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Mammalian Lgl Forms a Protein Complex with PAR-6 and aPKC Independently of PAR-3 to Regulate Epithelial Cell Polarity

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Summary

Background: Epithelial cells have apicobasal polarity and an asymmetric junctional complex that provides the bases for development and tissue maintenance. In both vertebrates and invertebrates, the evolutionarily conserved protein complex, PAR-6/aPKC/PAR-3, localizes to the subapical region and plays critical roles in the establishment of a junctional complex and cell polarity. In *Drosophila*, another set of proteins called tumor suppressors, such as Lgl, which localize separately to the basolateral membrane domain but genetically interact with the subapical proteins, also contribute to the establishment of cell polarity. However, how physically separated proteins interact remains to be clarified.

Results: We show that mammalian Lgl competes for PAR-3 in forming an independent complex with PAR-6/aPKC. During cell polarization, mLgl initially colocalizes with PAR-6/aPKC at the cell-cell contact region and is phosphorylated by aPKC, followed by segregation from apical PAR-6/aPKC to the basolateral membrane after cells are polarized. Overexpression studies establish that increased amounts of the mLgl/PAR-6/aPKC complex suppress the formation of epithelial junctions; this contrasts with the previous observation that the complex containing PAR-3 promotes it.

Conclusions: These results indicate that PAR-6/aPKC selectively interacts with either mLgl or PAR-3 under the control of aPKC activity to regulate epithelial cell polarity.

Introduction

Studies of cultured epithelial cells established the importance of an asymmetric junctional complex that includes adherens junctions (AJ) and tight junctions (TJ), which cap the most apical end of epithelial cells, in the establishment and maintenance of cell polarity [1, 2]. Studies of model genetic systems such as *Caenorhabditis elegans* (*C. elegans*) and *Drosophila* revealed a set of evolutionarily conserved polarity proteins that are required for asymmetric cell division during development and epithelial membrane polarity [3]. One example is the set of proteins, including PAR-6, aPKC, and PAR-3, which forms the PAR-6/aPKC/PAR-3 complex [4–8] and is involved in various biological contexts [9]. In mammals, the protein complex localizes to TJ in epithelial cells [4, 10, 11]. A series of studies with cultured epithelial cells established that the PAR-6/aPKC/PAR-3 complex promotes the asymmetric development of the epithelial junctional complex and cell polarity, in which aPKC kinase activity plays a critical role [4, 11–14]. In *Drosophila*, the PAR-6/aPKC/PAR-3 (Bazooka) complex localizes to the subapical region of the embryonic epidermis and is required for the development of AJ and apicobasal membrane polarity [15–17].

Genetic studies of *Drosophila* also identified other sets of proteins that establish both AJ and membrane polarity [18, 19]. These include Crumbs (Crb), Stardust (Std), and Discs lost (Dlt), which localize to the subapical region of epithelial cells, and Scribble (Scrib), Discs large (Dlg), and Lethal giant larvae (Lgl), which localize to the basolateral region. Recent studies have identified genetic interactions among these three sets of proteins. For example, Lgl or Dlg is required for apical localization of Bazooka in the epidermis [20], and Crb and Lgl, as well as Std and Dlg, cooperate in AJ formation through the competitive definition of membrane polarity [21, 22]. Mammalian homologs of Crb, Dlt, and Std localize to TJ [23–25], whereas homologs of Dlg and Lgl localize to the basolateral region in mammalian epithelial cells [26, 27]. Although these observations suggest the central importance of the PAR-6/aPKC/PAR-3 complex and functional interactions among these three sets of proteins in the establishment of the epithelial junctional complex and membrane polarity, the molecular nature of the interactions among these polarity complexes remains to be clarified. Particularly, how physically separated basolateral and apical complexes functionally interact is still a very big question.

Here, we identify the mammalian Lgl isoform, mLgl, as a PAR-6 binding protein. We provide evidence supporting the fact that mLgl is the major partner of the PAR-6/aPKC complex that plays roles in the initial phase of the establishment of epithelial cell polarity.

Results

PAR-6β and aPKCλ Form a Protein Complex with Mammalian Lgl Independently of PAR-3

Mammalian homologs of *Drosophila* Lgl, mLgl-2 and mLgl-1, were identified in immunoprecipitates (IPs) of PAR-6β (also called PAR-6B) from MDCK cells (see Figure S1 in the Supplemental Data available with this article online). Similar experiments with the anti-aPKCλ an-

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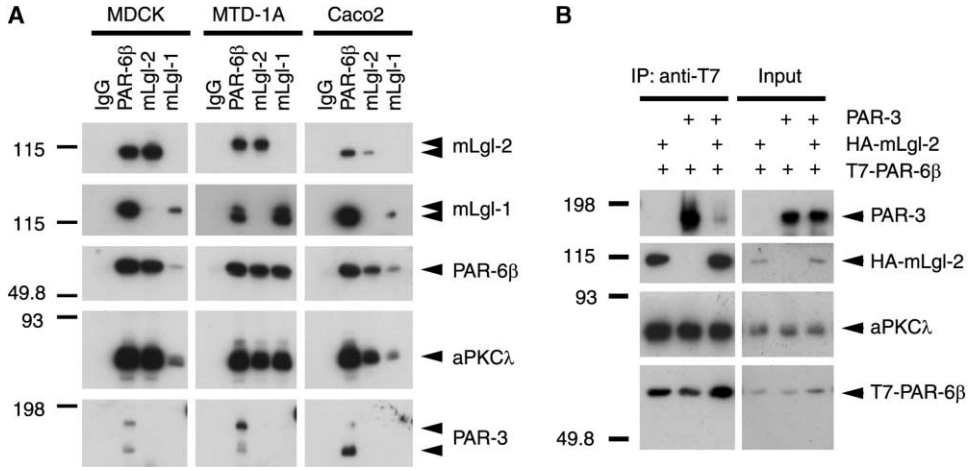

Figure 1. Identification of mLgl Isoforms as PAR-6β Binding Proteins

(A) The lysates of MDCK, MTD-1A, or $Caco_2$ (total 3 mg protein) were subjected to immunoprecipitation with 2 μg anti-PAR-6β (Beta2-4AP), anti-mLgl-1, or anti-mLgl-2 (N13AP) antibody or control IgG. Coprecipitated proteins were analyzed by using anti-PAR-6β (BCR12AP), anti-mLgl-1, anti-mLgl-2 (N13AP), anti-aPKCλ (ι), or anti-PAR-3 antibody. Note that two bands were detected for mLgl-1 of MTD-1A, suggesting the presence of splicing isoforms in MTD-1A cells.

(B) 293T cells were transfected with expression vectors as indicated (top). The cell lysates (Input) were subjected to immunoprecipitation with the anti-T7 tag antibody, followed by Western blot analysis with the anti-T7 tag (Omni), anti-HA tag, anti-PAR-3, or anti-aPKCλ (ι) antibody.

tibody also revealed that mLgl-1/2 coimmunoprecipitates with aPKCλ (data not shown). The interactions among PAR-6β, aPKCλ, and mLgl-1/2 were also observed for other epithelial cell lines, as shown in Figure 1A; PAR-6β IPs contain endogenous mLgl-1 and mLgl-2, in addition to aPKCλ and PAR-3. Interestingly, IPs of mLgl-2 or mLgl-1 contain PAR-6β and aPKCλ, but not PAR-3 (Figure 1A), and IPs for PAR-3 contain PAR-6β and aPKCλ, but not mLgl-1/2 (data not shown). These results indicate that the protein complexes of mLgl-1/2 contain PAR-6β and aPKCλ independent of PAR-3. These results are consistent with the following data showing that mLgl-2 competes with PAR-3 for its incorporation to the PAR-6β/aPKCλ complex in cDNA-transfected 293T cells (Figure 1B) and support the presence of two independent protein complexes containing PAR-6β/aPKCλ. It is interesting that the above interactions are not observed for PAR-6α (data not shown); this finding indicates that mLgl-2 is specific to the PAR-6β isoform.

A similar IP analysis using cDNA-transfected 293T cells reveals that the region containing the PDZ domain of PAR-6β is required for its interaction with mLgl-2. (Figures 2A and 2B). Consistently, a PDZ-affecting mutant of PAR-6β, M235W [28], fails to tightly interact with mLgl-2 (Figure 2B), whereas this mutant retains full activity to interact with the PDZ domain of PAR-3 (data not shown). A pull-down assay confirms these results (Figure 2C). A blot overlay assay further reveals that the interaction between PAR-6β and mLgl-2 is direct (Figure 2D). HA-mLgl-2 also coimmunoprecipitates with the kinase domain (KD), but not with the regulatory domain (RD), of HA-aPKCλ (Figure 2E); this finding suggests that the interaction between mLgl-2 and aPKCλ is PAR-6β independent. Taken together, these results indicate that mLgl-2 interacts with the PDZ domain of PAR-6β and with the kinase domain of aPKCλ and forms a protein complex independently of the one containing PAR-3 (Figure 2F).

aPKCλ Phosphorylates mLgl-2 at a Specific Serine Residue In Vivo

Drosophila Lgl from Sf9 cells and mammalian Lgl, mLgl-1, are phosphorylated by unknown copurified kinase(s) at their Ser residue(s) within an evolutionarily conserved region (see Figure 3A) [29, 30]. We revealed that GST fusion proteins with the 636–666 aa sequence of human Lgl-2 (GST-mLgl-2 636–666 wt) is phosphorylated by purified HA-aPKCλ in vitro, and substitution of Ser 649, 653, or 660 for Ala decreases this phosphorylation (Figure 3B). An antibody generated against phospho-Ser 653 reacts to GST-mLgl-2 636–666, but not to its S653A mutant (Figure 3C). Using this antibody, HA-mLgl-2 in T7-PAR-6β IPs from transfected 293T cells were revealed to be phosphorylated at Ser 653 in an aPKCλ activity-dependent manner (Figure 3D), indicating that aPKCλ can phosphorylate the full-length mLgl-2. Furthermore, the phospho-specific antibody reveals that the phosphorylation of Ser 653 of mLgl-2 and the corresponding Ser of mLgl-1 (Ser 659) occurs in MDCK cells and is enhanced by the expression of wild-type aPKCλ (aPKCλ wt), but not by that of the kinase-deficient mutant of aPKCλ (aPKCλ kn) (Figure 3E); this finding indicates that both mLgl-2 and mLgl-1 are phosphorylated in vivo in an aPKCλ activity-dependent manner.

Codistribution of PAR-6β and aPKCλ with mLgl-2 and Phosphorylation of mLgl-2 during an Early Phase of Epithelial Cell Polarization

As reported previously for mLgl-1 [27], mLgl-2 colocalizes with E-cadherin at the lateral region of polarized MDCK cells, whereas PAR-6β localizes to the apical periphery of MDCK cells in addition to the apical end

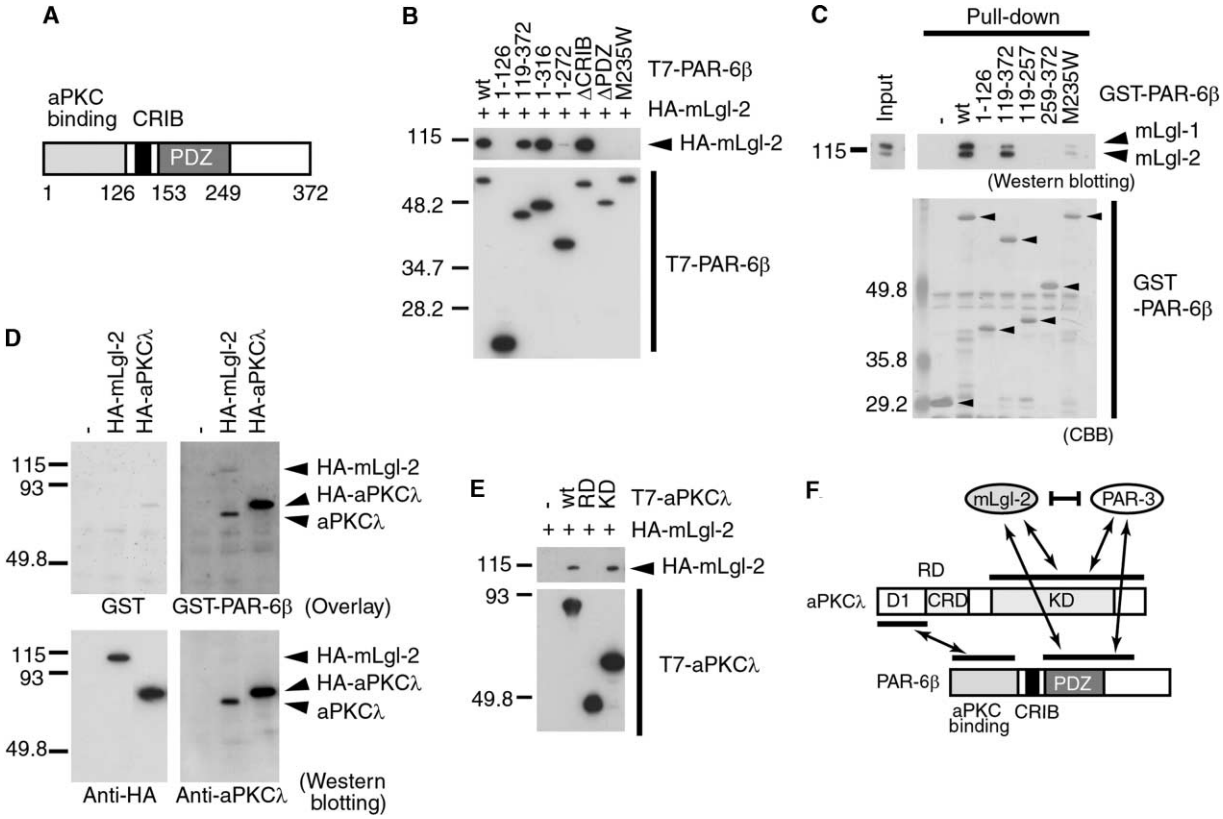

Figure 2. mLgl-2 Interacts with Both PAR-6β and aPKCλ

(A) Human PAR-6β has three conserved protein motifs: N-terminal 126 aa sequence, which interacts with the D1 region of aPKCλ; the CRIB motif, which interacts with the active form of Cdc42/Rac1 small GTPases; and the PDZ domain, which interacts with the PDZ1 domain of PAR-3 [9].

(B) 293T cells transfected with expression vectors as indicated (top) were subjected to immunoprecipitation with the anti-T7 tag antibody, followed by Western blot analysis with the anti-T7 tag (Omni) or anti-HA tag antibody.

(C) GST-PAR-6β or its mutants immobilized on glutathion-sepharose were coincubated with MDCK cell lysates (Input) and were eluted by glutathion solution, followed by Western blot analysis with the anti-mLgl-1 or anti-mLgl-2 (100–278 2-3AP) antibody. Eluted GST proteins were detected by staining of blotted membrane with CBB (arrowheads in lower panel).

(D) 293T cells transfected with expression vectors as indicated (top) were subjected to immunoprecipitation with the anti-HA tag antibody, followed by Western blotting. The blotted membranes were overlaid with GST or GST-PAR-6β, followed by analysis with the anti-GST antibody (upper panels). Immunoprecipitated proteins were analyzed by using the anti-HA-tag or anti-aPKCλ (ι) antibody (lower panels).

(E) 293T cells transfected with expression vectors as indicated (top) were subjected to immunoprecipitation with the anti-T7 tag antibody, followed by Western blot analysis with the anti-T7 tag (Omni) or anti-HA tag antibody.

(F) Summary of the interactions of PAR-6β/aPKCλ with mLgl-2 or PAR-3.

of the lateral region with a TJ marker, ZO-1 (Figure S2). Colocalization of PAR-6β with mLgl-2 was not observed (Figure S2). However, during MDCK cell polarization induced by calcium addition to the culture medium (calcium switch), PAR-6β and mLgl-2 together with ZO-1 show overlapping distribution (white) at the cell-cell contact region 0.5 hr after the calcium switch, and this codistribution was also detected at the apical end of the cell-cell contact region 3 hr after the calcium switch (Figure 4A). In contrast, at 20 hr, when the cells were fully repolarized, white staining was not detected, and the cells were costained for PAR-6β with ZO-1 (purple) at the apical end and single stained for mLgl-2 (green) at the lateral membrane. On the other hand, aPKCλ codistributes with PAR-6β and ZO-1 at the cell-cell contact region throughout the repolarization process (Figure 4B). The transient colocalization of PAR-6β and aPKCλ with mLgl-2 implies that an interaction takes place between mLgl-2 and the PAR-6β/aPKCλ complex during MDCK cell polarization. However, we failed to detect any changes of interactions between PAR-6β/aPKCλ and mLgl-2 after cell disruption (data not shown). These proteins are mainly recovered in the cytoplasmic fraction (data not shown), and this finding does not support the localization of these proteins revealed by immunostaining. It is possible that the cell lysis process alters the nature of the complex due to the instability of the PAR-3/PAR-6β/aPKCλ complex in lysates. Possible involvement of activated Cdc42 in the interaction might explain the instability (see the Discussion).

We also revealed that the phosphorylation of mLgl-2 was enhanced when the cells were subjected to the calcium switch (Figure 5A). The phosphorylated level increased about 2- to 3-fold within 30 min, and mLgl-2 remained phosphorylated 25 hr after the calcium switch (Figure 5B). A similar result was obtained for mLgl-1

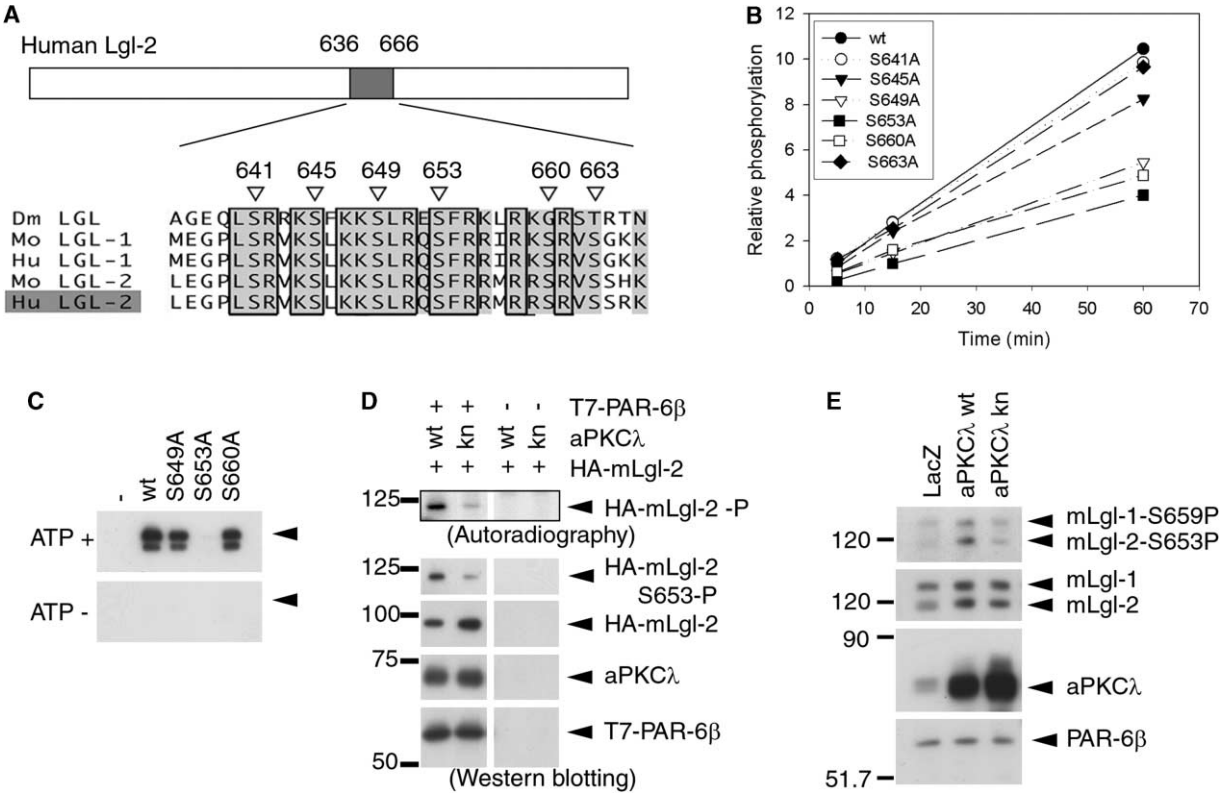

Figure 3. mLgl-2 Is Phosphorylated by aPKCλ In Vitro and In Vivo

(A) Sequence alignment of 636–666 aa of human Lgl-2 with the corresponding regions of mouse Lgl-2, human or mouse Lgl-1, and *Drosophila* Lgl. Conserved serine residues are indicated by arrowheads.

(B) GST-mLgl-2 636–666 (wt) or its serine-alanine mutants were coincubated with HA-aPKCλ for the indicated time, and γ-^{32}P incorporation into the GST fusion protein was examined by autoradiography. The level of GST-mLgl-2 636–666 wt phosphorylation at 5 min was taken as 1.

(C) GST-mLgl-2 636–666 or its serine-alanine mutants were coincubated with HA-aPKCλ for 120 min in the presence (ATP+) or absence (ATP−) of ATP. The products were subjected to SDS-PAGE, followed by Western blot analysis with the anti-phospho Ser 653 antibody.

(D) 293T cells transfected with expression vectors as indicated (top) were subjected to immunoprecipitation with the anti-T7-tag antibody (left) or control IgG (right). The IPs were incubated in the presence of [γ-^{32}P] ATP for 15 min and were subjected to SDS-PAGE followed by autoradiography. The reaction products were also analyzed by using the anti-phospho Ser 653 antibody together with the anti-HA-tag, anti-aPKCλ (ι), or anti-T7-tag (Omni) antibody.

(E) MDCK cells expressing LacZ, aPKCλ wt, or aPKCλ kn were subjected to calcium switch for 5 hr. The cells were lysed and subjected to Western blot analysis with the anti-phospho Ser 653 antibody, as well as the anti-mLgl-2 (100–278 2-3AP), anti-mLgl-1, anti-aPKCλ (ι), or anti-PAR-6β (Beta2-4AP) antibody.

(data not shown). These results suggest that phosphorylation of mLgl-1/2 by aPKCλ occurs during the cell-cell contact-induced cell polarization and that this is accompanied by dissociation of the interaction between mLgl-2 and the PAR-6β/aPKCλ complex.

Increased Amounts of mLgl-2 Suppress Functional TJ Formation

The above results suggest a possibility that the transient interaction between PAR-6β/aPKCλ and mLgl-2 contributes to the establishment of epithelial cell polarity. To test this, we overexpressed mLgl-2 in MDCK cells and evaluated its effects on TJ formation and function by examining the junctional localization of ZO-1 and the development of transepithelial electrical resistance (TER), respectively. As shown in Figures 6A and 6B, overexpressed HA-mLgl-2 localizes to the cell-cell contact region and affects calcium-induced TER development and ZO-1 localization. It should be noted that the effects of HA-mLgl-2 were not observed when the cells were not subjected to calcium switch (data not shown). Overexpressed T7-PAR-6β, but not its M235W, also localizes to the cell-cell contact region, where endogenous mLgl-2 localizes and affects calcium-induced TJ formation as described elsewhere [14] (Figure S3); this finding is in direct contrast with the data found for PAR-6α, which has a low affinity to mLgl and does not affect TJ formation (data not shown) [13]. These results indicate that enhanced localization of mLgl-2 or PAR-6β to the cell-cell contact region inhibits the cell-cell contact-mediated formation of the junctional complex.

Deregulation of aPKCλ Activity Causes Mislocalization of PAR-6β or mLgl-2 to the Entire Cell Periphery

The effects of HA-mLgl-2 or T7-PAR-6β resemble those observed for the kinase-deficient mutant aPKCλ (aPKCλ kn) [4]. These suggest that aPKCλ regulates the interac-

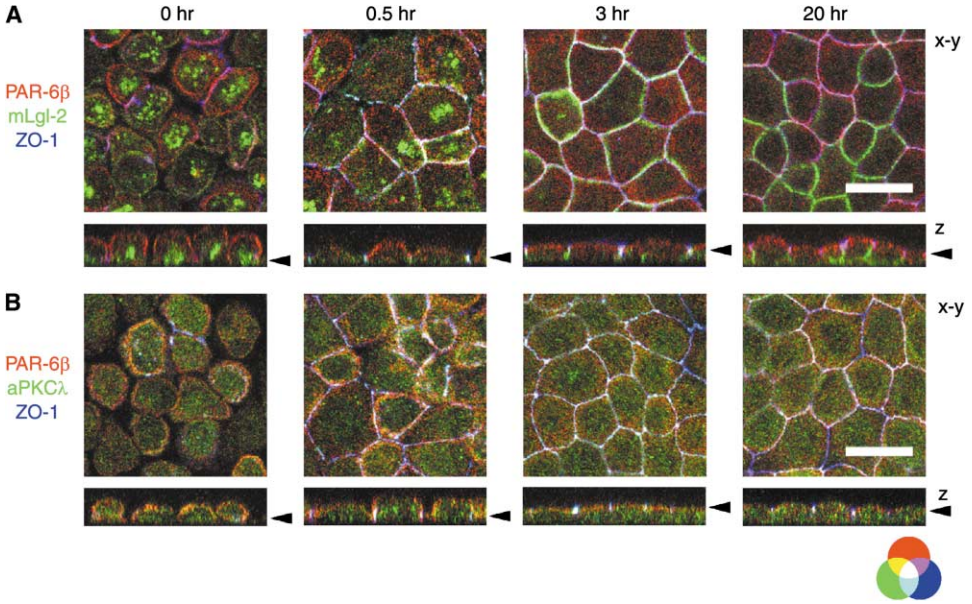

Figure 4. Codistribution of mLgl-2 with PAR-6β and aPKCλ at the Cell-Cell Contact Region of MDCK Cells during the Process of Polarization

(A and B) MDCK cells cultured in a low-calcium medium were subjected to calcium switch for 0, 0.5, 3, or 20 hr. The cells were fixed and triple stained with the anti-PAR-6β (red) and anti-ZO-1 (blue) antibodies together with the (A) anti-mLgl-2 or (B) anti-aPKCλ antibody (green) as indicated. The upper panels show single confocal x-y sections (x-y), and the lower panels show corresponding z sections (z). The arrowheads in the lower panels indicate the plane of x-y sections. (A) Partial triple staining for mLgl-2 with PAR-6β and ZO-1 (white) was observed at the cell-cell contact region of the cells at 0.5 and 3 hr after the calcium switch, whereas this triple staining was separated to a double staining for PAR-6β and ZO-1 (purple) and single staining for mLgl-2 (green) at 20 hr after the calcium switch. (B) Triple staining for aPKCλ with PAR-6β and ZO-1 (white) was observed at the cell-cell contact region of the cells 0.5, 3, and 20 hr after the calcium switch. The scale bars represent 20 μm.

tion of the PAR-6β/aPKCλ complex with mLgl-2 during epithelial cell polarization and contributes to the segregation of mLgl from the PAR-6β/aPKCλ complex. As shown in Figure 7A, overexpression of aPKCλ kn, but not its wt, causes mislocalization of both mLgl-2 and PAR-6β, resulting in the colocalization of these proteins at the apicolateral periphery. Importantly, these effects were not observed in MDCK cells not subjected to a calcium switch (Figure 7A), which is consistent with our previous observation that aPKCλ kn does not affect TJ formation when the cells are not subjected to a calcium switch [4]. These results indicate that, during epithelial cell polarization, the kinase activity of aPKCλ is required for segregation of PAR-6β and mLgl-2 localization and thus regulates TJ formation.

The overexpressed aPKCλ kn in MDCK cells colocalizes with endogenous PAR-6β at the apicolateral periphery, although most of aPKCλ kn localizes to the cytosol (Figure 7B). In addition, the kinase-deficient mutant of the kinase domain (KD) of aPKCλ (KD-kn) preferentially interacts with mLgl-2 (Figure 7C). Interestingly, the substitutions of Ser residues of mLgl-2 (649, 653, and 660) for Glu (3SE), but not Ala (3SA), decrease this interaction even if aPKCλ KD-kn is used instead of its KD (Figure 7C). These results suggest that aPKCλ regulates the interaction of the PAR-6β/aPKCλ complex with mLgl-2 through the phosphorylation of mLgl-2.

Discussion

Here, we present evidence showing that the PAR-6β/aPKCλ complex interacts with either mLgl or PAR-3 in a mutually exclusive manner, forming two independent protein complexes. Notably, overexpression of mLgl-2 inhibits TJ formation; this finding is in direct contrast with the data found for PAR-3, whose overexpression, but not that of its mutant lacking the aPKC binding region, promotes TJ formation [11]. This suggests that the two independent complexes have distinct functions in the establishment of epithelial cell polarity. This is consistent with the results of genetic studies of *Drosophila* in which Lgl is required for formation of the basolateral membrane domain through the inhibition of the formation of apical identity [21, 31], whereas subapical Bazooka (PAR-3) is required for the formation of the apical membrane domain [15, 22].

In polarized epithelial cells, mLgl localizes to the lateral region, in contrast to the PAR-6β/aPKCλ/PAR-3 complex that localizes to the apical end of the lateral domain. Interestingly, mLgl-2 transiently codistributes with PAR-6β and aPKCλ during the initial phase of epithelial cell polarization, whereas PAR-3 stably codistributes with them at the apical end of the cell-cell contact region [13]; this finding indicates that the balance between the two independent complexes changes during the initial phase of epithelial cell polarization. Further, overexpression of aPKCλ kn results in the abnormal codistribution of PAR-6β and mLgl-2 at the cell periphery; this finding suggests that aPKCλ activity is required for the segregation of PAR-6β and mLgl-2 localization during this process. Thus, our present results, as well as previous findings, lead us to hypothesize the following working model (Figure 8). The cell-cell contact initially

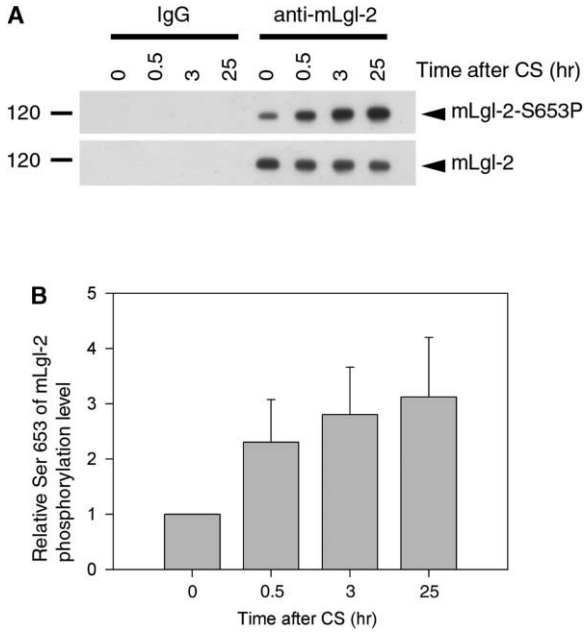

Figure 5. The Level of mLgl-2 Phosphorylation Increases during Calcium-Induced Cell Polarization

Confluent MDCK cells were subjected to the calcium switch for the indicated times. The cell lysates were subjected to immunoprecipitation with the anti-mLgl-2 (N13AP) or control IgG.

(A) Western blot analysis with the anti-phospho Ser 653 antibody as well as the anti-mLgl-2 (100–278 2-3AP) antibody.

(B) The relative Ser 653 of mLgl-2 phosphorylation level. Values are means (±SD) of three independent experiments, which were normalized with the amounts of mLgl-2. The phosphorylation level at 0 hr was taken as 1.

stimulates the localization of the protein complex containing PAR-6β, aPKCλ, and mLgl at the cell-cell contact region. The complex is "inactive" for TJ formation. Once aPKCλ is activated, mLgl segregates from the PAR-6β/aPKCλ complex. This triggers the formation of the "active" PAR-6β/aPKCλ/PAR-3 complex that promotes the formation of the epithelial junctional complex. Segregated mLgl remains in the lateral region and seems to contribute to the establishment of the basolateral membrane identity, because mLgl-1 has been reported to interact with syntaxin-4, a component of the basolateral exocytic machinery [27]. Although the mechanism for activation of aPKCλ remains to be clarified, Cdc42 and/or Rac1 are strong candidates as activators of aPKC in MDCK cells (Figure 8), since the GTP-bound form of Cdc42 activates aPKCλ kinase activity through PAR-6 in vitro [13] and cell-cell adhesion activates Cdc42 and Rac1 in epithelial cells [32, 33].

We also show that mLgl is phosphorylated by aPKCλ and that this phosphorylation increases in response to cell-cell adhesion-mediated cell polarization. Further, a phosphomimicking mutant of mLgl-2 (3SE) fails to bind to aPKCλ. These results imply that aPKCλ-dependent phosphorylation of mLgl is involved in the regulation of its interaction with the PAR-6β/aPKCλ complex (Figure 8). On the other hand, we could not detect any difference between mLgl-2 wt and its 3SE mutant in their interactions with the PDZ domain of PAR-6β (data not shown). In addition, overexpression of mLgl-2 mutants (3SA or 3SE) affects TJ formation similarly to that of its wt (data not shown). These results suggest the existence of another mechanism regulating the interaction of mLgl-2 with PAR-6β (see below).

Recently, Hurd et al. have revealed that mammalian Crumbs/Stardust (Pals1) can interact with the PDZ domain of PAR-6β and that this interaction is enhanced by activated Cdc42 [34]. Taken together with our present results, this suggests that the Crumbs/Pals1 complex might also be involved in the regulation of the interaction between mLgl and the PAR-6β/aPKCλ complex; the PAR-6β/aPKCλ complex, together with PAR-3, may involve the Crumbs/Pals1 complex to promote TJ formation (Figure 8). Thus, the dissociation of mLgl from the PAR-6β/aPKCλ complex likely triggers the interaction of the PAR-6β/aPKCλ complex with the Crumbs/Pals1 complex in addition to its interaction with PAR-3. The functional interactions proposed by this model are con-

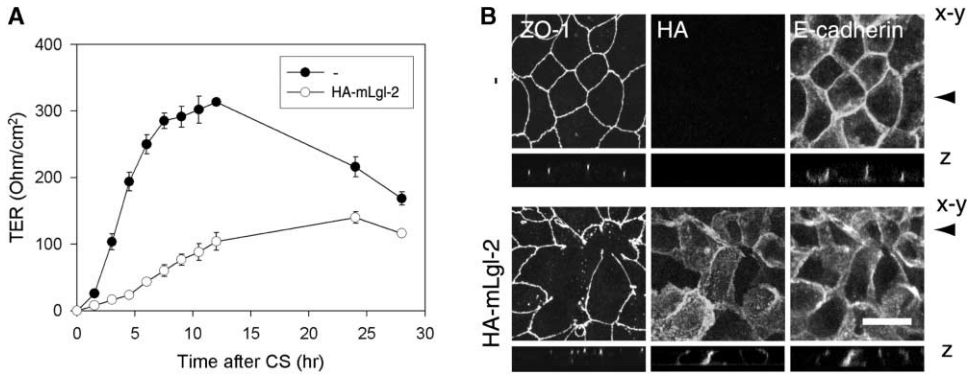

Figure 6. Overexpression of mLgl-2 Affects Calcium-Induced TER Development and ZO-1 Localization

Confluent MDCK cells were infected with adenovirus expression vectors encoding HA-mLgl-2. The empty vector was used as control (−). Cells were subjected to calcium switch 16 hr after viral infection.

(A) Measurement of TER development after calcium switch.

(B) The cells were fixed 5 hr after the calcium switch and were triple stained with the anti-HA, anti-ZO-1, and anti-E-cadherin antibodies. Photographs represent the projected views of optical sections from the apical to basal membrane of cells (x-y) and corresponding z-sections (z) at the planes indicated by arrowheads. The scale bar represents 20 μm.

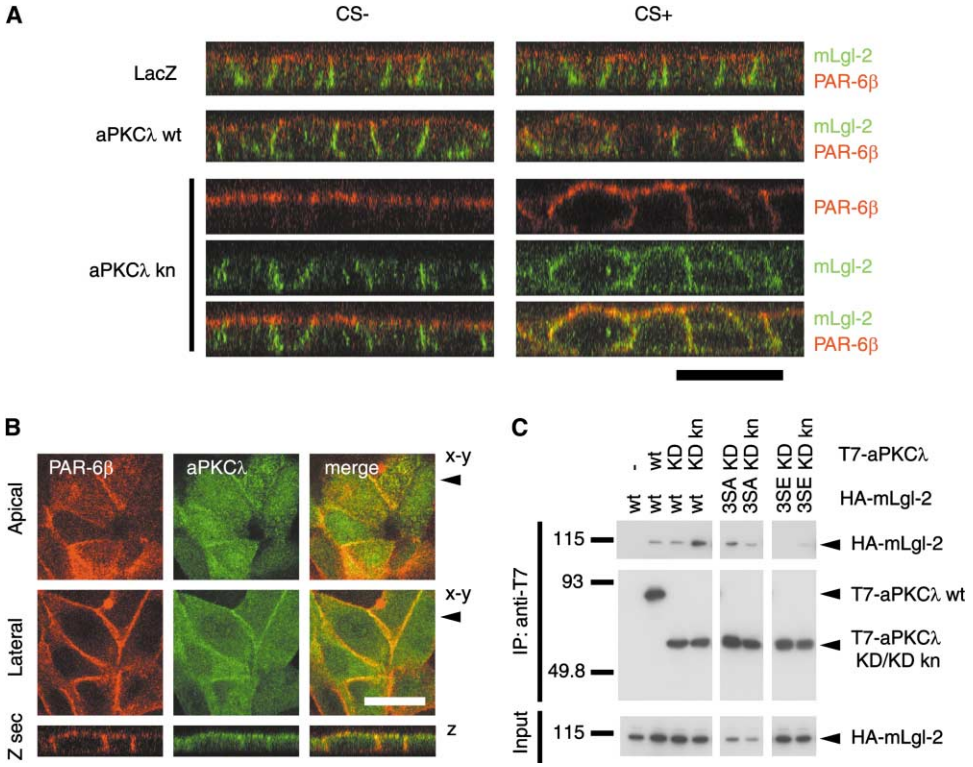

Figure 7. Overexpression of aPKCλ kn Results in Mislocalization of PAR-6β or mLgl-2 to the Entire Cell Periphery of MDCK Cells

(A and B) Confluent MDCK cells grown on coverslips were infected with adenovirus expression vectors encoding LacZ, aPKCλ wt, or aPKCλ kn. The cells were then subjected to calcium switch for 5 hr (CS+) or were left untreated (CS−). (A) Confocal z-sectional views of the cells double stained with the anti-PAR-6β (red) and anti-mLgl-2 (green) antibodies. (B) The cells expressing aPKCλ kn were subjected to the calcium switch and were double stained with the anti-aPKCλ (λ1) (green) and anti-PAR-6β (red) antibodies. Photographs represent the projected views of optical sections covering the apical region (Apical) or lateral region (Lateral), as well as corresponding z-sections (z) at the planes indicated by arrowheads.

(C) 293T cells were transfected with expression vectors as indicated (top), and the lysates (Input) were subjected to immunoprecipitation with the anti-T7 tag antibody, followed by Western blot analysis with the anti-T7 tag (Omni) or anti-HA tag antibody.

The scale bars represent 20 μm.

sistent with the results of recent genetic studies of *Drosophila* in which Lgl and Crumbs compete with each other to define respective membrane identity [21].

Our findings in this study suggest a notable analogy in the mechanism regulating epithelial polarity between *Drosophila* and mammals. This notion is supported by a recent observation in *Drosophila* that Lgl interacts with PAR-6 and aPKC and regulates the apicobasal polarity of *Drosophila* neuroblasts [35]. In mammalian fibroblasts, mLgl-1 has been reported to form a protein complex with PAR-6α (also called PAR-6C) and aPKC and is involved in the polarized migration of wounded MEF cells [36]. Further studies will help us to understand the molecular mechanism underlying the establishment of cell polarity in a variety of biological contexts.

Conclusions

We reveal that mammalian Lgl competes for PAR-3 in forming an independent protein complex with PAR-6 and aPKC in epithelial cells. During epithelial cell polarization, mLgl transiently colocalizes with PAR-6 and aPKC at the cell-cell contact region, and increased localization of mLgl and PAR-6 to the cell-cell contact region suppresses TJ formation. This finding contrasts with the data found for PAR-3, which promotes TJ formation and thus indicates that the balance between the two independent protein complexes regulates the establishment of epithelial cell polarity. We also suggest that aPKC activity-mediated phosphorylation of mLgl is involved in the regulation of mLgl's interaction with PAR-6/aPKC. Our findings provide new insight into the mechanism underlying the establishment of epithelial cell polarity.

Experimental Procedures

Expression Vectors

cDNAs encoding human PAR-6β [37] were subcloned into the SRHis/T7 vector [10]. PAR-6β mutants were generated using PCR. The following mutants were generated: 1–126, 119–372, 1–316, and 1–272, encoding the corresponding aa regions; ΔCRIB, lacking the CRIB region (126–138 aa); ΔPDZ, lacking the PDZ domain (159–259); and M235W, in which Met 235 was substituted for Trp. cDNA encoding human Lgl-2 (mLgl-2) (GenBank accession number: NM004524, X87342) was generated by fusing two cDNA fragments of human EST clones (BC010879 and AK025401) and one cDNA fragment generated from total RNA of HeLa cells by RT-PCR by using a OneStep RT-PCR kit (QIAGEN kk) and subcloned into the SRHA vector [38]. Expression vectors for mouse aPKCλ or its mutants, or rat PAR-3 (ASIP), were described previously [10, 39, 40]. Adenovirus

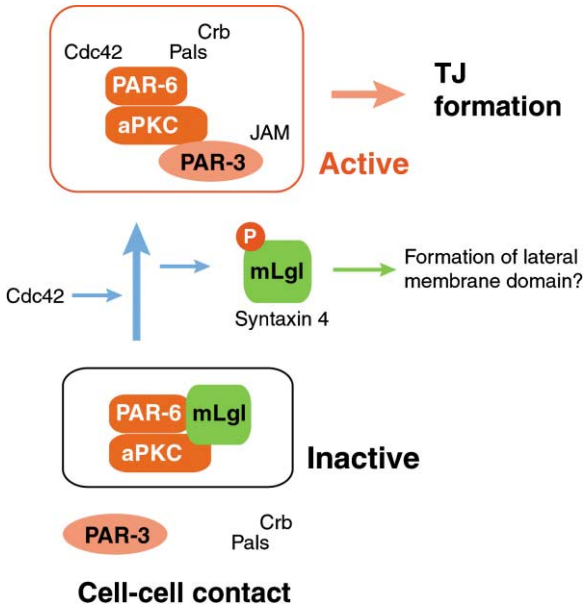

Figure 8. Schematic Model for Establishment of Epithelial Cell Polarity by PAR-6/aPKC and Its Interacting Proteins

The cell-cell contact initially localizes the "inactive" PAR-6β/aPKCλ/mLgl complex at the cell-cell contact region. Once aPKCλ is activated, presumably through the activated Cdc42, mLgl is phosphorylated and segregated from PAR-6β/aPKCλ, forming the "active" PAR-6β/aPKCλ/PAR-3 complex that promotes TJ formation with JAM. The Pals/Crb complex may also contribute to this process through its interaction with PAR-6β. On the other hand, the segregated mLgl remains in the lateral region and seems to be involved in the establishment of basolateral membrane identity with syntaxin-4.

expression vectors encoding mouse aPKCλ wt or kn and LacZ were described previously [4]. Adenovirus expression vectors encoding T7-PAR-6β wt or M235W, or HA-mLgl-2, as well as an empty vector, were generated using the Adeno-X expression system (Clontech).

Antibodies

Anti-PAR-6β antibodies, BC31AP and Beta2-4AP, were generated in rabbits, and BCR12AP was generated in rats; BC31AP and BCR12AP were raised against the C-terminal 14 aa of human PAR-6β, and Beta2-4AP was raised against the glutathione-S-transferase (GST) fusion protein with full-length human PAR-6β. Anti-mLgl-2 antibodies were generated in rabbits; N13AP was raised against the N-terminal 20 aa of human Lgl-2, and 100–278 2-3AP was raised against the GST fusion protein with 100–278 aa of human Lgl-2. The anti-mLgl-1 antibody, C-2 AP, was raised against the GST fusion protein with 808–1057 aa of mouse Lgl-1. Each antibody was affinity purified on the corresponding antigen-conjugated column. The phospho-specific antibody against serine 653 of human Lgl-2 was raised against a phospho-peptide (KSLRES[-P]FRKLR) and was purified on a phospho-peptide-conjugated column, after which it was coincubated with peptide (KSLRESFRKLR). Anti-PAR-3 pAb (C2-3AP), and anti-aPKCλ (λ_1) pAb were described previously [10, 41]. The following antibodies were also used: anti-aPKCλ (ι) mAb, anti-E-cadherin mAb, (Transduction Laboratories), anti-ZO-1 mAb/pAb (ZYMED Laboratories), anti-T7 mAb (Novagen), anti-T7 (Omni) pAb (Santa Cruz), anti-HA rabbit pAb (BETHYL), and anti-HA rat mAb (Roche).

Cell Culture and Adenovirus Infection

MDCKII (canine kidney), MTD-1A (mouse mammary tumor), $Caco_2$ (human colon cancer), and 293T cells were grown in DMEM containing 10% FCS, penicillin, and streptomycin under an air-5% CO_2 atmosphere at constant humidity. The calcium switch assay and adenovirus infection of MDCKII cells were performed as described previously [4]. Briefly, confluent MDCKII cells with or without adenovirus infection were incubated for 16 hr in the low-calcium medium (3 μM calcium) [42] and were then transferred to the normal calcium medium. Transepithelial electrical resistance (TER) was measured as described previously [4].

Immunoprecipitation and Western Blot Analysis

293T cells cultured on a 6-cm dish were transfected by using the PolyFect Transfection Reagent (QIAGEN). MDCK II, MTD-1A, or $Caco_2$ cells were cultured on a 10-cm dish. The cells were suspended in lysis buffer and subjected to immunoprecipitation, followed by SDS-PAGE and Western blotting as described previously [4].

In Vitro Protein Kinase Assays

To determine the phosphorylation site(s) of human Lgl-2 by aPKCλ, synthesized oligonucleotides encoding 636–666 aa of human Lgl-2 or its serine-alanine mutants were inserted into the pGEX-6P vector (Amersham Bioscience), and GST fusion proteins were purified by using the standard procedure. HA-aPKCλ was purified on anti-HA affinity beads (Roche) from transfected 293T cells as described previously [38]. GST-mLgl-2 636–666 or its serine-alanine mutants (2 μg) were coincubated with 0.05 μg HA-aPKCλ in 50 μl phosphorylation buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM EGTA, 40 μM ATP, and 2 μCi/50 μl [γ-^{32}P] ATP) at 30°C. The reaction was stopped by adding SDS sample buffer, and the products were subjected to SDS-PAGE, followed by autoradiography or Western blotting. To examine the phosphorylation of full-length mLgl-2 by aPKCλ, 293T cells were cotransfected with expression vectors encoding HA-mLgl-2, T7-PAR-6β, and aPKCλ and were subjected to immunoprecipitation with the anti-T7 tag antibody, followed by an in vitro phosphorylation assay using phosphorylation buffer as described previously [13].

In Vitro Binding Assay

GST-PAR-6β wt or its mutants were produced in *E. coli* and were purified by using the standard procedure. MDCK cells were suspended in lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin, 1 mM PMSF, and 1.8 μg/ml aprotinin, and the suspension was clarified by centrifugation. The lysates were coincubated for 1 hr with 5 μg GST fusion proteins immobilized to glutathione-sepharose 4B (Amersham Bioscience). After washing four times with lysis buffer, GST fusion proteins were eluted with 16 mM glutathion, and coeluted proteins were subjected to SDS-PAGE, followed by Western blotting or CBB staining.

Blot Overlay Assay

293T cells transfected with the expression vector encoding HA-mLgl-2 or HA-aPKCλ were lysed and subjected to immunoprecipitation with the anti-HA tag antibody. The IPs were then subjected to SDS-PAGE, and separated products were blotted on a PVDF membrane. The membrane was incubated with denaturing buffer (7 M guanidine-HCl, 50 mM DTT, 2 mM EDTA, 50 mM Tris-HCl [pH 8.3]) for 30 min and were then incubated with renaturing buffer (20 mM Tris-HCl [pH 7.4], 140 mM NaCl, 4 mM DTT, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 0.1% BSA, and 0.1% NP-40) for 16 hr. After treatment with 5% skim milk solution, the membrane was incubated with 2 μg/ml GST-PAR-6β or GST in renaturing buffer for 16 hr. After washing three times with renaturing buffer, GST proteins were detected by using the anti-GST antibody and chemiluminescence ECL (Amersham Bioscience).

Immunofluorescence Microscopy

MDCK cells were fixed with 2% paraformaldehyde and were stained as described previously [4]. The secondary antibodies used here were Alexa 488-conjugated goat anti-rabbit or anti-mouse antibody (Molecular Probes), Cy3-conjugated goat anti-rabbit or anti-rat antibody (Amersham Bioscience), or Cy5-conjugated goat anti-mouse antibody (Amersham Bioscience). The samples were mounted with VECTASHIELD (Vector Laboratories) and were viewed with a confocal microscope system (LSM 510; Carl Zeiss).

Supplemental Data

Supplemental Data including three additional figures are available at http://images.cellpress.com/supmat/supmatin.htm.

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