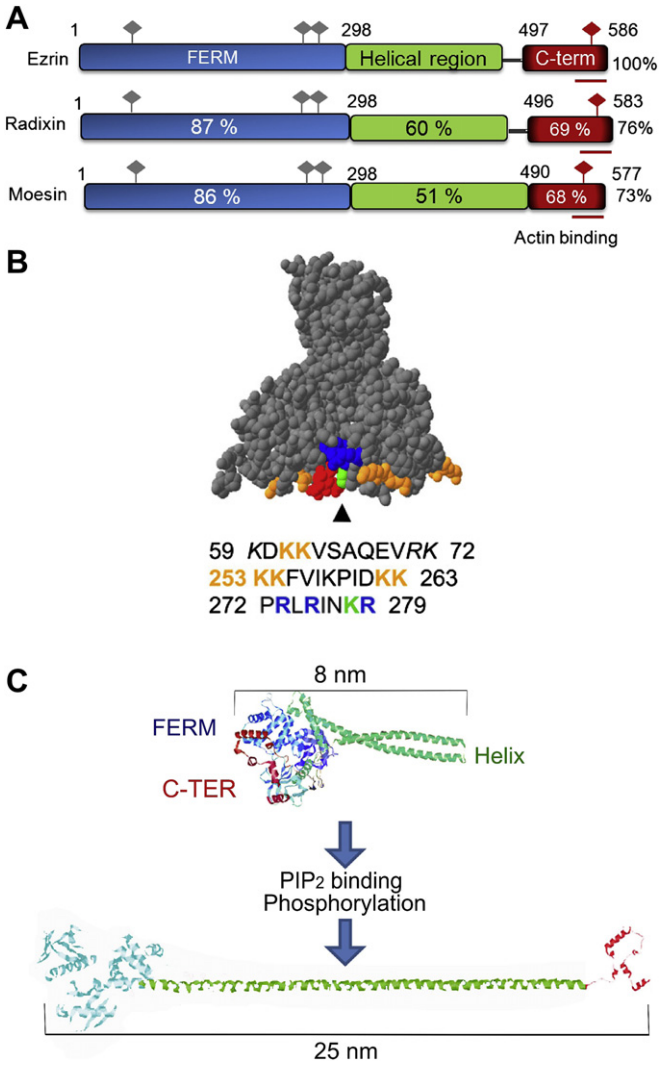

Fig. 2. Structure and activation of ERM. (A) Domain organization and sequence identity between human ezrin, moesin and radixin. The FERM domain appears in blue, the helical region in green and the C-terminal domain in red. Positively charged pairs of residues participating to PIP_2 binding. Major phosphorylation site. The F-actin binding domain is located at the end of the C-terminal domain. (B) Primary sequence of ezrin in the subdomains F1 and F3 of the FERM domain and three-dimensional visualization of the FERM domain (lateral view). Potential PIP_2 and IP_3 binding sites have been localized and are indicated in orange (lysine pairs K63, K64; K253, K254; K262, K263) (Protein Data Bank code, 1GC6, visualization with Rasmol) [50] and the basic residues (K278 in green and R273, R275, R279 in blue) being part of a basic cleft important for the interaction [15] are also indicated. (C) Closed conformation of ERM, as deduced from moesin from *Spodoptera frugiperda* structure (2I1K, visualization with Rasmol) and hypothetical open/activated structure based on the radixin protein [82] after phosphorylation and/or interaction with PIP_2.

a multifunctional trans-membrane receptor implicated in tumor cell invasion and metastasis. CD44 was shown to interact with ezrin during cell migration, cell adhesion, tumor progression and metastasis [37]. Another example is NHERF1, localized at the plasma membrane in normal (physiological) conditions. Depending on its subcellular distribution, NHERF1 may behave either as a tumor suppressor, when localized at the plasma membrane, or as an oncogenic protein, when shifted to the cytoplasm [38]. We can also cite ezrin interactions with podocalyxin, an anti-adhesive glycoprotein that has been associated with highly aggressive forms of cancers and was shown to increase the invasiveness of cancer cells [39]. Ezrin is also reported to interact with podoplanin, a small membrane mucin induced in oral squamous cell carcinomas, which colocalizes with ezrin [40] and induces an epithelial–mesenchymal transition in an ezrin-dependent manner [41]. Finally, we can mention that molecular interactions with ezrin were shown to allow membrane localization of Lamp-1 (also known as CD107a) [30], a heavily glycosylated transmembrane protein, involved in adhesion of cancer cells to the extracellular matrix. This membrane localization correlated with a high metastatic potential.

ERMs contribute to stiffening of the cell cortex during mitosis, thus facilitating the spindle morphogenesis [42]. Up-regulation or down-regulation of ERM leads to defects in chromosome positioning and gave rise to aneuploid cancer cells [43]. Recently, Estecha and co-workers [24] attributed a particular role to moesin in the early steps of melanoma tumor cell invasion of collagen matrices. Moesin was crucial for the flattening of cells and their attachment prior to invasion and migration. These authors hypothesized that ezrin was more present in dynamic structures such as the lamellipodium, while the role of moesin would be to organize more stable cortices.

In contrast to ezrin and moesin, the ERM-related protein merlin (also known as neurofibromatosis type 2 (NF2) tumor suppressor or schwannomin) has a tumor suppressing activity related to its inhibitory function in cell proliferation, by stabilizing cell to cell contacts and suppressing mitogenic signals at the membrane (for in depth review see Refs. [44–46]). It is worth mentioning that a conformational activation has also been proposed for merlin, and that the closed, dephosphorylated form is able to interact with proteins at the membrane thereby inhibiting proliferation. It has been suggested that ERMs and merlin may work in concert to regulate cellular interfaces and that tumor progression may result from deregulation of this equilibrium [45].

1.3.2. Virus entry in cells

The entry of HIV into target cell requires viral envelope interactions with receptors and co-receptor molecules. Polymerization of actin is required to assemble high concentrations of the co-receptors CD4 and CXCR4 at the plasma membrane of the target cell and to promote viral binding and entry. Three types of actin-interacting proteins were shown to play critical roles in this process: filamin, ERM and cofilin [47]. Among ERMs, Barrero-Villar *et al.* [48] found that moesin was phosphorylated after HIV-1 interaction and drove redistribution and clustering of CD4 and of CXCR4, through promotion of F-actin redistribution. Another study [49] showed, using an ezrin dominant negative mutant and knockdowns of ERM, that ERMs were involved in the cell infection by X4-tropic HIV-1 strain, a strain that requires the presence of the CXCR4 receptor for infection [49]. Moreover, this study came up with interesting differences between radixin, ezrin and moesin in the infection by the R5-tropic HIV-1 strain, i.e. a strain that uses only the CCR5 receptor. Whereas ezrin knockdown had no effect, radixin knockdown decreased and moesin knockdown rather increased the infection efficacy [49].

2. Biochemical and biophysical approach to study ERM interactions with plasma membrane and with actin filaments

2.1. Biochemical consideration of ERM activation at the plasma membrane

Experimental evidences have been obtained using mutated recombinant ezrin FERM domain that the lysine pairs (K253/K254, K262/K263 and K63/K64) located in subdomains F1 and F3 of the ezrin FERM domain play an important role in the binding of ezrin to multilamellar vesicles containing PIP_2 [50] (Fig. 2B). In addition to the lysine pairs implicated in the interaction between ERM and

PIP_2, the crystal structure of the radixin FERM domain complexed with IP_3, which is the headgroup of PIP_2, showed the presence of a basic cleft, residues K63, K278, which are located between the subdomains F1 and F3 [15]. All these residues are shown for ezrin in Fig. 2B. In native auto-inhibited moesin, this cleft is a cavity masked by a linker, which is called the "flap" by Ben Aissa *et al.* [51]. The "flap" is described as a functional auto-inhibitory region. Moreover, the same study showed that the pairs of lysine residues (K253/K254, K262/K263), which the authors named the "patch" and the K63, K278 residues, called "pocket", did not bind to PIP_2 simultaneously. The authors proposed a model of progressive conformational release of auto-inhibited moesin, in which binding of PIP_2 to the lysine patch initiates release of the flap region. This, in turn, facilitates subsequent opening via PIP_2 binding to the (K63/K278) pocket [51]. The PIP_2 binding site in the cleft is thus a "cryptic" site, which is accessible only after release of the auto-inhibition.

Similarly to the FERM domain interaction with the plasma membrane via phosphoinositides, the C-terminal domain interacts with actin filaments. The actin binding site was originally mapped to the last 34 residues of the C-terminal domain [13]. Punctual mutation of four residues in this region, T576, K577, R579 and I580, prohibited interaction between the C-term domain of ezrin and actin [52]. In particular, *in vitro*, R579A C-terminal mutant of ezrin did not bind the FERM domain. *In vivo* expression of the ezrin mutant altered the normal cell surface structure dramatically and inhibited cell migration [52]. Due to the high degree of homology of ERM, we can assume that similar mechanisms and similar actin binding properties will take place in the other ERMs. However, to date, the mechanism of ERM activation is not clearly elucidated. Two phenomena have extensively been discussed in literature: membrane binding, namely via interactions with PIP_2 [50], and/or phosphorylation on specific residues by small GTPases [53], but contribution of each event remains unclear. On one hand, several physiological processes involve ERM phosphorylation on conserved threonine residue in the actin binding site (T567 in ezrin, T564 in radixin, T558 in moesin). For example, *in cellulo*, moesin is phosphorylated by Rho kinase during platelet activation [53,54] or by lymphocyte-oriented kinase (LOK) which stimulates lymphocyte migration and polarization [55]. A number of other kinases in vertebrate cells can phosphorylate ERMs on this regulatory threonine, including protein kinase C (PKC) α and θ, Nck-interacting kinase (NIK), human serine/threonine kinase (Mst4) [56–59]. Phosphorylation of ERM proteins resulted in the localization of these proteins to the actin-rich membrane extensions [53,54,60,61]. Conversely, dephosphorylation of ERM proteins by processes such as anoxia [62] or following the early phase of Fas ligand-induced apoptosis [63] lead to the translocation of ERM proteins from the membrane to the cytoplasm. These data indicated that threonine phosphorylation plays an important part in the association of ERM proteins with the membrane and actin cytoskeleton [64].

On the other hand, it was shown [65] that i) ezrin activation process is dependent on binding to PIP_2 at the plasma membrane and that ii) phosphorylation in the C-terminal domain, occurred only in a second step, resulting in a conformational change of the protein and in its subsequent binding to F-actin. Thus, it is not clear whether phosphorylation is an absolute requirement for membrane binding or if this interaction with the plasma membrane is a primary step, which induces structural changes leading to full activation after phosphorylation.

The interactions between proteins and membranes, but also between actin and actin-binding proteins such as ERMs, are difficult to elucidate *in cellulo*. The presence of hundreds of actin and ERM-binding proteins may hide the true contribution of ERM proteins. The molecular mechanisms are complex and the use of biomimetic systems constituted of a limited number of components brings some light to this complex system. In addition to *in cellulo* and *in vivo* studies, *in vitro* experiments using well-defined lipid membranes and purified proteins can provide quantitative information about protein/membrane interactions and conformational changes. In the next part of this review, we will now focus on the most recent *in vitro* studies of ERM proteins and lipid membranes containing phosphoinositides, especially PIP_2.

2.2. Biomimetic membranes to study ERM/plasma membrane interactions and the activation mechanism

Three types of biomimetic membranes are mainly used to study ERM/membrane interactions: large unilamellar vesicles (LUVs) of ~100 nm in diameter, giant unilamellar vesicles (GUVs) of ~5–25 μm in diameter and supported lipid bilayers (SLBs) of ~5 nm in thickness. The size of GUVs is close to that of cells, which render them convenient for microscopic observations and micromanipulation. However, GUVs are fragile in medium containing salt, not highly stable, which renders study on GUVs rather tricky. LUVs are much smaller and their composition is more easily tunable. Furthermore, they can be stored for several days and be used to obtained quantitative information on binding affinity. They are also useful tools to study protein–membrane interactions by various spectroscopic methods. SLBs are obtained by spreading of LUVs onto a substrate to form lipid bilayers. They can also be used for microscopic visualization, including high resolution atomic force microscopy and quantitative binding measurements by means of quartz crystal microbalance. A survey of the literature shows that interactions of proteins with PIP_2 were studied *in vitro* using either PIP_2 inserted into PIP_2–LUVs [50,66–68], PIP_2–GUVs [69], PIP_2–SLBs [70–72], or PIP_2 directly introduced in solution [73] (Fig. 3 and Table 1).

2.3. Specificity of the interaction between ERM and phosphoinosides

The first proof of ERM interaction with PIP_2 was obtained in 1995 by Niggli *et al.*, who showed that ezrin co-sedimented with PIP_2-containing multilamellar vesicles and to a lesser extent with vesicles containing other negatively charged lipids [68]. Hirao *et al.*, observed that the affinity for CD44 increased in the presence of phosphoinositides and more particularly in the presence of PIP_2 [67], which indicated that not only did PIP_2 interact with ERMs, but also induced structural changes in the protein. The binding affinity (K_d) between ezrin and PIP_2 was first quantified by following the adsorption of the protein onto a PIP_2–SLB using quartz microbalance (QCM). In that case, the dissociation constant was found to be ~100 nM [70]. A quantitative measurement of K_d using cosedimentation assays or fluorescence correlation spectroscopy (FCS) gave higher values (about 50 fold lower affinity). In fact, the K_d of ezrin interacting with PIP_2–LUVs was found to be ~5 μM and was 20- to 70-fold lower for phosphatidylserine-containing LUV [66]. As far as moesin is concerned, its affinity for PIP_2–LUVs was also of 5 μM [74]. Ezrin affinity for other phosphoinositides such as PI(4)P and PI(3,4)P_2 was much lower, but a K_d in the μM range was also determined for PIP_3-containing LUVs [66].

Besides slight differences in binding affinities, some differences were also observed with respect to the cooperativity of the interaction. On SLBs, ezrin was shown to form lateral domains when the concentration of PIP_2 was increased [71], whereas no cooperativity was observed in the case of PIP_2–SLBs. Positive cooperativity in the case of SLBs means that a second protein binds to the lipid bilayer with higher affinity if it binds right next to another protein.

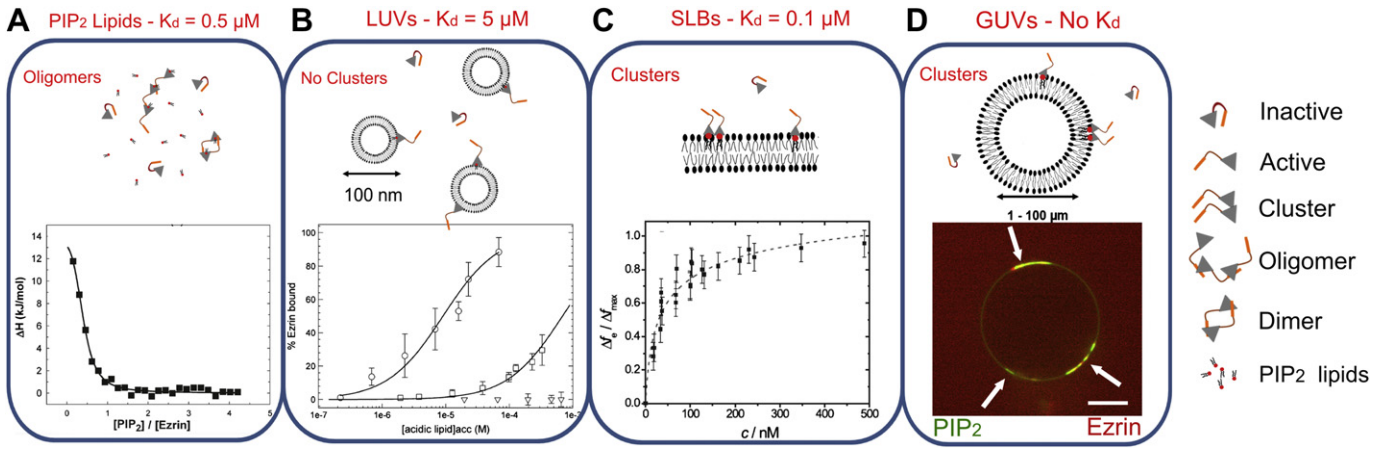

Fig. 3. Four different biomimetic systems used to analyze ERM–lipid interaction. Top: cartoon representing from left to right: PIP_2 dispersed in solution interacting with ezrin; PIP_2–LUVs interacting with ezrin; PIP_2–SLBs interacting with ezrin; PIP_2–GUVs interacting with ezrin. Bottom (from A to D): isothermal titration calorimetry curve: heat observed on each injection versus molar ratio of PIP_2 over ezrin. Data were fitted to a one-binding site model. Symbols are experimental data and lines represent the best fit [73] (reproduced with permission from Ref. [73] copyright (2010) American Chemical Society). (B) Fluorescence correlation spectroscopy measurements of ezrin binding to PC/PIP_2 (open circles), PC/PS (open squares), and PC LUVs (open triangles) (ezrin at 15 nM labeled with Alexa 488). The percentage of ezrin bound is plotted as a function of the accessible PIP_2 concentration for (PC/PIP_2, 95:5) (circles) (*x* axis being the PS *acc* concentration; and PC vesicles). The curves are the least squares fits of a 1:1 biding model to the data. Points are mean ± SD of seven measurements on each sample [66] (reproduced with permission from Ref. [66] copyright (2008) Elsevier). (C) Adsorption isotherms of ezrin binding to a PIP_2-doped (10 mol %) SLBs. Dashed lines are the results of fitting [70] (reproduced with permission from Ref. [70] copyright (2011) Elsevier). (D) Colocalization between PIP_2–GUVs labeled with FL–PIP_2 and ezrin-Alexa546. Scale bar: 10 µm [69] (reproduced with permission from Ref. [69] copyright (2008) Elsevier).

Furthermore, quenching experiments showed that no clustering of PIP_2 was observed in LUVs after ezrin binding [66].

Differences between LUVs and SLBs may arise from their different curvature with the possibility of clustering and lateral interactions between ezrin molecules in the case of planar SLBs. In the case of ~100 nm large LUVs of high curvature, the spatial hindrance of the FERM domain of ezrin (area of approximately 30 nm^2) binding to a single PIP_2 may impede other ezrin molecules to bind other PIP_2 molecules on the membrane. It should be noted that Steinem *et al.* also observed that the adsorption of ezrin on the PIP_2–SLBs membrane was quasi-irreversible [71]. Interestingly, when ezrin interacted with PIP_2–GUVs, protein domains were also formed at the membrane, which colocalized with PIP_2 clusters [69]. Thus, GUVs behaved in a very similar manner to SLBs. Of note, a study indicated that moesin was shown to induce and stabilize holes in GUV membranes [75], but no further experimental evidences support these *in vitro* findings.

These apparent puzzling results between LUVs, GUVs and SLBs reflect different behaviors of the ERM proteins with respect to membrane geometry. ERM proteins are known to interact with membrane structures of various curvature ranging from quasi flat, such as cell to cell junctions, to high curvature, either positive (internal compartments, endosomes, phagosomes, lysosomes) or negative (microvilli, filopodia…). Low curvature membranes such as GUV and SLB are good mimics of flat plasma membrane regions. A parallel can then be drawn between ERM clustering at the GUVs or SLB surface and the formation of ERM-rich regions during cell migration [76], the establishment of the immune synapse [77] or

Table 1
Overview of studies using biomimetic membranes, especially LUVs, GUVs and SLBs to study ERM interaction with PIP_2-containing membranes.

Biomimetic membrane	Main advantages	Protein studied	Main results	Ref.
LUVs	Wide range of lipid compositions	Ezrin	Presence of a PIP_2-binding site at FERM	[68]
	Stable	Ezrin	Mapping of PIP_2 binding site	[50]
		Ezrin	Quantitative affinity measurements	[66]
	Quantitative measurements	Moesin	Sequential binding of PIP_2 to 2 sites	[51]
	Compatible with spectroscopic measurements, but high membrane curvature	Ezrin, moesin	Structural changes after membrane binding Quantitative measurement of moesin affinity for PIP_2	[74]
GUVs	Fluorescence microscopy visualization	Ezrin	PIP_2–ezrin clustering	[69]
	Cell-sized	Ezrin, moesin, other FERM-containing proteins	Pore-forming oligomerisation	[75]
	Quasi flat membranes			
SLBs	Analyses and imaging by surface sensitive methods (QCM, SPR, AFM)	Ezrin	Protein clustering Ezrin binding to PIP_2 occurs in a positive cooperative manner	[71]
	Fluorescence microscopy imaging	Ezrin	Importance of PIP_2 interaction in ezrin activation: the work of adhesion between PIP_2-bound ezrin and F-actin is larger than between F-actin and ezrin bound to the membrane via a His-tag	[72]
		Ezrin	Actin binding of ezrin and its phosphomimetic mutant in interaction with PIP_2	[70]

the formation of high curvature blebs [78], where spatial distribution of PIP_2 may also be altered.

ERMs are also associated to positive curvature membranes of various internal compartments. For instance, ezrin was recently shown to play a role in maturation of endosomes [79], whereas moesin was shown to bind PIP_2 at the surface of clathrin-coated vesicles [80]. From results obtained with LUVs of comparable size (~100 nm) one can suggest that cluster formation is not favored in such cases, ERMs may bind to such membrane structures with lower affinity and cooperativity than to the flat membranes.

A piece of the puzzle is still missing as neither of the above mentioned models effectively mimics negative curvature structures such as microvilli, which are largely enriched in ERM proteins. Therefore, a promising direction in the analysis of ERM–membrane interactions could be to attempt to reconstitute *in vitro* microvilli-like structures and to study ERM activation in this context.

Last but not least, it has to be noticed that, due to experimental constraints, the protein/PIP_2 ratio is very different depending on the biomimetic system and type of experiments. In the case of GUVs, the protein concentration is high (~10 μM) and the maximum PIP_2 concentration was low (5 μM total lipids, 0.2 μM PIP_2) giving a maximum ratio of protein/PIP_2 around 50. In the case of LUVs, the protein concentration from 0.04 to 0.4 μM and the lipid concentration covered the range 1–100 μM PIP_2, giving a maximum protein/lipid ratio of 10^{-2}. Finally, in the case of SLBs, the protein concentration was ~2–4 μM for an estimated lipid concentration of few tens nM. So the protein/lipid ratio is comparable to that of GUVs. In light of such *in vitro* reports we may suggest that membrane local curvature and PIP_2 concentration may regulate ERM binding and activation.

2.4. Conformational changes of ERM upon PIP_2 binding

As discussed above, the closed inactive form of ERMs needs to undergo structural rearrangements to unmask the actin binding site. More precisely, the intramolecular association between the C-term and FERM needs to be released. The subsequent question is whether conformational changes arise upon PIP_2 binding and are sufficient to fully open the molecule leaving the phosphorylation stabilizing the open state. *In cellulo*, several studies have reported that mutants lacking the PIP_2 binding site show aberrant ERM distribution, with diminished membrane binding and translocation in the cytoplasm [50,65]. If the binding of PIP_2 to ERM contributes also to the activation then a spatially regulated process appears at the membrane: ERM are localized at the plasma membrane with PIP_2 weakening the intra-molecular interaction and the phosphorylation inducing full activation. These studies concluded that PIP_2 binding is the first step in ERM activation. But no evidence showing that interaction with PIP_2 is sufficient to activate the protein was provided. Some elements of response came from studies of ezrin interaction with PIP_2 dispersed in a uni-molecular way in buffer [73]. The fluorescence emission spectra of ezrin tryptophan residues, sensitive to the environment (hydrophobic or hydrophilic), were recorded in the presence or absence of PIP_2. Upon addition of PIP_2, there was a specific decrease in the fluorescence (quenching of fluorescence) of full-length ezrin, an a much lower decrease for the FERM domain alone [73]. The decrease of fluorescence was specific of PIP_2 binding, whereas the presence of SDS and other anionic lipids only induced a marginal decrease. This suggested a conformational change in the full length protein, which would lead to exposure to a more polar environment of the tryptophan residues. Proteolysis experiments confirmed this hypothesis. Indeed, the presence of PIP_2 noticeably enhanced the rate of proteolysis of ezrin to chymotrypsin (serine protease), implying a less compact state of the protein. Furthermore, isothermal titration calorimetry (ITC) data were also compatible with an opening of ezrin upon interaction with PIP_2. The interaction of PIP_2 with the FERM domain was enthalpy-driven, as expected for a charge to charge interaction between the phosphoinositol headgroup and the FERM domain. Conversely, the interaction between PIP_2 and ezrin was driven by favorable entropy, dominating an unfavorable enthalpy contribution. This thermodynamic signature was in agreement with an increased flexibility obtained through an opening of ezrin. ITC experiments gave an affinity between PIP_2 and ezrin or FERM of ~0.4–0.6 μM, which is comparable to the affinity measured on SLBs. Interestingly, the affinity for the full-length protein or for the FERM domain was comparable, indicating that the C-TER and the α-helix did not impair the interaction. Also another recent study from Jayasundar *et al.* [81] shows using small angle neutron scattering (SANS) that PIP2 in solution (micellar in this case) can interact with ezrin and lead to, more than a conformational change, to full activation, i.e. ezrin can interact with actin.

The conformational change of ezrin evidenced with PIP_2 dispersed in solution can be challenged in a cellular context, as PIP_2 is not available in its soluble form or even in its micellar form. Are such conformational changes present when PIP_2 is inserted in membranes? Ezrin and moesin tryptophan fluorescence showed specific quenching in the presence of PIP_2-containing LUV, which pointed out a change in the environment of the tryptophan when membrane binding occurred [74]. The membrane-bound proteins were also more sensitive to proteolysis by chymotrypsin, which confirmed that the two ERM proteins adopted a looser structure after binding to PIP_2-containing membranes. Infrared spectroscopy gave also a useful insight in conformational change of the proteins. Ezrin and moesin adopted mostly a helical structure (40–50%), together with random coil structures and β-sheets (~25%) [74]. The large fraction of helical structure was in agreement with crystallographic and circular dichroïsm data. Comparison of the infrared spectra of the lipid-bound versus soluble proteins showed that conformational changes were associated with the α-helical content, together with an increase in the random coil proportion. Several previously published experimental data may help analyzing this result. First, the isolated linker domain of radixin was described as a stable α-helical rod of an unusual length [82]. It is generally assumed that this fragment would keep its helical structure after "activation" [2]. Conformational changes may also take place in the FERM domain, which is directly affected by membrane binding. However, comparison of crystallographic data on the FERM domain in the absence [83] or presence of the C-terminal domain [84] or of IP_3 [15] showed essentially the same organization of the 3 subdomains, with important displacements observed locally, but with no obvious loss of secondary structure elements [83,84]. Thus, in light of the available crystallographic data, modifications in the secondary structure content arise from alterations in the C-terminal domain.

2.5. Formation of ezrin oligomers and their intriguing roles

The presence of ezrin oligomers has been evidenced *in vivo* and *in vitro*, but the mechanisms of their formation and their role are not completely established.

Berryman *et al.* first showed evidence of ezrin oligomers that correlated with physiological situations where ERMs are activated (formation of microvilli). The authors thus hypothesized that multimers were active forms of the protein [85]. They also indicated that ezrin dimers and monomers did not readily interconvert.

Gautreau *et al.* [86] reported that the phosphorylated monomer is the active form able to link the proteins to the membrane. Oligomers were formed through C-ERMAD–FERM domain interaction

between monomers. Phosphorylation disrupted this interaction, thus inducing a transition from oligomers to active monomers localized at the membrane [86]. The authors argued that the oligomers were disrupted during the activation. Thus, phosphorylation dissociates oligomers, the same way it does prevent the auto-inhibitory N-/C-ERMAD interaction.

In a recent study [73], we showed that PIP_2 dispersed in solution induced the formation of ezrin oligomers. Mutation of residues involved in PIP_2 binding (K63N, K64N, K253N, K254N, K262N, and K263N) also abolished oligomerization. Moreover, protein clusters were observed in planar membranes (SLBs or GUVs) [69,71]. The clustering of ezrin on planar membranes may be due to the oligomerization process as the key factor in lateral and cooperative interactions. One can then logically infer that ERM oligomers are required intermediates in the path to full activation. However, further work is needed to fully understand the underlying mechanism (dependence of PIP_2 or not, role of phosphorylation) and the exact role of oligomers in a cellular context.

2.6. ERM interactions with actin

As mentioned above, there are several experimental evidences showing that membrane binding via PIP_2 induces a conformation change in ERMs. Is this structural change sufficient to completely release the FERM domain from the C-terminal domain and allow interaction with actin filaments via amino acids located at the end of the C-term domain? Actin filament interaction with ezrin adsorbed onto PIP_2–SLBs was first investigated *in vitro* by Janshoff and co-workers [72]. Ezrin was coupled to membranes either through specific interactions with PIP_2 or via a His-tag to a lipid bilayer containing Ni-NTA. Using epifluorescence microscopy, the authors showed that accumulation of actin filaments occurred only on membranes with adsorbed ezrin in interaction with PIP_2. In a second set of experiments, actin filaments were firmly attached to colloidal spheres and the adhesion forces between ezrin and actin-coated spheres were measured using colloidal probe microscopy. The force–distance curves recorded differed depending on how ezrin was attached to the membrane, with adhesion forces larger when ezrin was specifically bound to PIP_2 when compared to ezrin bound with a His-tag. The authors discussed the existence of two regimes in the force–distance curves in the PIP_2 bound state: a first one in which both specific and non-specific interactions occurred and a second one which reflected both bending of actin filaments and stretching of ezrin. The role of the phosphorylation in the mechanism of ERM protein activation has been further investigated *in vitro* using SLBs and "constitutively phosphorylated" mutant of ezrin. The threonine residue T567 in the C-terminal domain was changed into an aspartate, mimicking the phosphorylation (T567D mutant) [70]. Confocal microscopy images showed higher coverage of the membrane containing PIP_2 by actin structures when T567D mutant was bound to the SLBs. The authors concluded that PIP_2 binding and phosphorylation state of ezrin function acted in synergy to induce protein activation and thus increased the affinity for filamentous actin. However, more studies are needed to understand the potential role of ezrin in actin dynamics.

3. Conclusions

Model membranes have shed some light on the molecular basis of ERM interactions with PIP_2-containg membranes. This interaction required at least the presence of two phosphates in positions 4 and 5 on the inositol headgroup. In the case of PIP_2-LUVs, the interaction was non cooperative with a low affinity ($K_d \sim 5$ μM), whereas the interaction on SLBs was strong and cooperative ($K_d \sim 120$ nM). Such differences may be attributed to a membrane curvature effect, which enable or not formation of ezrin oligomers. Formation of oligomers was observed in the case of low curvature membranes. In turn, this may induce segregation of PIP_2 in membranes. Given the importance of this lipid in actin dynamics (for review see Ref. [87]), ERM-induced PIP_2 clustering may reveal to be of high physiological importance.

At this stage, it is clear that membrane binding triggers conformational changes in ERM. Does this conformational change permit ERM interaction with actin and what is the part of phosphorylation in this process? Actin was found to accumulate on ezrin-covered SLBs, but this interaction seems to be increased when a phosphomimetic mutant was used. Thus, quantitative measurements of the interaction of ezrin with actin are still needed to understand this difference. The further studies will improve the biomimetic systems in order to integrate also the phosphorylation *in vitro*. Moreover, it is not very clear how ERMs bind actin filaments, whether they are able to orient filaments or what is the effect of ERM binding on the polymerization rate or nucleation (direct nucleation or interaction with co-factors). ERM proteins involvement in cancer or virus entry may be due to their role of scaffolding proteins at the plasma membrane. Studies with biomimetic membranes will bring on the front information about membrane-bound ERM interaction with other proteins.

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