Rab27 effector Slp2-a transports the apical signaling molecule podocalyxin to the apical surface of MDCK II cells and regulates claudin-2 expression

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ABSTRACT Most cells in tissues are polarized and usually have two distinct plasma membrane domains—an apical membrane and a basolateral membrane, which are the result of polarized trafficking of proteins and lipids. However, the mechanism underlying the cell polarization is not fully understood. In this study, we investigated the involvement of synaptotagmin-like protein 2-a (Slp2-a), an effector molecule for the small GTPase Rab27, in polarized trafficking by using Madin–Darby canine kidney II cells as a model of polarized cells. The results show that the level of Slp2-a expression in MDCK II cells increases greatly as the cells become polarized and that its expression is specifically localized at the apical membrane. The results also reveal that Slp2-a is required for targeting of the signaling molecule podocalyxin to the apical membrane in a Rab27A-dependent manner. In addition, ezrin, a downstream target of podocalyxin, and ERK1/2 are activated in Slp2-a–knockdown cells, and their activation results in a dramatic reduction in the amount of the tight junction protein claudin-2. Because both Slp2-a and claudin-2 are highly expressed in mouse renal proximal tubules, Slp2-a is likely to regulate claudin-2 expression through trafficking of podocalyxin to the apical surface in mouse renal tubule epithelial cells.

INTRODUCTION Cell polarity is a characteristic property of well-differentiated eukaryotic cells, and the physiological functions of polarized cells depend on the asymmetrical organization of cellular components, including the plasma membrane, organelles, and cytoskeletons. In epithelial cells—one of the most basic types of polarized cells—membranes and secretory proteins are delivered to specific regions of the plasma membrane, that is, the apical membrane and the basolateral membrane, which are separated by tight junctions, thereby defining and maintaining their unique identities and functions (Mostov et al., 2003; Rodriguez-Boulan et al., 2005; Rodríguez-Fraticelli et al., 2011). During the past few decades, more and more proteins involved in polarized membrane trafficking have been identified. The proteins have included members of the Rab-type small GTPases, conserved membrane-trafficking proteins in all eukaryotes (Fukuda, 2008; Stenmark, 2009), and their effectors, but their precise role in polarized trafficking is poorly understood.

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of the apical junctional region (AJR) of epithelial cells through recruitment to the AJR of moesin, one of the ezrin/radixin/moesin (ERM) family proteins that bind F-actin (Pilot et al., 2006). Because of the failure to form a stable, continuous network of actin filaments in the absence of Btzs, E-cadherin is not properly stabilized at the AJR of Drosophila embryonic epithelia (Pilot et al., 2006). Although there is no Btzs orthologue in mammals, since five homologous proteins, Slp1–5, have been reported to function as specific Rab27-binding proteins in secretory cells (Kuroda et al., 2002; Fukuda, 2005), certain Slp family members may have functions in mammalian epithelial cells that are similar to those of Btzs. However, involvement of mammalian Slp family members in polarized trafficking in epithelial cells has never been investigated.

In this study, we used Madin–Darby canine kidney (MDCK) II cells as a model of polarized cells and investigated the involvement of Slp2-a, originally described as a Rab27 effector in melanocytes and in particular secretory cells (Kuroda and Fukuda, 2004; Saegua et al., 2006; Yu et al., 2007; Holt et al., 2008), in polarized trafficking. The results show that Slp2-a regulates apical trafficking of podocalyxin-containing vesicles in MDCK II cells together with Rab27A and that podocalyxin transported to the apical surface transduces intracellular signals to express the tight junction protein claudin-2. We discuss the possible function of Slp2-a in claudin-2 expression in mouse renal epithelial cells based on our findings.

RESULTS
Slp2-a is specifically localized at the apical membrane in polarized MDCK II cells

We first investigated the expression of Slp2-a protein in MDCK II cells before and after the establishment of cell polarity. Immunoblot analysis with anti-Slp2-a–specific antibody indicated the presence of a much higher level of Slp2-a protein expression in confluent MDCK II cells than in subconfluent MDCK II cells (Figure 1A, top, and Supplemental Figure S1A, second panel). Furthermore, the level of Slp2-a protein expression in confluent MDCK II cells decreased when they were incubated with a Ca++-chelating reagent to disrupt cell–cell interaction (Supplemental Figure S1B), suggesting a close correlation between the level of Slp2-a protein expression and cell polarization. By contrast, the levels of expression of other Slp family members in subconfluent and confluent MDCK II cells were similar (Supplemental Figure S1A). Next we investigated the subcellular localization of endogenous Slp2-a protein by performing an immunofluorescence analysis. Although we were unable to detect any specific immunoreactive signals of endogenous Slp2-a protein in subconfluent MDCK II cells, presumably because of its lower expression level, immunoreactive signals of exogenously expressed enhanced green fluorescent protein (EGFP)–tagged Slp2-a were observed in the cytoplasm of subconfluent MDCK II cells, as well as at their plasma membrane (Figure 1B). By contrast, in polarized MDCK II cells immunoreactive signals of endogenous Slp2-a protein were observed specifically at the apical membrane. Moreover, Slp2-a was partially colocalized with its ligand Rab27A at the apical plasma membrane (Kuroda et al., 2002; Figure 1C, bottom right, arrowheads), suggesting that Slp2-a functions together with Rab27A at the apical membrane in polarized MDCK II cells.

Slp2-a regulates expression of the tight junction protein claudin-2

To investigate the role of Slp2-a in polarized MDCK II cells, we established two independent MDCK II cell lines with diminished Slp2-a expression by RNA interference technology using two different Slp2-a short hairpin RNAs (shRNAs; sites 1 and 2), which we named...
Slp2-a regulates claudin-2 expression

Slp2-a knockdown (KD) #1 cells and #2 cells, respectively. The levels of Slp2-a protein expression in Slp2-a KD #1 and #2 cells were S26 and 4.96%, respectively, of the level in parental MDCK II cells according to the results of an immunoblot analysis (Supplemental Figure S2A, lanes 3 and 4), whereas the level of Slp2-a protein expression in control shRNA-MDCK II cells (simply referred to as control cells later), which had been stably transfected with an empty shRNA expression vector, was similar to the level in the parental MDCK II cells (Supplemental Figure S2A, lane 2). Because the level of Slp2-a protein expression in the Slp2-a KD #2 cells was reduced much more than in the Slp2-a KD #1 cells, we used Slp2-a KD #2 cells for the subsequent analyses (unless otherwise specified, “Slp2-a KD cells” means Slp2-a KD #2 cells throughout the rest of this article). Staining of Slp2-a KD cells with phalloidin and anti-ZO-1 antibody revealed the typical cobblestone-like appearance of epithelial cells, although the surface area of the cells was slightly increased (control cells, 287.3 ± 6.7 μm²; Slp2-a KD cells, 448.4 ± 10.1 μm²; n = 70 cells from four independent experiments; p < 0.01; Supplemental Figure S2B, xy). Moreover, there was no difference in the distribution of the apical marker syntaxin-3 or the basolateral marker E-cadherin between the control cells and the Slp2-a KD cells (Supplemental Figure S2B, xz, third and fourth panels), indicating that the steady-state distribution of polarized membrane markers in the Slp2-a KD cells is normal.

We then evaluated the barrier function of the Slp2-a KD cells by measuring transepithelial electrical resistance (TER). Of interest, confluent monolayers of the Slp2-a KD cells had markedly higher TER values than the control cells (Figure 2A). Because alteration of the pattern of expression of tight junction proteins, especially of claudin-2 (Furuse et al., 2001), in MDCK cells has been shown to be related to different TER values, we investigated the protein expression of claudin-1 and claudin-2 by immunoblot analysis with specific antibodies. As expected, the level of claudin-2 protein expression was dramatically decreased in the Slp2-a KD cells, whereas the level of claudin-1 protein expression was the same as in the control cells (Figure 2B, second and third from top, lane 3). The claudin-2-deficiency in the Slp2-a KD cells is unlikely to have been attributable to off-target effects of the shRNAs, because a decreased claudin-2 protein level was also observed in another Slp2-a KD cell line (#1; Figure 2B, top and third from top, lane 2), and the level of claudin-2 protein expression was up-regulated by reexpression of an shRNA-resistant form of Slp2-a in the Slp2-a KD cells (Supplemental Figure S4, A and B). Note that the level of claudin-2 protein expression was proportional to the level of Slp2-a protein expression.

We proceeded to perform a reverse transcriptase (RT)-PCR analysis to determine whether the cause of the decreased level of claudin-2 protein expression was at the transcriptional level or at the translational level. As shown in Figure 2C, the level of claudin-2 mRNA expression, but not of claudin-1 mRNA expression, was dramatically decreased in both Slp2-a KD cell lines, indicating that Slp2-a is required for correct claudin-2 mRNA expression. Because a small amount of claudin-2 protein was still present in the Slp2-a KD cells (Figure 2B), we also investigated its localization in the Slp2-a KD cells by an immunofluorescence analysis. To our surprise, claudin-2 protein was correctly localized at ZO-1–positive and claudin-1–positive tight junctions even in the Slp2-a KD cells (Figure 2D, far upper right, arrowheads); however, the intensity of the claudin-2 immunoreactive signals in the Slp2-a KD cells was markedly decreased in comparison with the control cells. Thus Slp2-a is unlikely to directly regulate trafficking of claudin-2 protein to tight junctions in MDCK II cells.

Apical membrane localization of Rab27A is Slp2-a dependent

Because Slp2-a and claudin-2 were differentially localized at the apical membrane and at the tight junction, respectively, we hypothesized...
Slp2-a is required for the apical membrane localization of Rab27A in MDCK II cells. (A) Endogenous interaction between Slp2-a and Rab27A in MDCK II cells. Total cell lysates of confluent MDCK II cells were coimmunoprecipitated with anti–Slp2-a antibody and subjected to 10% SDS–PAGE, followed by immunoblotting with the antibodies indicated on the right. Note that Slp2-a interacted with Rab27A alone and not with any of the other Rabs tested. The positions of the molecular mass markers (in kilodaltons) are shown on the left. (B) Altered cellular localization of Rab27A in Slp2-a KD cells. Confluent control cells and Slp2-a KD cells were fixed, permeabilized, and stained with Texas red–conjugated phalloidin (red) and anti-Rab27A antibody (green). Although Rab27A was preferentially localized at the apical membrane in the control cells, the apical membrane localization of Rab27A was decreased in the Slp2-a KD cells. Nuclei were stained with DAPI (blue). Scale bars, 10 μm. (C) Requirement of Slp2-a binding of Rab27A for apical membrane localization. Control cells and Slp2-a KD cells were transfected with pmRFP-C1, pmRFP-C1-Rab27A(WT), or pmRFP-C1-Rab27A(L84I/F88Y/D91G). Two days after transfection, the cells were fixed, permeabilized, and stained with Alexa Fluor 488–conjugated phalloidin (green) and DAPI (blue). Although mRFP-Rab27A(WT) was preferentially localized at the apical membrane in the control cells, the apical membrane localization of Rab27A was decreased in the Slp2-a KD cells. Scale bars, 10 μm.
bind Slp2-a (Fukuda, 2006b), that is, Rab27B contributes to claudin-2 expression in Rab27A KD MDCK II cells (Supplemental Figure S4E). These results strongly indicated that Slp2-a is involved in the apical membrane targeting of Rab27A-bearing (and presumably Rab27B-bearing) vesicles in MDCK II cells via a direct interaction with Rab27A, as well as in the regulation of claudin-2 expression.

Slp2-a is required for the apical membrane targeting of podocalyxin in MDCK II cells

Next we searched the literature for candidate apical membrane proteins that induce claudin-2 expression and are transported to the apical membrane by Rab27-bearing vesicles. We focused on podocalyxin as a candidate signaling molecule because podocalyxin had been shown to be endogenously expressed (Meder, 2005; Cheng et al., 2005) and to alter TER when overexpressed (Takeda et al., 2000) in MDCK II cells. Podocalyxin is also known to transduce signals, for example, RhoA, Rac, and mitogen-activated protein kinase (MAPK, Takeda, 2003; Schmieder et al., 2004; Sizemore et al., 2007; Hsu et al., 2010), some of which are known to affect claudin-2 expression in MDCK II cells (Singh and Harris, 2004; Lipschutz et al., 2005; Guillemot and Citi, 2006; Guillemot et al., 2008; Flores-Benitez et al., 2009; Ikari et al., 2011). First, we performed an RT-PCR analysis to compare the level of podocalyxin mRNA expression in the control cells and the Slp2-a KD cells (Figure 4A). The RT-PCR analysis showed that podocalyxin mRNA was actually expressed in both the control cells and the Slp2-a KD cells and that the level of podocalyxin mRNA expression was similar in both of them. Next, to determine the subcellular localization of podocalyxin, we expressed C-terminal Venus (a variant of yellow fluorescent protein)-tagged podocalyxin in the control cells and the Slp2-a KD cells. As shown in Figure 4B (top), podocalyxin was localized at the apical membrane in the control cells, whereas the apical membrane localization of podocalyxin was dramatically decreased in the Slp2-a KD cells. Intriguingly, podocalyxin and Rab27A were colocalized in the cytoplasm of both the control cells and the Slp2-a KD cells (Figure 4B, bottom). The decreased apical membrane localization of podocalyxin was further confirmed biochemically by surface biotinylation assays, which showed that the surface expression of podocalyxin, but not of syntaxin-3, was significantly decreased in the Slp2-a KD cells (Figure 4C, D). These results indicated that podocalyxin is the most likely candidate for the cargo of Rab27-bearing vesicles, whose targeting to the apical membrane is mediated by the function of the Slp2-a–Rab27A complex.

Ezrin, a downstream target of podocalyxin, is activated in the absence of Slp2-a

ERM family proteins act both as linkers between the actin cytoskeleton and plasma membrane proteins and as signal transducers in response to the stimuli that induce cytoskeletal remodeling (Brettscher et al., 2002). Because ezrin has been shown to interact with podocalyxin (Takeda et al., 2001; Orlando et al., 2001; Schmieder et al., 2004) and to be involved in the regulation of the RhoA, MAPK, and phosphoinositide 3-kinase signaling pathways (Schmieder et al., 2004; Sizemore et al., 2007), we focused on ezrin as a candidate molecule downstream of podocalyxin and...
investigated the subcellular localization of endogenous ezrin in the Slp2-a KD cells. Although ezrin was predominantly localized at the apical membrane in the control cells (Figure 5A, left), reduced immunoreactive ezrin signals were evident at the apical membrane, and some ectopic ezrin signals were also observed at the basolateral membrane in the Slp2-a KD cells (Figure 5A, right, arrowheads), indicating that ezrin fails to accumulate at the apical membrane in the Slp2-a KD cells because of the decreased apical membrane localization of podocalyxin. Because the altered intracellular localization of ezrin in the Slp2-a KD cells was assumed to affect its activity, we next explored the activity of ezrin, that is, phosphorylation of ezrin, by an immunoprecipitation analysis with anti-ezrin–specific antibody (Figure 5B). Unexpectedly, phosphorylation of ezrin was found to be increased in the Slp2-a KD cells in comparison with the control cells. Moreover, an immunofluorescence analysis with anti–phospho-ERM antibody showed that ERM proteins were phosphorylated at the apical membrane in the control cells, whereas they were phosphorylated at both the apical membrane and the basolateral membrane in the Slp2-a KD cells (Figure 5C, arrowheads), suggesting that the phosphorylation of ezrin at the basolateral membrane in the Slp2-a KD cells affects intracellular signal transduction.

Epidermal growth factor–independent MAPK signaling is activated in the absence of Slp2-a

Because the increased phosphorylation of ezrin has been shown to modulate signaling pathways, such as the MAPK pathway (Sizemore et al., 2007), we analyzed the activity of the MAPK pathway by measuring the amount of phosphorylated, active ERK1/2. An immunoblot analysis showed that ERK1/2 phosphorylation was actually increased in the Slp2-a KD cells in comparison with the control cells (Figure 6A, middle). Given that it had been reported that activation of the MAPK pathway by epidermal growth factor (EGF) receptor (EGFR) activation decreases the mRNA and protein levels of claudin-2 (Singh and Harris, 2004; Lipschutz et al., 2005; Flores-Benitez et al., 2009; Ikari et al., 2011), the increased activity of the MAPK pathway in the Slp2-a KD cells might have been attributable to the EGFR activation.

We therefore explored the level of EGFR protein expression by an immunoblot analysis with anti-EGFR antibody to investigate the involvement of EGF signaling in claudin-2 expression in the Slp2-a KD cells. However, both the total level and the surface expression level of EGFR protein were unaltered in the absence of Slp2-a (Figure 6B), indicating that Slp2-a is not involved in the apical targeting of EGFR. Furthermore, EGF treatment increased ERK1/2 phosphorylation and additively decreased the level of claudin-2 protein expression in both the control cells and the Slp2-a KD cells (Figure 6C, middle three panels), suggesting that increased ERK1/2 phosphorylation in the Slp2-a KD cells is induced by ezrin activation and not by activation of EGF signaling.

Slp2-a is expressed in mouse kidney

Claudin-2 is highly expressed in the tight junctions of mouse renal proximal tubules and is involved in the reabsorption of NaCl and...
Slp2-a regulates claudin-2 expression

If Slp2-a is an upstream regulator of claudin-2 expression in mice, Slp2-a should be expressed in mouse renal proximal tubules, the same as claudin-2. Thus we stained mouse kidney with specific antibodies against Slp2-a, Rab27A, and claudin-2. The results of immunohistochemical analyses indicated that Slp2-a immunoreactive signals were present only in claudin-2–expressing tissue in mouse kidney (Figure 7, top), whereas Rab27A was expressed in all nephron segments (Figure 7, middle and bottom). Their coexpression in mouse renal proximal tubules suggested that Slp2-a regulates claudin-2 expression in mouse kidney, the same as in MDCK II cells.

DISCUSSION

Most organ systems (e.g., digestive, respiratory, genitourinary, and vascular systems) of multicellular animals are lined by a polarized monolayer of epithelial cells. The polarized epithelial cells have two surfaces—an apical surface that faces the lumen and a basal-lateral surface that is in contact with adjacent cells and the underlying connective tissue. These two surfaces are morphologically and functionally divided by tight junctions, which restrict the lateral diffusion of membrane proteins and lipids and function as a barrier between the cell and the extracellular milieu (Tsukita et al., 2001; Bryant and Mostov, 2008). Thus elucidation of the polarized targeting machinery is important to understanding how cell polarity and morphology are maintained. In the present study, we identified Slp2-a as a novel apical trafficking protein in MDCK II cells. Slp2-a is localized at the apical membrane in polarized MDCK II cells (Figure 1C) and together with Rab27A regulates apical

FIGURE 6: MAPK signaling is activated in Slp2-a KD cells independently of EGFR activation. (A) Increased phosphorylation of ERK1/2 in Slp2-a KD cells. Total cell lysates of confluent control cells and Slp2-a KD cells were subjected to 10% SDS–PAGE, followed by immunoblotting with the antibodies indicated on the right. Note that the phosphorylation level of ERK1/2 (P-ERK1/2) in the Slp2-a KD cells was markedly increased in comparison with the control cells, whereas the total level of ERK1/2 expression was the same. (B) Unaltered surface EGFR expression in Slp2-a KD cells. Total cell lysates and surface biotinylated proteins of confluent control cells and Slp2-a KD cells were subjected to 10% SDS–PAGE, followed by immunoblotting with the antibodies indicated on the right. Note that the level of EGFR expression and level of surface EGFR expression appeared to be the same. (C) Effect of EGF-stimulated EGFR activation. Confluent control cells and Slp2-a KD cells were treated with EGF for 16 h, and total cell lysates were subjected to 10% SDS–PAGE, followed by immunoblotting with the antibodies indicated on the right. Note that the ERK1/2 phosphorylation level in both the control cells and the Slp2-a KD cells was increased by EGF treatment and, as a result, the level of claudin-2 expression was further decreased. Actin was used as an internal control. The positions of the molecular mass markers (in kilodaltons) are shown on the left.
expression in the Slp2-a KD cells (right). The apical membrane localization of Slp2-a is mediated by the phospholipid-binding activity of the C2A domain. Phosphorylated ezrin, which activates MAPK signals (Sizemore et al., 2007), is recruited to and interacts with podocalyxin at the apical membrane, and ezrin is then dephosphorylated by phosphatase (e.g., PP2A) at the apical membrane (Nunbhakdi-Craig et al., 2002; Zeidan et al., 2008). As a result, phosphorylation of ERK1/2 is decreased and claudin-2 is expressed. By contrast, in the Slp2-a KD cells phosphorylated ezrin is ectopically localized at the basolateral membrane presumably because of the defect in podocalyxin trafficking to the apical membrane, and elevated ERK1/2 phosphorylation inhibits claudin-2 expression in the Slp2-a KD cells (right).

trafficking of podocalyxin-containing vesicles (Figure 4B). Bryant et al. (2010) reported that podocalyxin-containing vesicles are transported to the apical membrane through post-Golgi trafficking and a transcytosis pathway, in both of which Rab8 and Rab11 are involved. Given that Rab27A was partially colocalized with Rab8A and Rab11A (Supplemental Figure S5), it is tempting to speculate that the Slp2-a–Rab27A complex also regulates certain step of the post-Golgi trafficking and/or the transcytosis pathway. Because Slp2-a has a docking role of Rab27-bearing secretory vesicles (or melanosomes) in other cell types (Kuroda and Fukuda, 2004; Fukuda, 2006b), the Slp2-a–Rab27A complex is likely to regulate recruitment and docking of podocalyxin-containing vesicles to the apical membrane in MDCK II cells (Figure 8, control).

What is the function of the podocalyxin transported to the apical surface by the Slp2-a–Rab27A complex? Podocalyxin is a transmembrane sialomucin and has been shown to be involved in cell polarization and epithelial tubulogenesis (Rodríguez-Fraticelli et al., 2011). Consistent with the previous reports that podocalyxin associates with ezrin (Takeda et al., 2001; Orlando et al., 2001; Schmieder et al., 2004), apically localized endogenous ezrin was decreased in the absence of Slp2-a (Figure 5A). Our findings contrast somewhat with the previous finding in regard to the Drosophila Slp homologue Btsz, that is, moesin, the only member of the ERM protein family expressed in Drosophila, which is recruited to the AJR in a Btsz-dependent manner (Pilot et al., 2006). Given that mammalian Slp2-a is not a direct orthologue of Drosophila Btsz, it is not surprising that Slp2-a regulates polarized trafficking in epithelial cells in a different manner from Btsz. We especially noted the structural difference between the two molecules: Slp2-a contains an N-terminal Slp homology domain (SHD or Rab27-binding domain) and Btsz does not (Fukuda et al., 2001). Although Btsz is capable of being recruited to the AJR without an SHD (Pilot et al., 2006), our detailed deletion and mutational analyses clearly indicated that the SHD is required for apical targeting of Slp2-a in MDCK II cells (Supplemental Figure S6A). Although the EGFP-tagged, full-length Slp2-a was clearly localized at the apical membrane, the same as endogenous Slp2-a protein (Supplemental Figure S6B, top left, green), the Slp2-aΔSHD mutant, which lacks an SHD, did not exhibit apical membrane localization, although it is localized at both the apical membrane and the basolateral membrane (Supplemental Figure S6B, left, third panel). In addition to the SHD, the phospholipid-binding activity of the C2A domain of Slp2-a is also required for its apical localization, because EGFP-C2AΔKQ and EGFP-Slp2-aΔKQ mutants, both of which lack the phospholipid-binding activity of the C2A domain as a result of neutralization of key Lys residues (Kuroda and Fukuda, 2004), failed to be localized at the plasma membrane (Supplemental Figure S6B, right). Another difference between Slp2-a and Btsz lies in their ERM recruitment mechanism: Slp2-a recruits ezrin to the apical membrane indirectly through trafficking of podocalyxin, which binds ezrin (Figures 5A and 8), whereas Btsz recruits moesin to the AJR directly (Pilot et al., 2006). Actually, no interaction between mammalian Slp2-a and ezrin (or moesin) was observed in MDCK II cells under our immunoprecipitation conditions (unpublished data).

Although ezrin was decreased at the apical membrane in the Slp2-a KD cells (Figure 5A), we were surprised to find increased phosphorylation of ezrin in the absence of Slp2-a (Figure 5, B and C). Given that protein phosphatase 2A (PP2A) has been reported to be localized at the apical membrane in MDCK II cells (Nunbhakdi-Craig et al., 2002), PP2A may dephosphorylate ezrin at the apical membrane (Zeidan et al., 2008). In fact, when endogenous PP2A activity was inhibited by a specific PP2A inhibitor, okadaic acid (OA) (Haystead et al., 1989), phosphorylation of ezrin was increased and claudin-2 expression was decreased even in the control cells (Supplemental Figure S7). Thus ezrin is likely to have been dephosphorylated by PP2A at the apical membrane in the control cells (Figure 8, control). This scenario seems plausible in the Slp2-a KD cells, because some ectopic phospho-ezrin immunoreactive signals at the basolateral membrane were observed in the Slp2-a KD cells but not in the control cells (Figure 5C, bottom right, arrowheads). These findings, together with the fact that activated ezrin is involved in the regulation of the MAPK pathway (Sizemore et al., 2007), suggest that activation of the MAPK pathway (e.g., phosphorylation of ERK) by phospho-ezrin at the basolateral membrane inhibits claudin-2 expression (Figure 8, Slp2-a KD). Consistent with our model of ERK activation in Slp2-a KD cells, overexpression of a dominant-negative form of ERK(K52R) in Slp2-a KD cells clearly induced up-regulation of claudin-2 protein expression (Supplemental Figure S4, A and B). We also tried C-terminal–deleted ezrin, which has been reported to act as a dominant-negative form of ezrin (Ezrin-N; Bretscher et al., 2002), but the C-terminal–deleted ezrin had no effect on claudin-2 expression in Slp2-a KD cells (T.Y. and M.F., unpublished data), this mutant may not simply function as a dominant-negative mutant of ezrin in MDCK II cells. Alternatively, other downstream targets of podocalyxin might also be involved in the regulation of ERK1/2 phosphorylation.

In addition to MDCK II cells, we found that both Slp2-a and claudin-2 are coexpressed in mouse renal proximal tubules (Figure 7), and since podocalyxin is also expressed in renal tubules (Cheng et al., 2005; Lin et al., 2007), the same molecular mechanism regulated by Slp2-a and podocalyxin is likely to function in mouse renal proximal tubules. However, podocalyxin-deficient mice exhibit a severely impaired kidney development and die of anuric failure within 24 h after birth (Doyonnas et al., 2001), whereas Slp2-a-deficient
mice are viable and fertile (Saegusa et al., 2006). This discrepancy may be explained by the compensatory effect of Slp2-a-related proteins, such as Slp1, Slp2-b, Slp3-a, Slp4-a, and Slp5 (Fukuda, 2005, 2006b; Supplemental Figure S1A), in mice. Further work will be necessary to determine whether other Slp family members are also involved in the polarized trafficking that occurs in epithelial cells.

In conclusion, we demonstrated that Slp2-a is the first Slp member involved in polarized trafficking in mammalian epithelial cells, and we discovered that Slp2-a regulates apical targeting of podocalyxin and its binding partner ezrin in concert with Rab27A. In the absence of Slp2-a, the phospho-ezrin in MDCK II cells is ectopically localized at the basolateral membrane and then activates ERK by a largely unknown mechanism. The activated ERK then inhibits claudin-2 expression in the Slp2-a KD cells (a hypothetical model is summarized in Figure 8). Because previous studies on mammalian Slp family members have for the most part been limited to their secretory function—for example, exocytosis of hormones and enzymes—in secretory cells (Fukuda, 2006b), the findings in this study should provide a new paradigm for the function of Slp protein in the polarized trafficking of signaling molecules in epithelial cells.

MATERIALS AND METHODS

Materials
Horseradish peroxidase (HRP)-conjugated anti-GFP antibody was purchased from MBL (Nagoya, Japan). Anti-Rab3, anti-Rab8, anti-Rab11, anti-Rab27A, anti-moesin, and anti–E-cadherin mouse monoclonal antibodies were obtained from BD Transduction Laboratories (Lexington, KY). Anti–ZO-1 rat monoclonal antibody was from Chemicon International (Temecula, CA). Anti–claudin-1 and anti–claudin-2 rabbit polyclonal antibodies were from Invitrogen (Carlsbad, CA). Anti–syntxin-3 rabbit polyclonal antibody was from Synaptic Systems (Göttingen, Germany). Anti–ezrin mouse monoclonal antibody was from Abcam (Cambridge, MA). Anti–phospho-ezrin (Thr-567)/radixin (Thr-564)/moesin (Thr-558) (anti–phospho-ERM) rabbit polyclonal antibody, anti–p44/42 MAPK (ERK1/2) rabbit polyclonal antibody, and anti–phospho-p44/42 (ERK1/2; Thr-202/Tyr-204) mouse monoclonal antibody were from Cell Signaling Technology (Danvers, MA). Anti-EGFR sheep polyclonal antibody was from Fitzgerald (Concord, MA). Anti–Slp1, anti–Slp2-a, anti–Slp3-a, and anti–Slp4-a rabbit polyclonal antibodies were prepared as described previously (Imai et al., 2004). Two siRNAs against canine/human Rab27A site 1 (target site #1: 5′-GGAGAGGTTTCGTAGCTTA-3′, and target site #2, 5′-CGCTCAGATCCCATGTAAA-3′) were transfected into MDCK II cells (1 × 10⁵ cells) by using Lipofectamine 2000 as described. To establish cell lines stably expressing the pSilencer vectors, MDCK II cells were grown in DMEM containing 800 μg/ml G418 (Invitrogen). Stable MDCK II cells (referred to as control cells, Slp2-a KD #1 cells, and Slp2-a KD #2 cells, respectively) were plated on culture dishes, cultured for 72 h, and then subjected to immunoblotting and immunocytochemistry as described later.

Plasmid construction
pEGFP-C1 vectors (BD Biosciences Clontech, Palo Alto, CA) harboring mouse Slp2-a mutants or mouse Rab proteins and pmRFP-C1 vectors harboring mouse Rab27A mutants were prepared essentially as described previously (Kuroda and Fukuda, 2004; Fukuda, 2006a; Tsuboi and Fukuda, 2006). cDNA encoding human podocalyxin was amplified from Marathon-Ready adult human brain and testis cDNA (Tsuboi and Fukuda, 2006). Two siRNAs against canine/human Rab27A site 1 (target site #1: 5′-GGAGAGGTTTCGTAGCTTA-3′) and site 2 (target site #2: 5′-CGACACGGTGTTCTCAGAGA-3′) were synthesized by Nippon EGT (Toyama, Japan).

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Preparation of total RNA and RT-PCR
Total RNA was prepared from stable MDCK II cells with TRIzol reagent (Sigma-Aldrich), and reverse transcription was performed by using a ReverTra Ace-kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The canine glyceraldehyde 3-phosphate dehydrogenase (GAPDH), claudin-1, claudin-2, podocalyxin, and Rab27B were used as internal controls to adjust for unequal amounts of template cDNA in the different samples. The resulting full-length podocalyxin cDNA was subcloned into the pVe-nus-N1 vector. cDNA encoding ERK2(K52R) was prepared according to the procedure described by Robinson et al. (1996) and subcloned into the pEF-FLAG tag expression vector (Fukuda et al., 1999).

Cell culture, transfection, and EGF or OA treatment
MDCK II cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). Plasmids were transfected into MDCK II cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For EGF treatment, MDCK II cells were cultured in serum-free DMEM medium for 6 h and then treated with EGF (100 ng/ml; Invitrogen) for 16 h. For OA treatment, confluent MDCK II cells were treated with OA (20 or 100 nM; LC Laboratories, Woburn, MA) for 16 h.

Establishment of Slp2-a-knockdown cell lines by RNA interference
pSilencer-control plasmids and pSilencer-canine Slp2-a (cSlp2-a) #1 and #2 plasmids (target site #1, 5′-GGATGAACTCCCCACAGATT-3′, and target site #2, 5′-CGCTCAGATCCCATGTAAA-3′) were transfected into MDCK II cells (1 × 10⁵ cells) by using Lipofectamine 2000 as described. To establish cell lines stably expressing the pSilencer vectors, MDCK II cells were grown in DMEM containing 800 μg/ml G418 (Invitrogen). Stable MDCK II cells (referred to as control cells, Slp2-a KD #1 cells, and Slp2-a KD #2 cells, respectively) were plated on culture dishes, cultured for 72 h, and then subjected to immunoblotting and immunocytochemistry as described later.

Measurement of transepithelial electrical resistance
Stable MDCK II cells (4 × 10⁵) were seeded on a Transwell filter (0.4-μm pore size; 12-mm diameter; Corning, Corning, NY) and grown to confluence in DMEM containing 1.8 mM Ca²⁺ (normal calcium medium). Cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.4) and incubated for 2 h in S-MEM (Invitrogen) containing 2.1 mM Ca²⁺ (low-calcium medium). TER was monitored with a Millicel-ERS volt-ohm meter (Millipore, Bedford, MA) immediately after the addition of the normal calcium medium DMEM (time 0) and at 90- to 120-min intervals for up to 26 h. TER values were calculated after subtracting the blank value (an empty filter) and expressed in ohm cm², as described by Matter and Balda (2003).

Preparation of total RNA and RT-PCR
Total RNA was prepared from stable MDCK II cells with TRI reagent (Sigma-Aldrich), and reverse transcription was performed by using a ReverTra Ace-kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The canine glyceraldehyde 3-phosphate dehydrogenase (GAPDH), claudin-1, claudin-2, podocalyxin, and Rab27B were used as internal controls to adjust for unequal amounts of template cDNA in the different samples. The primers were used for GAPDH (forward primer, 5′-ATGGTGAAAGGTCGAGTCAA-3′, and reverse primer, 5′-GCCATGTAGACCATGAGGC-3′), claudin-1 (forward primer, 5′-ATTTAGTGCAAACCTCAGCC-3′, and reverse primer, 5′-GGTGGTCCCTCGGTGAGAGG-3′), claudin-2 (forward primer, 5′-TTGAGTGTAATCAGGGAGG-3′, and reverse primer, 5′-CGCACATACCCCTGAGC-3′), podocalyxin (forward primer, 5′-TTCTAAAGCTATTCTTCCAG-3′, and reverse primer, 5′-TGAAGGTTTCTCGAGG-3′), and Rab27B (forward primer, 5′-GAGGCATGAGGAGGCATAG-3′, and reverse primer, 5′-CTAACAGC-3′). cDNAs were amplified by PCR using rTaq DNA polymerase.
polymerase (Toyobo) with 25 or 50 (for Rab27B) cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1 min.

Immunofluorescence analysis

Stable MDCK II cells were cultured on glass-bottomed dishes (35-mm dish; MatTek, Ashland, MA) at (1–3) × 10^6 cells for 72 h and then fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. For immunostaining, cells were permeabilized with 0.3% Triton X-100 in PBS for 2 min and blocked with the following buffer (1% bovine serum albumin [BSA] and 0.1% Triton X-100 in PBS) for 1 h at room temperature. The cells were then incubated for 2 h at room temperature in the same buffer containing primary antibodies at the following concentrations: anti-Slp2-a antibody, 3 μg/ml; anti-ZO-1 antibody, 1/400 dilution; anti-moesin antibody, 1/100 dilution; anti-E-cadherin antibody, 1/100 dilution; anti-Rab8 antibody, 1/100 dilution; anti-Rab11 antibody, 1/100 dilution; anti-Rab27A antibody, 1/100 dilution; anti-claudin-1 antibody, 1/50 dilution; anti-claudin-2 antibody, 1/200 dilution; anti-syntenin-3 antibody, 1/100 dilution; anti-erlin antibody, 1/100 dilution; and anti–phospho-ERM antibody, 1/100 dilution. The cells were then incubated with secondary antibodies (Alexa 488-conjugated antibodies and Alexa 594-conjugated antibodies; Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI). To stain actin, cells were stained with Texas red-conjugated phalloidin or Alexa Fluor 488-conjugated phalloidin (each 1/100 dilution; Invitrogen). The stained cells were examined for immunofluorescence signals with a confocal fluorescence microscope (Fluoview 1000; Olympus, Tokyo, Japan).

Immunoblot analysis

MDCK II cells were plated on 10-cm culture dishes at 2 × 10^4 cells and 2 × 10^5 cells, cultured for 48 h, and harvested as subconfluent cells and confluent cells, respectively. Parental MDCK II cells and stable MDCK II cells were plated on 6-cm dishes at (1–3) × 10^5 cells and cultured for 48 h. After rinsing the cells with PBS and lysing them in a lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 1% Triton X-100, and a protease inhibitor cocktail [Roche, Mannheim, Germany]), we subjected the lysates to 10% SDS–PAGE, followed by immunoblotting with the antibodies indicated in the figures. The intensity of the immunoreactive bands was quantified with ImageJ, version 1.44c (National Institutes of Health, Bethesda, MD). The blots shown in the figures are representative of three or four independent experiments.

Cell surface biotinylation assay

Biotinylation assays were performed essentially as described previously (Shin et al., 2005). In brief, stable MDCK II cells grown on 10-cm dishes were transfected with podocalyxin-Venus expression vector and cultured for 48 h. Confluent MDCK II cells were washed three times with ice-cold PBS (+) (containing 0.1 mM CaCl2 and 0.1 mM MgCl2, pH 7.4) and incubated with 1 mg/ml Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) in PBS (+) for 30 min on ice to biotinylate cell surface proteins. The reaction was quenched by washing the dishes twice with ice-cold PBS (+) containing 100 mM glycine and 0.3% (wt/vol) BSA, and the dishes were subsequently washed twice with PBS (+). The cells were then lysed in 1 ml of the previously described lysis buffer, and the lysates were centrifuged at 15,000 × g for 20 min at 4°C in a microcentrifuge to remove cellular debris and insoluble materials. The supernatant was incubated with streptavidin-agarose beads (Pierce) overnight at 4°C with constant rotation. The beads were then washed three times with the lysis buffer, twice with a high-salt wash buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.1% Triton X-100), and, finally, once with 50 mM Tris-HCl, pH 7.5. The proteins were eluted from the beads by boiling in 30 μl of the SDS sample buffer, separated by 10% SDS–PAGE, and analyzed by immunoblotting with HRP-conjugated anti-GFP antibody, anti-syntenin-3 antibody, and anti-EGFR antibody.

Immunoprecipitation assay

Stable MDCK II cells were plated on 10-cm culture dishes at (1–3) × 10^4 cells and cultured for 48 h. The cells were rinsed with PBS and lysed in the lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich). The homogenate was rotated for 30 min at 4°C for solubilization, and the insoluble material was removed by centrifugation at 15,000 × g for 20 min at 4°C. The supernatant was incubated with 1 μg/ml anti-erlin antibody for 3 h at 4°C or with 1 μg/ml anti-Slp2-a antibody (or anti-Rab27A antibody) for 16 h at 4°C with constant rotation and then with protein G–Sepharose beads or protein A–Sepharose beads (GE Healthcare, Little Chalfont, United Kingdom) for 1 h at 4°C. After washing the beads with the lysis buffer, we subjected the immunoprecipitates to 10% SDS–PAGE, followed by immunoblotting with anti-erlin antibody and anti–phospho-ERM antibody. Mouse kidney, including renal epithelial cells, was similarly lysed in the foregoing lysis buffer, and the lysates were subjected to coimmunoprecipitation assays with anti-Rab27A antibody.

Immunohistological analysis

Immunohistochemical staining was performed essentially as described previously (Yasuda et al., 2010). In brief, the kidneys were removed from female ICR mice and immersion fixed in 4% PFA in 0.1 M phosphate buffer (pH 7.4) for 16 h at room temperature. The fixed kidneys were equilibrated at 4°C in 10% sucrose in PBS for 8 h and then in 20% sucrose in PBS overnight. They were then embedded in Tissue-Tek O.C.T. compound (Sakura, Tokyo, Japan) and frozen at −80°C until used. The frozen kidneys were cut into 10-μm sections in a cryostat (Carl Zeiss, Jena, Germany) and transferred to microscope slides (Matsunami Glass, Osaka, Japan). The samples were permeabilized with 0.3% Triton X-100 for 1 h at room temperature and then stained with anti–Slp2-a antibody, followed by immunoblotting with the antibodies indicated in the figures. The samples were subjected to coimmunoprecipitation assays with anti-Rab27A antibody.

ACKNOWLEDGMENTS

We thank Megumi Aizawa for technical assistance and members of the Fukuda Laboratory for valuable discussions. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology of Japan (to M.F.).

REFERENCES


Fukuda M (2002). Synaptotagmin-like protein (Slp) homology domain 1 of Slac2-a/melanophilin is a critical determinant of GTP-dependent specific binding to Rab27A. J Biol Chem 277, 40118–40124.