Cortical Actin Organization: Lessons from ERM (Ezrin/Radixin/Moesin) Proteins

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In recent years to clarify the molecular mechanism of dynamic organization of the cortical actin filaments, which is important not only for the determination of cell-surface structures but also for the functions of integral membrane proteins themselves, various types of submembrane proteins involved in cortical actin filament/plasma membrane interaction have been intensively studied.

In this minireview, we focus on ezrin/radixin/moesin (ERM) proteins, which are general cross-linkers between cortical actin filaments and plasma membranes and are involved in the formation of microvilli, cell adhesion sites, ruffling membranes, and cleavage furrows. ERM proteins have attracted a great deal of interest because their functions have been shown to be regulated by the Rho signaling pathway (for recent reviews, see Refs. 1–7).

Structure of ERM Proteins

The ERM family consists of three closely related proteins, ezrin, radixin, and moesin (ERM proteins) (8) (Fig. 1). Ezrin (~82 kDa) was first isolated from chicken intestinal brush borders as a component of microvilli (9). Molecular cloning revealed that ezrin was identical to cytovillin, which was enriched in microvilli of human placental syncytiotrophoblasts (10, 11). Radixin (~80 kDa) was isolated from rat liver as a component of adherens junctions (12). Moesin (~75 kDa) was isolated from bovine uterus abundant in smooth muscle cells as a heparin-binding protein (13). Homologues for ERM proteins have been found from Caenorhabditis elegans to human, although the number of family members appears to vary from one to three depending on species (2).

The sequences of their N-terminal halves are highly conserved (~85% identity) and similar to the N-terminal half of human erythroid band 4.1 protein (~78 kDa), indicating that the ERM family is included in the band 4.1 superfamily that contains merlin/schwannomin (a tumor suppressor molecule for neurofibromatosis type II), talin, PTP-H1, and PTP-MEG. Among these, merlin (isoforms I–III (~70 kDa)) is fairly similar to ERM proteins (~60% identity). The sequence, which is conserved among the members of the band 4.1 superfamily and referred to as the FERM (4.1 and ERM) domain, is a membrane-binding site in band 4.1 protein and the C-terminal domain of myosin VIIA. In ERM proteins, the N-terminal FERM domain is followed by an extended α-helical domain and a charged C-terminal domain, which includes a consensus sequence motif for actin binding. Thus, from their structure, ERM proteins have been suggested to function as cross-linkers between actin filaments and plasma membranes (1–7).

Subcellular Distribution and Functions of ERM Proteins

Immunoblotting analysis and immunofluorescence microscopy revealed that in most cultured cells all ERM proteins are co-expressed and co-localized but that in organs their expression and distribution pattern appear to be regulated in a cell type-specific manner (1–7). Immunofluorescence studies of cultured fibroblasts and epithelial cells have revealed that ERM proteins are co-expressed and co-concentrated at cell-surface structures such as microvilli, filopodia, uropods, ruffling membranes, retraction fibers, and cell adhesion sites where actin filaments are associated with plasma membranes (8, 14–17) (Fig. 2). ERM proteins are also concentrated specifically at cleavage furrows in dividing cells (14) but not along cytoplasmic actin filaments such as stress fibers, in contrast to filamin and a-actinin, which are concentrated in both sites (18). Suppression of the expression of all ERM proteins with antisense oligonucleotides in cultured fibroblasts/epithelial cells destroyed microvillus formation as well as cell-to-cell/cell-to-substrate adhesion (19). Similarly, in cultured neurons that contain mainly radixin and moesin, antisense oligonucleotides of radixin and moesin severely affected the morphology, motility, and process formation of growth cones (20). Specific ezrin ablation by Micro-CALI (chromatophore-assisted laser irradiation) blocked membrane ruffling and motility (21). Furthermore, overproduction of full-length ERM proteins appeared to enhance cell adhesion, whereas that of their C-terminal halves perturbed the cell-surface morphology and inhibited cytokinesis (22, 23). These findings suggested that ERM proteins were involved in the formation and/or maintenance of cortical actin organization through their cross-linking activity between actin filaments and plasma membranes.

Extensive functional analyses suggested the possible functional redundancy of ERM proteins at least at the cellular level. Recently, moesin-deficient mice were generated by gene targeting, and they appeared normal without any compensatory up-regulation of ezrin or radixin (24). Therefore, also at the whole body level ERM proteins appear to be functionally redundant, although ERM proteins are not necessarily co-localized and co-expressed at the organ level. Targeted disruption of ezrin and radixin genes will allow clarification of this redundancy problem in the near future.

Actin and Membrane Binding of ERM Proteins

The C-terminal halves of ERM proteins bind to F-actin through their major actin-binding sites, the C-terminal 34 amino acids, which are highly conserved among these proteins (25). In addition to this domain, two more actin-binding domains have recently been identified in their N-terminal and middle regions, which bind to F-actin and both F- and G-actin, respectively (26). Although the physiological relevance of these newly identified actin-binding sites in ERM proteins is not clear at present, the mode of association of actin filaments with ERM proteins does not appear to be simple. G-actin binding affinity in their middle region would explain the actin barbed-end-capping activity of ERM proteins, which was detected in radixin at low ionic strength (12).

On the other hand, the N-terminal halves of ERM proteins were reported to directly bind to the cytoplasmic domains of CD44 (27, 28) and other integral membrane proteins such as ICAM-1, -2, and -3 and CD43, which were co-localized with ERM proteins (17, 27–32). Although the cytoplasmic domains of these integral membrane proteins have no shared sequences, their juxtapamembrane positively charged amino acid clusters are thought to be responsible for their binding to ERM proteins (32, 33). This direct binding of ERM proteins with integral membrane proteins was shown to be essential for cell-surface morphogenesis such as microvillus formation (34, 35). Ezrin effected the function of ICAM-2 in thymoma cells for being targeted by natural killer cells (29).

The mechanism of indirect binding of ERM proteins to integral membrane proteins has also been reported. EBPs-50 (ERM-binding phosphoprotein of 50 kDa) was identified as a cytoplasmic protein,
Intramolecular Interdomain Interaction between N- and C-Terminal Halves of ERM Proteins: Molecular Mechanism for Inactivation of ERM Proteins

Given that the cortical actin filaments are dynamically organized in response to various signals, the cross-linking activity of ERM proteins between actin filaments and plasma membranes is expected to be dynamically regulated. Indeed, the pioneering work by Bretscher (42) or Hanzel et al. (43) showed that ROCK treatment of A431 cells or the secretion-stimulation of parietal cells rapidly recruited substantial amounts of ERM proteins to the cortical actin layer with concomitant phosphorylation of ERM proteins. When conventionally cultured cells were homogenized and centrifuged in physiological saline, ERM proteins were partitioned almost equally into the soluble and insoluble fractions (44). These findings suggested that there are active (insoluble) and inactive (soluble) forms of ERM proteins in terms of their cross-linking activity inside cells.

Evidence has accumulated in vitro and in vivo that the N- and C-terminal halves of ERM proteins mutually interact intramolecularly and suppress their actin filament and membrane binding activities, respectively (45–47). Recently, it was shown that when two amino acid residues were deleted from the C-terminal end of ezrin, which do not correspond to the EBP-50-binding domain, the interdomain interaction was affected, allowing ezrin to directly interact with EBP-50 (37). These findings indicated that conformational masking by intramolecular interdomain interaction is the molecular mechanism behind the inactivation of ERM proteins.

The region involved in the interdomain interaction was narrowed down to residues 1-297 and 480–586 (1-296 and 479–585 when methionine 1 is posttranslationally removed) as exemplified in ezrin, and these regions are called N- and C-ERMADs (ERM-association domains), respectively (46). Initially, these N- and C-ERMADs were thought to be responsible for oligomerization of ERM proteins, i.e., intermolecular interaction of ERM proteins, but it has been suggested that they are also important for intramolecular interaction (6, 7). Furthermore, yeast two-hybrid analyses identified several middle regions between N- and C-ERMADs that interact with N- and C-ERMADs (48). Although the molecular mechanism and physiological relevance of dimerization and oligomerization of ERM proteins remain elusive, it is now accepted that the intramolecular mutual suppression mechanism keeps ERM proteins in an inactive state and that some activation signal may release this suppression to activate ERM proteins inside cells.

Phosphorylation and PIP$_2$ Binding as Activation Signals of ERM Proteins

To date, two molecular events have been shown to generate and/or maintain the active form of ERM proteins in vitro: phosphorylation of their C-terminal threonine residue and PIP$_2$ binding to their N-terminal domains. The phosphorylation of ERM proteins, especially ezrin, has been examined in detail. EGF stimulation induced tyrosine and serine phosphorylation of ezrin in A431 cells with concomitant translocation from the cytoplasm to the cortical actin layer (42). The tyrosine residues that were phosphorylated in ezrin, the former of which was conserved in radixin and moesin (49). EBP-50 was shown to bind to a region of ezrin in which the intramolecular mutual suppression mechanism keeps ERM proteins in an inactive state and that some activation signal may release this suppression to activate ERM proteins inside cells.

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Secretion/stimulation in gastric parietal cells was reported to induce phosphorylation of ezrin in vivo (52). In platelets, thrombin activation induced the phosphorylation of moesin at a specific C-terminal threonine residue (Thr-558) (52), causing filopodia formation. This site was effectively phosphorylated in vitro by ROCK/ROCK-II/Rho-kinase in radixin (Thr-564) and by PKC-θ (53, 54). However, it is likely that ROCK kinases do not phosphorylate ERM proteins in vivo (55). In vitro functional analyses suggested that the C-terminal threonine phosphorylation maintains ERM proteins in the active state by suppressing the intramolecular interaction (53). Immunofluorescence microscopy with monoclonal antibodies specific for C-terminal threonine-phosphorylated ERM proteins

the C-terminal region of which binds to the N-terminal half of ezrin (36). Sequence analyses revealed that EBP-50 is identical to a Na’/H’ exchanger regulatory factor (NHE-RF). This NHE-RF and its isoform, E3KARP, bear two PDZ domains, which were shown to directly bind to the C terminus of NHE3 (36, 37). Thus, NHE-RF and E3KARP can function as adapters between NHE3 and ezrin (38). Interestingly, because NHE-RF regulates the NHE3 function in a protein kinase A (PKA)-dependent manner and because ezrin specifically binds to the RII subunit of PKA (38), ezrin appears to play an important role in recruiting PKA to the NHE3-RF complex to regulate the function of NHE3. Furthermore, NHE-RF and E3KARP were found to also be associated with other integral membrane proteins such as the β$_2$-adrenergic receptor and the cystic fibrosis transmembrane conductance regulator (39, 40), which are not always co-localized with ERM proteins (41). The physiological relevance of the existence of two mechanisms of binding of ERM proteins to integral membrane proteins, direct and indirect, is an interesting subject for future study.

Figure 1: ERM family members (ezrin, radixin, moesin), merlin/schwannomin, and band 4.1 protein. ERM proteins consist of three domains: a globular N-terminal membrane-binding domain (FERM domain or N-ERMAD), followed by an extended α-helical domain and a positively charged C-terminal actin-binding domain (C-ERMAD). Proteins as well as PI(3,4,5)P$_3$ that bind to ERM proteins are listed at the top (for details, see “Structure of ERM Proteins”). In some papers on ERM proteins the amino acid residues are numbered from proline 1, because methionine 1 is posttranslationally removed in human ezrin and moesin (10, 15). In this review, however, they are numbered from methionine 1. The percentage sequence identity with ezrin in each domain is indicated at the amino acid sequence level.

Figure 2: Immunofluorescence microscopy of cultured baby hamster kidney cells with anti-ERM polyclonal antibody. ERM proteins are co-concentrated at cell-surface structures such as microvilli (MV), cell-cell adhesion sites (C-C), and cleavage furrows (CF) where actin filaments are associated with plasma membranes. Bar, 20 μm.
proteins revealed that ERM proteins localized beneath plasma membranes were actually phosphorylated at the C-terminal threonine in vivo (53, 56). Taken all together, it seems that the threonine phosphorylation just maintains the activated ERM proteins.

Another candidate for the activation signal for ERM proteins is PIP₂, which has been shown to directly bind to the N-terminal halves of ERM proteins in vitro (28, 57). Recently, it has been shown that PIP₂ is a key factor for the activation of ERM proteins in vivo (65).

**ERM Proteins Downstream as Well as Upstream of Rho**

The question has thus arisen as to the identities of the upstream factors required for activation of ERM proteins. Rho, one of the small GTP-binding proteins, is now considered to be a general regulator of actin-based cytoskeletal organization. To date, in vitro as well as in vivo analyses have suggested an intimate relationship between the Rho signaling pathway and activation of ERM proteins. First, the binding ability of ERM proteins to the cytoplasmic domain of CD44 in crude cell homogenate was reported to be enhanced by activation of Rho (28). In semi-permeabilized Swiss 3T3 cells, at least one of the ERM proteins was shown to be required for Rho-dependent formation of stress fibers and focal contacts (58). Furthermore, transfection of the constitutively active mutant of RhoA (V14RhoA), but not that of Rac1 or Cdc42, induced microvillus formation to which ERM proteins were recruited (55, 59). Thus, it is now accepted that when Rho is activated in vivo, ERM proteins in the cytoplasm are activated and recruited to plasma membranes to form microvilli. Although ERM proteins are also suggested to be located downstream of Rac (58), the activation of ERM proteins to form microvilli specifically depends on Rho but not on Rac. Rho has been reported to activate several serine/threonine kinases such as ROKα/ROCK-II/Rho-kinase, ROKβ/ROCK-I, citron kinase, protein kinase N, and protein kinase C1 (58). Immunoblotting with a monoclonal antibody specific for C-terminal threonine-phosphorylated ERM proteins revealed that in serum-starved Swiss 3T3 cells Rho activation by lysophosphatidic acid stimulation increased the levels of the C-terminal threonine phosphorylation of ERM proteins (53). Rho-kinase, which effectively phosphorylates the C-terminal threonines of ERM proteins in vitro, is not responsible for this Rho-dependent threonine phosphorylation of ERM proteins in vivo.

PIP₂-producing phosphatidylinositol 4-phosphate 5-kinase (PI4P5K) has also been reported to be a direct Rho effector (for a review, see Ref. 60). Because activation of dormant ERM proteins was induced by PIP₂ in vivo as well as in vitro (28, 55), one possible pathway for the activation of ERM proteins is as follows. Rho may activate PI4P5K, which in turn increases the amount of PIP₂. PIP₂ then activates ERM proteins by inhibiting their interdomain interaction, which allows phosphorylation of their C-terminal threonine residue by some kinases. The C-terminally threonine-phosphorylated ERM proteins are stabilized at the activated forms, which function as actin filaments/plasma membranes/actin cross-linkers to form microvilli. Activated ERM proteins are associated directly with the adhesion molecules such as CD44 and ICAM-1, -2, and -3 and indirectly with other integral membrane proteins such as NHE3 through EBP-50/NHE-RF. Activated ERM proteins also bind to Rho-GDI at their N-terminal halves, suppressing GDI activity of Rho-GDI to release GDP-Rho, which is activated to GTP-Rho. This GTP-Rho can be used to activate ERM proteins just beneath the plasma membranes, providing a positive feedback pathway.

**ERM Proteins and Merlin**

Some, but not all, of the characteristics of ERM proteins appear to be shared by merlin. The subcellular localization of ERM proteins was similar to that of merlin in fibroblasts (microvilli and ruffling membranes) but different in epithelial cells; merlin, but not ERM proteins, is concentrated at lateral membranes together with E-cadherin (64). The N-terminal half of merlin bound to the cytoplasmic domains of CD44 (65) and NHE-RF (66) as well as Rho-GDI in vitro (64). Merlin has two major alternatively spliced isoforms, I and II, which differ at their C-terminal ends (67). Although neither of these C-terminal ends shows any similarity to those of ERM proteins, the major actin-binding domains, merlin has been reported to bind to actin filaments at its middle region (68). Interdomain interaction between the N- and C-terminal halves has been suggested in isoform-I, but the interaction does not appear to affect its binding affinity to actin filaments or to NHE-RF (66, 68). No interdomain interaction has been detected in isoform-II (69).

At present, there are three distinct possible explanations for the sequence similarity between ERM proteins and merlin. First, it is possible that merlin functions in cells by competing for shared binding partners such as CD44, NHE-RF, and Rho-GDI. However,

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2 Sa. Tsukita, unpublished data.
because the molar ratio of endogenous Merlin/ERM was calculated to be 0.05–0.15 in cultured fibroblasts and epithelial cells, the ERM-Merlin competition is not likely in vivo. Second, Merlin would be functionally redundant for ERM proteins. Data from the suppression experiments of ERM proteins by antisense oligonucleotides discussed above are inconsistent with this explanation. Furthermore, this explanation was not supported by the observation that a single deficient mouse showed an embryonic lethal phenotype (70). Third, it is also possible that Merlin shows sequence similarity to ERM proteins because both are involved in Rho signaling pathways (70). Finally, it is also possible that Merlin shows sequence similarity to ERM proteins because both are involved in Rho signaling pathways (70). It is reasonable to speculate that both neurofibromatosis type I and II are caused by some dysregulation of small GTP-binding protein-dependent signaling pathways.

**Perspective**

In the past decade, it has been established that ERM proteins function as general cross-linkers in the cortical layer, coupled with signal transduction pathways such as Rho signaling. Because ERM proteins have been expressed almost ubiquitously, this cross-linking system would be involved in various cellular events in various types of cells. Thus, in the coming decade, ERM proteins will attract increasing interest in many fields from not only biological but also medical researchers. For example, in the immune system ERM proteins are thought to play an important role in cell recognition of T lymphocytes by producing uropods (17, 29).

It will also be interesting to study the interactions between ERM proteins and microtubules. Radixin was characterized as a marginal microtubule band-associated protein in nucleated erythrocytes (72), and in activated T-lymphocytes tubulin was co-concentrated at the uropods together with ERM proteins (17). Furthermore, it was also shown that ERM proteins have some homology with Tea 1, which is localized on the ends of microtubules and is critical for polarization in yeast (73). Further studies of the ERM-proteins -microtubule interaction will provide new insight into the physiological functions of ERM proteins as well as the cortical actin layer.

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