EPI64 regulates microvillar subdomains and structure

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EPI64 is a TBC domain–containing protein that binds the PDZ domains of EBP50, which binds ezrin, a major actin-binding protein of microvilli. High-resolution light microscopy revealed that ezrin and EBP50 localize exclusively to the membrane-surrounded region of microvilli, whereas EPI64 localizes to variable regions in the structures. Overexpressing EPI64 results in its and EBP50’s relocalization to the base of microvilli, including to the actin rootlet devoid of ezrin or plasma membrane. Uncoupling EPI64’s binding to EBP50, expression of any construct mislocalizing its TBC domain, or knock down of EBP50 results in loss of microvilli. The TBC domain of EPI64 binds directly to Arf6-GTP. Overexpressing the TBC domain increases Arf6-GTP levels, and expressing dominant-active Arf6 results in microvillar loss. These data reveal that microvilli have distinct cytoskeletal subdomains and that EPI64 regulates microvillar structure.

Introduction

Microvilli are slender F-actin–containing structures on the apical surface of many epithelial cells. Perhaps the best-studied examples are the densely packed microvilli of brush borders on intestinal and kidney proximal tubule epithelial cells, where microvilli are believed to increase the surface area for absorption. Less well ordered microvilli, having a somewhat different complement of actin binding proteins, are found on the apical aspect of other epithelial cells, such as the placental syncytiotrophoblast (Bretscher, 1991; Bartles, 2000). In neither case is the regulation of these structures understood.

One protein common to both types of microvilli is ezrin, a member of the ezrin/radixin/moesin (ERM) family of membrane-cytoskeletal linking proteins. Members of this family have an ~300-residue N-terminal 4.1 ERM (FERM) domain, followed by a central ~150-residue region predicted to be largely α-helical, and terminate in an ~100-residue domain known as the C-ERMAD (C-terminal ERM association domain), as it has the ability to bind the FERM domain of all family members (Gary and Bretscher, 1993); an F-actin binding site lies in the last ~30 residues of the C-ERMAD (Turunen et al., 1994; Pestonjamaasp et al., 1995). ERM proteins are conformationally regulated, as the F-actin binding site in the C-ERMAD, and some sites for association with membrane proteins in the FERM domain, are masked in dormant molecules when these two domains are associated (Bretscher et al., 2002). Activation to release the association and expose these binding sites can occur through PIP2 binding and subsequent phosphorylation of a C-terminal threonine (567 in ezrin), found on the interface between the FERM domain and the C-ERMAD (Hirao et al., 1996; Matsui et al., 1998; Simons et al., 1998; Gautreau et al., 2000; Fievet et al., 2004).

The cytoskeletal-membrane linking properties of ERM proteins are attributed to their ability to bind F-actin through their C-terminal domain and membrane proteins, such as CD44, CD43, and ICAM-1-3, through their FERM domain (Tsukita et al., 1994; Helander et al., 1996; Hirao et al., 1996; Serrador et al., 1997, 1998; Heiska et al., 1998; Yonemura et al., 1998). In addition to this direct linkage with transmembrane proteins, the FERM domain binds the related scaffolding proteins EBP50 (ERM binding phosphoprotein of 50 kD)/NHERF1 and E3KARP/NHERF2, proteins enriched in epithelial microvilli (Reczek et al., 1997; Yun et al., 1997). These proteins consist of two N-terminal PDZ (postsynaptic density/95-discs large/zona occludens-1) domains and a C-terminal region that binds tightly to isolated FERM domains (Reczek et al., 1997). The EBP50 binding site on the FERM domain lies on the same surface occupied by the last helix of the C-ERMAD in the dormant protein, thereby providing a physical explanation for its masking in dormant ezrin (Reczek and Bretscher, 1998; Finnerty et al., 2004). EBP50 binds the C-terminal tails of many transmembrane proteins, including the CFTR, the β2-adrenergic receptor, and the PDGF receptor to regulate aspects of their function.
(Hall et al., 1998; Short et al., 1998; Cao et al., 1999; Moyer et al., 1999; Maudsley et al., 2000; James et al., 2004; Li et al., 2005). Previously, we identified EPI64 (EBP50-PDZ interactor of 64 kD) from extracts of placental microvilli, the richest known source of both ezrin and EBP50, as a protein that binds the PDZ domains of EBP50 (Reczek and Bretscher, 2001). EPI64 is a cytosolic protein of 508 residues containing an N-terminal Tre-2/Bub2/Cdc16 (TBC) domain and ending in the sequence DTYL, which binds preferentially to the first PDZ domain of EBP50 (Reczek and Bretscher, 2001).

The current model based on biochemical interactions (Fig. 1A) suggests that microvilli should exhibit a uniform distribution of phosphorylated ezrin, EBP50, and F-actin along their length. We used high-resolution fluorescence microscopy to assess this prediction and found that distinct subdomains exist in microvilli whose distribution can be regulated by EPI64. By exploring the effects of expressing different domains of EPI64, we found that when EPI64 is unable to be linked to ezrin, cells have a reduced number of microvilli. We have traced this phenomenon to the mislocalization of EPI64’s TBC domain, which we find binds directly to Arf6-GTP.

Results

Microvilli contain subdomains distinguished by the localization of ezrin and EPI64

To localize specific proteins in microvilli by immunofluorescence microscopy, 10–15 confocal sections 0.2 μm apart covering just the apical aspect of stained human JEG-3 syncytiotrophoblast cells were collected and merged. When the plasma membrane was stained with fluorescently tagged WGA and ezrin localized with specific antibodies, a high degree of colocalization was found. Comparing these with phalloidin staining for F-actin, the microvillar rootlet extending into the

Figure 1. Ezrin and phospho-ERM localization in microvilli. (A) Schematic representation of biochemical interactions between ezrin, EBP50, EPI64, and F-actin. The asterisk denotes the C-terminal T567 phosphorylation site in ezrin. (B) JEG-3 cells stained with antibodies to ezrin (blue), the plasma membrane with fluorescently conjugated WGA (green), and F-actin with fluorescently conjugated phalloidin (red). Individual channels of the boxed region are enlarged in the right panels. The arrows identify regions of ezrin localization with the plasma membrane, whereas the arrowheads identify the microvillar rootlet. (C) Fitted curves to the total dataset localizing ezrin, the plasma membrane, and F-actin regions in JEG-3 cell microvilli. Distance is measured from the microvillus tip to its base. (D) JEG-3 cells stained for pERM (blue), the plasma membrane (green), and F-actin (red); the boxed region is enlarged in the bottom panel. Arrows indicate membrane-bound regions weakly stained for pERM proteins. (E) Fitted curves to the total dataset localizing pERM, F-actin, and the plasma membrane regions in JEG-3 cell microvilli (top) or pERM, F-actin, and total ezrin (bottom). Unlike ezrin, pERM levels are more intense toward the microvillar tips and drop off before the plasma membrane (compare with C). (F) Schematic representation of microvilli depicting the localization of pERM (blue), F-actin (red), the plasma membrane, and total ezrin (green). Bars, 10 μm.
cell body was clearly visible, and this allowed us to determine the orientation of microvilli in triple-label studies (Fig. 1 B). To quantitate the degree of colocalization of three markers, the fluorescent intensity along the length of a minimum of 25 microvilli was determined, and the mean was assessed. It should be noted that because microvilli vary in length (from \( \approx 0.75 \) to \( 1.25 \) \( \mu m \)), this mean gives a measure of colocalization but does not accurately reflect sharp transitions seen in individual microvilli (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200604046/DC1).

As expected, the distribution of ezrin closely follows that of the plasma membrane (Fig. 1 C).

When the distribution of T567 phosphorylated ezrin (pERM) was compared with the plasma membrane, it was found that they were largely overlapping but that the pERM localization was slightly more enriched in the distal portion of microvilli and reduced toward their base (Fig. 1 D, arrows; and Fig. S1). Although this is a subtle effect in the means (Fig. 1 E), it is also seen in other cell types, such as intestinal Caco-2 epithelial cells and porcine kidney proximal tubule LLC-PK1 cells (not depicted). In similar analyses, EBP50 was found to precisely colocalize with the plasma membrane–like ezrin (Fig. 2 A).

In contrast to the localizations of ezrin and EBP50 in JEG-3 cell microvilli, the localization of EPI64 was more variable. Traces of individual microvilli never showed a clear colocalization with other markers but, rather, were enriched in different regions of individual microvilli (Fig. 2 B and Fig. S1). As such, the mean distribution curve shows no specific localization, though the peak of the curve is near the microvilli bases. Remarkably, EPI64 was sometimes enriched in the region of the microvillus rootlet with no ezrin or plasma membrane colocalization.

**Overexpression of EPI64 affects microvillar domains**

To begin examining how microvillar subdomains arise, we investigated the effects of overexpressing EPI64 and EBP50. Overexpressing EPI64 resulted in the majority of microvilli exhibiting enrichment of EPI64 in a region immediately below ezrin and associated with the microvillar rootlet (Fig. 3 A, arrows). Moreover, overexpressing EPI64 caused EBP50 to now be largely coincident with EPI64 and the distribution of both EBP50 and EPI64 to be distinct from that of ezrin (Fig. 3 B). Thus, overexpressing EPI64 results in redistribution of EBP50 and enhances the distinction between microvillar subdomains. In contrast to EPI64, overexpressing EBP50 had no discernable effect on the distribution of ezrin, pERM, EBP50, or EPI64 in JEG-3 cells (unpublished data). We therefore focused our studies on the role of EPI64.

**The TBC domain of EPI64 is a regulator of microvilli**

To further investigate the role of EPI64, we generated several N-terminally tagged mutants (Fig. 4 A). Because the C-terminal DTYL sequence of EPI64 binds the PDZ domains of EBP50, we made a mutant (EPI64-LA) with an additional C-terminal alanine known to abolish this interaction (Reczek and Bretscher, 2001). Some TBC domains are known to accelerate the catalytic activity of Rab GTPases through a catalytic arginine finger motif (Neuwald, 1997; Albert et al., 1999), so we mutated this residue, arginine 160 in EPI64, to generate EPI64-R160A.
In addition, we made an N-terminal construct (EPI64-NT) containing the TBC domain and a C-terminal construct (EPI64-CT) consisting of the C-terminal 189 residues. The expression of these constructs modestly enhanced the level of EPI64 expression as revealed by Western blotting (Fig. 4 B).

Overexpressing EPI64-LA resulted in the reduction or loss of microvilli from JEG-3 cells as seen by ezrin or F-actin staining, without noticeably affecting the levels of ezrin, pERM, or EBP50 in these cells (Fig. 4 B). Instead of being enriched in the apical aspect of cells, like overexpressed wild-type EPI64, EPI64-LA was found diffuse in the cytoplasm (Fig. 4 C). To quantify the microvillar loss phenotype, at least 200 transfected cells were assessed for the presence of microvilli, with 46% of the transfected cells lacking or exhibiting greatly reduced numbers of microvilli, compared with 25% of untransfected or wild-type EPI64 overexpressing cells that had reduced numbers of microvilli. In addition, a small percentage of EPI64 and EPI64-LA overexpressing cells contained vacuoles (Fig. 4 D), which are discussed in the next section. In contrast to EPI64-LA, EPI64-R160A overexpression was similar to overexpressing wild-type EPI64.

To investigate the mechanism of microvillar loss by EPI64-LA, we overexpressed domains of the protein. Overexpressing EPI64-CT resulted in a reduction of microvilli and an increase in the number of ruffling cells (Fig. 4 D), with the ruffles enriched in the transfected protein (not depicted). Similar to overexpressing EPI64-LA, overexpressing EPI64-NT, containing the TBC domain, resulted in microvillar loss. A similar effect was seen in cells expressing a mutant TBC domain, EPI64-NT-R160A (Fig. 4 D). To explore whether the loss of microvilli resulted from a defect in cell polarity, the localization of the tight junction marker ZO-1 and the adherens junction marker E-cadherin was examined in cells overexpressing EPI64 or EPI64-LA. In neither case did cell junctions appear significantly different between transfected and nontransfected cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200604046/DC1).

The data imply that mislocalizing the TBC domain from the apical region results in loss of microvilli. Because perturbing the binding of EBP50 to ezrin should reproduce this effect, we explored the consequences of overexpressing EBP50-PDZ1-PDZ2, which lacks the C-terminal ezrin binding site. Consistent with this notion, whereas overexpressing wild-type EBP50 had no effect on microvilli, overexpressing EBP50-PDZ1-PDZ2 resulted in the loss of microvilli (Fig. 5, A and B). Moreover, knock down of EBP50 by siRNA treatment for 48 h also resulted in the loss of surface microvilli, whereas treatment with control siRNA did not (Fig. 5, C and D). Interestingly, the microvilli located above the adherens junctions were the most resistant to EBP50 knockdown, perhaps suggesting that the lateral border provides a signal for microvillus formation that can normally be propagated across the apical surface.

Overexpressing EPI64 and EPI64-LA can cause the production of F-actin–coated vacuoles

Overexpressing the full-length EPI64 constructs caused a small percentage of cells to contain intracellular vacuoles often decorated by EPI64 and F-actin both in JEG-3 cells but more dramatically in HeLa cells (Fig. 6 A). Because similar F-actin–decorated structures are seen in cells overexpressing dominant-active Arf6 (Brown et al., 2001), we cotransfected tagged EPI64 and Arf6 and found that the vacuoles were also enriched in Arf6 (Fig. 6 B), suggesting a relationship between Arf6 and EPI64. We thus examined cells expressing EPI64 and wild type or dominant-active (Q67L) or dominant-negative (T27N) mutants of Arf6. EPI64 colocalized on vacuoles with both Arf6 and...
Arf6 Q67L, but not with Arf6 T27N (Fig. 6 B). An enrichment of endogenous EPI64 is seen in the region of vacuoles produced by Arf6 Q67L overexpression and on areas of the plasma membrane to which it localizes, suggesting that Arf6 may have the ability to recruit EPI64 to these regions (Fig. 6 C).

The TBC domain of EPI64 binds active Arf6

It has been found that the TBC domain of TRE17 binds Arf6-GDP (Martinu et al., 2004). Thus, we explored the possibility that Arf6 binds to the TBC domain of EPI64. Cells were transfected with Xpress-tagged EPI64 and HA-tagged Arf6, Arf6 T27N, or Arf6 Q67L. Immunoprecipitates of EPI64 contained a small amount of wild-type Arf6, were enriched in Arf6 Q67L, and lacked Arf6 T27N (Fig. 7 A). Immunoprecipitates from cells expressing EPI64-LA or -NT recovered both Arf6 and Arf6 Q67L efficiently, whereas Arf6 T27N was absent (Fig. 7 A). Notably, there was a consistent increase in the relative amount of wild-type Arf6 recovered by these constructs when compared with EPI64, indicating that the mutated proteins have increased access, or bind more efficiently, to Arf6-GTP. In addition, both constructs also decorated Arf6 Q67L–induced vacuoles, as is seen for EPI64 (unpublished data). In coimmunoprecipitation experiments, EPI64-R160A behaved indistinguishably from EPI64 (Fig. 7 A), suggesting that the R160A mutation has no effect on Arf6 binding.

An in vitro binding assay using purified recombinant proteins was used to determine whether the interaction between EPI64 and Arf6-GTP is direct (Fig. 7 B). Immobilized EPI64 bound Arf6-GTP with a greater affinity than Arf6-GDP, whereas no binding was observed to immobilized BSA. Collectively, these results indicate that, unlike the TBC domain of TRE17, which preferentially binds Arf6-GDP (Martinu et al., 2004), the TBC domain of EPI64 specifically binds Arf6-GTP.

To evaluate whether EPI64 might be a GTPase activating protein (GAP) for Arf6, we assessed the level of Arf6-GTP in...
cells by making use of the ability of GST-GGA3 to bind selectively to Arf6-GTP (Dell’Angelica et al., 2000). Cells were transfected with HA-tagged Arf6, together with full-length EPI64 or mutants, and lysed. Lysates were incubated with GST-GGA3 beads, and the amount of bound Arf6-GTP was determined. Expression of all constructs containing the TBC domain did not reduce the level of Arf6-GTP and, in fact, enhanced it, when compared with the vector alone control (Fig. 7 C), making it unlikely that the TBC domain of EPI64 is a GAP for Arf6.

Arf6 regulates the abundance of microvilli

The connection between EPI64 and Arf6 prompted us to explore the effect of expressing wild-type, dominant-active, or dominant-negative Arf6 on microvilli of JEG-3 cells. Overexpressing wild-type Arf6 and Arf6 Q67L both led to microvillar loss, whereas the dominant-negative Arf6-T27N had no effect (Fig. 8 A). Dominant-active Arf6 also greatly enhanced the percentage of cells with vacuoles, as reported previously (Brown et al., 2001). In cells transfected to express HA-Arf6 yet still containing microvilli, the Arf6 colocalized with ezrin and EPI64 in

Figure 5. Uncoupling EBP50 from ezrin results in loss of microvilli. (A) Cells were transfected with either Xpress-tagged EBP50 or EBP50-PDZ1-PDZ2 and stained for the tag, ezrin, and F-actin, and scored as described in Materials and methods. (Right) Western blot of total cell lysates probed with antibodies to the Xpress tag epitope. (B) Cells were transfected to Xpress-tagged EBP50-PDZ1-PDZ2 and stained for the tag, ezrin and F-actin. (C) Cells were transfected with siRNA to EBP50 (siEBP50) or luciferase (siGL2) and either stained for EBP50, ezrin, and actin or collected for Western blot analysis. (Left) Results of scoring cells for the presence of microvilli; (Right) Western blot of total cell lysates probed with antibodies to EBP50 and β-tubulin. Error bars indicate mean ± SD. (D) Localization of the indicated proteins in cells treated with siEBP50 (top) and siGL2 (bottom). Bars, 10 μm.
the apical microvilli (Fig. 8 B), confirming observations of Arf6 at the apical surface of epithelial cells (Altschuler et al., 1999).

**Discussion**

We have uncovered two unexpected findings relating to the organization of the apical domain of epithelial cells. First, using high-resolution light microscopy, we found that the microvilli of epithelial cells do not have a uniform composition but, rather, have distinguishable subdomains that are influenced by mild overexpression of EPI64. Second, we have found that expressing an EPI64 construct unable to bind the PDZ domains of EBP50 results in a dramatic loss of microvilli. Moreover, knock down of EBP50, or expression of an EBP50 construct that binds EPI64 but not ezrin, also results in the loss of microvilli. Thus, our studies suggest that mislocalization of EPI64 results in loss of microvilli. Because a major recognizable feature in EPI64 is the presence of a TBC domain that we show binds directly to Arf6-GTP, it is likely that Arf6 is also involved, directly or indirectly, in regulating microvilli on cells.

As far as we are aware, all previous studies have suggested that microvilli are of uniform cytoskeletal composition along...
membrane-cytoskeletal linking role might treadmilling actin and become dephosphorylated as it nears the form (depending on the rates of dissociation from actin and the comes dephosphorylated it takes time to return to its closed molecules (Reczek and Bretscher, 1998). Perhaps as ezrin be\

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prising because EBP50 is proposed to only bind activated ezrin molecules (Reczek and Bretscher, 1998). Perhaps as ezrin be\

comes dephosphorylated it takes time to return to its closed form (depending on the rates of dissociation from actin and the membrane) and so retains some ability to bind EBP50 after dephosphorylation.

Analysis of cells overexpressing EPI64 reinforces the idea that microvilli have different domains: ezrin is found colocaliz-

ing with the plasma membrane of microvilli, whereas EBP50 and EPI64 are now enriched on the rootlet ~0.25 μm below the membrane; how this enrichment might occur remains unknown. To investigate how EPI64 alters the distribution of proteins in microvilli, we began to study its functional domains. To evaluate the importance of the C-terminal linkage of EPI64 to the PDZ domains of EBP50, we examined cells expressing EPI64 with an additional single alanine residue to abolish binding to EBP50. Surprisingly, the transfected cells lost microvilli, indicating that the proper localization of EPI64 is necessary for microvillar maintenance. By expressing domains of EPI64, we found that expressing any construct mislocalizing the TBC domain resulted in microvillar loss.

Sequence comparisons suggest that the TBC domain of EPI64 might have RabGAP activity (Reczek and Bretscher, 2001). However, biochemical studies did not identify any GAP activity on several purified Rab proteins using full-length recombinant EPI64 (unpublished data; see the following paragraph). A clue to the possible role of the TBC domain came from two sources. First, we noticed that in 5–10% of cells overexpressing EPI64 vacuoles formed, often associated with F-actin, highly reminiscent of the vacuoles seen in dominant-active Arf6-expressing cells (Brown et al., 2001). Second, it was recently reported that the TBC domain of TRE17 binds to Arf6-GDP (Martinu et al., 2004). Immunoprecipitation experiments and in vitro studies using recombinant proteins revealed that the TBC domain of EPI64 also binds Arf6, but with a preference for Arf6-GTP over Arf6-GDP. Initially, we wondered if EPI64 was a GAP for Arf6. RabGAPs have a conserved arginine residue necessary for enhancing the hydrolysis of the γ-phosphate of GTP (Albert et al., 1999). Cells expressing EPI64-R160A, a construct in which this catalytic arginine was mutated to alanine, showed no reduction in microvilli, and the level of Arf6 associated with EPI64 was not diminished. Indeed, overexpression of either EPI64 or EPI64-R160A resulted in an enhanced level of Arf6-GTP. Therefore, EPI64 is not a GAP for Arf6 and, because EPI64 has a preference for Arf6-GTP, it is not a gua-

nne nucleotide exchange factor for Arf6 either. At present, we believe that EPI64 is an effector of Arf6-GTP and that, by binding Arf6-GTP, it protects it from inactivation by a GAP.

While this manuscript was in the final stages of review, Itoh and Fukuda (2006) reported the important finding that EPI64 is a GAP for Rab27A and that this activity is abolished by a R160K mutation. Combining their results with ours implies that the TBC domain of EPI64 has two distinct functions: as a RabGAP for Rab27A and as a binding domain for Arf6-GTP that is unaffected by the R160A mutation.

Why does expression of EPI64 defective in binding EBP50 result in microvillar loss? Loss of microvilli is also seen in cells transfected to express just the PDZ domains of EBP50 or in cells with EBP50 levels reduced by siRNA treatment, thereby breaking the linkage to ezrin. Furthermore, overexpressing wild-type Arf6 or its mutant that binds GTP constitutively
also results in microvillar loss. Because overexpression of the dominant-active Arf6 results in the formation of actin-containing vacuoles in the majority of cells, it is possible that the loss of microvilli is simply due to sequestration of F-actin internally. However, we do not think this is very likely, as overexpression of wild-type Arf6, which induces a very much lower level of vacuoles, also results in microvillar loss, including cells where no vacuoles are evident. Although these data suggest a correlation between elevated levels of mislocalized Arf6-GTP and the loss of microvilli, we also attempted to explore the consequences of Arf6 depletion by siRNA. Although we were able to knock down expression of transfected HA-Arf6 about fivefold, no effect on microvilli was seen in siRNA-treated HA-Arf6 expressing or otherwise wild-type cells (unpublished data).

Whether these data suggest that microvilli can exist in the absence of Arf6 function, which is consistent with the lack of interference by expression of the dominant-negative Arf6-T27N mutant, or that Arf6 was not sufficiently reduced, remains an open question. If Arf6 is directly relevant to the regulation of microvilli, an attractive scenario is that Arf6-GDP is returned to the plasma membrane through a membrane-recycling pathway, where it is acted upon at the plasma membrane by Arf6 guanine nucleotide exchange factors such as ARNO or EFA6 (Franco et al., 1998, 1999) and then captured by EPI64. Microvillar formation or maintenance might first require capture of Arf6-GTP by EPI64, followed by Arf6 inactivation, when it is brought in close proximity to a microvillar ARF-GAP. Consistent with this model, we found that the association of Arf6-GTP with EPI64 was elevated in mutants defective in microvillar localization. Moreover, in a preliminary report, a novel Arf6 GAP (ARF-GAP) was shown to be associated with the activated form of ezrin (Wu, F., H. Deng, R. Zhou, Z. Guo, Z. Fan, K. Yuan, X. Cao, J. Forte, and X. Yao. 2005. American Society for Cell Biology Annual Meeting. Abstr. 1804).

Among the known effectors of Arf6 is phosphatidylinositols-tol-4-phosphate 5 kinase-α (PI(4)P5Kα), which generates PI(4)P, at the plasma membrane (Honda et al., 1999). Excess PI(4)P5Kα, or by either overexpression of the protein or overexpression of dominant-active Arf6, induces the formation of vacuoles rich in PI(4)P and F-actin (Brown et al., 2001). This appears to be a consequence of greatly enhanced endocytosis and decreased membrane recycling through an actin-dependent mechanism, thereby depleting PI(4)P from the plasma membrane, ultimately resulting in multiple phenotypic consequences (Brown et al., 2001; Aikawa and Martin, 2003). The similar phenotype conferred by EPI64 overexpression suggests that it drives this system by enhancing the level of Arf6-GTP, to hyperactivate PI(4)P5Kα to generate excess PI(4)P. Our results suggest that localized capturing or cycling of EPI64-Arf6-GTP is necessary for the maintenance of microvilli. If this is the case, one possibility is that PI(4)P5K has to be locally activated to maintain microvilli. As it has been reported that PI(2)P binding to ezrin is the first step in its activation (Fievet et al., 2004), one attractive model is that transient locally elevated levels of PI(2)P might be responsible for initiating or maintaining active ezrin necessary for microvilli.

In summary, we have shown that microvilli have subdomains and uncovered an involvement of EPI64 in regulating the presence of microvilli on the cell surface. We have also shown that the EPI64’s TBC domain binds Arf6-GTP, which is the second example of a TBC domain interacting with Arf6, indicating that the function of TBC domains may extend beyond their RabGAP activities to encompass other small GTPases involved in membrane trafficking. A recent report suggested that actin organization and clathrin-mediated endocytosis at the apical surface of polarized epithelial cells are regulated by Arf6 (Hyman et al., 2006). The collected data suggest that EPI64 integrates actin cytoskeletal organization and membrane trafficking events mediated by Arf6 and Rab27A, thereby placing it at a pivotal point in balancing these processes. It will be fascinating to further unravel the connection between microvillar structure, endocytosis, and these proteins; such studies are under way.

Materials and methods

Cell culture

JEG-3 and HeLa cells [American Type Culture Collection] were maintained in a 5% CO₂ humidified atmosphere at 37°C in MEM (Invitrogen) with 10% FBS (Invitrogen).

Antibodies and other reagents

Antisera and affinity-purified antibodies against human ezrin and EBP50 were described previously (Bretcher, 1999; Reczek et al., 1997). Hexahistidine-tagged EPI64 was expressed in insect cells, purified, and used to elicit antibody production in rabbits. The affinity-purified antibody recognized a single band at 64 kD in total extracts of several cultured cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200604046/DC1). Specific antibodies for phospho-T567 ezrin/T564 radixin/T558 moesin were obtained from Cell Signaling Technology or generated Cell Signaling Technology or generated against the peptide CRDKYKT possibilité (Matsui et al., 1998). Antibodies against the Xpress epitope were purchased from Invitrogen (anti-Xpress) or Santa Cruz Biotechnology, Inc. (OminiProbe M-21). Anti-HA [HA.11] was obtained from Covance Research Products. Mouse anti-Arf6 was provided by C. D’Souza-Schorey (University of Notre Dame, Notre Dame, Indiana). Mouse anti–ZO-1 and anti–E-cadherin were obtained from BD Biosciences. The antibody against tubulin (N356) was purchased from GE Healthcare. Donkey anti–rabbit Alexa Fluor 488, goat anti–mouse Alexa Fluor 568, goat anti–rabbit Rhodamine RedX, fluorescently conjugated phallolidin, and WGA were obtained from Invitrogen. Donkey anti–rabbit and anti–mouse Cy5 were obtained from Jackson Immunoresearch Laboratories. Goat anti–rabbit HRP was obtained from MP Biomedicals. GTPyS and GDPyS were obtained from Sigma-Aldrich. The siRNAs targeting human EBP50 (S′CCGGCCAAAACGTTGGAGAG3′) and Luciferase GLZ (S′CGUACCGGAAUACUUCUGA3′) were obtained from Dharmacon.

Western blotting and immunofluorescence

Protein samples were separated by SDS-PAGE and transferred to Immobilon-P (Millipore). Western blotting was done as described previously (Reczek and Bretcher, 2001). For immunofluorescence, cells grown on glass coverslips were fixed in 3.7% formaldehyde/PBS for 10 min at room temperature. Cells were permeabilized in 0.2% Triton X-100/PBS for 5 min at room temperature, rinsed in PBS, and incubated with primary antibodies in PBS/2% FBS. After washing in PBS, secondary antibodies and additional markers [phalloidin and/or WGA] were added in PBS/2% FBS (unless WGA was included, in which case, FBS was omitted). Cells were mounted on glass slides in Vectashield (Vector Laboratories) and observed on a microscope (Eclipse TE-2000U; Nikon) using a 100 × 1.4 NA lens (Nikon) on a confocal imaging system (UltraView LCI; PerkinElmer). Z-series images of single focal planes were observed on a microscope (Eclipse TE-2000U; Nikon) using a 100 × 1.4 NA lens (Nikon) on a confocal imaging system (UltraView LCI; PerkinElmer). Z-series images of single focal planes were observed on a microscope (Eclipse TE-2000U; Nikon) using a 100 × 1.4 NA lens (Nikon) on a confocal imaging system (UltraView LCI; PerkinElmer). Z-series images of single focal planes were observed on a microscope (Eclipse TE-2000U; Nikon) using a 100 × 1.4 NA lens (Nikon) on a confocal imaging system (UltraView LCI; PerkinElmer). Z-series images of single focal planes were observed on a microscope (Eclipse TE-2000U; Nikon) using a 100 × 1.4 NA lens (Nikon) on a confocal imaging system (UltraView LCI; PerkinElmer).

Measurements of microvillar protein distribution

Fluorescence intensity grayscale levels along the length of at least five random microvilli on each of at least five different cells were measured by selecting a linear region of interest along the length of each microvillus from...
its tip to actin rootlet (or the opposite end of staining if not stained for actin) using the UltraView software and recorded in Excel (Microsoft).

References

Aikawa, Y., and T.F. Martin. 2003. ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis. J. Cell Biol. 162:647–659.

Albert, S., E. Will, and D. Gallwitz. 1999. Identification of the catalytic domains of the isolated microvillus cytoskeleton, and its localization in nonmuscle cells. J. Cell Biol. 147:7–12.

Barlet, J.R. 2000. Parallel actin bundles and their multiple actin-binding proteins. Curr. Opin. Cell Biol. 12:72–78.

Bretsch, A. 1983. Purification of a 50,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in nonmuscle cells. J. Cell Biol. 97:425–432.

Bretsch, A. 1989. Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. J. Cell Biol. 108:921–930.
Bretscher, A. 1991. Microfilament structure and function in the cortical cytoskeleton. Annu. Rev. Cell Biol. 7:337–374.

Bretscher, 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.

Brown, F.D., A.L. Rozelle, H.L. Yin, T. Balla, and J.G. Donaldson. 2001. Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. J. Cell Biol. 154:1007–1017.

Cao, T.T., H.W. Deacon, D. Reczek, A. Bretscher, and M. von Zastrow. 1999. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. Nature. 401:286–290.

Dell’Angelica, E.C., R. Puertollano, C. Mullins, R.C. Aguilar, L.M. Hartnell, and J.S. Bonifacino. 2000. GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. J. Cell Biol. 149:81–94.

D’Souza-Schorey, C., R.L. Boshans, M. McDonough, P.D. Stahl, and L. Van Aelst. 1997. A role for POR1, a Rac-1-interacting protein, in ARF6-mediated cytoskeletal rearrangements. EMBO J. 16:3445–3454.

Durocher, Y., S. Perret, and A. Kamen. 2002. High-level and high-throughput recombiant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. 30:E9.

Fievet, B.T., A. Gautreau, C. Roy, L. Del Maestro, P. Mangeat, D. Louvard, and M. Arpin. 2004. Phosphoinositide binding and phospholipid activation sequentially in the activation mechanism of ezrin. J. Cell Biol. 164:653–659.

Finnerty, C.M., D. Chambers, J. Ingraffea, H.R. Faber, P.A. Karplus, and A. Bretscher. 2004. The EPB50-moesin interaction involves a binding site regulated by direct masking on the FERM domain. J. Cell Sci. 117:1547–1552.

Franco, M., P.J. Peters, J. Boretto, E. van Donselaar, A. Neri, C. D’Souza-Schorey, and P. Chavrier. 1999. EF-A6, a sec7 domain-containing exchange factor for ARF6, coordinates membrane recycling and actin cytoskeleton organization. EMBO J. 18:1480–1491.

Frank, S., U. Perper, S.H. Hansen, and J.E. Casanova. 1998. ARNO is a guanine nucleotide exchange factor for ADP-ribosylation factor 6. J. Biol. Chem. 273:23–27.

Gary, R., and A. Bretscher. 1993. Heterotypic and homotypic associations between ezrin and moesin, two putative membrane-cytoskeletal linking proteins. Proc. Natl. Acad. Sci. USA. 90:10846–10850.

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.

Gorelik, J., A.I. Shevchuk, G.I. Frolenkov, I.A. Diakonov, M.J. Lab, C.J. Kros, G.P. Richardson, I. Vodyaynov, C.R. Edwards, D. Klenerman, and Y.E. Korchev. 2003. Dynamic assembly of surface structures in living cells. Proc. Natl. Acad. Sci. USA. 100:5819–5822.

Ha, V.L., G.M. Thomas, S. Stauffer, and P.A. Randazzo. 2005. Preparation of apical PDZ protein anchors the brush border Na\(^{+}/H\(^{+}\) exchanger regulatory protein and the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. J. Biol. Chem. 280:37634–37643.

Loomis, P.A., L. Zheng, S. Sekerkova, B. Changyalekut, E. Muginski, and J.R. Bartles. 2003.Espin cross-links cause the elongation of microvillus-type parallel actin bundles in vivo. J. Cell Biol. 163:1045–1055.

Martinu, L., J.M. Masuda-Robens, S.E. Robertson, L.C. Santy, J.E. Casanova, and M.M. Chou. 2004. The TBC (Tor1a/2/Bub2/Cdc16) domain protein TRE17 regulates plasma membrane-endoosomal trafficking through activation of Arf6. Mol. Cell. Biol. 24:9752–9762.

Matsu, T., M. Maeda, Y. Doi, S. Yonemura, M. Amano, K. Kauchi, 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.

Maudsley, S.A., A.M. Zaham, R. Nahman, J.T. Blitzer, L.M. Luttrell, R.J. Letkowitz, and R.A. Hall. 2000. Platelet-derived growth factor receptor association with Na\(^{+}/H\(^{+}\) exchanger regulatory factor potentiates receptor activity. Mol. Cell. Biol. 20:8352–8363.

Moyer, B.D., J. Benton, K.H. Karlson, D. Reynolds, S. Wang, J.E. Mickle, M. Milewski, G.R. Cutting, W.B. Guggino, M. Li, and B.A. Stanton. 1999. A PDZ-interacting domain in CFTR is an apical membrane polarization signal. J. Clin. Invest. 104:1333–1361.

Neuwald, A.F. 1997. A shared domain between a spindle assembly checkpoint protein and Ypt/Rab-specific GTase-activators. Trends Biochem. Sci. 22:244–248.

Pestonjamasp, K., M.R. Amieva, C.P. Strassel, W.M. Nauseef, H. Furthay, and E.J. Luna. 1995. Moesin, ezrin, and p205 are actin-binding proteins associated with neutrophil plasma membranes. Mol. Biol. Cell. 6:247–259.

Peters, P.J., W.W. Hsu, C.E. Ooi, D. Finazzi, B.S. Teal, V. Oorschot, J.G. Donaldson, and R.D. Kaiser. 1995. Overexpression of wild-type and mutant ARF1 and ARF6: distinct perturbations of nonoverlapping membrane compartments. J. Cell Biol. 128:1003–1017.

Reczek, D., and A. Bretscher. 1998. The carboxyl-terminal region of EPB50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule. J. Cell Biol. 139:169–179.

Santy, L.C., and J.E. Casanova. 2001. Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. J. Cell Biol. 154:599–610.

Serrador, J.M., J.L. Alonso-Lebrero, M.A. del Pozo, H. Furthay, R. Schwartz-Albeiz, J. Calvo, F. Lozano, and F. Sanchez-Madrid. 1997. Moesin interacts with the cytoplasmic region of intercellular adhesion molecule-3 and regulates its redistribution to the uropods of T lymphocytes. J. Cell Biol. 138:1409–1423.

Serrador, J.M., M. Nieto, J.L. Alonso-Lebrero, M.A. del Pozo, J. Calvo, H. Furthay, R. Schwartz-Albeiz, F. Lozano, R. Gonzalez-Amaro, P. Sanchez-Mateos, and F. Sanchez-Madrid. 1998. CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell-cell contacts. Blood. 91:4632–4644.

Short, D.B., K.W. Trotter, D. Reczek, S.M. Kreda, A. Bretscher, R.C. Boucher, M.J. Stutts, and S.L. Milgram. 1998. An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. J. Biol. Chem. 273:18452–18458.

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.

Tsukita, S., K. Oishi, N. Sato, J. Sagara, and A. Kawai. 1994. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J. Cell Biol. 126:391–401.

Turunen, O., T. Wahlstrom, and A. Vaheri. 1994. Ezrin has a COOH-terminal actin-binding site that is conserved in the ezrin family protein. J. Biol. Chem. 269:14545–14553.

Tsyk, M.J., and M.S. Moosker. 2002. MOY1 (brush border myosin I) dynamics in the brush border of LLC-PK1-CL4 cells. Biol. Cells. 82:1869–1883.

Yonemura, S., M. Hirao, Y. Doi, N. Takahashi, T. Kondo, and S. Tsukita. 1998. Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-cytoplasmic membrane component of CD44, CD43, and ICAM-2. J. Cell Biol. 140:885–895.

Yun, C.H., S. Oh, M. Zizak, D. Steplock, S. Tsao, C.M. Tse, E.J. Weinmann, and M. Donowitz. 1997. A cAMP-mediated inhibition of the epithelial brush border Na\(^{+}/H\(^{+}\) exchanger, NHE3, requires an associated regulatory protein. Proc. Natl. Acad. Sci. USA. 94:3010–3015.