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Dynamics of ezrin and EBP50 in regulating microvilli on the apical aspect of epithelial cells

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Abstract

Microvilli are found on the apical surface of epithelial cells. Recent studies on the microvillar proteins ezrin and EBP50 (ezrin/radixin/moesin-binding phosphoprotein of 50 kDa) have revealed both the dynamics and the regulation of microvillar components, and how a dynamic ezrin phosphocycle is necessary to confine microvilli to the apical membrane. In the present review, we first summarize the background to allow us to place these advances in context.

Keywords

ezrin; ezrin/radixin/moesin-binding phosphoprotein of 50 kDa (EBP50); moesin; phosphoprotein; radixin

Introduction

Microvilli are finger-like structures confined to the apical aspect of all metazoan columnar epithelial cells where they serve as platforms for signal transduction, nutrient absorption and immunity. Specialized microvilli are required for photoreception in fruitflies, and structures similar to microvilli called stereocilia are essential for audition. Microvilli contain bundled F-actin (filamentous actin) cores surrounded by plasma membrane. The actin core is attached to the membrane in part by the regulated cytoskeletal linker ezrin, and the scaffolding protein EBP50 associates with ezrin to regulate microvillar structure. In the present short review, we first discuss ezrin and how its regulation confines microvilli to the apical domain, and then introduce EBP50 (ezrin/radixin/moesin-binding phosphoprotein of 50 kDa) and its role in both signal transduction and microvilli formation and discuss its unusual dynamics for a scaffolding protein. Figure 1 provides a summary of the present review.

Ezrin in microvilli

Ezrin was originally identified as a component of intestinal brush border microvilli, and later shown to be a major component of microvilli from placenta [1, 2]. EM studies showed that

ezrin is at the interface of the F-actin core and the plasma membrane [3, 4]. Moreover, expression of truncation mutants has shown that the N-terminal region of ezrin is directed towards the plasma membrane, whereas the C-terminal region associates directly with F-actin [5, 6]. In mammals, ezrin is homologous with two other proteins, radixin and moesin, and together these make up the ERM (ezrin/radixin/moesin) family, but, in general, only ezrin is expressed in epithelial cells [4]. Fruitflies and nematodes each have a single essential ERM protein. These proteins have now emerged as central regulators of polarity in numerous contexts [7].

ERMs are key regulators of microvillus formation in tissue culture epithelial cells. Cells lacking ERMs or overexpressing the dominant-negative N-terminal domain have reduced numbers of microvilli [8, 9]. Mouse and fruitfly knockout studies have supported this role as intestinal epithelial cell microvilli are shortened in mouse knockout and absent from fruitfly photoreceptor cells [10, 11], but, owing to their incomplete penetrance, these phenotypes also demonstrate that additional cell-type-specific mechanisms might exist to cross-link F-actin to the plasma membrane in microvilli *in situ*.

Conformational regulation of ezrin

The C-terminal region of ezrin was found to bind F-actin [6]. However, full-length recombinant ezrin is unable to bind F-actin, and this was traced to the ability of the C-terminal region to bind to the N-terminal FERM (4.1/ERM) domain with high affinity [12]. This observation and subsequent studies led to a model of ERM regulation. The FERM domain harbours a lipid–protein and protein–protein interaction domain, separated by a coiled-coil region from the C-terminal domain that binds directly to the FERM domain to mask the F-actin-binding site [13, 14]. ERMs are thought to localize to the cytoplasm in this closed dormant form.

ERMs are activated at the plasma membrane by inner leaflet phospholipid PtdIns(4,5) P_2 and ezrin phosphorylation. Binding of PtdIns(4,5) P_2 to the FERM domain induces a conformation change releasing the C-terminal domain [15, 16] (Figure 1, steps 1–3).

Although PtdIns(4,5) P_2 is sufficient to create complexes of ERMs and binding partners *in vitro* a critical point of regulation of ERM activation in cells is the phosphorylation of membrane-bound ERMs to stabilize the open state [15]. Phosphorylation occurs at the interface between the N- and C-terminal regions of ERMs, blocking their reassociation through electrostatic changes [13]. In cells, phosphorylation leads to longer-lived membrane occupancy as demonstrated by FRAP of GFP-tagged phosphomimetic mutants [17]. In epithelial cells, the critical phosphorylation site is Thr⁵⁶⁷ in ezrin, which is homologous with Thr⁵⁶⁴ in radixin and Thr⁵⁵⁸ in moesin, a site which is also conserved in both fruitflies and nematodes. The identity of the Thr⁵⁶⁷ kinase has been controversial. In fruitflies and cultured fruitfly cells, a variety of genetic evidence has shown that this role is fulfilled by the Sterile-20-related kinase Slik [18–20]. Its homologues in mammalian cells, LOK (lymphocyte-oriented kinase) and SLK (Sterile-20-like kinase), phosphorylate ERMs in lymphocytes and are clearly the relevant kinases in epithelial cell lines [21, 22] (Figure 1, step 2). Earlier studies have also implicated PKC (protein kinase C) ι or the germinal

centrerelated kinase Mst4 in ERM phosphorylation in epithelial cells [23, 24]. These discrepancies remain to be resolved. The regulation of ERM dephosphorylation, which is carried out in human tissue culture cells and fruitflies by a protein phosphatase 1 complex [25, 26] (Figure 1, steps 5 and 6), may also be important for microvilli.

At issue is why conformation change occurs. For instance, why does ezrin have a dormant form at all? Recent results reveal that ezrin undergoes a cycle of phosphorylation/dephosphorylation every 2 min. Inhibiting phosphorylation leads to a rapid loss of microvilli, whereas inhibition of dephosphorylation results in a loss of apical microvilli and mislocalization of ezrin to the basolateral domain. Thus apical microvilli require dynamic activation/deactivation cycles of ezrin. Appropriately, LOK and SLK are both localized and activated at the apical membrane, so they can phosphorylate ezrin locally, which is then subject to global dephosphorylation. In this way, ezrin activity, and thereby the presence of microvilli, is confined apically [22]. Analysis of cells expressing constitutively active ERMs supports this model as they localize to the plasma membrane in a depolarized manner, unable to distinguish the apical from the basolateral membrane [22, 27]. We have proposed that LOK and SLK possess a regulatory domain that restricts their activity to the apical domain. Thus plasma membrane recruitment by PtdIns(4,5) P_2 and subsequent local ezrin phosphorylation promotes and restricts microvilli to the apical membrane [22]. How the kinases are themselves regulated in any system remains a mystery.

Regulation of microvillus formation by ERMs

No coherent model has yet emerged for how microvilli are formed after ERMs are concentrated in the apical domain, but two hypotheses have been put forth. The first proposes that ERMs locally recruit specific proteins necessary for cytoskeletal and/or membrane remodelling, but cannot provide sufficient mechanical strength alone. Candidates for such a role would be ERM-binding proteins which also play a role in microvillus formation. These include EBP50 (discussed below), Eps8 (epidermal growth factor receptor kinase substrate 8) [28], CLIC4 (chloride intracellular channel protein 4) [29, 30], β -dystroglycan [31], TACSTD2 (tumour-associated calcium signal-transducer 2) [32] and Crumbs3 [33]. It should be noted that ezrin is far more abundant than any of these interacting proteins, so many may contribute simultaneously.

A second hypothesis is that cytoskeletal remodelling is a consequence of activated ERM proteins, without the need for highly specific interactions. By virtue of their general plasma membrane–F-actin cross-linking ability, ERM proteins could modulate the mechanical rigidity of the cortex and this has been suggested to lead to alterations in cell protrusions [34–36].

A more likely scenario is that both models may coexist: specific ERM-binding proteins may help to localize ERM proteins so that they may mould the membrane in discrete locations.

Discovery of EBP50

EBP50 was initially identified in epithelial cells as a protein that binds active, but not dormant, ezrin and is precisely localized with ezrin in microvilli [37] (Figure 1, step 4).

EBP50 has two PDZ (postsynaptic density 95/discs large/zonula occludens-1) domains that bind to a large variety of proteins implicated in diverse signalling pathways, and a C-terminal tail that binds to the FERM domain of active ERM proteins [7]. EBP50 was also discovered independently as a factor necessary for conferring PKA (protein kinase A) regulation on NHE3 (Na^+/H^+ exchanger 3), and is the founding member of the NHERF (NHE3 regulatory factor) family [38]. Mice lacking EBP50 have short aberrant microvilli on their epithelia, similar to the phenotype of ezrin-knockout mice, suggesting that the two proteins function together in microvillus structure or regulation [10, 39]. EBP50-null mice also show defects in urinary phosphate secretion due to mislocalization of the Npt2a (sodium phosphate IIa cotransporter), suggesting that EBP50 plays a role in its apical targeting through its PDZ domains [40].

EBP50 is an important regulator of signalling

EBP50 is involved in the scaffolding and regulation of membrane proteins such as CFTR (cystic fibrosis transmembrane conductance regulator), and the $\beta_2\text{AR}$ (β_2 -adrenergic receptor) [41–43] (Figure 1, step 4). EBP50 has also been shown to interact with the RabGAP (GTPase-activating protein) EPI64 (EBP50-PDZ interactor of 64kD), the RhoGAP nadrin and β -Pix [PAK (p21-activated kinase)-interacting exchange factor] through its first PDZ domain [44, 45], and PDZK1 (PDZ kidney containing 1) through either domain [46].

There is growing evidence for the importance of the NHERF family of adapter proteins in regulation of cellular signalling and trafficking pathways [47]. One well-studied example of regulation by NHERF proteins is that of CFTR. The most common mutation in patients with cystic fibrosis is a deletion of Phe⁵⁰⁸ (F508) which causes defects in chloride secretion because of its retention in the endoplasmic reticulum and ERAD (endoplasmic reticulum-associated degradation) [48]. Interestingly, overexpression of EBP50 is able to rescue CFTR-F508 function in cultured cells [49]. Additionally, the different CFTR signalling complexes assembled by either EBP50 or another NHERF family member, E3KARP (NHE3 kinase A regulatory protein), can regulate its activity by grouping proteins with overlapping signalling pathways in close proximity. EBP50 is able to bind to CFTR through its PDZ1 domain, and $\beta_2\text{AR}$ through its PDZ2 domain. Once active, $\beta_2\text{AR}$ increases cAMP levels which in turn activate PKA, which is able to bind to ezrin; active PKA is then able to phosphorylate and activate CFTR, thereby stimulating chloride secretion [48]. However, when CFTR is bound to the PDZ1 domain of E3KARP, it is instead grouped with the LPA2R (lysophosphatidic acid 2 receptor), which binds to the PDZ2 domain of E3KARP. Active LPA2R reduces cAMP levels, which then suppress PKA activity, thereby inhibiting CFTR activity [48]. There are many other examples of EBP50 and other NHERF family members having a critical role in regulating signalling pathways, as nicely summarized in earlier more extensive reviews [47, 50, 51].

EBP50 and microvilli

EBP50 is required for the presence of microvilli on cultured epithelial cells [52]. More specifically, it must bind to both a PDZ1 ligand and ezrin in order to form microvilli [53]. EBP50 is subject to phosphorylation by several kinases, and these modifications have been

suggested to alter its binding activity and downstream signalling events [54–57]. When EBP50 is phosphorylated by either Cdc2 (cell division cycle 2) or PKC, it is unable to bind to PDZ1 and PDZ2 ligands simultaneously and is unable to form microvilli perhaps due to unidentified PDZ2 ligand(s) which preferentially bind to its phosphorylated form [53]. The phosphoregulation of EBP50 activity is also involved in regulating EBP50's localization and activity in endothelial cells and other epithelial cell lines [57, 58].

EBP50, like many other scaffolding proteins, is thought to stabilize protein complexes. However, EBP50 shows an incredibly rapid exchange rate (recovery in 5–10 s) in microvilli compared with ezrin and the PDZ1 ligand podocalyxin (ezrin recovery in 1–2 min, podocalyxin in 2–4 min) [59]. Surprisingly, PDZ ligand binding enhances EBP50 exchange instead of stabilizing its association with ezrin inside microvilli. Phosphorylation by PKC enhances EBP50 exchange from microvilli, and reduces its membrane localization by reducing its association with ezrin [57, 59]. Interestingly, EBP50's closest related family member, E3KARP, is not as dynamic and this is due to differences in their tail regions [60, 61]. Taken together, this suggests that the EBP50 tail dynamics are repressed when EBP50 PDZ domains are unbound to ligands. Upon PDZ ligand binding, the inhibition is released and EBP50 tail's association with ezrin can now undergo rapid association/dissociation cycles which account for its dynamic nature (Figure 1).

How can a scaffolding protein provide important structural linkages in a structure like a microvillus amid such molecular turbulence? Brush border myosin I also provides an important structural link between the plasma membrane and the underlying actin cytoskeleton in intestinal epithelial cells and is relatively dynamic compared with the actin core of microvilli [62, 63], but clearly not nearly as dynamic as EBP50. One possibility is that EBP50 dynamics might present a more tunable (via phosphoregulation and PDZ-ligand-binding-induced exchange) structural linkage that can more rapidly adapt to changing physical forces in a microvillus. EBP50 dynamics might also serve to regulate the number and duration of any PDZ ligand's stabilization within a microvillus which could have important consequences for downstream signalling pathways.

Ezrin phosphocycling might also play an important role in regulating the duration of ezrin's association with EBP50, although perhaps on a slightly longer timescale. This might be advantageous in that it could provide the cell with multiple levels of regulation of the important macromolecular complexes mediated by ezrin and EBP50. In support of this, it has been suggested that EBP50 might also recruit factors that influence the phosphorylation status of ezrin (and other ERM proteins) [64, 65]. The identification of factors that regulate the dynamics of the EBP50–ezrin interaction is an important subject for future studies.

Concluding remarks

Recent work on the components, function and regulation of microvilli on the apical aspect of epithelial cells has shown that they are subject to much more regulation, and are more dynamic, than heretofore appreciated. Their restriction to the apical surface is at least in part due to a rapid ezrin phosphocycle, although the mechanism of localization of the ezrin kinases LOK and SLK, and with which membrane proteins active ezrin interacts, is still

unclear. Also unclear are the dynamics of active ezrin in microvilli; one possibility is that it binds treadmilling actin and moves down the microvillus to become dephosphorylated towards the base [22, 52] (Figure 1). The properties and dynamics of the scaffolding protein EBP50 has uncovered unanticipated regulatory mechanisms through phosphorylation, PDZ ligand binding, and a regulatory region in the C-terminal domain. It will be fascinating to discover the relevant factors and ligands that regulate this protein. What emerges is a highly dynamic and complex system; far from the boring static structures often depicted on epithelial cells.

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Abbreviations

β₂AR	β ₂ -adrenergic receptor; CFTR, cystic fibrosis transmembrane conductance regulator
EBP50	ezrin/radixin/moesin-binding phosphoprotein of 50 kDa
E3KARP	NHE3 kinase A regulatory protein
ERM	ezrin/radixin/moesin
F-actin	filamentous actin
FERM	4.1/ERM
GAP	GTPase-activating protein
LOK	lymphocyte-oriented kinase
LPA2R	lysophosphatidic acid 2 receptor
NHE3	Na ⁺ /H ⁺ exchanger 3
NHERF	NHE3 regulatory factor
PDZ	postsynaptic density 95/discs large/zonula occludens-1
PKA	protein kinase A
PKC	protein kinase C
SLK	Sterile-20-like kinase

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Figure 1. Sequential recruitment and dynamics of ezrin and EBP50 in microvilli

(1) Ezrin is recruited to microvilli in the dormant autoinhibited form by its affinity for membrane PtdIns(4,5) P_2 (pink). (2) PtdIns(4,5) P_2 binding induces a conformation change in ezrin partially transitioning it to the open state, which is stabilized further by threonine phosphorylation within the C-terminal domain by LOK/SLK. (3) Open ezrin then engages in interaction with the F-actin core through its C-terminal domain and the basic juxtamembrane region of an ERM-binding transmembrane protein through its N-terminal FERM domain. (4) Open ezrin also binds to EBP50 through its N-terminal FERM domain. Binding to ezrin

reverses an autoinhibitory interaction between the EBP50 tail and its second PDZ domain. EBP50 is then free to bind PDZ ligands, but, interestingly, instead of forming a stable complex, PDZ ligand binding promotes exchange of ezrin-bound EBP50 with the unbound form (teal arrow) (5) As ezrin treadmills towards the microvillus base, it is dephosphorylated by the PP1 (protein phosphatase 1) complex. (6) Dephosphorylated ezrin and inactive EPB50 recycle into the cytoplasm. (7) The cycle repeats. Note that the ezrin phosphocycle and membrane occupancy cycle rates are lower than the rate of EBP50 exchange between the cytoplasm and microvillar membrane.