PDZ-domain-directed basolateral targeting of the peripheral membrane protein FRMPD2 in epithelial cells

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Summary

Multi-PDZ (PSD-95/Discs large/Zonula-occludens-1) domain proteins play a crucial role in the establishment and maintenance of cell polarization. The novel multi-PDZ domain protein FRMPD2 is a potential scaffolding protein consisting of an N-terminal KIND domain, a FERM domain and three PDZ domains. Here we show that FRMPD2 is localized in a polarized fashion in epithelial cells at the basolateral membrane and partially colocalizes with the tight-junction marker protein Zonula-occludens-1. Downregulation of FRMPD2 protein in Caco-2 cells is associated with an impairment of tight junction formation. We find that the FERM domain of FRMPD2 binds phosphatidylinositols and is sufficient for membrane localization. Moreover, we demonstrate that recruitment of FRMPD2 to cell-cell junctions is strictly E-cadherin-dependent, which is in line with our identification of catenin family proteins as binding partners for FRMPD2. We demonstrate that the FERM domain and binding of the PDZ2 domain to the armadillo protein p0071 are required for basolateral restriction of FRMPD2. Moreover, the PDZ2 domain of FRMPD2 is sufficient to partially redirect an apically localized protein to the basolateral membrane. Our results provide novel insights into the molecular function of FRMPD2 and into the targeting mechanism of peripheral membrane proteins in polarized epithelial cells.

Supplementary material available online at
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Introduction

Proper subcellular localization of proteins and lipids is crucial for the establishment and maintenance of cell polarization (Shin et al., 2006; Bryant and Mostov, 2008). One of the best cellular models for cell polarization are polarized epithelial cells, which exhibit an apical surface facing the lumen and a basolateral surface facing neighbouring cells or the extracellular matrix. The asymmetric distribution of proteins to basolateral and apical membranes is an important feature of epithelial cell polarity. Apical and basolateral surfaces are separated by tight- and adherens-junctions and contain a different composition of membrane lipids and a different set of integral and peripheral membrane proteins (Shin et al., 2006; Martin-Belmonte and Mostov, 2008). Elucidating mechanisms for polarized localization of integral and peripheral membrane proteins is pivotal for the understanding of cell polarization.

For integral membrane proteins, vesicular trafficking routes and structural requirements for specific polarized targeting have been well documented and some common themes identified (Rodriguez-Boulan et al., 2005; Folsch, 2008). Membrane proteins can be either selectively delivered to the apical or basolateral membrane. Alternatively, membrane proteins can be first delivered to the basolateral membrane and then, after endocytosis, targeted to the apical domain. Finally, membrane proteins can be delivered in a non-polarized fashion to the plasma membrane but are subsequently stabilized and enriched either at the apical or basolateral membrane.

PDZ (PSD-95/Discs large/Zonula-occludens-1)-domain-mediated interactions play an important role in the polarized localization of many integral membrane proteins (Kim, 1997; Muth et al., 1998; Perego et al., 1999; Moyer et al., 1999; Milewski et al., 2001). PDZ domains are protein-protein interaction modules of about 80-90 amino acids in length and bind preferentially to the extreme C-terminus of their target proteins (Sheng and Sala, 2001; Nourry et al., 2003); some PDZ domains can also bind to phosphatidylinositols (Zimmermann, 2006; Wu et al., 2007). A very simplified categorization of PDZ domains has established four different classes according to the relative PDZ-binding motif (Harris and Lim, 2001): type I, X-(S/T)-X-Φ; type II, X-Φ-X-Φ; type III, X-(D/E)-X-Φ; and type IV, X-X-Ψ-(D/E), where Φ and Ψ represent a hydrophobic or an aromatic residue, respectively. Alternative classifications for PDZ domains have been suggested that take into account the observation that PDZ domains often bind to more than one consensus sequence (Bezprozvanny and Maximov, 2001; Kang et al., 2003). Recent proteome-wide PDZ-ligand interaction studies have led to a substantial revision of PDZ domain classifications. Tonikian et al. suggest at least 16 different specificity classes, whereas Stiffler et al. have given up the individual class model and suggest instead that PDZ domains lie on a binding continuum where the canonical PDZ domain classes reside only in select portions of this continuum (Stiffler et al., 2007; Tonikian et al., 2008).

Very often a protein contains multiple PDZ domains, leading to the establishment of large protein complexes with the multi-PDZ domain protein as a central scaffolding unit (Harris and Lim, 2001). In the case of integral membrane proteins, the PDZ-domain-mediated interactions either lead to a selective stabilization of the protein at the apical or basolateral membrane, or they play a role in selective receptor recycling to a specific membrane domain

(Perego et al., 1999; Milewski et al., 2001; Swiatecka-Urban et al., 2002). However, in many cases, integral membrane proteins require another localization signal in addition to a PDZ-domain-binding motif to ensure correct polarized protein distribution (Milewski et al., 2005). Finally, it has been suggested that PDZ-domain-mediated interactions play a direct role in sorting of membrane proteins at the trans-Golgi network (Maday et al., 2008).

Whereas the targeting mechanisms of many basolateral and apical integral membrane proteins are well documented, the knowledge of targeting mechanisms and structural requirements for polarized localization of peripheral membrane proteins is sparse. However, PDZ domains might also play an important role in localizing peripheral membrane proteins. In zebrafish, the apical localization of the ASIP/PAR-3 protein requires an oligomerization domain (CR1) and the PDZ domains (von Trotha et al., 2006). The *Drosophila* Scribble protein requires its PDZ2 domain for apical localization in neuroblasts (Albertson et al., 2004). However, localization mechanisms not directly involving PDZ-domain-mediated interactions exist as well. Recently, it has been shown for the LAP [LRR (leucine-rich repeats) and PDZ domain] protein LET-413 that the leucine-rich repeat domain is sufficient for its basolateral localization in *Caenorhabditis elegans* (Legouis et al., 2003). Alternatively, SAP97, a member of the membrane-associated guanylate kinase family (MAGUK) and the adapter protein mLin-7 are targeted to the basolateral membrane by interaction of their N-termini with the L27N domain of Lin-2/CASK (Lee et al., 2002; Alewine et al., 2007), which is another member of the MAGUK-family.

Here we present a novel mode for basolateral localization of the peripheral membrane protein FRMPD2 (FERM and PDZ-domain-containing 2). FRMPD2 is a novel potential scaffolding protein consisting of an N-terminal KIND (Kinase non-catalytic C-lobe domain) followed by a FERM (Four-point-one, ezrin, radixin, moesin) domain and three PDZ domains. Its modular structure resembles the modular structure of the protein tyrosine phosphatase PTP-BL but lacks two PDZ domains and a tyrosine phosphatase domain (Erdmann, 2003). The protein tyrosine phosphatase PTP-BL is highly expressed in polarized cells such as epithelial cells and neurons. It is involved in the regulation of cell migration, ephrin-B receptor signalling and cytokinesis (Palmer et al., 2002; Herrmann et al., 2003; Lai et al., 2007). In polarized epithelial cells it is localized at the apical membrane (Cuppen et al., 1998; Cuppen et al., 1999).

We show that FRMPD2 is expressed in many cell lines of epithelial origin and is selectively localized at the basolateral membrane in polarized epithelial cells. We demonstrate a potential role in tight junction formation. We show that basolateral localization of FRMPD2 requires the membrane binding activity of the FERM domain and the basolateral steering activity of its PDZ2 domain. We have identified a family of catenin proteins as binding partners for the PDZ2 domain. Calcium switch and knockdown experiments reveal that recruitment of FRMPD2 to cell-cell contacts is strictly dependent on E-cadherin. Thus, our data further extend the concept of PDZ-domain-mediated polarized localization of peripheral membrane proteins.

Results

The modular structure of the protein tyrosine phosphatase PTP-BL comprises an N-terminal KIND domain, followed by a FERM domain and several PDZ domains as well as a C-terminally located tyrosine phosphatase domain. In order to identify PTP-BL-related proteins we searched the human genome for proteins containing coexistent KIND, FERM and PDZ domains. This search identified the protein FRMPD2. FRMPD2 is a predicted protein (NP_001018081) encoded by a predicted mRNA (NM_001018071) derived from analysis of the human genome. Similarly to PTP-BL, the protein FRMPD2 consists of an N-terminal KIND domain followed by a FERM domain and three PDZ domains; however, in contrast to PTP-BL it lacks two PDZ domains and a protein tyrosine phosphatase domain (Fig. 1A). To our knowledge no further studies regarding this predicted protein are available, and because of its similarity to PTP-BL we decided to analyze this protein in more detail.

We cloned a cDNA corresponding to the predicted open reading frame of human FRMPD2 from brain- and kidney-cDNA libraries. 5′ and 3′ RACE PCRs did not reveal any additional coding regions

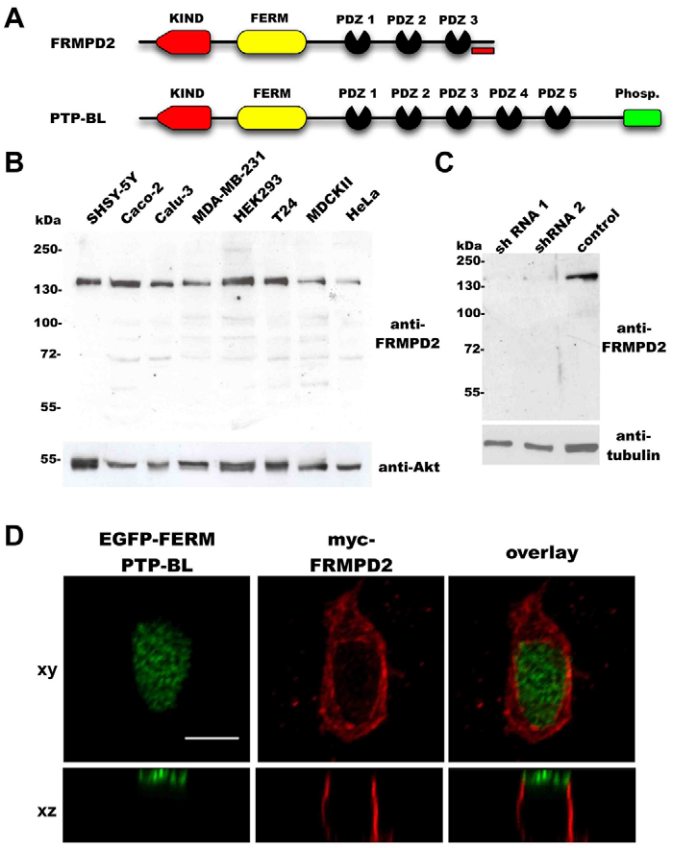

Fig. 1. Expression and localization of FRMPD2. (A) Modular structure of FRMPD2 and PTP-BL (Phosp., tyrosine phosphatase domain). The red bar indicates the region used to produce FRMPD2-specific polyclonal antibodies. (B) Endogenous expression of FRMPD2 in several cell lines. Total lysates of indicated cell lines were separated by SDS-PAGE and the proteins transferred to nitrocellulose. FRMPD2 was detected using a polyclonal anti-FRMPD2 antibody. (C) Downregulation of FRMPD2 using shRNA. SHSY-5Y cells were transiently transfected with FRMPD2-specific shRNA and corresponding control shRNA. Total lysates were separated by SDS-PAGE and the proteins transferred to nitrocellulose and probed with an anti-FRMPD2 antibody. (D) FRMPD2 is localized at the basolateral membrane in polarized MDCKII cells and does not colocalize with PTP-BL. MDCKII cells were grown on Transwell filters and transiently co-transfected with expression constructs for EGFP-FERM (PTP-BL) (green) and Myc-FRMPD2 (red). Cells were fixed and immunostained for Myc-FRMPD2. The orientation of the respective fluorescence microscopic cross-section is indicated on the left. Scale bar: 10 μm.

for FRMPD2. To prove the presence of the corresponding protein, we developed polyclonal antibodies raised against a GST-fusion protein comprising the last 150 amino acids of FRMPD2 (see Fig. 1A) and analyzed its potential expression in several cell lines. Using this antibody, we were able to detect an immunoreactive band in western-blots at 140 kDa in lysates of several cell lines. The molecular mass at 140 kDa matched the predicted molecular mass of 144kDa for FRMPD2 (Fig. 1B). To add further evidence that the immunoreactive band at 140 kDa corresponds to endogenous FRMPD2, we transfected SHSY-5Y cells with short hairpin RNA (shRNA) specifically designed to downregulate FRMPD2. We observed significant downregulation of the immunoreactive band at 140 kDa after transfection of FRMPD2-specific shRNA but not after transfection of a corresponding control shRNA (Fig. 1C). This supports the idea that the immunoreactive band indeed corresponds to endogenous FRMPD2.

We noticed that expression of FRMPD2 was observed in cell lines of epithelial origin; moreover the closely related protein PTP-BL is expressed mainly in epithelial cells and shows a restricted localization to the apical membrane domain. Interestingly, transfection of expression constructs for EGFP-tagged FRMPD2 into polarized MDCKII epithelial cells showed basolateral localization of the protein. To clearly demonstrate complementary localization of PTP-BL and FRMPD2 in epithelial cells and to exclude any potential cellular variation, we co-transfected MDCKII cells with expression constructs for the FERM domain of PTP-BL [the FERM domain is necessary and sufficient for apical localization of PTP-BL (Cuppen et al., 1999)] and for Myc-FRMPD2. As expected, the EGFP-FERM construct showed exclusive apical localization, whereas Myc-FRMPD2 was localized at the basolateral membrane (Fig. 1D). This localization of FRMPD2 was independent of its type of tagging (EGFP, Myc). The basolateral localization of EGFP-FRMPD2 was further corroborated using immunofluorescence microscopy, which showed its colocalization with endogenous β-catenin (Fig. 2A).

To reveal the localization of endogenous FRMPD2, we performed immunofluorescence experiments on human epithelial Caco-2 cells. Double immunofluorescence analysis using an anti-FRMPD2 antibody and an antibody against E-cadherin showed significant colocalization of both proteins at the basolateral membrane (Fig. 2B, top). However, some apical staining was also detected using the anti-FRMPD2 antibody. To test the specificity of our FRMPD2 staining in Caco-2 cells we pre-adsorbed the anti-FRMPD2 antibody with the corresponding antigen. Subsequent immunofluorescence analysis revealed complete loss of the basolateral staining but retention of the apical staining portion, suggesting the apical staining to be nonspecific (Fig. 2B, bottom). Furthermore, similar basolateral staining was observed using an unrelated commercially available antibody against FRMPD2 (see supplementary material Fig. S1).

We noticed that localization of EGFP-FRMPD2, although clearly overlapping with β-catenin localization, extends further to the apical region. Thus, we tested colocalization of EGFP-FRMPD2 with the tight-junction marker protein Zonula occludens-1 (ZO-1) and observed a significant overlap of both proteins, suggesting that FRMPD2 is also localized at tight junctions (Fig. 3A). In order to analyze a potential function of FRMPD2 at tight junctions we performed a calcium switch experiment on Caco-2 cells grown on Transwell filters and monitored reestablishment of tight junctions via measurements of the transepithelial electrical resistance (TER) in shRNA-treated cells. We observed a clear delay in the

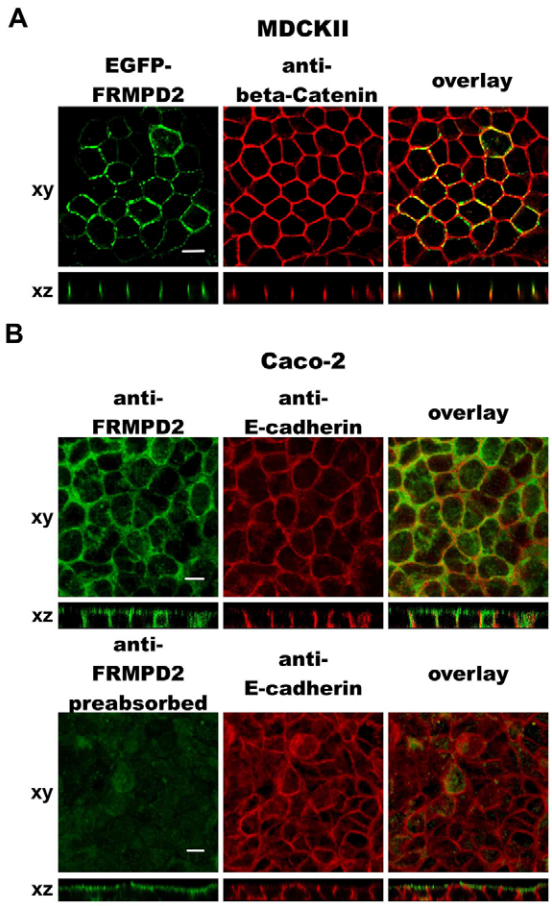

Fig. 2. Colocalization of FRMPD2 with basolateral marker proteins. (A) MDCKII cell lines stably expressing EGFP-FRMPD2 were grown on filters, fixed and immunostained for β-catenin. (B) Localization of endogenous FRMPD2 in Caco-2 cells. Caco-2 cells were grown on filters, fixed and immunostained with anti-FRMPD2 antibody (top panels) or anti-FRMPD2 antibody pre-absorbed with a GST-fusion protein comprising the last 150 C-terminal amino acids of FRMPD2 (lower panels) and anti-E-cadherin (red) antibody. Confocal images are shown; the orientation of the respective cross-section is indicated on the left. Scale bars: 10 μm.

reestablishment of tight junctions, as indicated by reduced TER in cells treated with FRMPD2-specific shRNA, compared to Caco-2 cells transfected with control shRNA (Fig. 3B). Successful downregulation of FRMPD2 protein was confirmed in parallel experiments using western blotting (Fig. 3C).

The similar modular structure of FRMPD2 and PTP-BL but their striking different subcellular localization in epithelial cells prompted us to perform a detailed analysis of the molecular determinants for basolateral localization of FRMPD2. First, we tested the individual domains fused to EGFP for subcellular localization (Fig. 4A,B). The KIND domain alone was found in a diffused pattern throughout the cytoplasm (Fig. 4B). Moreover, deletion of the KIND domain did not alter subcellular localization of FRMPD2 (see supplementary material Fig. S2). The FERM domain alone showed plasma membrane localization, but without any preference to the apical or basolateral membrane. The PDZ1-3 domains showed a high cytosolic pool with some minor enrichment at the plasma membrane. However, fusion of the FERM domain with the PDZ2 domain led to almost complete localization of the FERM-PDZ domain construct

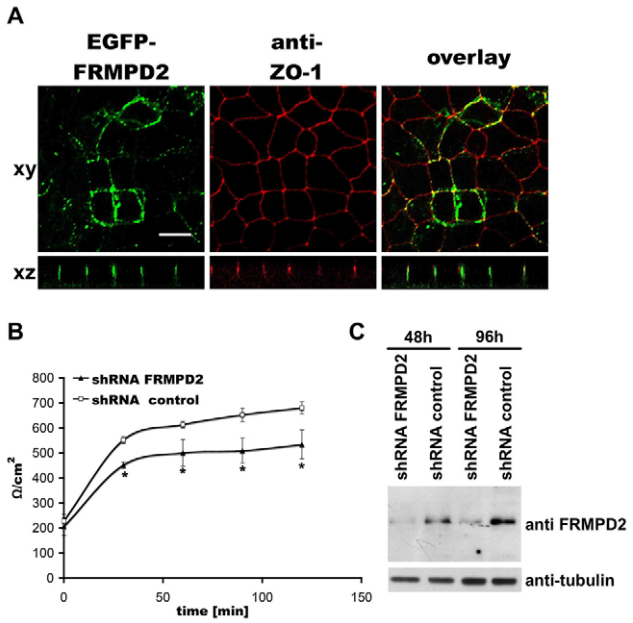

Fig. 3. Colocalization of FRMPD2 with tight-junction marker. (A) MDCKII cell lines stably expressing EGFP-FRMPD2 were grown on filters, fixed and immunostained for ZO-1. The orientation of the respective fluorescence microscopic cross-section is indicated on the left. Scale bar: 10 µm. (B) Tight-junction formation is delayed in Caco-2 cells with reduced FRMPD2 expression levels. Caco-2 cells were transfected with the indicated shRNA expression vectors and grown on filters. After 4 days, tight junctions were dissolved by addition of EDTA and reassembly was allowed by changing back to calcium-containing medium. Reassembly of tight junctions was monitored by measuring the transepithelial electrical resistance (TER). The zero time point corresponds to the time point when EDTA-containing medium was replaced by normal calcium-containing medium. Values are the mean ± s.e.m., n=4 for siRNA control, n=5 for siRNA FRMPD2. Asterisks indicate time points with significant difference (P<0.01). (C) Specific downregulation of FRMPD2 in Caco-2 cells. Cells were transfected with the indicated shRNA expression vectors, total lysates were prepared at indicated time points after transfection, and FRMPD2 knockdown was determined by western blotting using anti-FRMPD2 antibody.

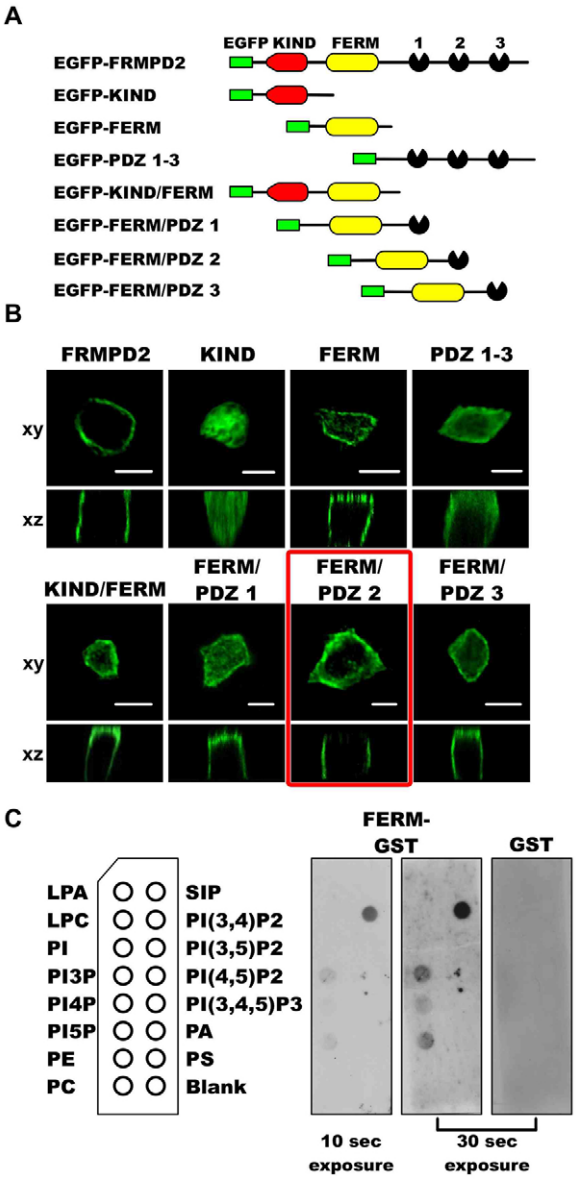

Fig. 4. Domain-specific localization analysis of FRMPD2. (A) Modular structure of FRMPD2 and corresponding deletion constructs used for localization analysis of FRMPD2. (B) The FERM and PDZ2 domains of FRMPD2 are sufficient for basolateral localization. Confocal images of EGFP constructs transfected into MDCKII cells are shown as summarized in A. MDCKII cells were grown on filters and transiently transfected with the indicated expression constructs. Cells were fixed and EGFP-derived fluorescence monitored using confocal microscopy. The orientation of the respective cross-section is indicated on the left. Scale bars: 10 µm. (C) The FERM domain of FRMPD2 binds to phosphatidylinositols. PIP-Strips with the indicated immobilized phospholipids were incubated with GST-FERM protein or GST protein alone. Bound proteins were detected using anti-GST antibody followed by secondary antibody and chemiluminescence detection.

to the basolateral domain, whereas fusion with either PDZ1 or PDZ3 domain did not influence distribution of the FERM domain (Fig. 4A,B). The PDZ2 domain alone appeared to be cytosolic (see supplementary material Fig. S2). As described above, the FERM domain of FRMPD2 is sufficient for plasma membrane localization. Previously, membrane targeting of PTP-BL has been shown to depend on phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] binding of its FERM domain. To test whether the FERM domain of FRMPD2 also binds to phosphatidylinositols we performed a PIP-Strip overlay assay. Interestingly, the FERM domain of FRMPD2 binds specifically to PtdIns(3,4)P_2) and to a lesser extent also to phosphatidylinositol monophosphates (Fig. 4C), no binding instead was detected to PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3.

In summary, the minimal molecular unit that confers basolateral targeting is the membrane-binding activity of the FERM domain together with the PDZ2 domain.

To further address the importance of the PDZ2 domain for basolateral targeting of FRMPD2, we introduced a point mutation into the carboxylate-binding loop of the PDZ2 domain, exchanging lysine 955 to glutamate (Fig. 5A). Within PDZ domains this position is usually occupied by a basic amino acid and is crucial for the binding of the extreme C-terminus of the target protein. Introduction of this point mutation into full-length FRMPD2 led to complete abolishment of the basolateral restriction of FRMPD2 and to localization of FRMPD2 at the apical and the basolateral domains (Fig. 5B). The localization of FRMPD2 that harbours this point mutation is indistinguishable from the localization of the FERM domain of FRMPD2 alone (see Fig. 4B). Similarly, overexpression of an N-terminal truncated version of the FRMPD2 interaction

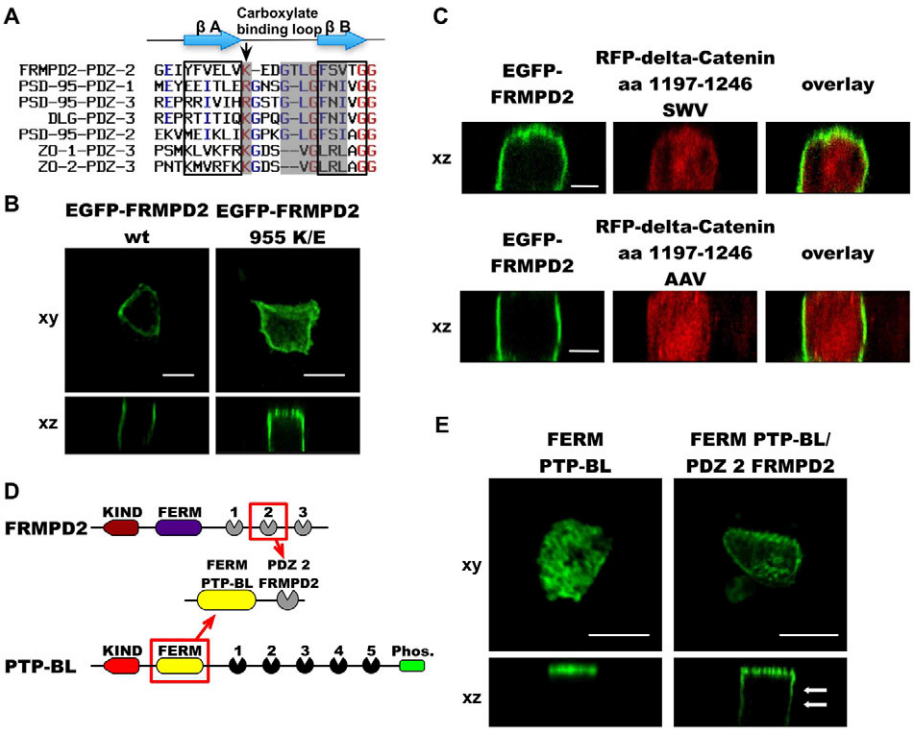

Fig. 5. The PDZ2 domain of FRMPD2 exerts basolateral steering activity. (A) Sequence comparison of the carboxylate-binding loop of different PDZ domains. The corresponding protein and the respective PDZ domain are indicated. The conserved basic amino acid within the carboxylate-binding loop is indicated by an arrow. (B) Point mutation of lysine 955 to glutamate leads to complete abolishment of basolateral restriction of FRMPD2. MDCKII cells were grown on filters and transiently transfected with EGFP–wild-type-FRMPD2 or with a mutant version of FRMPD2 harbouring a lysine-to-glutamate point mutation within the carboxylate-binding loop of the PDZ2 domain. Cells were fixed and EGFP-derived fluorescence was monitored using confocal microscopy. (C) Overexpression of the extreme C-terminus of δ-catenin leads to loss of basolateral restriction of EGFP-FRMPD2. MDCKII cells were co-transfected with expression vectors for EGFP-FRMPD2 and RFP-δ-catenin (amino acids 1197-1246 SWV) or RFP-δ-catenin (amino acids 1197-1246 AAV) harbouring an inactivated PDZ-domain-binding motif at the extreme C-terminus (SWV, wild type; AAV, mutant). Cells were fixed and analyzed using confocal microscopy. The *xz* view is shown. (D) Construction scheme for FERM (PTP-BL)-PDZ2 (FRMPD2). (E) The PDZ2 domain redirects the apically localized FERM domain of PTP-BL partially to the basolateral membrane. MDCKII cells were grown on filters and transiently transfected with EGFP-FERM (PTP-BL) or a construct coding for EGFP-FERM (PTP-BL) fused to the PDZ2 domain of FRMPD2. Cells were fixed and EGFP-derived fluorescence was monitored using confocal microscopy. Scale bars: 10 μm in B,E; 5 μm in C.

partner, δ-catenin (see Fig. 6), designed to block endogenous proteins from binding to PDZ2, led to abolishment of basolateral restriction of EGFP-FRMPD2 (Fig. 5C, upper panel). Using a corresponding δ-catenin construct that harboured point mutations to alanine at positions –1 and –2, which does not bind to FRMPD2 (see Fig. 6C), did not show any effect on FRMPD2 localization (Fig. 5C, lower panel).

Next, we asked whether the PDZ2 domain can also exert its basolateral steering activity on other FERM domains. We fused the FERM domain of PTP-BL, which is normally exclusively localized at the apical membrane, with the PDZ2 domain of FRMPD2 and analyzed the subcellular localization of this fusion construct in MDCKII cells (Fig. 5D,E). In clear contrast to the situation with the FERM domain of PTP-BL alone, we observed a significant portion of the fusion protein localized at the basolateral membrane (Fig. 5D). Thus, PDZ2 is able to partially redirect an apical protein to the basolateral membrane. To elucidate the potential mechanism by which the PDZ2 domain exerts its basolateral targeting activity, we searched for potential binding partners. Using the yeast two-hybrid system, we screened a human brain cDNA library with the PDZ1-3 domains of FRMPD2 as a bait. Among several potential interacting proteins, we identified one clone coding for the protein δ-catenin. δ-catenin has been shown before to interact with PDZ-domain-containing proteins and its basolateral localization as part of the cell-cell adhesion cadherin complex has been described (Lu et al., 1999), which made this protein a possible candidate for further analysis.

First, we looked at which PDZ domain of the bait protein was responsible for interaction with δ-catenin. Each individual PDZ domain was analyzed for interaction with δ-catenin using yeast two-hybrid assays (Fig. 6A). Only the PDZ2 domain showed an interaction with δ-catenin, and this interaction could be confirmed by pull-down assays using glutathione-*S*-transferase fusion proteins of the PDZ domains (Fig. 6B). Moreover, this interaction was highly specific for δ-catenin because no interaction could be detected using corresponding regions of α-, β- or γ-catenin (see supplementary material Fig. S3). The interaction is dependent on the extreme C-terminus of δ-catenin. Deletion of the last three amino acids or mutation of the –0 position to alanine completely abolished the interaction, as demonstrated by yeast two-hybrid assays (Fig. 6C). The proteins ARVCF and p0071, two additional catenin family members, are highly similar to δ-catenin and share significant sequence homology at the C-terminus (Fig. 6D). Using a yeast two-hybrid assay, we observed interaction of the PDZ2 domain of FRMPD2 with both ARVCF and p0071 (Fig. 6D). Interaction of full-length proteins was demonstrated for FRMPD2 with δ-catenin and with p0071 via co-immunoprecipitation using the corresponding transfected cells (Fig. 6E). These interactions were also dependent

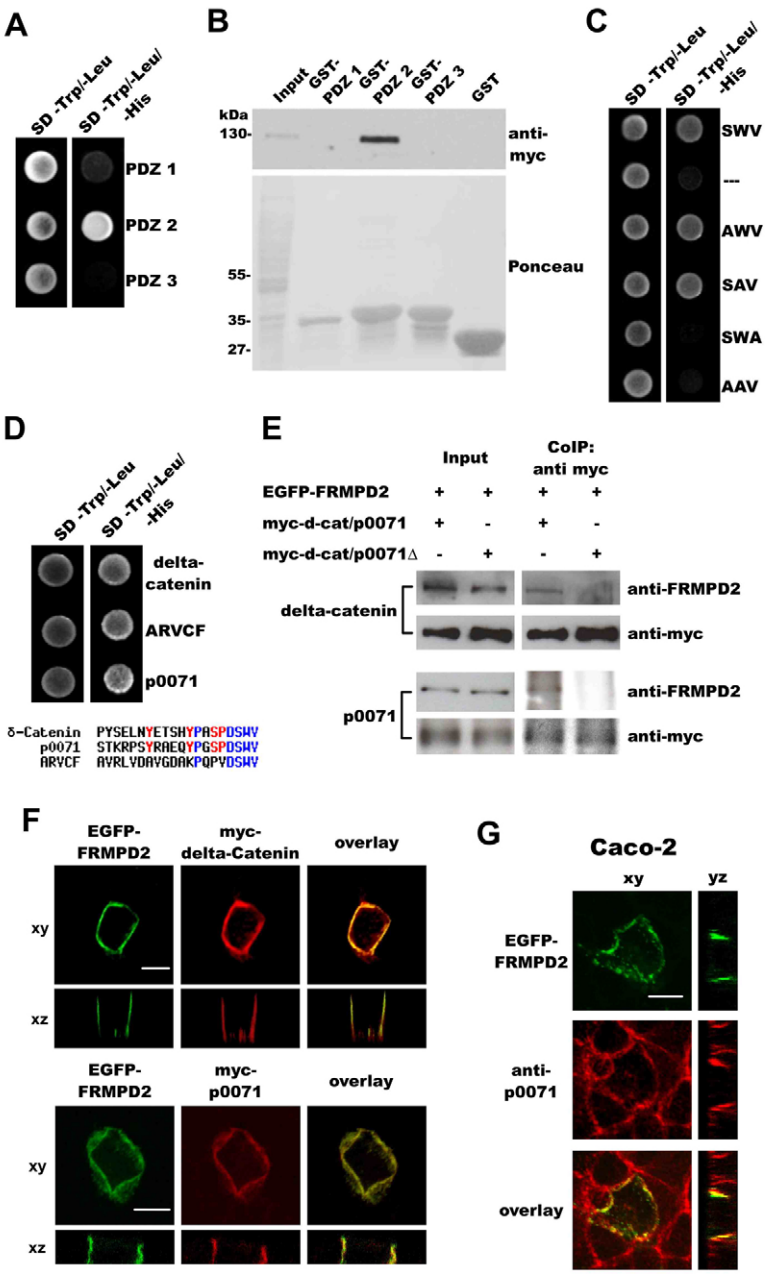

Fig. 6. FRMPD2 interacts with members of the catenin-family: (A) The PDZ2 domain of FRMPD2 interacts with δ-catenin. An expression construct for δ-catenin was co-transformed with expression vectors for the individual PDZ domains of FRMPD2 into yeast and plated on the indicated selective media. Growth on media deficient for trytophan (Trp), leucine (Leu) and histidine (His) indicates interaction of the prey- and bait-protein. (B) Pull-down experiment. HeLa cells were transiently transfected with an expression vector for Myc-δ-catenin and corresponding lysates were subjected to pull-down analysis using GST-fusion proteins of the indicated individual PDZ domains. Protein complexes were separated by SDS-PAGE, transferred to nitrocellulose and detected using an anti-Myc antibody. Corresponding GST-fusion proteins were visualized using Ponceau staining. (C) The C-terminus of δ-catenin is essential for FRMPD2 binding. Expression constructs for PDZ2 and for δ-catenin harbouring the indicated C-termini were co-transformed into yeast and plated on selective media as described in A. (D) The PDZ2 domain of FRMPD2 interacts with δ-catenin, ARVCF and p0071. Upper panel: Yeast two-hybrid assay. Expression constructs for PDZ2 and the indicated catenin members (the last C-terminal 150 amino acids) were co-transformed into yeast and two-hybrid assays were performed as above. Lower panel: Sequence comparison of the extreme C-terminal region of the indicated catenin family members. (E) Interaction of full-length FRMPD2 with full-length δ-catenin or p0071. Lysates of HeLa cells transiently transfected with EGFP-FRMPD2 and Myc-tagged δ-catenin or p0071 or corresponding proteins lacking the last three amino acids (Myc-d-cat/p0071Δ) were subjected to immunoprecipitation using anti-Myc antibody. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose and detected with the indicated antibodies. (F) Colocalization of FRMPD2 with δ-catenin and p0071 in MDCKII cells. MDCKII cells were grown on filters and co-transfected with expression constructs for EGFP-FRMPD2 and Myc-δ-catenin (upper panel) or Myc-p0071 (lower panel). Cells were fixed and immunostained using anti-Myc antibody. (G) Colocalization of FRMPD2 and p0071 in Caco-2 cells. Caco-2 cells were grown on filters and transfected with expression construct for EGFP-FRMPD2. Cells were fixed and stained for endogenous p0071 using anti-p0071 antibody. Confocal images are shown. Scale bars: 10 μm.

on the presence of the PDZ-binding motifs in the respective catenins because deletion of the last three amino acids led to abolishment of co-immunoprecipitation (Fig. 6E). Furthermore, interaction of δ-catenin and p0071 with FRMPD2 was supported by colocalization of these proteins at the basolateral membrane after co-transfection of expression constructs for Myc-δ-catenin or Myc-p0071 and for EGFP-FRMPD2 into MDCKII cells (Fig. 6F). Finally, EGFP-FRMPD2 also showed strong colocalization with endogenous p0071 in epithelial Caco-2 cells (Fig. 6G).

Next, we tested whether p0071 or ARVCF are responsible for basolateral restriction of FRMPD2 (δ-catenin is not expressed in epithelial cells but is expressed in neural cells). Using an siRNA approach, we downregulated ARVCF and p0071 individually or together in Caco-2 cells and monitored localization of EGFP-FRMPD2. Whereas downregulation of ARVCF did not change the distribution of EGFP-FRMPD2, we observed a strong delocalization of EGFP-FRMPD2 to the apical membrane domain after knockdown of p0071 (Fig. 7A). Western blot analyses performed in parallel confirmed an almost complete knockdown for ARVCF and p0071 (Fig. 7B). In fact, we completely lost any specific immunofluorescence signal for p0071 in Caco-2 cells transfected with siRNA directed to p0071 (data not shown). The abolishment of basolateral restriction of FRMPD2 in the case of p0071 knockdown was not due to a complete disturbance of the epithelial sheet architecture because basolateral E-cadherin localization appeared to be normal (Fig. 7C). Thus, we conclude that basolateral restriction of FRMPD2 requires the presence of p0071 in Caco-2 cells.

Given the binding of p0071 to E-cadherin and its role in cell-cell adhesion (Hatzfeld et al., 2003), we speculated that FRMPD2 is preferentially recruited to positions of cell-cell contact. In fact,

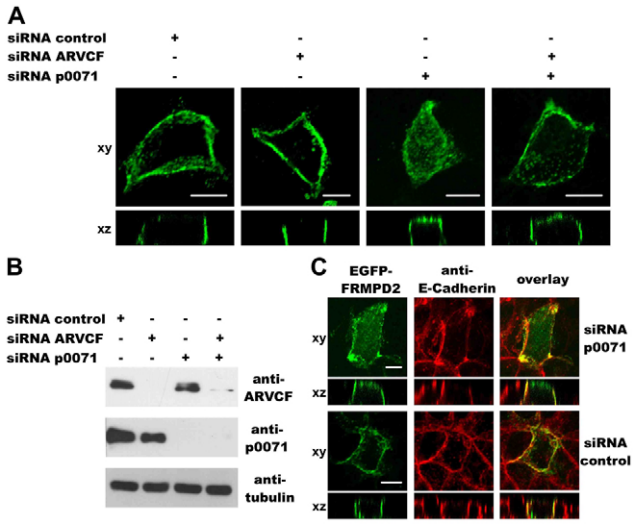

Fig. 7. Knockdown of p0071 protein but not knockdown of ARVCF leads to abolishment of basolateral restriction of FRMPD2. (A) Caco-2 cells were transfected with the indicated siRNAs. After two days, siRNA transfection was repeated and co-transfection with an expression vector for EGFP-FRMDP2 was performed. Cells were grown on Transwell filters, fixed 96 hours after the first transfection and EGFP-derived fluorescence monitored using confocal microscopy. (B) Western blots corresponding to transfections shown in A confirm successful downregulation of p0071 and ARVCF in Caco-2 cells. Caco-2 cells were transfected as described in A and total lysates were separated by SDS-PAGE and transferred to nitrocellulose. Blots were detected using the indicated antibodies. (C) Cells with reduced p0071 expression levels retain basolateral restriction of E-cadherin but lose basolateral restriction for FRMPD2. Cells were processed as described in A and stained for endogenous E-cadherin. The orientation of the respective fluorescence microscopic cross-sections is indicated on the left. Scale bars: 10 μm.

we observed strong accumulation of FRMPD2 at positions of cell-cell contact in MDCKII cells, whereas membrane localization of FRMPD2 in the same cells at positions without cell-cell contacts was weak (supplementary material Fig. S4). In order to analyze the potential relevance of E-cadherin engagement for basolateral restriction and cell-cell contact recruitment of FRMPD2 in epithelial cells, we performed a calcium switch experiment using MDCKII cells stably expressing EGFP-FRMPD2. The disengagement of E-cadherin induced by incubation of MDCKII cells in low calcium medium led to cell rounding and delocalization of EGFP-FRMPD2 (Fig. 8A). EGFP-FRMPD2 became partially cytosolic and distributed along the entire cell membrane. Increasing the calcium concentration led to re-recruitment of FRMPD2 to the basolateral membrane (Fig. 8A, lower panels).

To obtain further evidence that junctional localization of FRMPD2 is E-cadherin dependent, we analyzed its localization in HeLa cells before and after transfection of E-cadherin. HeLa cells do not express endogenous E-cadherin and transiently transfected EGFP-FRMPD2 showed a homogenous distribution along the entire plasma membrane according to the membrane binding capability of the FERM domain (Fig. 8B, top). However, after expression of E-cadherin in HeLa cells by transient transfection we observed a striking redistribution of EGFP-FRMPD2 with strong accumulation of EGFP-FRMPD2 at E-cadherin-positive cell-cell contacts (Fig. 8B, bottom). Next, we tested the reciprocal approach by downregulating endogenous E-cadherin in Caco-2 cells. In Caco-2 cells, endogenous FRMPD2 is strongly accumulated at cell-cell contacts positive for E-cadherin (Fig. 8D). However, siRNA-mediated knockdown of E-cadherin to almost undetectable levels led to a redistribution of FRMPD2 along the plasma membrane with no enrichment at cell-cell contacts (Fig. 8C,D). In summary, the results strongly support an E-cadherin-dependent localization of FRMPD2 at sites of cell-cell contacts in epithelial cells.

Discussion

Many integral membrane proteins rely on PDZ-domain-mediated interactions with peripheral membrane proteins for proper polarized localization (Moyer et al., 1999; Muth et al., 1998; Perego et al., 1999; Peng et al., 2000; Kuwahara et al., 2005). However, the mechanisms and structural requirements for polarized localization of these peripheral membrane proteins are often unclear. Here we have revealed the localization mechanism of the novel multi-PDZ domain protein FRMPD2. This protein was predicted from sequence analysis of the human genome. The modular structure of FRMPD2 resembles the structure of the protein tyrosine phosphatase PTP-BL but lacks two PDZ domains and the catalytic tyrosine phosphatase domain (Erdmann, 2003). FRMPD2 does not represent a truncated version of a longer protein for the following reasons: Firstly, we performed 5′ and 3′ RACE PCRs for FRMPD2 using brain and kidney cDNAs but could not detect any additional coding regions; in fact, we confirmed the stop codon of the predicted mRNA. Secondly, no expressed sequence tags of longer versions were identified in the several data bases analyzed. Thirdly, our polyclonal antibody does not recognize any additional bands larger than the predicted molecular weight. However, alternative splice variants might exist.

PTP-BL expression is largely restricted to polarized cells (Cuppen et al., 1998; Thomas et al., 1998). Given the similarity of FRMPD2 and PTP-BL, we analyzed the expression of FRMPD2 in several cell lines of epithelial or neuronal origin. In all cell lines examined, we were able to detect an immunoreactive band of about 140 kDa although to various extent. This immunoreactive band could be downregulated using shRNA specific for FRMPD2, indicating that it corresponds to endogenous FRMPD2. Our transfection experiments using tagged FRMPD2 expression constructs revealed a basolateral localization of FRMPD2 in polarized MDCKII cells. This localization was confirmed for endogenous FRMPD2 using Caco-2 epithelial cells. We also observed partial colocalization of EGFP-FRMPD2 with ZO-1 at the very apical region in MDCKII cells, indicating a potential localization at tight junctions as well. We have observed a delayed reconstitution of tight junctions after calcium switch in Caco-2 cells with downregulated expression levels of FRMPD2, suggesting a positive role of FRMPD2 in the regulation of tight junction formation.

A similar positive role has been already described for other PDZ domain proteins, like the multi-PDZ domain protein PATJ [protein associated with Lin seven 1 (PALS1)/Stardust and PALS1-associated tight junction protein] or the MAGUK protein MPP7 (Shin et al., 2005; Stucke et al., 2007). Both PATJ and MPP7 facilitate tight junction assembly; however, the precise function of FRMPD2 with respect to tight junction formation is currently not clear but it could be related to the dynamic stability of the tight junctions, either directly or more indirectly by regulating the dynamic of adherens junctions.

The basolateral localization observed here for FRMPD2 is in clear contrast to the localization of the closely related PTP-BL protein, which is an apical peripheral membrane protein in polarized epithelial cells (Cuppen et al., 1998; Cuppen et al., 1999). Our

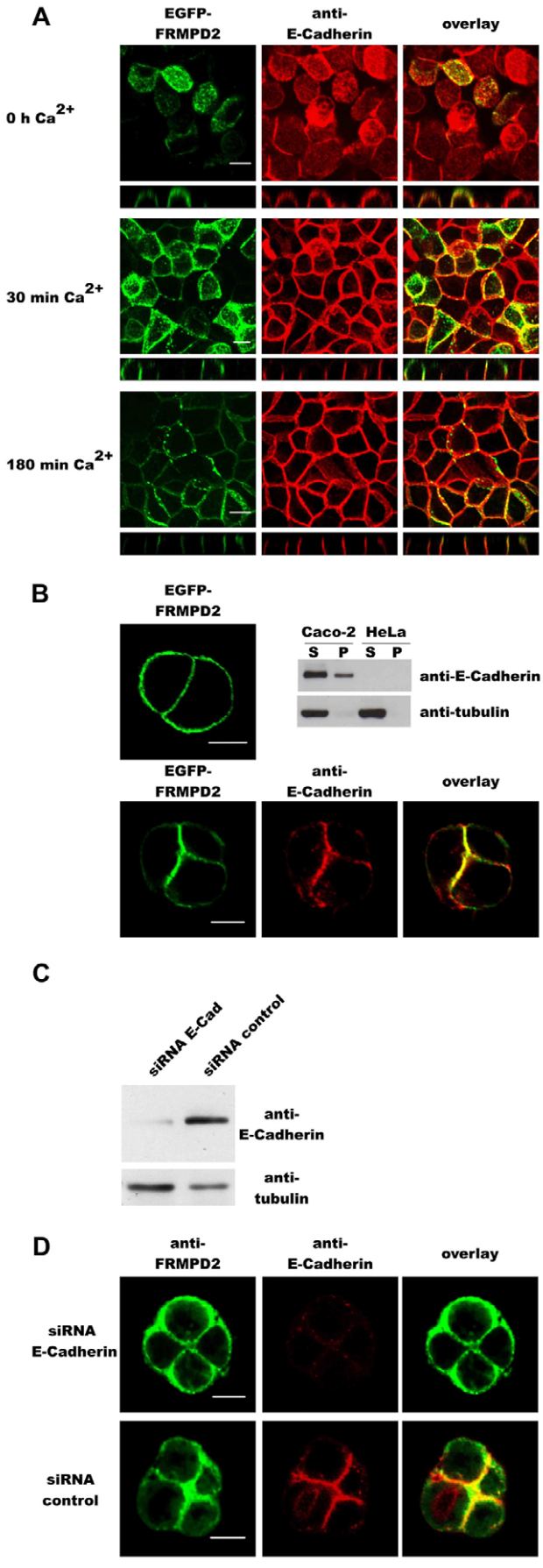

Fig. 8. FRMPD2 is recruited to sites of cell-cell adhesion in an E-cadherin-dependent manner. (A) Calcium switch experiment. MDCKII cells stably expressing EGFP-FRMPD2 were grown overnight in media containing low calcium. Medium was exchanged to medium containing normal calcium concentration (1.8 mM) and cells were fixed and immunostained for E-cadherin. The time after changing the medium is indicated. Combined stacks of confocal images are shown. (B) Ectopic expression of E-cadherin in HeLa cells leads to accumulation of EGFP-FRMPD2 at E-cadherin-positive junctions. Upper right panel: HeLa cells do not express endogenous E-cadherin. Triton-soluble (S) and Triton-insoluble (P) fractions of Caco-2 and HeLa cells were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-E-cadherin antibody or anti-tubulin antibody, respectively. Upper left panel: HeLa cells were transfected with an expression vector for EGFP-FRMPD2, and EGFP-FRMPD2 distribution monitored by confocal microscopy. Lower panel: HeLa cells were co-transfected with an expression vector for E-cadherin and EGFP-FRMPD2. Cells were fixed and immunostained for E-cadherin. Confocal images are shown. (C) Caco-2 cells were transfected twice with the indicated siRNAs. After 96 hours, cells were lysed and lysates separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-E-cadherin antibody or anti-tubulin antibody, respectively. (D) Caco-2 cells were transfected twice with the indicated siRNAs, 96 hours after the first transfection, cells were fixed and immunostained for endogenous FRMPD2 (green) and E-cadherin (red). Scale bars: 10 μm.

domain-specific localization analysis identified the FERM domain together with the PDZ2 domain as the minimal unit sufficient for basolateral localization. FERM domains have been shown to link the plasma membrane with the cytoskeleton (Chishti et al., 1998; Bretscher et al., 2002). To exert this function, many FERM domains can bind to integral membrane proteins and/or to phosphatidylinositols (Niggli et al., 1995; Yonemura et al., 1998). In the case of PTP-BL, the FERM domain is sufficient to target the protein to the apical membrane, and this membrane targeting is mediated by binding to PtdIns(4,5)P_2 (Bompard et al., 2003). In addition, association of the FERM domain of PTP-BL with filamentous actin (F-actin) has been described (Herrmann et al., 2003), which could also contribute to its localization at the F-actin-rich apical membrane compartment. Here we show that the FERM domain of FRMPD2 also binds to phosphatidylinositols but, in contrast to PTP-BL, the preferred phosphatidylinositol is PtdIns(3,4)P_2. This is of particular interest since phosphatidylinositols are important regulator of epithelial cell polarization. Whereas PtdIns(4,5)P_2 is enriched at the apical domain (which fits with apical localization of PTP-BL), PtdIns(3,4,5)P_3 is enriched at the basolateral domain of epithelial cells (Martin-Belmonte et al., 2007; Gassama-Diagne et al., 2006). One major pathway to synthesize PtdIns(3,4)P_2, which binds to the FERM domain of FRMPD2, is by dephosphorylation of PtdIns(3,4,5)P_3 using inositol-5-phosphatases. Thus, the complementary localization of FRMPD2 and PTP-BL is also reflected by the different phosphatidylinositol-binding specificities of their respective FERM domains.

As shown above, basolateral restriction of FRMPD2 also requires its PDZ2 domain. In line with this finding, a point mutation in the PDZ2 domain, which interferes with target binding, abolishes basolateral restriction of full-length FRMPD2. Here we have shown specific binding of the PDZ2 domain to a group of highly related members of the catenin family (p0071, ARVCF, δ-catenin), implicating a cadherin-dependent localization mechanism for FRMPD2 (Hatzfeld and Nachtsheim, 1996; Sirotkin et al., 1997; Lu et al., 1999). In fact, direct interaction with the cytoplasmic domain of E-cadherin has been demonstrated for each of these catenins (Calkins et al., 2003; Mariner et al., 2000; Lu et al., 1999).

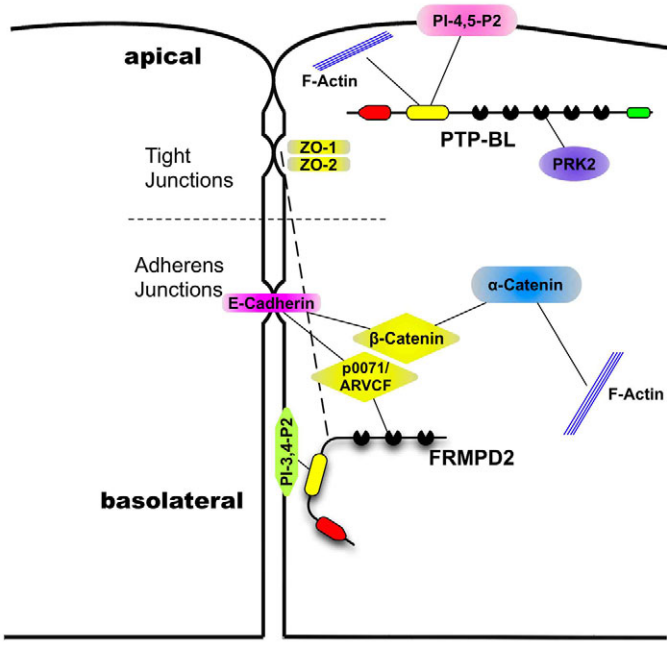

Fig. 9. Overview of the localization of FRMPD2 and PTP-BL in an epithelial cell and illustration of the corresponding molecular interaction network. PRK2, protein-kinase-C-related kinase 2; PI-4,5-P2, phosphatidylinositol-4,5-bisphosphate; PI-3,4-P2, phosphatidylinositol-3,4-bisphosphate.

The catenin family members p0071, ARVCF and δ-catenin share all an identical extreme C-terminus (DSWV) and we have demonstrated that this C-terminus is important for FRMPD2 binding. However, other PDZ-domain-containing proteins have been identified as binding partners for p0071, ARVCF and δ-catenin. For instance, ARVCF binds ZO-1, which leads to a partial localization of ARVCF at tight junctions (Kausalya et al., 2004). Interestingly, we also observed partial colocalization of FRMPD2 with ZO-1. All three catenin members bind to the LAP protein erbin. δ-catenin, which is highly expressed in the nervous system, interacts with the synaptic scaffolding multi-PDZ domain protein S-Scam/Magi-2 (Izawa et al., 2002; Jaulin-Bastard et al., 2002; Laura et al., 2002; Ide et al., 1999). Here we have used an siRNA approach to demonstrate that the interaction between FRMPD2 and p0071 protein is required for basolateral restriction of FRMPD2 in Caco-2 cells. Interestingly, although ARVCF also binds to FRMPD2 we did not observe an effect on FRMPD2 localization after ARVCF knockdown. A possible explanation for this difference could be different expression levels of both proteins in Caco-2 cells or subtle differences in binding affinity to FRMPD2.

Interaction with members of the catenin family implied a potential E-cadherin-dependent basolateral restriction of FRMPD2. We performed a calcium switch experiment to look at a role for E-cadherin in FRMPD2 localization. Disengagement of E-cadherin by lowering the calcium concentration in the medium led to redistribution of FRMPD2 to the entire plasma membrane. This effect was reversible, supporting an E-cadherin-dependent localization of FRMPD2. Furthermore, we present two additional lines of evidence for E-cadherin-mediated junctional recruitment of FRMPD2. First, redistribution of FRMPD2 with strong accumulation at cell-cell junctions was demonstrated after transient transfection of E-cadherin in HeLa cells. Second, specific downregulation of endogenous E-cadherin led to loss of accumulation of endogenous FRMPD2 at cell-cell contacts. Previously, it has been reported that mammalian scribble, another peripheral multi-PDZ domain membrane protein, relies on E-cadherin-mediated cell-cell adhesion for junctional recruitment (Navarro et al., 2005). However, how mammalian scribble is connected to the E-cadherin complex at the molecular level remains to be shown. The fact that peripheral membrane proteins provide binding sites for many integral membrane proteins important for their polarized localization raises the issue of how these peripheral membrane proteins themselves are anchored at the plasma membrane in a polarized fashion. Obvious candidates that could serve as suitable anchor points for basolateral peripheral membrane complexes are cell adhesion molecules like cadherins or nectins. Cadherin function is tightly regulated by cell-cell contacts, and cell-cell contact is one of the first triggers for establishing cellular asymmetry, which leads eventually to cell polarization (Takai and Nakanishi, 2003). Thus, we propose a model in which the membrane-binding activity of the FERM domain, together with the binding capacity of the PDZ2 domain to p0071, determines E-cadherin-dependent basolateral localization of FRMPD2 (Fig. 9). This model is reminiscent of the localization model proposed for scribble in fly neuroblasts, where a combination of the cortical targeting activity of the leucine-rich repeat domain together with the PDZ2 domain leads to apical localization of scribble (Albertson et al., 2004). Interestingly, the basolaterally localized LAP proteins erbin and densin-180 also interact via their PDZ domains with p0071, ARVCF and δ-catenin (Izawa et al., 2002; Jaulin-Bastard et al., 2002; Laura et al., 2002). This suggests that molecular connection to cell-cell adhesion systems might be a common mechanism for basolateral restricted localization of many peripheral membrane proteins, as shown here for FRMPD2.

Materials and Methods

DNA constructs

Full-length cDNA of FRMPD2 was cloned from foetal human brain and human kidney cDNA libraries (Clontech). Subcloning was performed using standard PCR-techniques using PfuUltr II Fusion HS DNA polymerase (Stratagene). Cloning vectors were pEGFP-C1 (Clontech) or pcDNA3 vector (Invitrogen) harbouring an N-terminal Myc-Tag. Subcloning was performed using SacII/XmaI or BamHI/NotI sites. Insertion of point mutations was performed using splicing by the overlapping-PCR technique. N-terminal-truncated δ-catenin was generated by fusing amino acids 1197-1246 of mouse δ-catenin to monomeric red fluorescence protein (RFP). EGFP–δ-catenin was a generous gift from Kenneth S. Kosik (University of California, Santa Barbara, CA). cDNA of full-length p0071 was kindly provided by Mechthild, Hatzfeld (Martin Luther University, Halle, Germany). The plasmid containing mouse E-cadherin [plasmid18804, (Onder et al., 2008)] was obtained from Addgene (Cambridge, MA). Plasmids containing shRNA against FRMPD2 and control shRNA were purchased from Origene (Rockvillle, MD). A small interfering RNA (siRNA) to inhibit E-cadherin expression was generated according to Hayashida et al. with the target sequence 5′-CAGACAAAGACCAGGACTA-3′ (Hayashida et al., 2005). A siRNA for ARVCF was generated with the target sequence 5′-CGTATGGCTTGGAGGATGA-3′. For knockdown of p0071 a siRNA was generated with the target sequence 5′-GATAACGATAGAGTTGTTTCT-3′ as published (Wolf et al., 2006). A non-targeting siRNA was used as control for all experiments. All siRNAs were purchased from Dharmacon.

Yeast two-hybrid analysis

The Matchmaker GAL4 yeast two-hybrid system (Clontech) was used to identify interacting proteins for FRMPD2. Bait constructs were cloned into the expression vector pGBT9 and corresponding prey constructs were made in pGADGH vector. The yeast strain YRG2 was co-transformed with the indicated bait, and prey plasmids and double transformants were selected on minimal agar plates lacking leucine and tryptophan (SD –Leu/–Trp). Activation of the *HIS3* reporter gene was monitored by re-plating individual colonies on minimal medium agar plates lacking leucine, tryptophan, histidine and containing 0.5 mM 3-aminiotriazol. Activation of the *lacZ* reporter gene was analyzed using a β-galactosidase colony-lift filter assay

Antibodies

Mouse anti-E-cadherin (BD Biosciences), mouse anti-Myc (BD Biosciences), mouse anti-α-catenin (BD Biosciences), rabbit anti-ZO-1 (Zymed), rabbit anti-FRMPD2 (Sigma-Aldrich), mouse anti-p0071 (Clone 7.7.9, Progen), guinea pig anti-ARVCF (Progen) and anti-rabbit- or anti-mouse-Alexa-Fluor-488 and -594 (Molecular Probes) were purchased from commercial sources. Polyclonal antibodies against FRMPD2 were generated by immunizing rabbits with a glutathione-*S*-transferase fusion protein of the C-terminal region of human FRMPD2 (amino acids 1169-1309).

Cell culture and transfection

Madin-Darby canine kidney cells (MDCKII cells) were purchased from European Collection of Cell Cultures (ECACC) and maintained in minimal essential medium (MEM) containing 2 mM glutamine supplemented with 5% foetal calf serum (FCS), penicillin and streptomycin in 5% CO_2 atmosphere. MDA-MB-231 (American Type Culture Collection, ATCC), HeLa, HEK293 and T24 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose and containing 2 mM glutamine supplemented with 10% FCS and antibiotics in 10% CO_2 atmosphere. Caco-2 (ECACC) and Calu-3 (ATCC) cells were maintained in MEM supplemented with 2 mM glutamine, 15% FCS, antibiotics and non-essential amino acids in 5% CO_2 atmosphere. SHSY-5Y cells were cultured in a 1:1 mixture of DMEM high glucose and Ham's F12 with 10% FCS, glutamine and antibiotics in 5% CO_2 atmosphere. MDCKII and HeLa cells were transfected with Polyfect (Quiagen) according to the manufacturer's protocol. Localization analysis and western blot analysis, if not otherwise indicated, were performed 48 hours after transfection. MDCKII cells stably expressing EGFP-FRMPD2 or EGFP were generated by transfection with the corresponding expression vectors followed by selection using 700 μg/ml G418. Stable transfectants were subsequently enriched using fluorescence automated cell sorting (FACS) analysis. For depletion of proteins using siRNA, Caco-2 cells were transfected twice with Nucleofector Kit T (LonzaCologne, Cologne, Germany) within 48 hours. Cells were lysed or fixed 48 hours after second transfection.

Immunoprecipitation and immunoblotting

MDCKII and HeLa cells were lysed in lysis buffer [50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, protease inhibitors (Complete Tabs, Roche)]. For GST-pulldown assays, equal amounts of GST fusion proteins bound to glutathione Sepharose 4B (GE Healthcare) were incubated with cell lysates of HeLa cells transfected with the relevant expression constructs for 4 hours at 4°C. After three washing steps with lysis buffer, bound proteins were eluted in 2× Laemmli buffer for 10 minutes at 95°C and separated by SDS-polyacrylamide electrophoresis (SDS-PAGE).

For co-immunoprecipitation experiments, cell lysates were incubated for 2 hours with the indicated antibodies following 1.5 hours incubation with protein A Sepharose at 4°C. Washing and elution steps were carried out as described above. Proteins were transferred to nitrocellulose membrane, incubated with the relevant antibodies and detected by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Calcium switch experiments

For calcium switch experiments, MDCKII cells were grown to confluence on Transwell polyester membrane inserts (Corning Costar) for 5 days; medium was changed every second day. The MDCKII monolayers were washed twice with phosphate-buffered saline (PBS), followed by one washing step with PBS containing 5 mM EDTA and two washing steps with PBS, and maintained overnight in MEM Eagle (Spinner modification, Sigma-Aldrich) containing 5% dialysed FCS and 5 μM Ca^{2+} to dissociate cell-cell contacts. After 16 hours of incubation, the low calcium medium was removed and replaced with normal calcium media containing 1.8 mM Ca^{2+}. Cells were fixed and stained at the indicated time points.

Immunostaining and confocal microscopy

Confluent MDCKII and Caco-2 cells grown on Transwell filters or HeLa cells grown on cover slips were fixed in PBS containing 4% paraformaldehyde (PFA). For immunofluorescence analysis, cells were treated with 0.5% Triton X-100 for permeabilization for 15 minutes at room temperature and blocked with 1.5% bovine serum albumin overnight at 4°C. Filters were incubated with the relevant antibodies for 2 hours at room temperature followed by extensive washes with 1× PBS and mounted on glass slides using Vectashield Mounting Medium (Vector Laboratories). Confocal images were taken with a Leica TCS SP2 Laser Scanning Spectral Confocal Microscope. The provided Leica confocal software was used to create *xy* views that represent maximum or average projections of 30-50 horizontal sections, and *xz* or *xy* views that represent vertical sections through the cells. Images were further processed using Corel Photo Paint X3.

Protein-lipid overlay assay

PIP-Strip (Echelon) binding assays were performed according to the manufacturer's protocol using 3 μg of GST-FERM fusion protein or GST as control. After 1 hour of incubation in 3% nonfat dry milk, PIP-Strips were incubated with protein for 2 hours at room temperature followed by incubations with anti-GST antibody and horseradish-peroxidase-conjugated secondary antibody. Detection was performed using Super Signal West Pico Chemilumiscent Substrate (Pierce)

Measurement of transepithelial electrical resistance

Transepithelial electrical resistance (TER) was measured as described previously (Seth et al., 2007). In short, Caco-2 cells were transfected with shRNA-expressing vectors and cultured on 12-mm Transwell inserts for 4 days. Cells were treated with 1 mM EDTA in both the apical and basolateral compartment of the Transwell filters. TER was monitored using a Millicell-ERS electrical resistance electrode (Millipore, Bedford, MA) until the values were reduced to 15% of the basal values. The cells were washed three times with normal medium and incubated for varying times with continuous measurement of TER. Values are calculated in Ω/cm^2 and normalized for the Transwell surface. The resistance of the plain polyester membrane without cells was subtracted from all readings.

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