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Requirement of ZO-1 for the formation of belt-like adherens junctions during epithelial cell polarization

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The molecular mechanisms of how primordial adherens junctions (AJs) evolve into spatially separated belt-like AJs and tight junctions (TJs) during epithelial polarization are not well understood. Previously, we reported the establishment of ZO-1/ZO-2–deficient cultured epithelial cells (1[ko]/2[kd] cells), which lacked TJs completely. In the present study, we found that the formation of belt-like AJs was significantly delayed in 1(ko)/2(kd) cells during epithelial polarization. The activation of Rac1 upon primordial AJ formation is severely impaired in 1(ko)/2(kd) cells. Our data indicate that ZO-1 plays crucial roles not only in TJ formation, but also in the conversion from "fibroblastic" AJs to belt-like "polarized epithelial" AJs through Rac1 activation. Furthermore, to examine whether ZO-1 itself mediate belt-like AJ and TJ formation, respectively, we performed a mutational analysis of ZO-1. The requirement for ZO-1 differs between belt-like AJ and TJ formation. We propose that ZO-1 is directly involved in the establishment of two distinct junctional domains, belt-like AJs and TJs, during epithelial polarization.

Introduction

Epithelial cells play fundamental roles in separating compositionally different compartments to regulate homeostasis and maintain physiological functions in multicellular organisms. These functions are established by organized junctional complexes, cytoskeletal architecture, and highly polarized membrane domains (Nelson, 2003).

During epithelial cell polarization, E-cadherin– and nectin-mediated cell–cell contacts induce the formation of primordial "spot-like" adherens junction (AJ) complexes (Irie et al., 2004). Through interaction between actin filaments and components of primordial AJs, these junctions are gradually fused side by side and finally become "belt-like" AJs (Yonemura et al., 1995; Vasioukhin et al., 2000). In parallel with this event, tight junctions (TJs) are formed at the apical side of AJs. However, how belt-like AJs and TJs are evolved from primordial AJs and sorted during the polarization process of epithelial cells remains mostly to be clarified.

The molecular architecture of AJs and TJs has been unraveled rapidly in recent years (Nagafuchi, 2001; Tsukita et al., 2001; Matter and Balda, 2003; Anderson et al., 2004; Furuse and Tsukita, 2006). Among them, ZO-1 and Par-3–Par-6–aPKC are unique in that they localize at primordial AJs in the initial phase of epithelial polarization (Ando-Akatsuka et al., 1999; Suzuki et al., 2002), but they eventually localize at TJs and not at belt-like AJs after the maturation of epithelial polarization (Stevenson et al., 1986; Itoh et al., 1993). Par-3–Par-6–aPKC protein complexes are known to be required for the formation of belt-like AJ and TJ formation in epithelial polarization (Macara, 2004); however, our knowledge about the functional roles of ZO-1 in cell polarization is limited.

ZO-1/ZO-2/ZO-3 is a membrane-associated guanlyate kinase (MAGUK) protein composed of the following domains: three PDZ (PSD95/Dlg/ZO-1) domains, an SH3 domain, a GK domain, an acidic domain, and an actin binding region (Gonzalez-Mariscal et al., 2000). The PDZ1 domain binds to claudins. ZONAB is localized to TJ plaque by binding to the SH3 domain. The GK domain is the binding site for occludin (Matter and Balda, 2003). SH3-GK domains are responsible for the binding to α-catenin and afadin (Yamamoto et al., 1997; Imamura et al., 1999). In addition to diverse interactions, the SH3-GK domain is thought to play a role in the dimerization of MAGUK proteins as reported for other MAGUK proteins, especially as shown by PSD-95 (McGee and Bredt, 1999) and Dlg/SAP90/SAP102 (Masuko et al., 1999), but direct evidence

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Abbreviations used in this paper: AJ, adherens junction; DA, dominant-active; MAGUK, membrane-associated guanlyate kinase; TJ, tight junction.

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is lacking in the case of ZO-1. The acidic domain has not been well characterized in previous studies. As ZO-1 binds to not only TJ proteins (such as claudins and occludin), but also to AJ proteins (such as α-catenin and afadin), we speculate that ZO-1 may orchestrate the behavior of binding partners during epithelial cell polarization and play a role in sorting belt-like AJs and TJs from primordial AJs.

We have previously established an epithelial cell line lacking the expression of all ZO-1/ZO-2/ZO-3 to clarify their function. Using mouse EpH4 epithelial cells in which ZO-3 was not expressed, we established cell lines with a knocked-out ZO-1 gene (ZO-$1^{-/-}$ cells) with homologous recombination (Umeda et al., 2004). As the next step, clones with suppressed ZO-2 expression (1[ko]/2[kd] cells) were obtained from ZO-$1^{-/-}$ cells by stably expressing short interfering RNAs (Umeda et al., 2006). We previously reported that these cells possessed well-polarized cell architecture in terms of the differentiation of apical/basolateral membranes and formation of belt-like AJs but lacked TJs completely in the confluent state. The exogenous expression of N-terminal PDZ1-3 domains of ZO-1 was inefficient to rescue the formation of TJs in 1(ko)/2(kd) cells; however, when N-terminal PDZ1-3 domains of ZO-1 were forcibly recruited to the lateral membrane by adding a myristoylation signal and dimerized using the FKBP system, claudins were polymerized in 1(ko)/2(kd) cells, indicating that dimerization of the PDZ domains of ZO-1 determine whether and where claudins are polymerized in epithelial cells (Umeda et al., 2006).

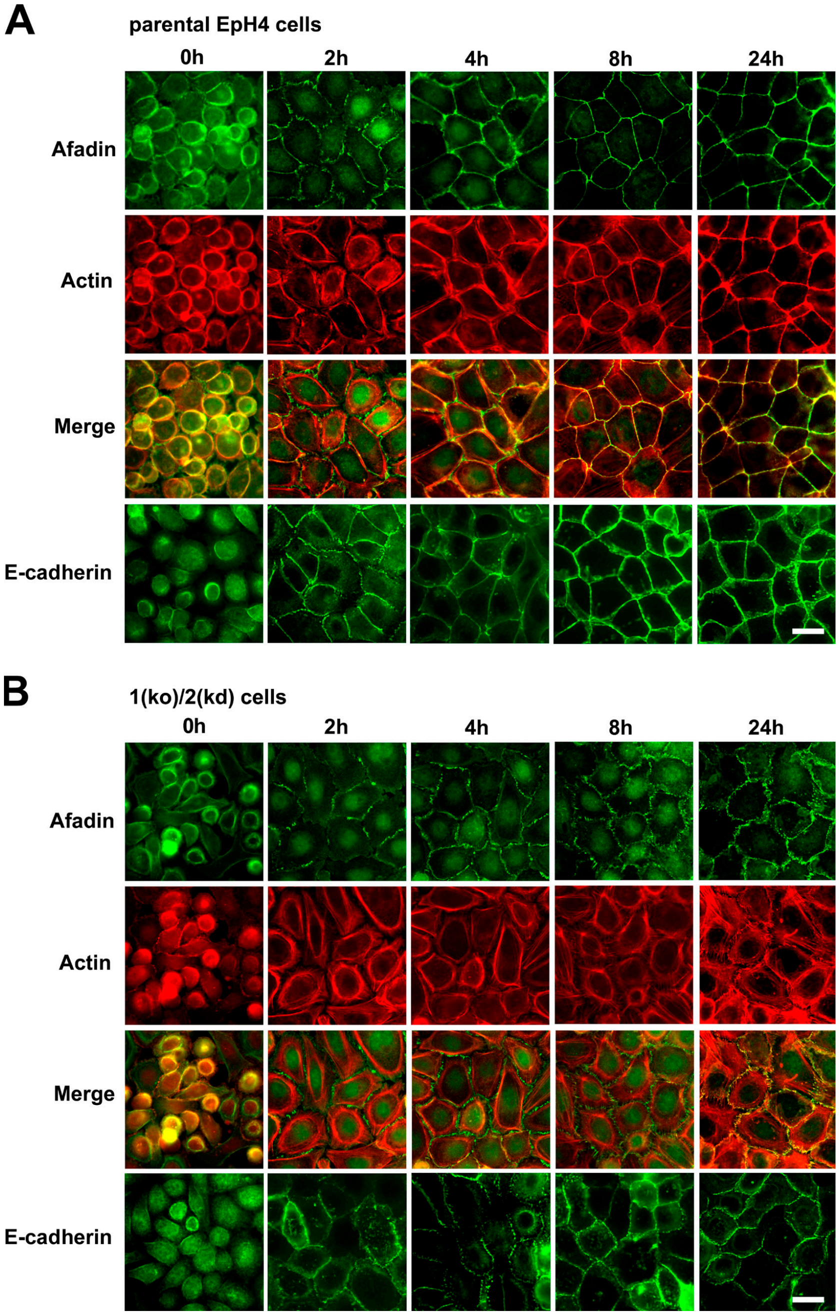


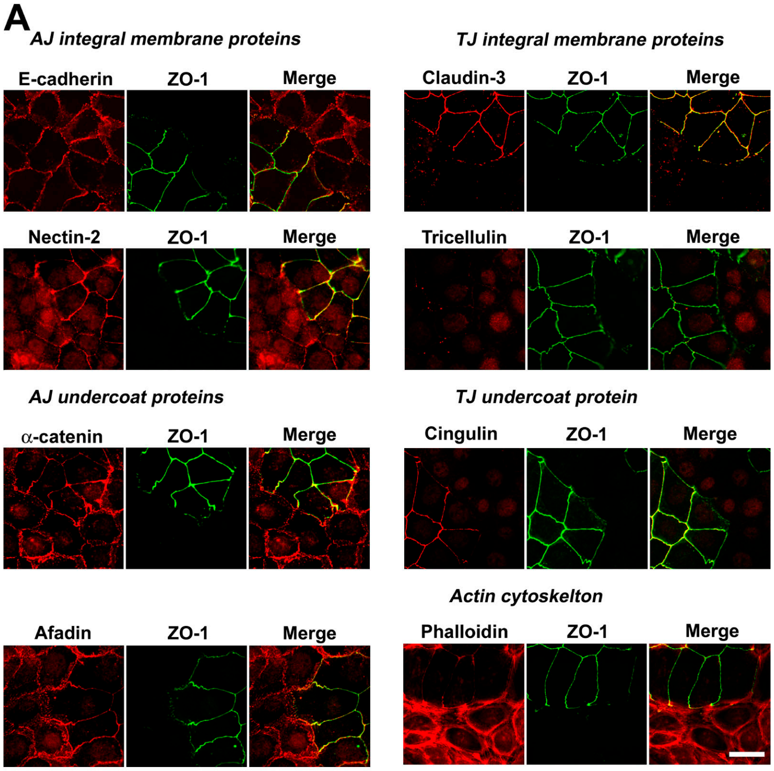
Figure 1. **Retardation of the formation of belt-like AJs and linear actin cables in 1(ko)/2(kd) cells during epithelial polarization.** Parental EpH4 cells (A) and 1(ko)/2(kd) cells (B) were cultured overnight in low Ca^{2+} medium overnight, and their polarization was initiated by transferring to normal Ca^{2+} medium. After a 0, 2-, 4-, 8-, or 24-h incubation, cells were fixed and stained with anti-afadin mAb, anti-E cadherin mAb, and phalloidin. Bars, 10 μm.

In the present study, we carefully observed the formation process of junctional complexes in 1(ko)/2(kd) cells and parental EpH4 cells using the Ca^{2+} switch assay and examined the roles of ZO-1 in the formation of belt-like AJs and junction-associated linear actin cables besides TJs during epithelial polarization. Our data indicate that ZO-1 plays crucial roles not only in TJ formation, but also in the conversion from “fibroblastic” AJs to belt-like “polarized epithelial” AJs during epithelial polarization. Furthermore, to examine whether ZO-1 itself mediates the formation of both belt-like AJs and TJs, we performed a mutational analysis of ZO-1.

Results and discussion

Formation of belt-like AJs and reorganization of actin filaments during cell polarization are retarded in 1(ko)/2(kd) cells

We examined AJ formation carefully during epithelial cell polarization in 1(ko)/2(kd) cells and parental EpH4 cells using the Ca^{2+} switch assay. The cells were cultured in a low Ca^{2+} medium containing 5 μM Ca^{2+} overnight under confluent conditions, and their polarization was initiated by transferring to a normal Ca^{2+} medium. The degree of AJ formation was



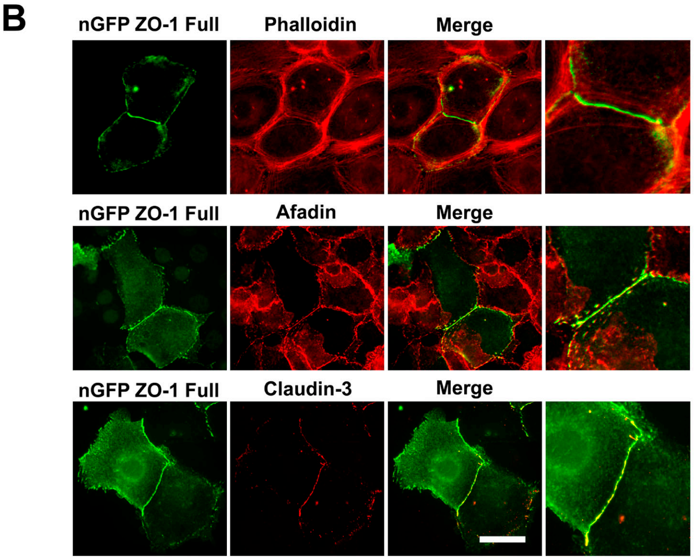


Figure 2. **Molecular assembly of junctions in 1(ko)/2(kd) cells.** (A) Parental EpH4 cells and 1(ko)/2(kd) cells were cocultured and doubly stained with the indicated antibodies 24 h after relpating. Bar, 10 μm. (B) The retardation of the formation of belt-like AJs, TJs, and linear actin cables were canceled by the exogenous expression of GFP-tagged full-length ZO-1. Cells were fixed and double stained with the indicated antibodies 24 h after transfection. Bar, 15 μm.

evaluated by immunofluorescence staining with anti-afadin mAb and anti–E-cadherin mAb. As shown in Fig. 1 A, parental EpH4 cells began to form belt-like AJs at 2 h and appeared to have mostly completed the process at 4 h after being transferred to normal Ca^{2+} medium. In clear contrast, in 1(ko)/2(kd) cells, even after a 24-h incubation in normal Ca^{2+} medium, AJ staining was still punctate. The number of spots of primordial AJs increased in 1(ko)/2(kd) cells along the time course, but each spot of primordial AJs was not fused (Fig. 1 B).

The rearrangement of the actin filaments during junctional maturation was also significantly delayed in 1(ko)/2(kd) cells compared with parental cells. In parental cells, the cortical actin cytoskeleton was aligned in a linear fashion along the cell–cell junction 4 h after the Ca^{2+} switch. In contrast, actin bundles were not organized at the cell cortex even 24 h after Ca^{2+} repletion in 1(ko)/2(kd) cells.

To confirm the retardation of junction maturation in 1(ko)/2(kd) cells, 1(ko)/2(kd) cells were cocultured with parental EpH4 cells and double stained with antibodies against components of AJs or TJs, phalloidin and anti–ZO-1 antibody, 24 h after replating (Fig. 2 A). We examined the behavior of integral membrane proteins of AJs (E-cadherin and nectin) and undercoat proteins of AJs (α-catenin and afadin) in 1(ko)/2(kd) cells 24 h after replating. Judging from their staining, belt-like AJs were formed between parental EpH4 cells; however, spot-like AJs were still present in 1(ko)/2(kd) cells, representing a general defect in the assembly of belt-like AJs in 1(ko)/2(kd) cells. Afadin and β-catenin normally colocalized at primordial AJs in 1(ko)/2(kd) cells, indicating that the E-cadherin–catenin and nectin–afadin complexes were associated even in the absence of ZO-1/ZO-2 (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200612080/DC1). In addition, Par-3 is also normally colocalized with afadin at primordial AJs in 1(ko)/2(kd) cells (Fig. S1 B), and we concluded that molecular assembly of primordial AJs is normal in 1(ko)/2(kd) cells. TJ components (claudin-3, tricellulin, and cingulin) were not present at cell–cell contacts of 1(ko)/2(kd) cells (Fig. 2 A).

These phenotypes of 1(ko)/2(kd) cells, the absence of TJ formation and delayed formation of belt-like AJs and junction-associated linear actin cables, were rescued by recovery of ZO-1 or -2 (Fig. 2 B and not depicted). The same results were obtained in the case of F9 cells lacking ZO-1 and -2 by homologous recombination (unpublished data). The discontinuity of AJs in 1(ko)/2(kd) cells was decreased by further culture in normal

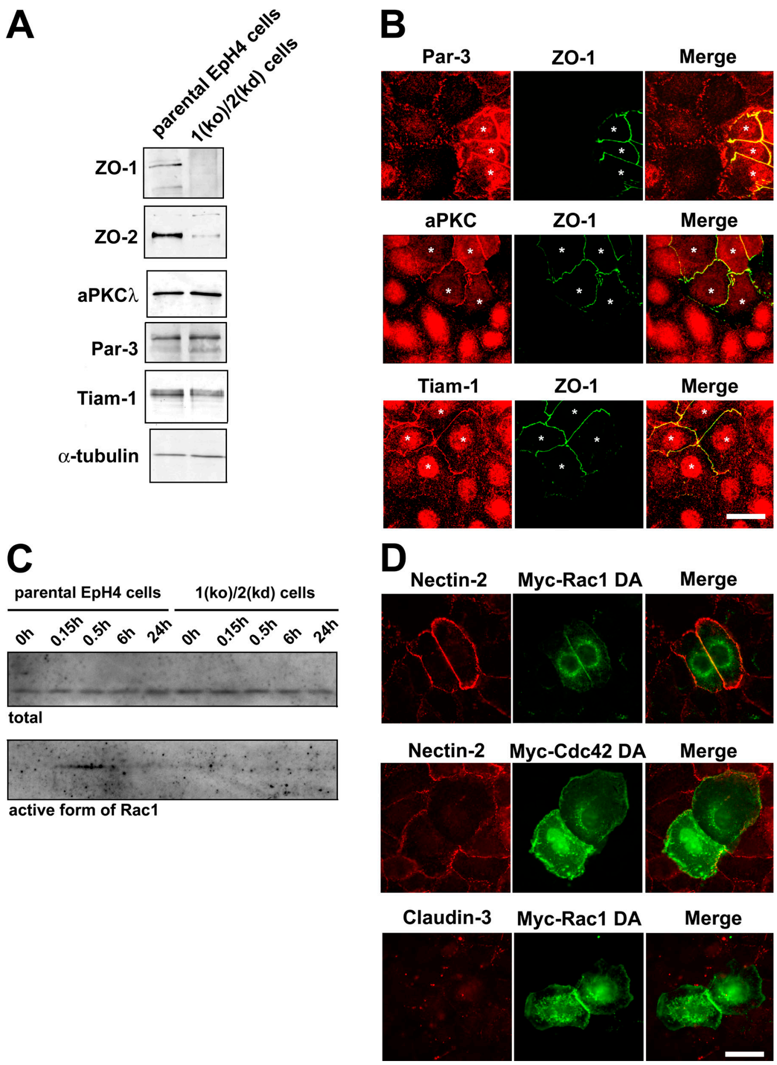


Figure 3. **Impaired activation of Rac1 in 1(ko)/2(kd) cells during epithelial polarization.** (A) Immunoblotting of whole-cell lysates of parental EpH4cells and 1(ko)/2(kd) cells with the indicated antibodies. (B) Parental EpH4 cells and 1(ko)/2(kd) cells were cocultured and doubly stained with the indicated antibodies 24 h after replating. Parental cells are indicated by asterisks. (C) Rac activity assays from parental EpH4 cells and 1(ko)/2(kd) cells after a Ca^{2+} switch for the indicated times. (D) 1(ko)/2(kd) cells were transiently transfected with myc-Rac1-DA or Cdc42-DA. Cells were double stained for myc and anti–nectin -2 antibody (as an AJ marker) or anti–claudin-3 antibody (as a TJ marker) 24 h after transfection. Bars, 10 µm.

Ca^{2+} medium judging from the staining of afadin, Par-3, and E-cadherin (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200612080/DC1). Cortical actin cables were normally formed in 1(ko)/2(kd) cells 72 h after Ca^{2+} switch; however, junction-associated actin cables were not observed (Fig. S2 B). The staining of actin filaments became sharp in more confluent state as shown in our previous paper (Umeda et al., 2006). The formation of TJs was not restored by long culture, and we confirmed that ZO-1/ZO-2 is a structurally essential component of TJs.

ZO-1/ZO-2 is required for the activation of Rac1 during cell polarization

Although 1(ko)/2(kd) cells finally became well-polarized epithelial cells in a confluent state after Ca^{2+} switch (Fig. S2 A), the retardation of belt-like AJ formation during cell polarization in 1(ko)/2(kd) cells clearly indicated that the loss of ZO-1/ZO-2 affected the initial phase of epithelial cell polarization. The polarity protein complex, which consists of Par-3, Par-6, and aPKCλ/ζ, has been shown to be required for the maturation of belt-like AJs and TJs from primordial AJs in epithelial cells (Suzuki et al., 2002; Macara, 2004). The newly discovered phenotype of 1(ko)/2(kd) cells, the persistence of primordial AJs, is similar to a previously reported phenotype of the dominant-negative mutant of aPKCλ overexpressing epithelial cells (Suzuki et al., 2002). During epithelial cell polarization, the polarity protein complex is known to be recruited to primordial AJs, and its activation at primordial AJs triggers belt-like AJ and TJ formation (Suzuki et al., 2002). In addition to the polarity protein complex, several groups reported that the Rac1-specific guanine nucleotide exchange factor, Tiam-1, acts upstream of Par-3, Par-6, and aPKCλ/ζ during epithelial polarization (Chen and Macara, 2005; Mertens et al., 2005; Nishimura et al., 2005). We first examined whether molecular assembly of the Par-3–Par-6–aPKC complex and Tiam-1 at primordial AJs was changed in 1(ko)/2(kd) cells. There was no obvious difference in the expression levels of Par-3, Par-6, aPKCλ/ζ, and Tiam-1 between parental Eph4 cells and 1(ko)/2(kd) cells (Fig. 3 A). 24 h after replating, Par-3, Par-6, aPKCλ/ζ, and Tiam-1 localized at primordial AJs in 1(ko)/2(kd) cells, indicating that the localization of them at primordial AJs was not affected by the loss of ZO-1/ZO-2 (Fig. 3 B and not depicted).

A small G protein, Rac1, is known to be activated upon E-cadherin and nectin mediated cell–cell contact formation (Yap and Kovacs, 2003; Irie et al., 2004). The activation of Rac1 is required for the activation of aPKC and subsequent cell polarization (Mertens et al., 2005). We examined whether the activation of Rac1 during cell polarization was altered in 1(ko)/2(kd) cells. We analyzed Rac1 activation in parental EpH4 cells and 1(ko)/2(kd) cells upon a Ca^{2+} switch. In parental EpH4 cells, Rac1 activation occurred within 10–30 min, whereas Rac1 activity was hardly stimulated in 1(ko)/2(kd) cells, suggesting that delayed cell polarization in 1(ko)/2(kd) cells was due to the impaired activation of Rac1 in primordial AJs (Fig. 3 C). Indeed, the exogenous expression of dominant-active (DA) Rac1 (but not Cdc42-DA) led to the maturation of belt-like AJs in 1(ko)/2(kd) cells, whereas the polymerization of claudins was not restored by the exogenous expression of Rac1-DA (Fig. 3 D). These data demonstrated that ZO-1 plays a critical role in the establishment of belt-like AJs through the activation of Rac1. The relationship between the activation of Rac1 and ZO-1 upon cell–cell contact should be clarified in future studies.

ZO-1 functions as a molecular machine to segregate TJs and AJs

The aforementioned findings suggested that ZO-1 plays crucial role in the conversion from fibroblastic AJs to belt-like polarized epithelial AJs through activation of Rac1 at primordial AJs in the initial phase of epithelial polarization. On the other hand, 1(ko)/2(kd) cells completely lacked TJs (Umeda et al., 2006). Because a previous study suggested that AJ formation was a prerequisite for the assembly of TJs (Gumbiner et al., 1988), we examine whether ZO-1 is directly involved in the establishment of two distinct junctional domains, belt-like AJs and TJs, during epithelial polarization. To determine whether ZO-1 itself might mediate the formation of both belt-like AJs and TJs, we performed a mutational analysis of ZO-1. We tested whether these mutants could rescue belt-like AJ and/or TJ formation by transiently expressing them in 1(ko)/2(kd) cells.

We first examined whether the N-terminal half of ZO-1 containing three PDZ domains, an SH3 domain, and a GK

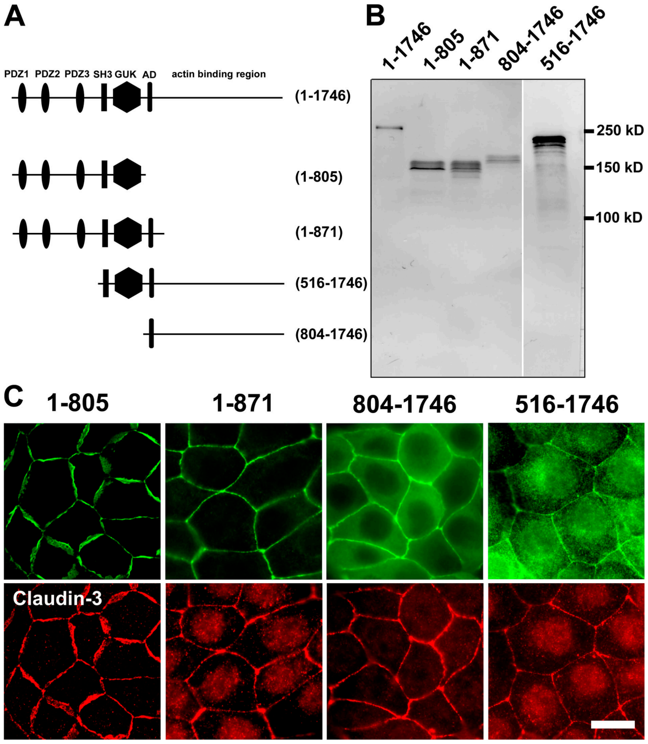
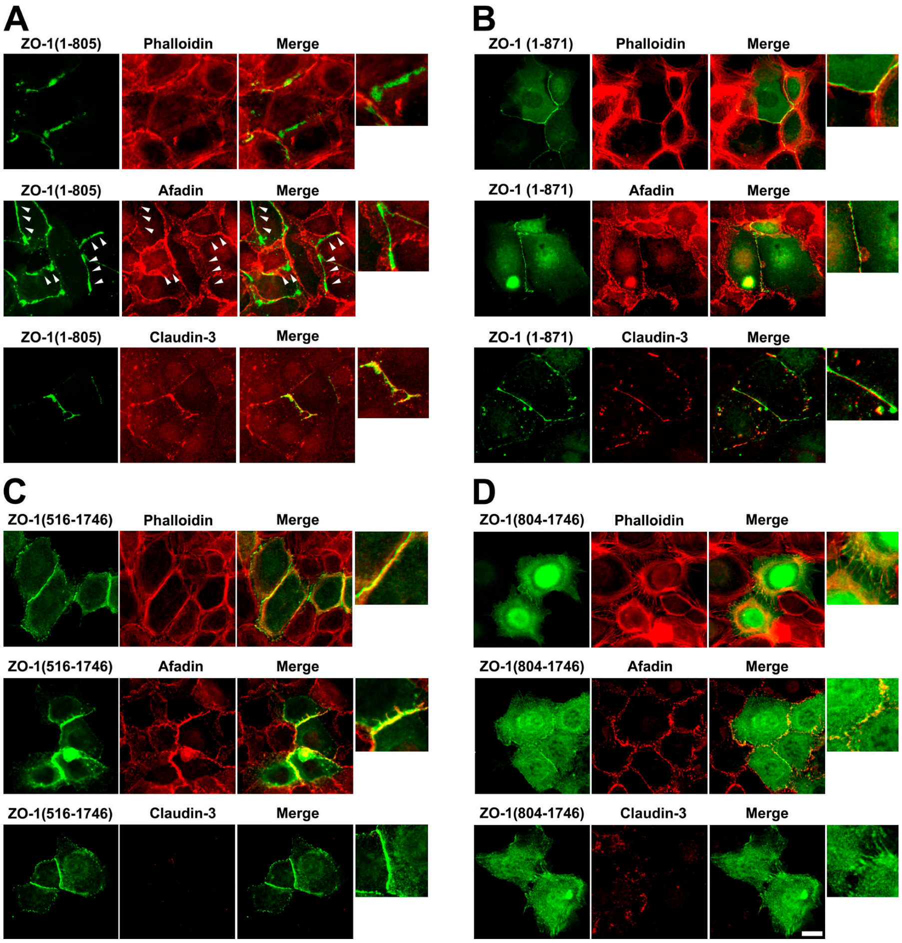


Figure 4. **Schematic representation of ZO-1 deletion mutants.** (A) Schematic drawings of deletion constructs of ZO-1. Amino acid residues of ZO-1 are shown in parentheses. (B) Total cell lysates derived from HEK293 cells transiently expressing each construct were separated by SDS-PAGE and immunoblotted with anti-GFP pAb. (C) Stable EpH4 lines expressing the deletion constructs of ZO-1 were stained with antibodies against GFP and claudin-3. Note that ZO-1$^{1-871}$, ZO-1$^{516-1746}$, and ZO-1$^{804-1746}$ were incorporated into TJs and did not affect the localization of claudin-3, whereas ZO-1$^{1-805}$ was distributed along the lateral plasma membrane and induced ectopic TJs. Bar, 10 μm.

Figure 5. **Rescuing belt-like AJ and/or TJ formation by deletion constructs of ZO-1 in 1(ko)/2(kd) cells.** 1(ko)/2(kd) cells were transfected with GFP-tagged ZO-$1^{1\text{-}805}$ (A), ZO-$1^{1\text{-}871}$ (B), ZO-$1^{516\text{-}1746}$ (C), and ZO-$1^{804\text{-}1746}$ (D). Cells were fixed and stained with the indicated antibodies 24 h after transfection. The formation of belt-like AJs was not observed at cell–cell contacts between ZO-$1^{1\text{-}805}$ expressing 1(ko)/2(kd) cells (arrowheads). Bar, 10 µm.



domain (ZO-$1^{1\text{-}805}$) restored the formation of linear actin cables and belt-like AJs and TJs in 1(ko)/2(kd) cells. When ZO-$1^{1\text{-}805}$ was expressed in parental EpH4 cells, ZO-$1^{1\text{-}805}$ localized at lateral membranes (Fig. 4 C and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200612080/DC1). Claudin-3 and occludin were incorporated into the ectopic TJs formed at cell–cell contacts between ZO-$1^{1\text{-}805}$ expressing parental EpH4 cells (Fig. 4 C and not depicted). ZO-$1^{1\text{-}805}$ localized at lateral membranes in 1(ko)/2(kd) cells and did not induce reorganization of actin filaments and formation of belt-like AJs, whereas ZO- $1^{1\text{-}805}$ induced claudin polymerization in 1(ko)/2(kd) cells (Fig. 5 A). TJs formed at cell–cell contacts between ZO-$1^{1\text{-}805}$ expressing 1(ko)/2(kd) cells were abnormal in that the TJs were discontinuous and expanded along the lateral membrane. Although ZO-$1^{1\text{-}805}$ was an artificial construct, ZO-$1^{1\text{-}805}$ induced TJ formation without belt-like AJ formation, indicating that ZO-1 has a potential to form TJs independently of fully blown belt-like AJ formation in epithelial cells.

Because SH3 and GK domains of PSD-95, another member of MAGUK homologues, were reported to interact with each other and dimerize through the intermolecular association, we considered that SH3-GK domains of ZO-1 also function as a dimerization module and contribute to the formation of a MAGUK network and the subsequent polymerization of claudins. We confirmed that ZO-$1^{1\text{-}805}$ (but not ZO-$1^{804\text{-}1746}$) formed a self-dimer in vitro (Fig. S3 C). The ability to form a self-dimer of ZO-$1^{1\text{-}805}$ is consistent with the idea that SH3-GK domains of ZO-1 function as a dimerization unit and contribute to the formation of a MAGUK network.

Previously, we reported that the N-terminal half of ZO-1 containing three PDZ domains, an SH3 domain, and a GK domain was enough to induce the formation of normal TJs in 1(ko)/2(kd) cells (Umeda et al., 2006). The construct used as the N-terminal half of ZO-1 in the previous study was characterized by Itoh et al. (1997) and encoded 1–862 amino acid residues of ZO-1. More precisely, 805–871 amino acids of ZO-1 comprise an acidic domain, and the construct contained most of an acidic domain.

Therefore, as the next step, a longer construct including ZO-$1^{1\text{-}805}$ and an acidic domain (ZO-$1^{1\text{-}871}$) was introduced into parental EpH4 cells and 1(ko)/2(kd) cells. In parental EpH4 cells, exogenously expressed ZO-$1^{1\text{-}871}$ was incorporated into TJs efficiently (Fig. 4 C). In 1(ko)/2(kd) cells, formation of belt-like AJs and TJs was restored completely by exogenous expression of ZO-$1^{1\text{-}871}$ (Fig. 5 B). Our knowledge about the function of the acidic domain is limited so far. In the present study, for the first time, we demonstrate that the acidic domain is required for the proper segregation of belt-like AJs and TJs during epithelial polarization.

As PDZ domains of ZO-1 directly bind to the C terminus of claudins, we examined whether PDZ domains of ZO-1 are required for proper formation of belt-like AJs. The construct of

ZO-1 lacking PDZ1-3 (ZO-$1^{516\text{-}1746}$) did not rescue TJ formation but restored the formation of belt-like AJs and linear actin cables, judging from the staining of phalloidin and afadin in 1(ko)/2(kd) cells (Fig. 5 C). The lines of actin cables induced by ZO-$1^{516\text{-}1746}$ in 1(ko)/2(kd) cells were less sharp than those of parental EpH4 cells. This indicated that ZO-$1^{516\text{-}1746}$ was insufficient for the formation of junction-associated actin cables. Finally, the construct containing an acidic domain and actin binding region (ZO-$1^{804\text{-}1746}$) was not effective for the recovery of both belt-like AJs and linear actin cables, indicating that SH3-GK domains are indispensable for the formation of belt-like AJ formation (Fig. 5 D).

Collectively, these data demonstrate that ZO-$1^{1\text{-}805}$ rescued at least polymerization of claudins independently of AJ formation, whereas ZO-$1^{516\text{-}1746}$ rescued only belt-like AJ formation. Thus, the required domains for belt-like AJ and TJ formation differed. The SH3-GK domains of ZO-1 are essential for both belt-like AJ and TJ formation. As ZO-$1^{1\text{-}871}$ rescued both belt-like AJ and TJ formation, future studies have to clarify how the acidic domain regulates the proper segregation of belt-like AJs and TJs during epithelial polarization.

In conclusion, our data demonstrate that ZO-1/ZO-2 is essential for the formation of both belt-like AJs and TJs during epithelial polarization. ZO-1/ZO-2 plays a crucial role in the conversion from fibroblastic AJ to belt-like polarized epithelial AJs through Rac1 activation upon cell–cell contact formation. Furthermore, through domain analyses of ZO-1, we found that the requirement for ZO-1 differs between belt-like AJ and TJ formation. These findings favor the notion that ZO-1 is directly involved in the establishment and sorting out of two distinct junctional domains, belt-like AJs and TJs, during epithelial polarization.

Materials and methods

Materials

Mouse anti–ZO-1 mAb (Itoh et al., 1993), rat anti-occludin mAb (Saitou et al., 1997), rat anti-tricellulin mAb (Ikenouchi et al., 2005), and rat anti-cingulin mAb (Ohnishi et al., 2004) were raised and characterized previously. Rat anti–E-cadherin mAb (ECCD2) and rabbit anti-PAR-3/ASIP pAb were provided by M. Takeichi (Center for Developmental Biology, Kobe, Japan) and S. Ohno (Yokohama City University, Yokohama, Japan), respectively. Mouse anti-afadin mAb and rat anti–nectin-2 mAb were provided by Y. Takai (Osaka University, Osaka, Japan). Rabbit anti–α-catenin and mouse anti–α-tubulin (DM1A) were purchased from Sigma-Aldrich. Rabbit anti–claudin-3 pAb and rabbit anti–ZO-2 pAb were purchased from Zymed Laboratories. Rat anti-HA mAb was purchased from Roche Applied Science. Rabbit anti-aPKC pAb and anti-Tiam1 pAb were purchased from Santa Cruz Biotechnology, Inc. Mouse anti-Rac1 mAb was purchased from Upstate Biotechnology.

Plasmids

pGEX4T1-CRIB-Pak for pull-down assay of Rac1 has been described previously (Matsuo et al., 2002) and was provided by F. Oceguera-Yanez and S. Narumiya (Kyoto University, Kyoto, Japan). pEF-BOS-myc-Rac-DA and pEF-BOS-myc-Cdc42-DA were provided by Y. Takai (Osaka University, Osaka, Japan) and T. Sasaki (University of Tokushima, Tokushima, Japan). A diagram of the expression constructs of deletion mutants used in this study is shown in Fig. 4 A. Each fragment was amplified by PCR and subcloned into the vector pCAGGS-NGFP or pCAGGS-NHA.

Cell culture and transfection

Mouse EpH4 epithelial cells, 1(ko)/2(kd) cells, and MDCK II cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. EpH4 cells were a gift from E. Reichmann (Institute Suisse de Recherches, Lausanne, Switzerland). Transfection was performed using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's instructions.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously (Ikenouchi et al., 2005). In brief, cells cultured on coverslips were fixed with 3% formalin in PBS for 10 min at RT, treated with 0.2% Triton X-100 in PBS for 5 min, and washed with PBS. Blocking was done by incubating the fixed cells with 5% BSA in PBS for 30 min at RT. After the antibodies had been diluted with the blocking solution, the cells were incubated at RT for 1 h with the primary antibody and for 30 min with the secondary antibody. For actin staining, Alexa Fluor 568 phalloidin (Invitrogen) was added to the secondary antibody. Specimens were observed at RT with a photomicroscope (BX51; Olympus) and with a confocal microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) equipped with a Plan-APOCHROMAT (60/1.40 N.A. oil-immersion objective) with appropriate binning of pixels and exposure time. The images were analyzed with IPLab version 3.9.5 (BD Biosciences) and LSM510 Meta version 3.0 (Carl Zeiss MicroImaging, Inc.).

In vitro binding assay, gel electrophoresis, and immunoblotting

The in vitro binding assay was performed as previously described (Itoh et al., 1997; Matsuo et al., 2002). Samples were resolved by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell). This membrane was incubated successively with primary antibodies, which were visualized using a blotting detection kit (GE Healthcare).

Online supplemental material

Fig. S1 shows the colocalization of β-catenin, afadin, and Par-3 at primordial AJs in 1(ko)/2(kd) cells. Fig. S2 shows that 1(ko)/2(kd) cells restore the formation of belt-like AJs and cortical actin cables 72 h after the Ca^{2+} switch. Fig. S3 shows that exogenous expression of ZO-$1^{1\text{-}805}$ induced aberrant TJs also in MDCK II cells and that ZO-$1^{1\text{-}805}$ formed a self-homodimer in vitro. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200612080/DC1.

This paper is dedicated to the memory of the late Shoichiro Tsukita.

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