FRMD4A regulates epithelial polarity by connecting Arf6 activation with the PAR complex

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The Par-3/Par-6/aPKC/Cdc42 complex regulates the conversion of primordial adherens junctions (AJs) into belt-like AJs and the formation of linear actin cables during epithelial polarization. However, the mechanisms by which this complex functions are not well elucidated. In the present study, we found that activation of Arf6 is spatiotemporally regulated as a downstream signaling pathway of the Par protein complex. When primordial AJs are formed, Par-3 recruits a scaffolding protein, termed the FERM domain containing 4A (FRMD4A). FRMD4A connects Par-3 and the Arf6 guanine-nucleotide exchange factor (GEF), cytohesin-1. We propose that the Par-3/FRMD4A/cytohesin-1 complex ensures accurate activation of Arf6, a central player in actin cytoskeleton dynamics and membrane trafficking, during junctional remodeling and epithelial polarization.

Results

Considering that primordial AJs are formed between mesenchymal cells but formation of belt-like AJs occurs only in epithelial cells, it may be that unidentified components involved in this process are enriched in epithelial cells. To find previously unidentified components involved in this process, we compared the expression profiles of cultured epithelial cells (EpH4 cells) and EpH4 cells transformed to fibroblastic cells by exogenous expression of Snail (12). Among the genes up-regulated in epithelial cells, we searched for genes encoding proteins with a pleckstrin homology (PH) domain, because phosphatidyl inositol signaling has been implicated in cellular polarization and the determination of membrane identity (13, 14). In the clones that fulfilled both of the above criteria, we identified cytohesin-1 as a PH domain-containing protein (Fig. 1A). Cytohesin-1 was expressed at a fivefold or greater rate in EpH4 cells compared to EpH4-Snail cells (Fig. S1 A and B).

The expression profile of cytohesin-1 suggested by analysis of EST counts confirmed that cytohesin-1 was ubiquitously expressed in epithelial tissues (http://www.ncbi.nlm.nih.gov/UniGene/EST-ProfileViewer.cgi?uglist=Mm.86413). We raised a polyclonal antibody against cytohesin-1. Immunoblotting of EpH4 cell lysate with the cytohesin-1 pAb revealed a strong signal at 48 kDa, the expected molecular weight of cytohesin-1 (Fig. 1B). Then, we examined the subcellular localization of cytohesin-1. In mouse intestinal epithelial cells, cytohesin-1 was colocalized with ZO-1 at TJs (Fig. 1C). Next, we examined the behavior of cytohesin-1 during epithelial polarization, using the Ca2+ switch assay. At 0.15 h after Ca2+ repletion, cytohesin-1 was colocalized with ZO-1 at primordial AJs (Fig. 1 D and E). These observations indicate that cytohesin-1 is a component of both primordial AJs and TJs in epithelial cells.

Cytohesin-1 was originally characterized as a binding protein for the cytoplasmic domain of the integrin beta2 chain in Jurkat T cells (15); however, its function in epithelial cells remains totally unknown. Cytohesin-1 contains the amino (NH2)-terminal coiled-coil domain, the central Sec7 domain, and the carboxy (COOH)-terminal PH domain (Fig. 1D). Cytohesin-1 is a GEF for Arf6, and the Sec7 domain is the catalytic domain that stimulates guanine-nucleotide exchange of Arf6, a member of the Arf family small GTPases (16). Therefore, we examined whether cytohesin-1 and Arf6 are involved in epithelial polarization.

We first examined whether Arf6 was actually activated during epithelial polarization using affinity precipitation with GST-GGA3 (amino acids 1–316), which is known to bind to the GTP-bound form of Arf6 (17). Using the Ca2+ switch assay, we

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found that the GTP-bound Arf6 transiently increased during 0.15–0.25 h after Ca²⁺ repletion (Fig. 2 A and B). As the conversion of primordial AJs to belt-like AJs coincided with the activation of Arf6 (shown in Fig. S2), we next tested whether the activation of Arf6 is essential for epithelial polarization. We established EpH4 cells stably expressing shRNA against Arf6. Arf6 protein expression was suppressed by >75% as determined by Western blot analysis (Fig. 2C). Arf6-knockdown EpH4 cells showed retardation of belt-like AJ formation during epithelial cell polarization, as compared to the control-knockdown EpH4 cells (Fig. 2D). Furthermore, EpH4 cells transiently expressing a GFP-tagged dominant-negative, GTP binding-defective Arf6 T27N mutant also showed retardation of belt-like AJ formation during epithelial cell polarization, as compared to EpH4 cells transfected with GFP-tagged Arf6 (Fig. S3 A and B). Collectively, these data suggested that activation of Arf6 is involved in the epithelial polarization.

Cytohesin-1 is a member of the cytohesin family, which also includes ARNO/cytohesin-2, Grp-1/cytohesin-3, and cytohesin-4, and these proteins share the same domain organization and show remarkably high sequence similarities (~70–82% amino acid identity) (18). All four cytohesins were expressed in EpH4 cells (Fig. S1A). In addition to cytohesin-1, ARNO and Grp-1 were also concentrated at spot-like AJs and TJs in EpH4 cells (Figs. S1C and S3). To test the possibility that the GEF activities of these cytohesin family proteins are responsible for the activation of Arf6 during epithelial polarization, we took advantage of SecinH3, the newly identified chemical inhibitor of all cytohesin family proteins (17). We treated EpH4 cells with 20 μM SecinH3 during polarization after a Ca²⁺ switch. Treatment with SecinH3 blocked the transient activation of Arf6 in the initial stage of epithelial polarization (Fig. 2B). The formation of belt-like AJs was delayed in EpH4 cells treated with SecinH3 as compared to the control EpH4 cells (Fig. 2D).

Next, we attempted to determine which cytohesin(s) is responsible for this process. Previously, several groups reported that substitution of a Lys residue for a conserved Glu residue in the Sec7 domains of cytohesin family proteins abrogates GEF activity of cytohesin family proteins in vitro, and these catalytically inactive mutants of cytohesin family proteins act in a dominant-negative manner (19, 20). Overexpression of dominant-negative forms of cytohesin-1 and Grp-1, but not those of ARNO and cytohesin-4, blocked the formation of belt-like AJs after a Ca²⁺ switch, implying that cytohesin-1 and Grp-1 play essential roles in the epithelial polarization among cytohesin family proteins (Fig. S3 A and B).

We assessed the effect of inactivation of Arf6 and cytohesin family proteins on epithelial polarization by measurement of
transepithelial electrical resistance (TER). Arf6-knockdown EpH4 cells and control knockdown EpH4 cells were cultured in Transwell chambers under confluent conditions and submitted to a Ca\(^{2+}\) switch. After readdition of Ca\(^{2+}\), the TER developed slowly in Arf6-knockdown EpH4 and EpH4 cells treated with SecinH3, as compared to its development in the control EpH4 cells (Fig. 2F).

Thus, inactivation of both Arf6 and cytohesin family proteins affected maturation of functional TJs, suggesting that activation of Arf6 is restrictively controlled via cytohesin family proteins in the process of epithelial polarization.

How, then, is the activation of Arf6 by cytohesin family proteins regulated spatially and temporally during epithelial polarization? To identify the binding partner of cytohesin-1 at spot-like AJs and TJs, we employed a yeast two-hybrid system. Using full-length TJs, we employed a yeast two-hybrid system. Using full-length cytohesin-1 tagged at the C terminus, we identified FRMD4A as a binding partner of cytohesin-1 (Fig. 3A).

Because the coiled-coil domain of cytohesin-1 is necessary and sufficient for the localization at primordial AJs and TJs (Fig. S5), we examined whether the FRMD4A protein binds directly to the coiled-coil domain of cytohesin-1. The results obtained from the yeast two-hybrid assay indicated that amino acids 343–405 of FRMD4A are responsible for the interaction of cytohesin-1 (Fig. 3A). We confirmed the direct interaction between GST fusion proteins encoding the coiled-coil domain of cytohesin-1 (amino acids 1–100) and GFP-tagged FRMD4A (Fig. 3B). GFP-tagged FRMD4A was colocalized with cytohesin-1 at primordial AJs and TJs in EpH4 cells (Fig. 3 C and D). In addition to cytohesin-1, ARNO and Grp-1 also bound to FRMD4A (Fig. S6C). Collectively, these results demonstrated that FRMD4A binds cytohesin-1/ARNO/Grp-1 at primordial AJs and TJs in epithelial cells.

We next attempted to identify the binding partners of FRMD4A at primordial AJs and TJs. We examined the direct interaction between FRMD4A and proteins previously known as components of both primordial AJs and TJs, including ZO-1/ZO-2/ZO-3, Tiam-1, Par-3, AKPC, and Par6. Among them, only Par-3 showed binding to FRMD4A. Anti-FRMD4A antibodies coprecipitated Myc-tagged Par-3 in EpH4 cells that stably expressed a low level of Myc-tagged Par-3 (Fig. 3F). During the process of epithelial polarization, GFP-tagged FRMD4A and Par-3 were colocalized at primordial AJs and TJs (Fig. 3 G and H).

To determine the domains of FRMD4A responsible for the localization at primordial AJs and TJs, we examined the localization of a series of deletion mutants of FRMD4A. Among these deletion mutants, the amino acids 565–920 region of FRMD4A was necessary and sufficient for the localization at primordial AJs and TJs (Fig. 3E and Fig. S7). Therefore, it was expected that the amino acids 565–920 region of FRMD4A would directly interact with Par-3. An in vitro binding assay showed that the amino acids 565–920 region of Par3 bound to the amino acids 565–920 region of FRMD4A (hereafter, FRMD4A Par3BD) (Fig. 4 A and B). The fact that FRMD4A binds to both Par-3 and cytohesin-1 led us to postulate that FRMD4A mediates the interaction between Par-3 and cytohesin-1. To examine whether cytohesin-1, FRMD4A, and Par-3 form a ternary complex, HEK293 cells were transfected with the Myc-tagged amino acids 931–1,334 region of Par3, HA-tagged cytohesin-1, and GFP-tagged FRMD4A deletion constructs. HA-tagged cytohesin-1 was coimmunoprecipitated with the Myc-tagged amino acids 931–1,334 region of Par3 in the presence of full-length FRMD4A (Fig. 4C).

We then examined whether exogenous expression of FRMD4A can recruit exogenously expressed cytohesin-1 to Par-3-containing AJs in L fibroblasts stably expressing E-cadherin (EL cells) (21). Endogenous Par-3 was recruited to the ectopic AJs (Fig. 4D). FRMD4A lacking a Par-3 binding domain was localized in the cytoplasm together with cytohesin-1. Cytohesin-1 was recruited to Par-3-containing AJs in an FRMD4A-dependent manner (Fig. 4E).

Finally, we used siRNA against FRMD4A to evaluate the significance of the ternary complex composed of Par3, FRMD4A, and cytohesin-1 in epithelial polarization. Depletion of FRMD4A only slightly affected the formation of belt-like AJs and TJs (Fig. 5 B and D). To explore the possibility that there are other proteins that compensate for the lack of the FRMD4A protein, we performed a BLAST homology search and found that the FERM domain of FRMD4A shows high sequence similarity to the Grp-1 signaling partner (GRSP-1) (Fig. 5A).

GRSP-1 has been reported to bind to Grp-1 and colocalize with F-actin in membrane ruffles upon stimulation with insulin in CHO-T cells (22). The GFP-tagged GRSP-1 localized together with anti-GFP mAb (green) and anti-Par-3 pAb (red). (Scale bar, 10 μm.) (D) Xz section of a confocal image depicting colocalization of cytohesin-1 and FRMD4A. (E) Schematic drawings of deletion constructs of FRMD4A. The subcellular localization of these mutants is shown in Fig. S7. FRMD4A (amino acids 565–920) was sufficient and necessary for the localization at spot-like AJs and TJs. (F) Cell lysates derived from EpH4 cells stably expressing Myc-tagged Par-3 at a low expression level were subjected to immunoprecipitation with preimmune sera (control IgG) and anti-FRMD4A pAb. The immunoprecipitates were then analyzed with anti-Myc mAb. (G) After Ca\(^{2+}\) switch and a 0.15- or 24-h incubation, stable transfecants expressing GFP-FRMD4A were fixed and stained with anti-GFP mAb (green) and anti-cytohesin-1 pAb (red). (Scale bar, 10 μm.) (H) Xz section of a confocal image depicting colocalization of Par-3 and FRMD4A.

Fig. 3. Identification of FRMD4A as a binding partner of cytohesin-1 and Par-3. (A) The domain structure of mouse FRMD4A. (B) Interaction of FRMD4A and cytohesin-1. An in vitro binding assay was performed between GST fusion proteins encoding the coiled-coil domain of cytohesin-1 (amino acids 1–100) and GFP-tagged FRMD4A (Fig. 3B). GFP-tagged FRMD4A was colocalized with cytohesin-1 at primordial AJs and TJs in EpH4 cells (Fig. 3 C and D). In addition to cytohesin-1, ARNO and Grp-1 also bound to FRMD4A (Fig. S6C). Collectively, these results demonstrated that FRMD4A binds cytohesin-1/ARNO/Grp-1 at primordial AJs and TJs in epithelial cells.

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with Par-3 at spot-like AJs and TJs in EpH4 cells and at AJs in NIH 3T3 cells (Fig. S6A).

We tested the subcellular localization of a series of deletion mutants of GRSP-1 and found that the amino acids 542–972 region of GRSP-1 was necessary and sufficient for the localization at primordial AJs and TJs (Fig. S6D). Furthermore, the GST-tagged amino acids 542–972 region of GRSP-1 bound to Myc-tagged Par-3. GRSP-1 also bound directly to cytohesin-1 and ARNO in addition to Grp-1, suggesting that GRSP-1 can compensate for the loss of FRMD4A in FRMD4A-knockdown cells (Fig. S6D).

Therefore, we examined the effects of double knockdown of FRMD4A and GRSP-1 during epithelial polarization. We performed a Ca\(^{2+}\) switch assay in cells treated with FRMD4A siRNA, GRSP-1 siRNA, or both. We found that double knockdown of FRMD and GRSP-1 affected the formation of belt-like AJs and TJs (Fig. 5B). As expected, the localization of cytohesin-1 at primordial AJs disappeared from the cell–cell boundaries between double knockdown cells, supporting the idea that FRMD4A and GRSP-1 cooperatively recruit cytohesin-1 to primordial AJs (Fig. 5C).

We assessed the effect of depletion of FRMD4A and GRSP-1 on epithelial polarization by measuring the TER. The TER experiment clearly showed that double knockdown of FRMD4A and GRSP-1 in EpH4 cells led to the delayed formation of functional TJs (Fig. 5D).

Next, we quantified the percentage of cells that showed delayed epithelial polarization in siRNA-treated cells (Fig. 5E). EpH4 cells, EpH4 cells stably expressing GFP-tagged human FRMD4A, and EpH4 cells stably expressing GFP-tagged human GRSP-1 were treated with siRNA against FRMD4A and siRNA against GRSP-1. These cells were subjected to a Ca\(^{2+}\) switch assay. After Ca\(^{2+}\) switch and a 1-h incubation, the percentage of cells arrested at the “spot-like AJs” state was significantly increased in double-knockdown cells when compared with control knockdown cells (from 4.1% ± 0.83%–17.4% ± 4.2%) (P = 0.021) (Fig. 5E). Exogenous expression of GFP-tagged human FRMD4A or GFP-tagged human GRSP-1 totally rescued the phenotype caused by depletion of both FRMD4A and GRSP-1 (Fig. 5E).

These data clearly showed that the cooperative recruitment of cytohesin-1 to primordial AJs by FRMD4A and GRSP-1 and subsequent activation of Arf6 at primordial AJs are essential for the epithelial polarization.
Discussion
In the present study, we showed that Arf6 activation is spatially and temporally regulated during epithelial polarization by cytoskeleton family proteins, FRMD4A/GRSP-1, and the Par-3 complex (Fig. 5F).

Recently, several lines of evidence have suggested that Arf6 and its GEFs regulate the cell polarity in various systems, such as the cell polarization of neurons (23). Arf6 is reported to regulate dendritic branching in hippocampal neurons and neurite outgrowth in PC12 cells (19, 24). Moreover, in yeast, the Arf6 family proteins, FRMD4A/GRSP-1, and the Par-3 complex molecules (such as E-cadherin) that mediated cell-cell contacts induce the formation of primordial AJs. FRMD4A and GRSP-1 are recruited to primordial AJs through domain structures between mouse FRMD4A and human FRMD4A (18). One of the causes of this discrepancy may be the difference in cell lines between the two studies, but the precise reason is unclear. We cannot exclude the possibility that other GEFs for Arf6 cooperate in the formation of epithelial polarity together with cytoskeleton family proteins.

Finally, we briefly discuss the possible functions ascribed to Arf6 in epithelial polarization. First, Arf6 has been shown to activate PIP5-kinase and phospholipase D (30, 31). Phosphatidic acid, the product of phospholipase D, has been shown to function as a cofactor in the activation of PIP5-kinase (32). PIP5-kinase is responsible for generating PI(4,5)P2, which recruits numerous actin-binding proteins and induces drastic changes in the cortical membrane of TJs in MDCK cells (29). However, in EpH4 cells, EFA6 was localized throughout the entire plasma membrane and did not colocalize with ZO-1 at primordial AJs and TJs. On the other hand, cytoskeleton-1/Grp-1 colocalized precisely with ZO-1 at primordial AJs and TJs (Figs. S1D and S4). One of the causes of this discrepancy may be the difference in cell lines between the two studies, but the precise reason is unclear. We cannot exclude the possibility that other GEFs for Arf6 cooperate in the formation of epithelial polarity together with cytoskeleton family proteins.
actin network (33). Furthermore, a previous study reported that Arf6 changes the actin structure at the plasma membrane through activation of Rac1 (34). We previously found that Rac1 activation occurred upon the formation of primordial AJs (35). The ternary complex described in this study may be involved in the formation of linear actin cables and belt-like AJs through spatially restricted activation of PIP2-kinase and/or Rac1. Another important function of Arf6 is its role in endosome recycling (36). Recently, several lines of evidence indicated that Arf6 is involved in the membrane trafficking of E-cadherin (37, 38). Elucidation of the roles of Arf6 at primordial AJs is needed to understand the molecular mechanisms underlying epithelial polarization.

**Materials and Methods**

**Cell Culture, Ca2+ Switch Assay, and Transfection.** Mouse EpiH4 epithelial cells (generously provided by E. Reichmann, Institut Suisse de Recherches, Lausanne, Switzerland), EL cells (generously provided by A. Nagafuchi, Kumamoto University, Kumamoto, Japan), and NIH 3T3 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS. The low Ca2+ medium was the DMEM containing 5 μM calcium. In the Ca2+ switch assay, the cells were cultured in a low Ca2+ medium containing 5 μM Ca2+ overnight under confluent conditions, and their polarization was initiated by transferring to a normal Ca2+ medium.

For TER measurement and immunostaining of polarized EpiH4 cells, 1 x 10^6 cells were plated on Transwell polycarbonate filter supports with a pore size of 0.4 μm (Costar). Transfection was performed using Lipofectamine-Plus Reagent (Invitrogen) and Nucleofactor II (Akit, G-016 program; Amnaxa Biosystems) according to the manufacturer’s instructions.

Details of expression vectors, antibodies, reagents, immunofluorescence microscopy, yeast two-hybrid screening, TER measurement, in vitro binding assay, gel electrophoresis, and immunoblotting are provided in the **SI Text**.

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