Cooperative roles of Par-3 and afadin in the formation of adherens and tight junctions

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Summary

Par-3 is a cell-polarity protein that regulates the formation of tight junctions (TJs) in epithelial cells, where claudin is a major cell-cell adhesion molecule (CAM). TJs are formed at the apical side of adherens junctions (AJs), where E-cadherin and nectin are major CAMs. We have revealed that nectin first forms cell-cell adhesions, and then recruits cadherin to nectin-based cell-cell adhesion sites to form AJs and subsequently recruits claudin to the apical side of AJs to form TJs. The cytoplasmic tail of nectin binds afadin and Par-3. Afadin regulates the formation of AJs and TJs cooperatively with nectin. Here, we studied the role of Par-3 in the formation of these junctions by using Par-3-knockdown MDCK cells. Par-3 was necessary for the formation of AJs and TJs but was not necessary for nectin-based cell-cell adhesion. Par-3 promoted the association of afadin with nectin, whereas afadin was not necessary for the association of Par-3 with nectin. However, the association of afadin with nectin alone was not sufficient for the formation of AJs or TJs, and Par-3 and afadin cooperatively regulated it. We describe here these novel roles of Par-3 in the formation of junctional complexes.

Key words: Adherens junctions, Tight junctions, Nectin, Par-3, Afadin

Introduction

In epithelial cells, cell-cell adhesion is mediated through a junctional complex composed of tight junctions (TJs) and adherens junctions (AJs), and is essential for cell polarization (Farquhar and Palade, 1963). These junctional structures are typically aligned from the apical to basal sides. At TJs, claudin is a key member of the Ca^{2+}-independent cell-cell adhesion molecule (CAM) family, which comprises more than 27 members (Tsukita and Furuse, 1999; Tsukita et al., 2001). Occludin is another CAM at TJs, but its function has not been established. Claudin and occludin are associated with the actin cytoskeleton through peripheral membrane proteins, such as ZO-1, -2, and -3, which are actin filament (F-actin)-binding proteins. Junctional adhesion molecule (JAM), which is one of four members of the Ca^{2+}-independent immunoglobulin (Ig)-like CAM family, also localizes at TJs and interacts with ZO proteins (Bazzoni et al., 2000; Ebnet et al., 2000). At AJs, E-cadherin, one of over 80 cadherin superfamily members, is a key Ca^{2+}-dependent CAM (Gumbiner, 2000; Yagi and Takeichi, 2000). E-cadherin is associated with many peripheral membrane proteins, including $p120^{ctn}$, β-catenin, α-catenin, vinculin and α-actinin. The latter three proteins are F-actin-binding proteins. E-cadherin is associated with the actin cytoskeleton through these proteins and this association strengthens the cell-cell adhesion activity of E-cadherin (Nagafuchi, 2001).

Nectin, a member of the Ca^{2+}-independent Ig-like CAM family, localizes to AJs (Takai et al., 2003; Takai and Nakanishi, 2003). All nectins are associated with the actin cytoskeleton through afadin, which is an F-actin- and nectin-binding protein. Each nectin first forms homo-cis-dimers and then homo- or hetero-trans-dimers through the extracellular region in a Ca^{2+}-independent manner, causing cell-cell adhesion. Our series of studies have revealed that nectin first forms cell-cell adhesions, which then induce the activation of Rap1, Cdc42 and Rac small G proteins through Src (Fukuhara et al., 2004; Fukuyama et al., 2005; Kawakatsu et al., 2005; Kawakatsu et al., 2002). Cdc42 and Rac reorganize the IQGAP1-dependent actin cytoskeleton, which then recruits non-trans-interacting cadherin to the nectin-based cell-cell adhesion sites (Katata et al., 2003; Sato et al., 2006). Rap1 binds to afadin, which then binds to $p120^{ctn}$ associated with non-trans-interacting cadherin (Hoshino et al., 2005). This Rap1-dependent binding of afadin to $p120^{ctn}$ inhibits the endocytosis of non-trans-interacting cadherin and enhances the accumulation of non-trans-interacting cadherin at the nectin-based cell-cell adhesion sites and the cell-cell adhesion activity of cadherin, which eventually results in the formation of AJs (Hoshino et al., 2005; Sato et al., 2006). In addition, Cdc42 activated in this way increases the number of filopodia and cell-cell adhesion sites, whereas activated Rac induces the formation of lamellipodia and efficiently seals the cell-cell adhesion between filopodia like a zipper. Thus, the trans-interaction of nectin induces the formation of AJs in a complex mechanism. By contrast, after or during the formation of AJs, nectin recruits, first, JAM and then claudin and occludin to the

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apical side of AJs in cooperation with cadherin, which results in the formation of TJs (Takai et al., 2003). It remains unknown how nectin-based cell-cell adhesion recruits the TJ components to the apical side of AJs.

Evidence is accumulating that a set of polarity proteins, including Par-3, Par-6 and atypical protein kinase C (aPKC), are required for apico-basal polarization of epithelial cells (Ohno, 2001; Roh and Margolis, 2003). These three proteins directly interact together to form a ternary complex. Lgl2 forms a ternary complex with aPKC and Par-6, which prevents aPKC and Par-6 from forming a ternary complex with Par-3. Binding of Cdc42 to Par-6 induces the activation of aPKC, which then phosphorylates Lgl2, releasing phosphorylated Lgl2 from the aPKC–Par-6 complex and allowing this complex to bind to Par-3. In this complex, the PDZ domain of Par-3 binds to the C-terminal four amino acids of the cytoplasmic tail of JAM and localizes at TJs. Many reports have shown that the dynamic formation of the Par-3–aPKC–Par-6 complex, regulated by Lgl2 and Cdc42, is essential for the formation of TJs (Macara, 2004; Plant et al., 2003; Yamanaka et al., 2003), but it remains unknown how these cell polarity proteins regulate the formation of TJs, following the formation of AJs.

It has not been fully studied in mammals whether these cell polarity proteins affect the formation of AJs. It has been shown in epithelial cells of *Drosophila* that the Par-3 homologue bazooka is involved in the formation of the cadherin-based AJs and septate junctions, structures analogous to TJs (Harris and Peifer, 2004), and that knockdown of bazooka results in mislocalization of E-cadherin and disruption of septate junctions (Pinheiro and Montell, 2004). In mammalian MDCK cells, overexpression of the CR1 domain of Par-3, which disrupts the distribution of Par-3, has been shown to perturb the staining pattern of E-cadherin, ZO-1 and F-actin (Mizuno et al., 2003). By contrast, it has been shown that knockdown of Par-3 does not affect the staining pattern of E-cadherin or β-catenin, although it perturbs the reorganization of the actin cytoskeleton associated with epithelial polarization and the staining pattern of occludin and ZO-1 in MDCK II cells (Chen and Macara, 2005). Thus, Par-3 is consistently involved in the formation of TJs, but its role in the formation of AJs is controversial.

We have previously shown that Par-3 directly binds to nectin-1 and -3, but not to nectin-2: the first PDZ domain of Par-3 binds to the C-terminal four amino acids of the cytoplasmic tails of nectin-1 and -3 (Takekuni et al., 2003). It has not been established how Cdc42, which binds to Par-6, is activated, but it is probably by the action of the nectin-based cell-cell adhesion (Takai et al., 2003). In addition, we have recently found that nectin-based cell-cell adhesion is essential for the formation of TJs but, in contrast to previous findings (Gonzalez-Mariscal et al., 1985; Gumbiner et al., 1988; Watabe et al., 1994; Yap et al., 1997), cadherin-based cell-cell adhesion is not always essential for it under some conditions (Yamada et al., 2006). We have examined the possibility that Par-3 regulates initially the formation of AJs and thereby secondarily the formation of TJs and found that Par-3 regulates the formation of both AJs and TJs. One mode of action of Par-3 was to regulate the association of afadin with nectin. However, this association alone was not sufficient for the formation of AJs or TJs, and Par-3 and afadin cooperatively regulated it.

Results

Necessity of Par-3 for the formation of AJs and TJs

We first examined whether Par-3 is necessary for the formation of AJs and TJs in MDCK cells. Wild-type MDCK cells were transfected with pBS-H1-Par-3 for knockdown of Par-3 or pBS-H1-scramble as a control. Western blotting showed that the amount of Par-3 was markedly reduced in Par-3-knockdown MDCK cells (Fig. 1Aa). The amounts of other major components of AJs and TJs, including afadin, E-cadherin, $p120^{ctn}$, β-catenin, α-catenin, claudin-1, occludin, JAM-A, ZO-1, aPKC, Par-6β and actin, remained unchanged. Immunofluorescence microscopy revealed that the signal for Par-3 was markedly reduced at cell-cell adhesion sites between two Par-3-knockdown MDCK cells that expressed GFP (Fig. 1Ab). When the cells pre-cultured at 2 μM Ca^{2+} were re-cultured at 2 mM Ca^{2+}, the immunofluorescence signals for E-cadherin and occludin were concentrated at the cell-cell adhesion sites in the wild-type MDCK cells that did not express GFP, whereas those for E-cadherin and occludin were markedly reduced at the cell-cell adhesion sites between two Par-3-knockdown MDCK cells that expressed GFP (Fig. 1Ac). In this experiment, we stained occludin as a marker of TJs, but essentially the same results were obtained for claudin-1, JAM-A and ZO-1 (see also Fig. 5A). The signals for the components of AJs and TJs between wild-type and Par-3-knockdown MDCK cells were still present but were slightly reduced compared with those between two wild-type MDCK cells. The reason why these signals did not completely disappear might be because Par-3 was not completely knocked down. The signals for the components of AJs and TJs were not concentrated at any sites on the free plasma membrane in both wild-type and Par-3-knockdown MDCK cells (data not shown). To confirm that the effects observed in Par-3-knockdown MDCK cells are indeed mediated by Par-3, wild-type MDCK cells were co-transfected with pBS-H1-Par-3 and pEGFP-Par-3 (GFP-Par-3) and subjected to the Ca^{2+} switch experiments. GFP–Par-3 was resistant to the RNA interference (RNAi) of Par-3. Western blotting showed that endogenous Par-3 was markedly reduced, but that GFP–Par-3 was expressed in Par-3-knockdown MDCK cells (Fig. 1Bb). The signals for E-cadherin and occludin as well as that for GFP–Par-3 were re-concentrated at the cell-cell adhesion sites by re-expression of Par-3 (Fig. 1Ba). Taken together, these results indicate that Par-3 is necessary for the formation of not only TJs but also AJs in MDCK cells.

To confirm that AJs were incompletely formed in Par-3-knockdown MDCK cells, we looked at cell dissociation activity in these cells. Par-3-knockdown MDCK cells formed smaller aggregates than those of control cells, indicating that the cell-cell adhesion activity of E-cadherin in Par-3-knockdown MDCK cells is weaker than that of control cells (Fig. 1Ca). To confirm that TJs were incompletely formed in Par-3-knockdown MDCK cells, we looked at the paracellular diffusion activity in these cells. Diffusion activity using FITC-conjugated dextran was higher in Par-3-knockdown MDCK cells than in control cells (Fig. 1Cb).

No requirement of Par-3 for nectin-based cell-cell adhesion

We have previously shown that nectin-based cell-cell adhesion is necessary for the formation of both AJs and TJs (Sato et al.,

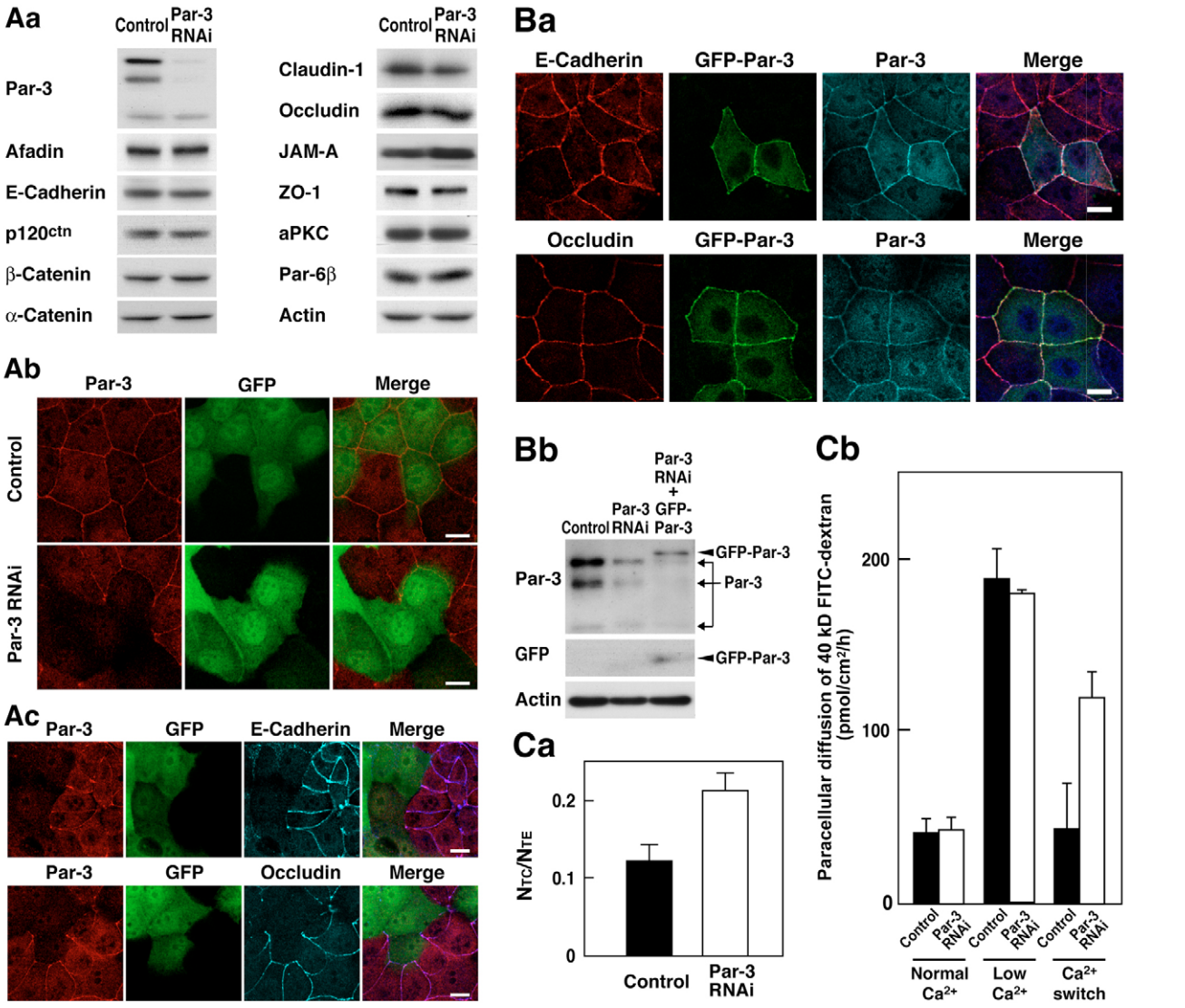

Fig. 1. Necessity of Par-3 for the formation of AJs and TJs. (Aa) Reduction in the amount of Par-3 by RNAi in MDCK cells. The cell lysates were subjected to SDS-PAGE, followed by western blotting with the indicated Abs. (Ab) Reduction in the immunofluorescence signal for Par-3 at cell-cell adhesion sites between two Par-3-knockdown MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with the anti-Par-3 pAb. (Ac) Reduction in the immunofluorescence signals for E-cadherin and occludin at cell-cell adhesion sites between two Par-3-knockdown MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm. (Ba) Re-concentration of the immunofluorescence signals for E-cadherin and occludin at cell-cell adhesion sites in the Par-3-knockdown MDCK cells that re-expressed GFP–Par-3. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm. (Bb) Exogenous expression of GFP–Par-3 in Par-3-knockdown MDCK cells. MDCK cells were co-transfected with pBS-H1-Par-3 and pEGFP-Par-3. The cell lysates were subjected to SDS-PAGE, followed by Western blotting with anti-Par-3 pAb. (Ca) Weak cell-cell adhesion activity of Par-3-knockdown MDCK cells. After Ca^{2+} switch, the wild-type MDCK cells transfected with pBS-H1-Par-3 or pBS-H1-scramble as a control were trypsinized in the presence of 1 mM $CaCl_2$ (TC treatment) or 1 mM EGTA (TE treatment) for 1 hour and dissociated through pipetting ten times. The extent of the cell dissociation was represented by the index N_{TC}/N_{TE}, where N_{TC} and N_{TE} were the total particle numbers after the TC and TE treatments, respectively. The results shown are representative of three independent experiments. (Cb) The paracellular diffusion of FITC-conjugated dextran (average 40 kDa) in Par-3-knockdown MDCK cells. Data are expressed as the means±s.d. of three independent experiments.

2006). Next, we examined whether Par-3 is necessary for the formation of nectin-based cell-cell adhesion. For this purpose, we knocked down Par-3 in MDCK cells stably expressing nectin-1 (nectin-1-MDCK cells), because the signals for nectin-1 and -3 in wild-type MDCK cells were hardly detected by their available antibodies (Abs) in immunofluorescence microscopy. In the nectin-1-MDCK cells that did not express GFP, the immunofluorescence signals for nectin-1, E-cadherin, and occludin were concentrated at the nectin-based cell-cell adhesion sites (Fig. 2). However, in the Par-3-knockdown nectin-1-MDCK cells that express GFP, the signals for E-cadherin and occludin were markedly reduced at the nectin-

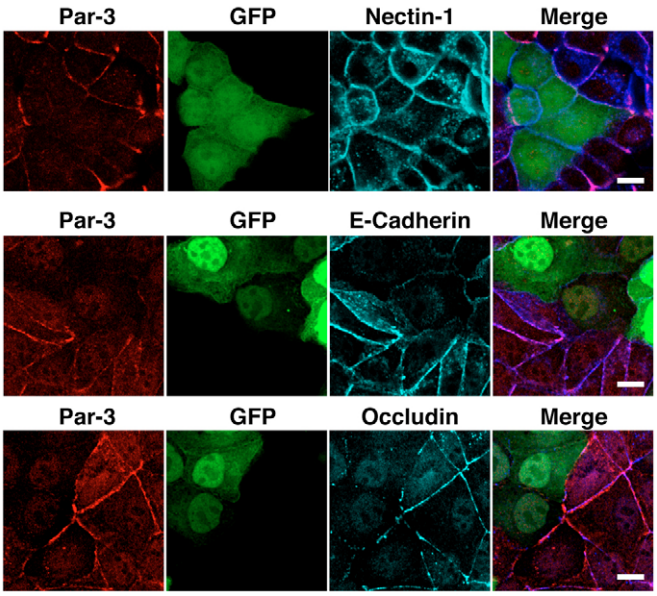

Fig. 2. No requirement of Par-3 for nectin-based cell-cell adhesion. After Ca^{2+} switch, Par-3-knockdown nectin-1-MDCK cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm.

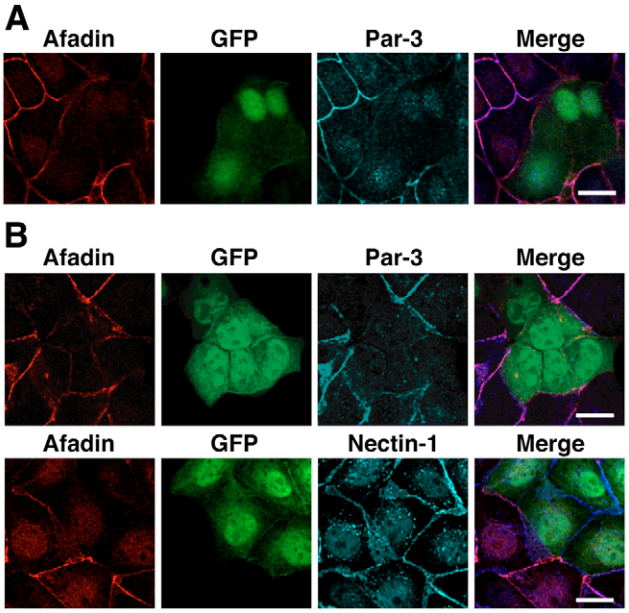

Fig. 3. Requirement of Par-3 for co-localization of afadin with nectin. (A) Reduction in the immunofluorescence signal for afadin at cell-cell adhesion sites in Par-3-knockdown MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm. (B) Reduction in the immunofluorescence signal for afadin, but not that for nectin-1, at the nectin-based cell-cell adhesion sites in Par-3-knockdown nectin-1-MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm.

based cell-cell adhesion sites, but the signal for nectin-1 remained unchanged there. These results indicate that Par-3 is not necessary for the formation of nectin-based cell-cell adhesion, at least when nectin is sufficiently expressed.

Necessity of Par-3 for co-localization of afadin with nectin

We have previously shown that nectin directly binds afadin (Takahashi et al., 1999), and that afadin is not necessary for the formation of nectin-based cell-cell adhesion but essential for the formation of AJs and TJs, at least when nectin is sufficiently expressed (Sato et al., 2006). Therefore, we examined whether Par-3 affects the co-localization of afadin with nectin. After Ca^{2+} switch, the immunofluorescence signals for afadin and Par-3 were concentrated at the cell-cell adhesion sites in the wild-type MDCK cells that did not express GFP, but the signals for afadin and Par-3 were markedly reduced there in the Par-3-knockdown MDCK cells that expressed GFP (Fig. 3A). Similarly, the signals for afadin and Par-3 were concentrated at the nectin-based cell-cell adhesion sites in the nectin-1-MDCK cells that did not express GFP, but the signals for afadin and Par-3 were markedly reduced there in the Par-3-knockdown nectin-1-MDCK cells that expressed GFP (Fig. 3B). The signal for nectin-1 remained unchanged at the cell-cell adhesion sites in Par-3-knockdown nectin-1-MDCK cells. These results indicate that Par-3 is necessary for the association of afadin with nectin at nectin-based cell-cell adhesion sites.

No requirement of afadin for co-localization of Par-3 with nectin

We next examined whether afadin is necessary for the association of Par-3 with nectin. Nectin-1-MDCK cells were co-transfected with pBS-H1-afadin for knockdown of afadin or pBS-H1-luciferase as a control. Western blotting showed that the amount of afadin was markedly reduced in afadin-knockdown nectin-1-MDCK cells (Fig. 4A). The amounts of other major components of AJs and TJs, including Par-3, nectin-1, E-cadherin, occludin and actin, remained unchanged. After Ca^{2+} switch, the immunofluorescence signals for nectin-1 and Par-3 remained unchanged at the cell-cell adhesion sites in the afadin-knockdown nectin-1-MDCK cells that expressed GFP (Fig. 4B), but the signal for E-cadherin, occludin, or afadin was not concentrated there. We have previously shown that the signals for the components of AJs and TJs, including E-cadherin, claudin-1, occludin, JAM-A and ZO-1, were markedly reduced at the cell-cell adhesion sites in afadin-knockdown MDCK cells (Sato et al., 2006). The signal for Par-3 was hardly detected at the cell-cell adhesion sites in afadin-knockdown MDCK cells (data not shown). These results indicate that afadin is not necessary for the association of Par-3 with nectin at the nectin-based cell-cell adhesion sites, at least when nectin is sufficiently expressed to form nectin-based cell-cell adhesion, even in the absence of afadin.

Similar phenotypes in Par-3- and afadin-knockdown MDCK cells

We have previously shown in afadin-knockdown MDCK cells that the immunofluorescence signals for $p120^{ctn}$, β-catenin and α-catenin are concentrated at the nectin-based cell-cell adhesion sites, whereas the signal for E-cadherin, claudin-1, occludin, JAM-A, or ZO-1 is not concentrated there (Sato et al., 2006). We then stained these molecules in Par-3-

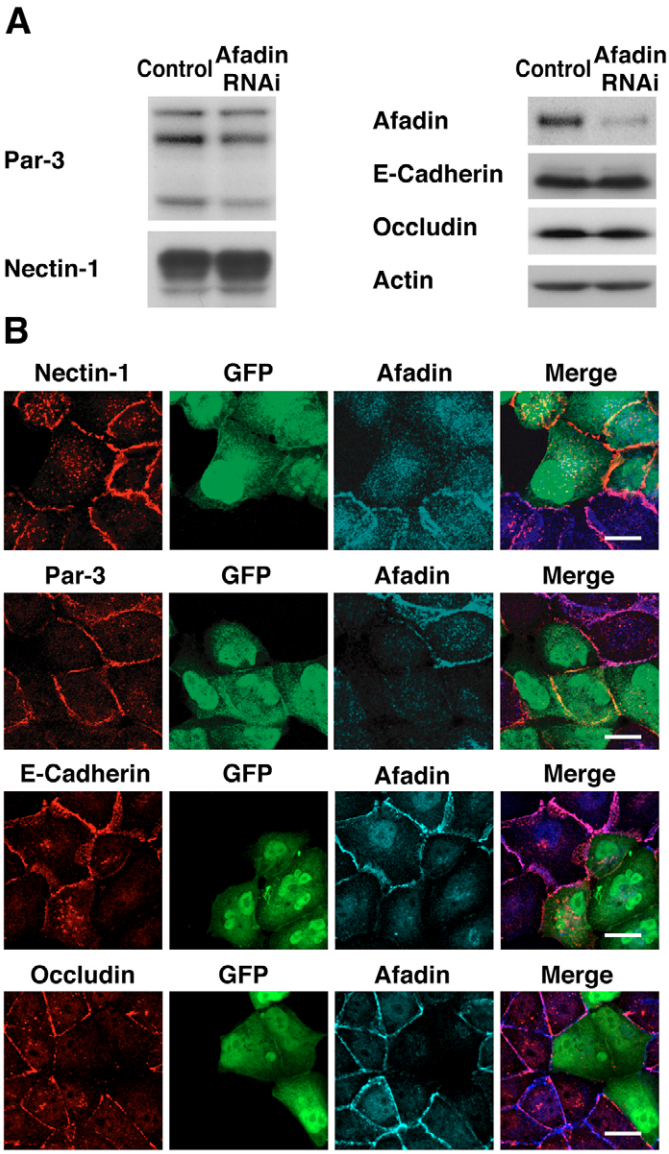

Fig. 4. No requirement of afadin for co-localization of Par-3 with nectin. (A) Reduction in the amount of afadin by RNAi. The lysates of afadin-knockdown nectin-1-MDCK cells were subjected to western blotting with the indicated Abs. (B) Concentration of the immunofluorescence signals for Par-3 and nectin-1 at nectin-based cell-cell adhesion sites in afadin-knockdown nectin-1-MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm.

knockdown MDCK cells after Ca^{2+} switch. The signals for claudin-1, JAM-A, and ZO-1 as well as those for E-cadherin and afadin were markedly reduced at the cell-cell adhesion sites, but those for $p120^{ctn}$, β-catenin and α-catenin remained unchanged in the Par-3-knockdown MDCK cells that expressed GFP or GFP–β-tubulin (Fig. 5A). Since GFP is a cytosolic protein in MDCK cells, acetone/methanol fixation tends to wash out GFP easily from MDCK cells. By contrast, since GFP–β-tubulin is incorporated into microtubule networks in MDCK cells, acetone/methanol fixation does not wash out GFP–β-tubulin from MDCK cells. Therefore, we used GFP–β-tubulin, instead of GFP, as a morphological marker of the co-transfection, when we fixed MDCK cells in acetone/methanol. The staining patterns of these molecules in Par-3-knockdown MDCK cells were apparently similar to those of afadin-knockdown MDCK cells. To confirm that the effects observed in Par-3-knockdown MDCK cells are indeed mediated by Par-3, wild-type MDCK cells were co-transfected with pBS-H1-Par-3 and pEGFP-Par-3 and subjected to the Ca^{2+} switch experiments. Western blotting showed that endogenous Par-3 was markedly reduced, but that GFP–Par-3 was expressed in Par-3-knockdown MDCK cells (Fig. 1Bb). When GFP–Par-3 was re-expressed in Par-3-knockdown MDCK cells, the signals for afadin, claudin-1, JAM-A and ZO-1, as well as that for Par-3, were re-concentrated at the cell-cell adhesion sites (Fig. 5B). These results suggest that Par-3 regulates the association of afadin with nectin and thereby the formation of AJs and TJs.

Necessity of aPKC and Par-6 for the co-localization of afadin with nectin

It has been shown that Par-3 forms a ternary complex with aPKC and Par-6 (Ohno, 2001; Roh and Margolis, 2003). The observations described above suggest that aPKC and Par-6β are also necessary for the association of afadin with nectin. We first knocked down Par-6β in MDCK cells. Wild-type MDCK cells were transfected with pBS-H1-Par-6β for knockdown of Par-6β or pBS-H1-scramble as a control. Western blotting showed that the amount of Par-6β was markedly reduced in Par-6β-knockdown MDCK cells (Fig. 6A). The amounts of other major components of AJs and TJs, including Par-3, afadin, E-cadherin, $p120^{ctn}$, β-catenin, α-catenin, claudin-1, occludin, JAM-A, ZO-1, aPKC, Par-6α and actin, remained unchanged. After Ca^{2+} switch, the immunofluorescence signals for E-cadherin, afadin, claudin-1, occludin, JAM-A, ZO-1, and Par-3 were markedly reduced at the cell-cell adhesion sites in the Par-6β-knockdown MDCK cells that expressed GFP or GFP–β-tubulin (Fig. 6B). The signals for $p120^{ctn}$, β-catenin, and α-catenin remained unchanged at the cell-cell adhesion sites (Fig. 6B and data not shown). When similar experiments were performed using nectin-1-MDCK cells, essentially the same results were obtained except that the signal for nectin-1 remained unchanged at the cell-cell adhesion sites in Par-6β-knockdown nectin-1-MDCK cells (Fig. 6C).

We next knocked down aPKCλ in MDCK cells, because MDCK cells express a much higher amount of aPKCλ than aPKCζ, as described previously (Suzuki et al., 2004). Wild-type MDCK cells were transfected with pBS-H1-aPKC for knockdown of aPKCλ or pBS-H1-scramble as a control. Western blotting showed that the amount of aPKC was markedly reduced in aPKC-knockdown MDCK cells (Fig. 7A). The amounts of other major components of AJs and TJs, including Par-3, afadin, E-cadherin, $p120^{ctn}$, β-catenin, α-catenin, claudin-1, occludin, JAM-A, ZO-1, Par-6β, and actin, remained unchanged. After Ca^{2+} switch, the signals for E-cadherin, afadin, claudin-1, occludin, JAM-A, ZO-1, and Par-3 were markedly reduced at the cell-cell adhesion sites in the aPKC-knockdown MDCK cells that expressed GFP (Fig. 7B). The signals for $p120^{ctn}$, β-catenin, and α-catenin remained unchanged at the cell-cell adhesion sites (data not shown). When similar experiments were performed using nectin-1-MDCK cells, essentially the same results were obtained except that the signal

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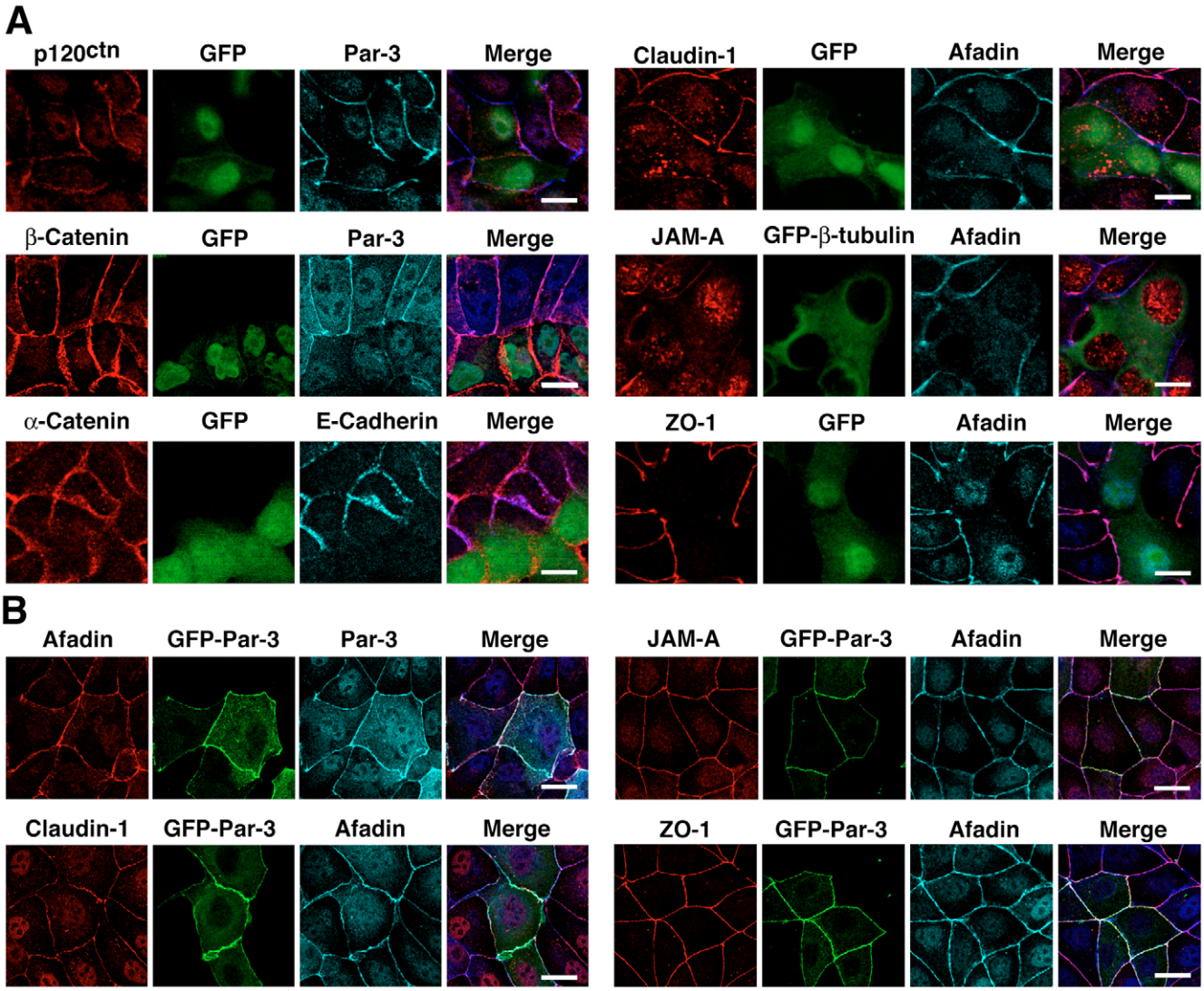

Fig. 5. Similar phenotypes between Par-3- and afadin-knockdown MDCK cells. (A) Reduction in the immunofluorescence signals for claudin-1, JAM-A and ZO-1, as well as those for E-cadherin and afadin, but not those for p120^{ctn}, β-catenin, and α-catenin, at cell-cell adhesion sites in Par-3-knockdown MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm. (B) Re-concentration of the immunofluorescence signals for claudin-1, JAM-A and ZO-1, as well as that for afadin at cell-cell adhesion sites in the Par-3-knockdown MDCK cells that re-expressed GFP–Par-3. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm.

for nectin-1 remained unchanged at the cell-cell adhesion sites in aPKC-knockdown nectin-1-MDCK cells (Fig. 7C).

The phenotypes of Par-6β- and aPKC-knockdown MDCK cells were similar to those of Par-3- and afadin-knockdown MDCK cells (Sato et al., 2006). Taken together, it is likely that not only Par-3 but also Par-6 and aPKC are necessary for the formation of AJs and TJs and the association of afadin with nectin.

Cooperative actions of Par-3 and afadin in the formation of AJs and TJs

We examined whether the Par-3-dependent association of afadin with nectin is sufficient for the formation of AJs and TJs. Wild-type MDCK cells were co-transfected with pBS-H1-Par-3 and pEGFP-afadin (GFP-afadin) and subjected to the Ca^{2+} switch experiments. In the Par-3-knockdown MDCK cells that expressed GFP-afadin, the immunofluorescence signals for GFP-afadin, p120^{ctn}, β-catenin and α-catenin were concentrated at the cell-cell adhesion sites, but the signal for E-cadherin, claudin-1, occludin, JAM-A or ZO-1 was not concentrated there (Fig. 8). Whereas the signal for endogenous afadin was reduced at the cell-cell adhesion sites between neighboring Par-3-knockdown MDCK cells, the signal for GFP-afadin was concentrated at the cell-cell adhesion sites between neighboring Par-3-knockdown MDCK cells that expressed GFP-afadin (Fig. 3A). Since afadin can bind directly to nectin in the absence of Par-3, as shown previously (Takahashi et al., 1999), an excess of GFP-afadin, which was overexpressed in Par-3-knockdown MDCK cells, could associate with nectin at cell-cell adhesion sites in

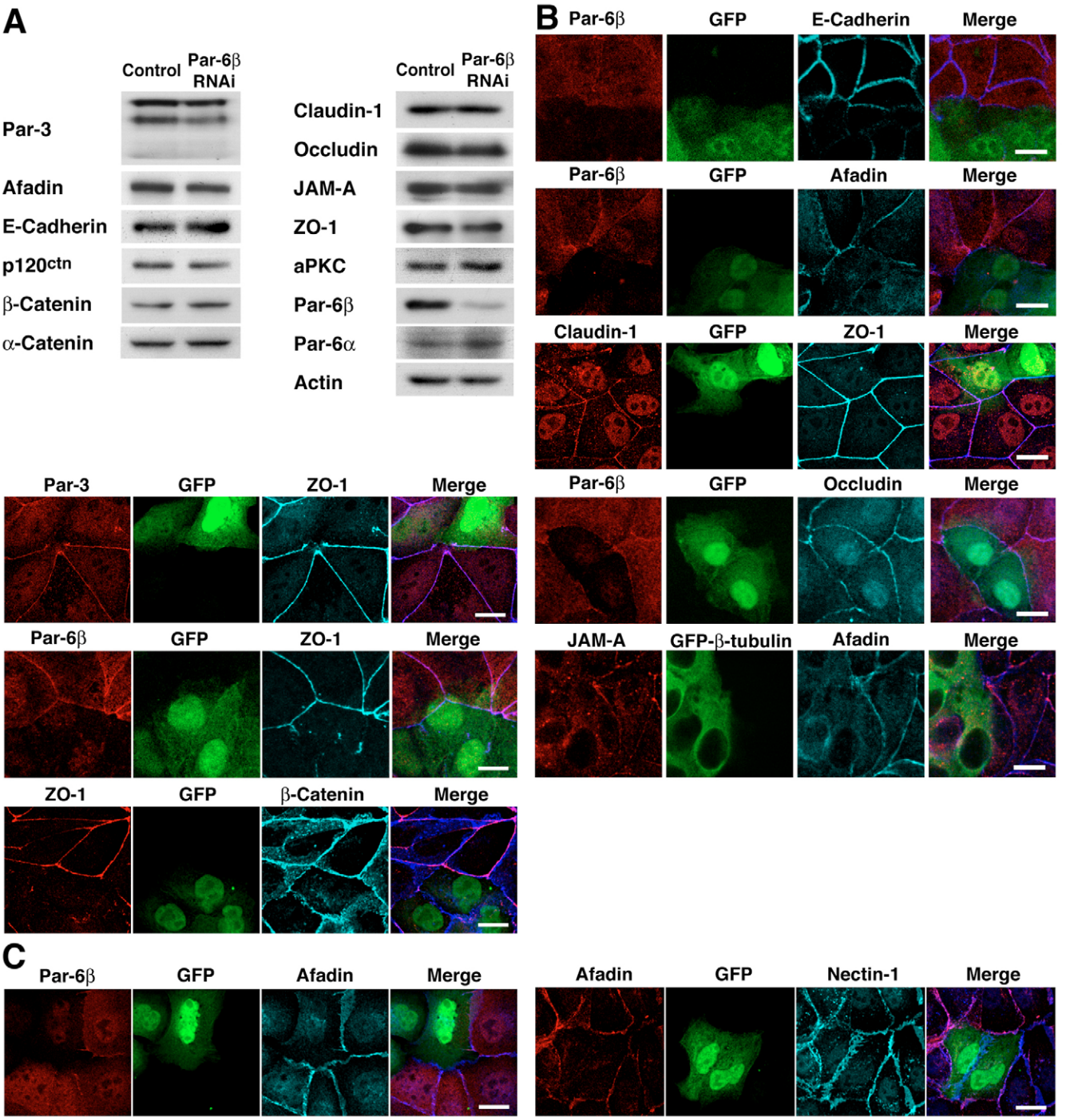

Fig. 6. Requirement of Par-6β for co-localization of afadin with nectin. (A) Reduction in the amount of Par-6β by RNAi. The lysates of Par-6β-knockdown MDCK cells were subjected to SDS-PAGE, followed by western blotting with the indicated Abs. (B) Reduction in the immunofluorescence signals for E-cadherin, afadin, claudin-1, occludin, JAM-A, Par-3 and ZO-1 at cell-cell adhesion sites in Par-6β-knockdown MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. (C) Reduction in the immunofluorescence signal for afadin, but not that for nectin-1, at the nectin-based cell-cell adhesion sites in Par-6β-knockdown nectin-1-MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm.

a Par-3-independent manner. By contrast, since the amount of endogenous afadin in Par-3-knockdown MDCK cells was much smaller than that of GFP-afadin in GFP-afadin-expressing Par-3-knockdown MDCK cells, association of endogenous afadin and nectin were strictly regulated by Par-3, as shown in Fig. 3A. These results suggest that Par-3 regulates the association of afadin with nectin but that the association of afadin with nectin alone in the absence of Par-3 is not sufficient for the formation of AJs or TJs and that Par-3 and afadin cooperatively regulate it.

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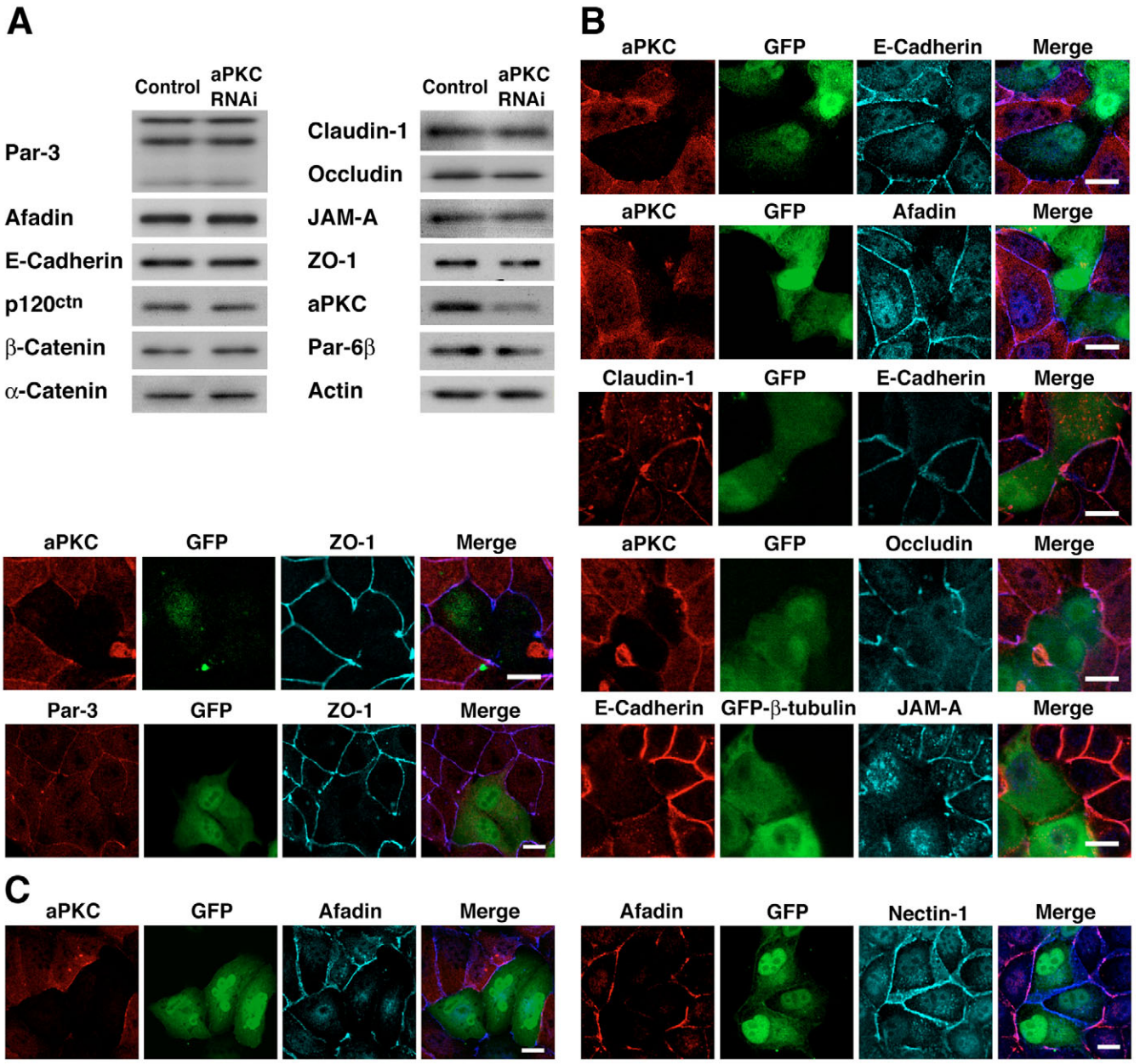

Fig. 7. Requirement of aPKC for the association of afadin with nectin. (A) Reduction in the amount of aPKC by RNAi. The lysates of aPKC-knockdown MDCK cells were subjected to SDS-PAGE, followed by western blotting with the indicated Abs. (B) Reduction in the immunofluorescence signals for E-cadherin, afadin, claudin-1, occludin, JAM-A, Par-3 and ZO-1 at cell-cell adhesion sites in aPKC-knockdown MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. (C) Reduction in the immunofluorescence signal for afadin, but not that for nectin-1, at the nectin-based cell-cell adhesion sites in aPKC-knockdown nectin-1-MDCK cells. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm.

Involvement of Par-3 in the trans-interacting E-cadherin-induced, but not nectin-induced, activation of Rac

It has been shown that Rac is activated by trans-interacting nectin and E-cadherin (Fukuyama et al., 2006; Kawakatsu et al., 2005; Kawakatsu et al., 2002; Noren et al., 2001). It has been further shown that Par-3 directly binds to the Rac-specific guanine nucleotide exchange factor Tiam1/STEF and regulates the activity of Rac (Chen and Macara, 2005; Nishimura et al., 2005). We examined the possibility that knockdown of Par-3 reduces the activation of Rac and thereby affects the formation of AJs. In the Ca^{2+}-switch experiments, re-addition of Ca^{2+} stimulated the activation of Rac1 in wild-type MDCK cells as estimated by pull-down assay, consistent with earlier observations (Noren et al., 2001). However, Rac1 activation was markedly reduced in Par-3-knockdown MDCK cells (Fig. 9A). When nectin-1-MDCK cells were cultured on coverslips coated with an extracellular fragment of nectin-3 fused to human IgG Fc (Nef-3), Nef-3 mainly trans-interacted with cellular nectin-1 and enhanced cell-spreading with lamellipodia and filopodia (Fig. 9B), consistent with earlier

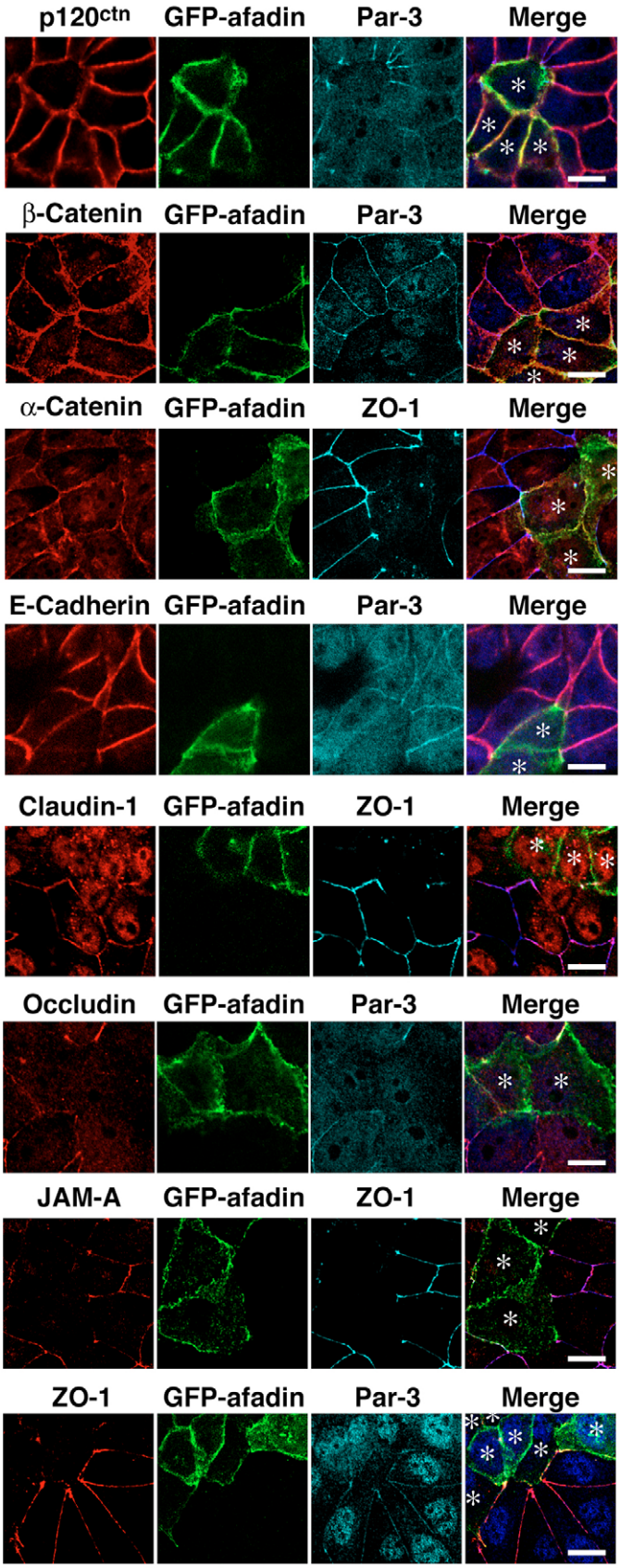

Fig. 8. Cooperative actions of Par-3 and afadin in the formation of AJs and TJs. After Ca^{2+} switch, the Par-3-knockdown MDCK cells that expressed GFP-afadin were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm. Asterisks indicate the Par-3-knockdown MDCK cells that expressed GFP-afadin.

observations (Fukuhara et al., 2004; Honda et al., 2003; Kawakatsu et al., 2005). This effect of Nef-3 was not changed in Par-3-knockdown nectin-1-MDCK cells. Because the spreading assay reflects the activation of Rac, these results suggest that the nectin-induced activation of Rac is not affected by Par-3. By contrast, when MDCK cells stably expressing E-cadherin (E-cadherin-MDCK cells) were cultured on coverslips coated with an extracellular fragment of E-cadherin fused to human IgG Fc (Cef), Cef mainly trans-interacted with cellular E-cadherin and enhanced cell-spreading with lamellipodia (Fig. 9C), consistent with earlier observations (Fukuyama et al., 2006; Hoshino et al., 2004). However, this effect of Cef was markedly reduced in Par-3-knockdown E-cadherin-MDCK cells, which suggests that the cadherin-induced activation of Rac requires Par-3. Consistently, expression of GFP-V12Rac1, a constitutively active mutant of Rac1, restored cell-spreading with lamellipodia in Par-3-knockdown E-cadherin-MDCK cells. Because we previously showed that afadin enhances the trans-interaction of E-cadherin (Sato et al., 2006) and showed here that Par-3 is necessary for the co-localization of afadin with nectin, the effect of Par-3 on the E-cadherin-induced activation of Rac might be mediated by afadin. However, knockdown of afadin did not affect the Cef-induced activation of Rac as estimated by the cell-spreading assay (Fig. 9D). Taken together, these results indicate that Par-3 affects the E-cadherin-induced activation of Rac in an afadin-independent manner.

Finally, we examined whether the Par-3-dependent activation of Rac is sufficient for the formation of AJs and TJs. Wild-type MDCK cells were co-transfected either with pBS-H1-Par-3 and pEGFP-V12Rac1 or with pBS-H1-Par-3, pEGFP-V12Rac1 and pCMV-FLAG-afadin (FLAG-afadin) and then subjected to the Ca^{2+} switch experiments. In the Par-3-knockdown MDCK cells that expressed GFP-V12Rac1, the immunofluorescence signal for GFP-V12Rac1 was concentrated at the cell-cell adhesion sites, but the signal for E-cadherin or occludin was not concentrated there (Fig. 9Ea). Essentially the same results were obtained in the Par-3-knockdown MDCK cells that expressed GFP-V12Rac1 and FLAG-afadin (Fig. 9Eb). Thus, overexpression of V12Rac1 alone or together with afadin did not restore the formation of AJs or TJs in Par-3-knockdown MDCK cells. Essentially the same results were obtained in Par-6β-knockdown and aPKC-knockdown MDCK cells (data not shown). Taken together, these results indicate that Par-3 regulates a site(s) other than the association of afadin with trans-interacting nectin and the E-cadherin-induced activation of Rac.

Discussion

We show here that knockdown of Par-3 impaired the staining pattern and cell-cell adhesion activity of E-cadherin in MDCK cells. In addition, Par-6 and aPKC showed similar effects. Essentially the same results were obtained using other Par-3, Par-6 and aPKC RNAi sequences (data not shown). In addition, re-expression of RNAi-resistant Par-3 in Par-3-knockdown MDCK cells restored the staining of E-cadherin at AJs. Our

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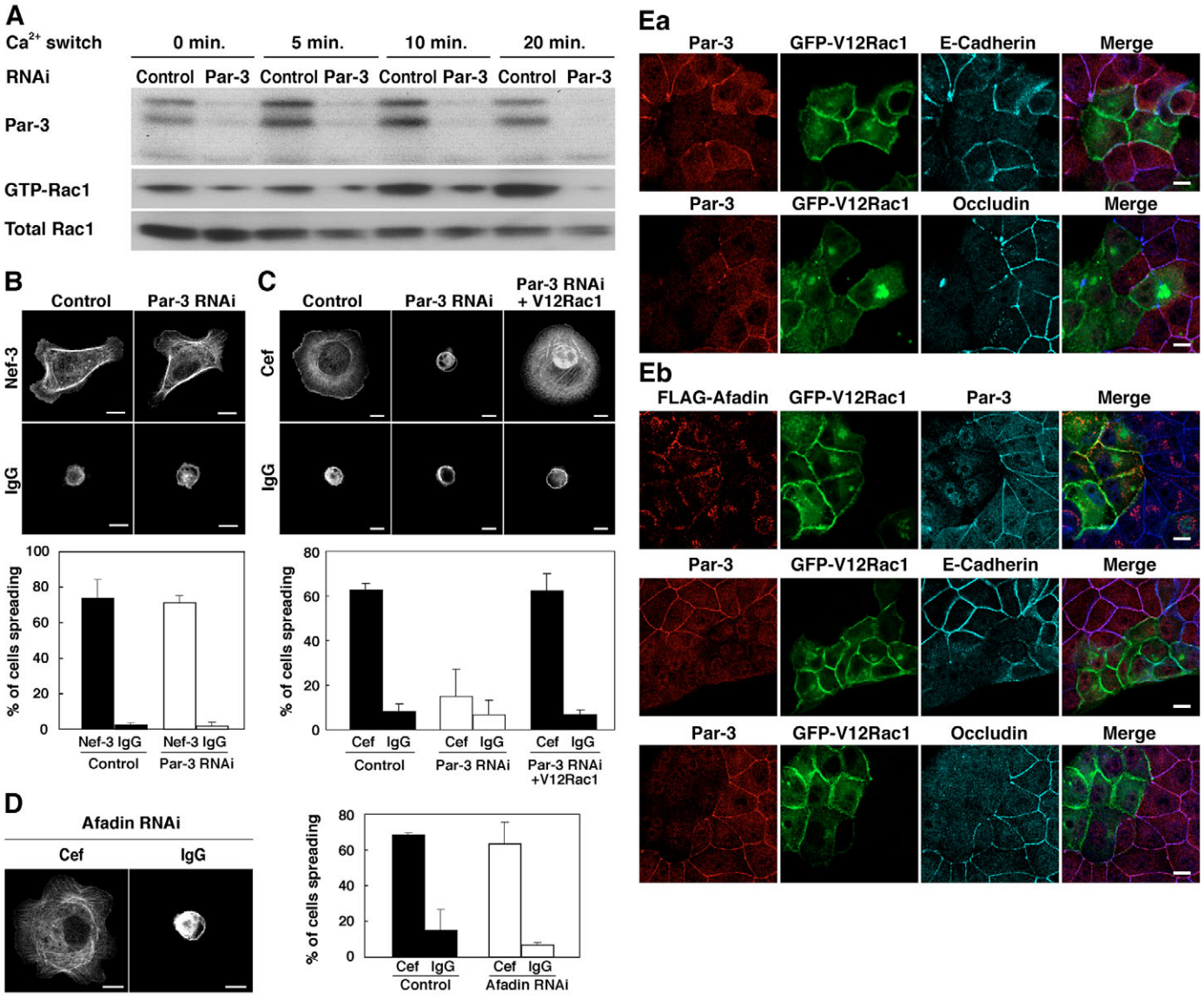

Fig. 9. Involvement of Par-3 in the trans-interacting E-cadherin-induced, but not nectin-induced, activation of Rac. (A) Reduction in the activation of Rac1 in Par-3-knockdown MDCK cells. After Ca^{2+} switch, the cell lysates were subjected to pull-down assay using GST-PAK-CRIB, followed by western blotting using the anti-Rac1 mAb. The results shown are representative of three independent experiments. (B) No effect of Par-3 knockdown on nectin-induced cell-spreading. The nectin-1-MDCK cells co-transfected either with pBS-H1-Par-3 and pEGFP or with pBS-H1-scramble and pEGFP as a control were cultured on the Nef-3- or IgG-coated coverslips for 2 hours. The cells were fixed and stained for F-actin with rhodamine-phalloidin. Bars, 10 μm. Bars in the graph represent the percentage of spreading cells with lamellipodial cell protrusions of the 100 GFP-positive cells counted, and are expressed as means±s.d. of three independent experiments. (C) Reduction of E-cadherin-induced cell-spreading by Par-3 knockdown. The E-cadherin-MDCK cells co-transfected either with pBS-H1-Par-3 and pDsRed, with pBS-H1-Par-3, pEGFP-V12Rac1, and pDsRed, or with pBS-H1-scramble and pDsRed as a control were cultured on the Cef- or IgG-coated coverslips for 2 hours. The cells were fixed and stained for F-actin with Cy5-phalloidin. Bars, 10 μm. Bars in the graph represent the percentage of spreading cells with lamellipodial cell protrusions of the 100 DsRed-positive cells counted, and are expressed as means±s.d. of three independent experiments. (D) No effect of afadin knockdown on E-cadherin-induced cell-spreading. The E-cadherin-MDCK cells co-transfected either with pBS-H1-afadin and pDsRed or with pBS-H1-scramble and pDsRed as a control were cultured on the Cef- or IgG-coated coverslips for 2 hours. The cells were fixed and stained for F-actin with Cy5-phalloidin. Bars, 10 μm. Bars in the graph represent the percentage of spreading cells with lamellipodial cell protrusions of the 100 DsRed-positive cells counted, and are expressed as means±s.d. of three independent experiments. (Ea) Reduction in the immunofluorescence signals for E-cadherin and occludin, but not that for GFP-V12Rac1, at cell-cell adhesion sites in the Par-3-knockdown MDCK cells that expressed GFP-V12Rac1. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm. (Eb) Reduction in the immunofluorescence signals for E-cadherin and occludin, but not that for GFP-V12Rac1 and FLAG-afadin, at cell-cell adhesion sites in the Par-3-knockdown MDCK cells that expressed GFP-V12Rac1 and FLAG-afadin. After Ca^{2+} switch, the cells were fixed and stained with various combinations of the indicated Abs. Bars, 10 μm.

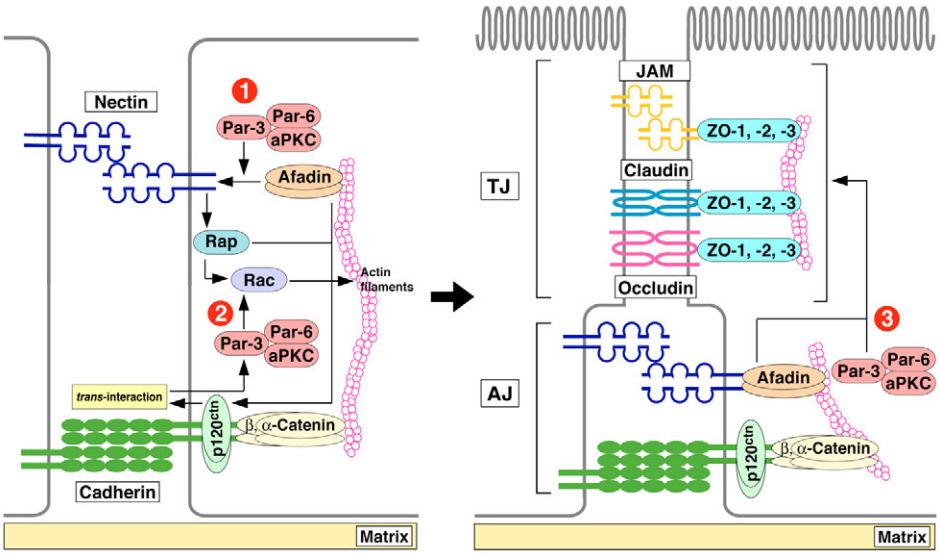

Fig. 10. The mode of action of Par-3 in the process of the formation of AJs and TJs. Par-3 regulates at least three sites of the junctional formation of epithelial cells: (1) the association of afadin with nectin; (2) the E-cadherin-induced activation of Rac; and (3) a site other than these first two sites.

results are consistent with earlier observations in epithelial cells of *Drosophila* (Harris and Peifer, 2004; Pinheiro and Montell, 2004) and MDCK II cells (Mizuno et al., 2003) in which the Par-3 homologue bazooka and Par-3 regulate the formation of AJs, but inconsistent with earlier observations in MDCK II cells (Chen and Macara, 2005) in which Par-3 does not affect AJs. The cell lines and Ca^{2+}-switch experimental conditions used in this study are the same in Ohno's and Macara's groups. Although MDCK cells, which were obtained from W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany) (Behrens et al., 1985; Imhof et al., 1983), and the assay conditions used in our group are different from those in Ohno's and Macara's groups, the results obtained from our group are the same as those obtained from Ohno's group but different from those obtained from Macara's group. The exact reason for this discrepancy is unknown, but when Par-3 was knocked down in MTD-1A cells, essentially the same results were obtained as those obtained in Par-3-knockdown MDCK cells (data not shown). In contrast to these epithelial cell lines, knockdown of Par-3 did not affect the staining pattern of N-cadherin in NIH3T3 cells. Taken together, it is likely that Par-3 is involved in the formation of the cadherin-based AJs in epithelial cells.

We have previously shown in afadin-knockdown MDCK cells that the immunofluorescence signals for $p120^{ctn}$, β-catenin and α-catenin are concentrated at nectin-based cell-cell adhesion sites, whereas the signal for E-cadherin, claudin-1, occludin, JAM-A or ZO-1 is not concentrated there (Sato et al., 2006). Although the signal for E-cadherin, which is associated with catenins, is hardly detected by the anti-E-cadherin Abs used in the study, it localizes at the nectin-based cell-cell adhesion sites in afadin-knockdown MDCK cells. This E-cadherin shows weak cell-cell adhesion activity and is presumably the non-trans-interacting form. The reason why the signal for E-cadherin is not detected at the nectin-based cell-cell adhesion sites may be due to a decrease in the sensitivity of the E-cadherin Abs used, resulting from the conformational change of E-cadherin. We show here that the phenotypes of Par-3-knockdown MDCK cells were similar to those of afadin-knockdown MDCK cells. Studies on the mode of action of Par-3 in the formation of AJs revealed that Par-3 is not necessary for the formation of the nectin-based cell-cell adhesion but is necessary for the association of afadin with nectin, and that afadin is not necessary for the association of Par-3 with nectin. However, expression of afadin did not restore the formation of AJs in Par-3-knockdown MDCK cells. Taken together, it is likely that Par-3 regulates the association of afadin with nectin but that this association alone in the absence of Par-3 is not sufficient for the formation of AJs. Thus, Par-3 and afadin cooperatively regulate the formation of AJs.

It remains unknown how Par-3 regulates the association of afadin with nectin. We have previously shown several lines of evidence for the direct association of afadin with nectin through the PDZ domain of afadin and the C-terminal four amino acids of nectin: (1) immunofluorescence and immunoelectron microscopy have revealed co-localization of these two proteins at AJs in epithelial cells and fibroblasts, puncta adherentia junctions at synapses, Sertoli-cell–Sertoli-cell junctions and Sertoli-cell–spermatid junctions in the testis, and the apex-apex junctions between the pigment and non-pigment epithelia of the ciliary body (Inagaki et al., 2005; Mandai et al., 1997; Mizoguchi et al., 2002; Nishioka et al., 2000; Ozaki-Kuroda et al., 2002; Takahashi et al., 1999); (2) bead-cell contact assays have revealed that when latex-sulfate microbeads coated with Nef-3 (an extracellular fragment of nectin-3 fused to the Fc portion of human IgG) were placed on the surface of cells, Nef-3 trans-interacts with cellular nectin-1, and nectin-1 and afadin are recruited to the bead-cell contact sites (Honda et al., 2003); and (3) biochemical assays have revealed that purified recombinant protein of the cytoplasmic tail of nectin-1 stoichiometrically binds the fragment of afadin that contains the PDZ domain (Takahashi et al., 1999). In addition, we have previously shown that afadin is co-immunoprecipitated with nectin from the cell lysates of the bile canaliculi-rich fraction of mouse liver and MTD-1A cells (Takahashi et al., 1999). However, when afadin was co-immunoprecipitated with nectin from the cell lysates of nectin-1–MDCK cells, the stoichiometry of afadin to nectin was less than 5% (data not shown). Taken together, it is likely that afadin associates with trans-interacting nectin, but not with non-trans-interacting nectin. However, it is practically difficult to obtain biochemical evidence, using immunoprecipitation assays, of how Par-3 affects the association of afadin with trans-interacting nectin, because the trans-interaction of nectin is very sensitive to detergents, such

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as Triton X-100, and we have not yet succeeded in preparing the lysates in which the trans-interaction of nectin remained intact.

We further examined whether the effect of Par-3 on the association of afadin with nectin and the AJ and TJ formation is mediated by Rac, because it has been shown that Rac is activated by trans-interacting nectin and E-cadherin (Fukuyama et al., 2006; Kawakatsu et al., 2005; Kawakatsu et al., 2002; Noren et al., 2001). It has been further shown that Par-3 directly binds to Tiam1/STEF and regulates the activity of Rac (Chen and Macara, 2005; Nishimura et al., 2005). We showed here that the activity of Rac was stimulated in the Ca^{2+}-switch experiments, consistent with earlier observations (Noren et al., 2001), but that knockdown of Par-3 markedly reduced the activation of Rac induced by trans-interacting E-cadherin, but not by trans-interacting nectin. Because it has been shown that afadin enhances the trans-interaction of E-cadherin (Sato et al., 2006), we further examined the effect of Par-3 on E-cadherin-induced activation of Rac and found that it was afadin independent. These results indicate that Par-3 regulates at least two sites: one is the association of afadin with trans-interacting nectin and the other is the E-cadherin-induced activation of Rac (Fig. 10). In addition, overexpression of Rac1-CA alone or together with afadin did not restore the formation of AJs or TJs in Par-3-knockdown MDCK cells, which indicates that Par-3 regulates a site(s) other than the above two sites.

It still remains unclear how nectin recruits the TJ components to the apical side of the nectin-based cell-cell adhesion sites, but we have previously shown that afadin is necessary for the recruitment of claudin-1, occludin, and JAM-A to the apical side of nectin-based cell-cell adhesion sites to form TJs (Irie et al., 2004; Takai and Nakanishi, 2003). It had been believed that the formation and maintenance of TJs are dependent on cadherin-based cell-cell adhesion (Yap et al., 1997). However, we have recently found that nectin-based cell-cell adhesion is necessary for the formation of TJs but that cadherin-based cell-cell adhesion is not always necessary for it under some conditions, such as in annexin-II-knockdown MDCK cells (Yamada et al., 2006). In addition, it has been shown that ZO proteins are involved in the formation of TJs (Umeda et al., 2006; Wittchen et al., 2000). In non-epithelial cells, which are deficient of TJs, ZO proteins have been shown to associate with cadherin through α-catenin (Itoh et al., 1997), but we have previously shown that ZO-1 associates with nectin through afadin in an α-catenin-independent manner (Yokoyama et al., 2001). Therefore, nectin may recruit the TJ components through afadin and ZO proteins irrespectively of the dependency or independency of the cadherin system. We have confirmed the earlier observation (Harris and Peifer, 2004; Mizuno et al., 2003; Pinheiro and Montell, 2004) that Par-3 is necessary for the formation of TJs in wild-type MDCK cells. In addition, we have shown that the association of afadin with nectin alone, in the absence of Par-3, was not sufficient for the formation of AJs and TJs. Thus, Par-3 and afadin cooperatively regulate the formation of TJs, similarly to their roles in the formation of AJs.

Materials and Methods

Cell culture and transfection

MDCK cells were supplied by W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). MDCK cells stably expressing FLAG-tagged nectin-1 (nectin-1-MDCK cells) were prepared as described (Takahashi et al., 1999). MDCK cells stably expressing GFP-tagged E-cadherin (E-cadherin-MDCK cells) were prepared as described (Hoshino et al., 2004). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For DNA transfection, Lipofectamine 2000 reagent (Invitrogen) and an Amaxa Nucleofector kit (Amaxa) were used. When we prepared the samples of immunofluorescence microscopy, lipofection was always employed.

Antibodies and expression vectors

A mouse anti-afadin monoclonal antibody (mAb) and a rabbit anti-afadin polyclonal antibody (pAb) were prepared as described (Mandai et al., 1997; Sakisaka et al., 1999). A rabbit anti-nectin-1 pAb was prepared as described (Takahashi et al., 1999). A rat anti-E-cadherin mAb (ECCD2) was supplied by M. Takeichi (Center for Developmental Biology, RIKEN, Kobe, Japan). A rabbit anti-Par-6β pAb was supplied by S. Ohno (Yokohama City University, Yokohama, Japan). A mouse anti-FLAG M1 mAb, a mouse anti-FLAG M5 mAb, and a rabbit anti-α-catenin pAb were purchased from Sigma. A mouse anti-$p120^{ctn}$ was purchased from BD Transduction Laboratories. A mouse anti-β-catenin mAb, a rabbit anti-Par-6α pAb, and a goat anti-aPKC pAb, which recognizes both aPKCλ and aPKCζ, were purchased from Santa Cruz Biothechnology. A rabbit anti-Par-3 pAb and mouse anti-Rac1 mAb were purchased from Upstate Biotechnology. A rabbit anti-claudin-1 pAb, a rabbit anti-JAM-A pAb, and a mouse anti-occludin mAb were purchased from Zymed. A rat anti-occludin mAb and a mouse anti-ZO-1 mAb were purchased from SANKO JUNYAKU. A rabbit anti-GFP pAb was purchased from MBL. Horseradish peroxidase-conjugated and fluorophore-conjugated secondary Abs were purchased from Amersham Biosciences and Chemicon, respectively. The full-length cDNA of rat Par-3 was supplied by S. Ohno (Yokohama City University, Yokohama, Japan). The full-length cDNA of rat Par-3 was subcloned into pEGFP-C1 (Clontech) and the RNAi-resistant mutant of pEGFP-Par-3 (GFP–Par-3) was generated by mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene). The full-length cDNA of rat afadin was subcloned into pCMV-FLAG (FLAG-afadin). The expression vectors for pEGFP-afadin (GFP-afadin) and pEGFP-V12Rac (GFP-V12Rac) were constructed as described (Hoshino et al., 2005).

Knockdown experiments

For knockdown of Par-3 and afadin by a short hairpin RNA (shRNA) method, pBS-H1 containing the H1 promoter was used for the expression of the shRNA. To generate vectors for knockdown of Par-3 (pBS-H1-Par-3), aPKCλ (pBS-H1-aPKC), Par-6β (pBS-H1-Par-6β) and afadin (pBS-H1-afadin), a specific insert for Par-3, aPKCλ, Par-6β or afadin was subcloned into pBS-H1 as described previously (Yamada et al., 2005). The inserts for canine Par-3, mouse Par-3, canine aPKCλ, canine Par-6β, and canine afadin were 5′-GACACAGAAGAAAGTTCAA-3′, 5′-GACAGACTGGTAGCAGTAT-3′, 5′-AGTTCTGTTGGTGCGAATA-3′, 5′-GACTTCAGACCTGTGTCTT-3′ and 5′-GACAATCCTGCTGTCTACC-3′, respectively (Sato et al., 2006; Suzuki et al., 2004). For the control experiments, scrambled (pBS-H1-scramble) and luciferase (pBS-H1-luciferase) sequences were subcloned into pBS-H1. The scrambled sequences against canine Par-3, mouse Par-3, canine aPKCλ, canine Par-6β and luciferase were 5′-AAGAAGAAGAAGCTCTCA-3′, 5′-GGTGGTAACAACGGTAACT-3′, 5′-GCATCTATCGTTGTCATCG-3′, 5′-GTGTAGTGTCGTGTACTAA-3′ and 5′-CGTACGCGGAATACTTCGA-3′, respectively. The cells were co-transfected with pBS-H1 and pEGFP (GFP), pDsRed, pEGFP-Tub (GFP-β-tubulin; Clontech), GFP–par-3 or GFP-afadin using the Lipofectamine 2000 reagent. The GFP-, DsRed-, or GFP-β-tubulin-positive cells were monitored as a marker of the co-transfection.

Ca^{2+} switch experiments

Ca^{2+}-switch experiments using nectin-1-MDCK, E-cadherin-MDCK, or wild-type MDCK cells were carried out as described previously (Kartenbeck et al., 1991). Briefly, the cells (1×10^5) were plated on 18 mm glass coverslips in 12-well culture dishes. At 72 hours after transfection, the cells were washed with phosphate-buffered saline (PBS) and cultured at 2 mM Ca^{2+} in DMEM without serum for 1 hour. Next, the cells were pre-cultured at 2 μM Ca^{2+} in DMEM with 5 mM EGTA for 3 hours, and then re-cultured at 2 mM Ca^{2+} for 4 hours.

Immunofluorescence microscopy

Cells were fixed in a mixture of 50% acetone and 50% methanol at –20°C for 1 minute or in PBS containing 1% formaldehyde for 15 minutes, followed by PBS containing 0.2% Triton X-100 for 15 minutes at room temperature. After being blocked in Tris-buffered saline (TBS) containing 1% bovine serum albumin and 1 mM Ca^{2+} for 1 hour, the cells were incubated in the same buffer containing various combinations of Abs for 1 hour. The samples were washed three times with TBS containing 1 mM Ca^{2+} for 5 minutes and incubated for 30 minutes in TBS containing 1% bovine serum albumin and 1 mM Ca^{2+} with the secondary pAbs. The samples were then washed three times with TBS containing 1 mM Ca^{2+} for 5 minutes and mounted in GEL/MOUNT (Biomeda). The samples were analyzed using a Radiance 2100 confocal laser scanning microscope (Bio-Rad Laboratories) and an LSM 510

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META confocal microscope (Carl Zeiss). All the immunofluorescence images shown in the present study are representative of three independent experiments.

Other procedures

Cell dissociation assays were carried out as described (Nagafuchi et al., 1994; Sato et al., 2006). The barrier function of TJs in Par-3-knockdown MDCK cells grown on transwell filters was evaluated as described previously (Fukuhara et al., 2002). The cell-spreading assay was performed as described (Hoshino et al., 2004; Kawakatsu et al., 2002). The pull-down assay for Rac1 was performed as described (Fukuyama et al., 2006; Noren et al., 2001). Protein concentrations were determined with BSA as a reference protein (Bradford, 1976). SDS-PAGE was carried out as previously described (Laemmli, 1970).

We are grateful to W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany) for MDCK cells, M. Takeichi (Center for Developmental Biology, RIKEN, Kobe, Japan) for the anti-E-cadherin mAb, S. Ohno (Yokohama City University, Yokohama, Japan) for the anti-Par-6β pAb and the cDNA of rat Par-3, and H. Shibuya (Tokyo Medical and Dental University, Tokyo, Japan) for pBS-H1. We also thank Sh. Tsukita for helpful comments on the manuscript. Lastly, on a sad note, we record our debt to Sh. Tsukita who died of cancer in December 2005. The investigation at Osaka University was supported by a grant-in-aid for Scientific Research and for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (2006, 2007).

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