Regulation of the Assembly and Adhesion Activity of E-cadherin by Nectin and Afadin for the Formation of Adherens Junctions in Madin-Darby Canine Kidney Cells*

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The Ca\(^{2+}\)-independent immunoglobulin-like molecule nectin first forms cell-cell adhesion and then assembles cadherin at nectin-based cell-cell adhesion sites, resulting in the formation of adherens junctions (AJs). Afadin is a nectin- and actin filament-binding protein that connects nectin to the actin cytoskeleton. Here, we studied the roles and modes of action of nectin and afadin in the formation of AJs in cultured MDCK cells. The trans-interaction of nectin assembled E-cadherin, which associated with p120\(^{CTN}\), \(\beta\)-catenin, and \(\alpha\)-catenin, at the nectin-based cell-cell adhesion sites in an afadin-independent manner. However, the assembled E-cadherin showed weak cell-cell adhesion activity and might be the non-trans-interacting form. This assembly was mediated by the IQGAP1-dependent actin cytoskeleton, which was organized by Cdc42 and Rac small G proteins that were activated by the action of trans-interacting nectin through c-Src and Rap1 small G protein in an afadin-independent manner. However, Rap1 bound to afadin, and this Rap1-afadin complex then interacted with p120\(^{CTN}\) associated with non-trans-interacting E-cadherin, thereby causing the trans-interaction of E-cadherin. Thus, nectin regulates the assembly and cell-cell adhesion activity of E-cadherin through afadin, nectin signaling, and p120\(^{CTN}\) for the formation of AJs in Madin-Darby canine kidney cells.

In polarized epithelial cells, cell-cell adhesion is mediated through junctional complexes composed of tight junctions (TJs)\(^{1,2}\) and adherens junctions (AJs) (1). These junctional structures are typically aligned from the apical to basal sides. TJs are likely to serve as barriers that prevent solutes and water from passing through the paracellular pathway and as fences between the apical and basolateral plasma membranes in epithelial cells. AJs are composed of closely apposed plasma membrane domains reinforced by a dense cytoplasmic plaque with actin filament (F-actin) bundles underneath. The formation and disruption of these junctional complexes are dynamically regulated by many extracellular and intracellular signals, and AJs play key roles in the formation and maintenance of TJs (2). However, the mechanisms responsible for the dynamic organization of these junctional complexes are not fully understood.

At TJs, claudin is a key Ca\(^{2+}\)-independent cell-cell adhesion molecule (CAM), which comprises a family consisting of more than 27 members (3, 4). Ocludin is another CAM at TJs, but its function has not yet been established. Claudin and ocludin are associated with the actin cytoskeleton through peripheral membrane proteins, such as ZO-1, -2, and -3. Junctional adhesion molecule (JAM), which belongs to the Ca\(^{2+}\)-independent immunoglobulin-like (Ig)-like CAM family, also localizes at TJs and interacts with ZO proteins (5, 6). JAM comprises a family consisting of four members. At AJs, E-cadherin, a member of the cadherin superfamily consisting of more than 80 members, is a key Ca\(^{2+}\)-dependent CAM (7–14). E-Cadherin is associated with the actin cytoskeleton through peripheral membrane proteins, including \(\alpha\)-catenin, \(\beta\)-catenin, vinculin, and \(\alpha\)-actinin, and this association strengthens the cell-cell adhesion of AJs. p120\(^{CTN}\) also binds to the juxtamembrane region of E-cadherin and stabilizes it on the cell surface plasma membrane by inhibiting the endocytosis of E-cadherin (15).

Nectin has recently been identified as a Ca\(^{2+}\)-independent Ig-like CAM at AJs (16–21). Nectin constitutes a family consisting of four members, designated nectin-1, -2, -3, and -4. All nectins are associated with the actin cytoskeleton through the F-actin-binding protein afadin. Each nectin first forms homo-cis dimers and then homo- or hetero-trans dimers through the extracellular regions in a Ca\(^{2+}\)-independent manner, resulting in cell-cell adhesion. Nectin then assembles cadherin at the nectin-based cell-cell adhesion sites to form AJs. In addition, each nectin induces the activation of Cdc42 and Rac small G proteins. Nectin first forms cell-cell adhesion, recruits c-Src to the nectin-based cell-cell adhesion sites, and activates it. Activated c-Src then tyrosine-phosphorylates FRG, a Cdc42-GDP/GTP exchange factor (GEP). In addition, c-Src induces the activation of C3G (a Rap1-GEF)-catalyzed Rap1 small G protein through Crk, and Rap1 then induces local activation of tyrosine-phosphorylated FRG at the nectin-based cell-cell adhesion sites, eventually causing the activation of Cdc42. Moreover, c-Src tyrosine phosphorylates Vav2, a Rac-GEF. Cdc42 then activates tyrosine-phosphorylated Vav2 locally at the nectin-based cell-cell adhesion sites, eventually causing the activation of Rac. Cdc42 induces the formation of filopodia and increases the number of cell-cell contact sites. Rac induces the formation of lamellipodia, which efficiently zip the cell-cell adhesion between the filopodia, acting like a "zipper." In these ways, the small G proteins enhance the formation of AJs.

In addition, during or after the formation of AJs, nectin recruits first JAM and then claudin and ocludin to the apical side of AJs in cooperation with cadherin, resulting in the formation of TJs (21). JAM binds a cell-polarity protein complex, consisting of Par-3, atypical PKC, and...
Par-6, by directly binding Par-3 and recruits this complex to TJs (21–23). This complex is essential for the formation of TJs. However, it remains unknown how nectin recruits the TJ components to the apical side of AJs, although it should be noted that nectin-1 and -3, but not nectin-2, directly bind Par-3 (24). Cdc42 activated by the action of trans-interacting nectin is likely to bind to Par-6 and activate the polarity protein complex (20, 22).

Afadin is an F-actin-binding protein that connects each nectin to the actin cytoskeleton (25). It consists of multiple domains, namely two Ras-binding domains, a forkhead-associated domain, a dilute domain (DIL), a PDZ domain, three proline-rich domains, and an F-actin-binding domain found in that order from the NH2 terminus (21). The PDZ domain binds to the COOH-terminal four amino acids of each nectin, and the F-actin-binding domain binds to F-actin (25, 26). In addition, the third proline-rich domain binds ponsin, which interacts with vinculin (27). Vinculin binds to both F-actin and α-catenin (28). The Ras-binding domain was originally shown to bind Ras small G protein (29) but subsequently was shown to bind Rap1 more efficiently than Ras (30). The DIL domain binds afadin DIL domain-interacting protein (ADIP), which interacts with α-actinin (31). α-Actinin binds to both F-actin and α-catenin (32, 33). The COOH-terminal region including the F-actin-binding domain binds LIM domain only 7 (LMO7), which interacts with α-actinin (34). The NH2-terminal region including the Ras-binding and forkhead-associated domains binds ZO-1 (29, 35). Thus, afadin binds many proteins through its multiple domains, although the physiological roles of these interactions remain to be established. Furthermore, afadin has been shown to bind to α-catenin, but this binding is weak, and the region of afadin responsible for the binding remains unknown (36, 37). Afadin has been reported to bind to JAM (38), but this result has not been reproduced (39).

Several lines of evidence for the roles of afadin in the formation of AJs and TJs have been reported as follows. 1) Nectin assembles the E-cadherin-catenin complex at nectin-based cell-cell adhesion sites in a manner that depends on its COOH-terminal PDZ binding-motif for afadin (37); 2) nectin recruits JAM and ZO-1 to the nectin-based cell-cell adhesion sites in a manner that depends on its COOH-terminal PDZ-binding motif for afadin (35, 39); 3) in the ectoderm of afadin-deficient mice and embryoid bodies, the organization of not only AJs but also TJs is highly impaired (40); 4) afadin interacts with p120ctn in a Rap1-dependent manner, and this interaction inhibits the endocytosis of non-trans-interacting E-cadherin, thereby causing its accumulation at the nectin-based cell-cell adhesion sites for the formation of AJs (41). However, definitive evidence for these roles of afadin has not yet been obtained. Furthermore, the modes of action of afadin in these roles have not yet been elucidated either. In this study we knocked down afadin in cultured MDCK cells using an RNA interference (RNAi) method to investigate the roles and modes of action of afadin in the formation of AJs and TJs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—MDCK cells were supplied by Dr. 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 (26). MDCK cells stably expressing GFP-tagged E-cadherin (GFP-E-cadherin-MDCK cells) were prepared as described (42). These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. For DNA transfection, the Lipofectamine 2000 reagent (Invitrogen) and an Amaxa Nucleofector kit (Amaxa) were used.

**Antibodies, Chemicals, and Expression Vectors**—A mouse anti-afadin monoclonal antibody (mAb) and rabbit anti-afadin polyclonal antibody (pAb) were prepared as described (43). A rabbit anti-nectin-1 pAb was prepared as described (26). A rat anti-E-cadherin mAb (ECCD2) was supplied by Dr. M. Takeichi (Center for Developmental Biology, RIKEN, Kobe, Japan). A rabbit anti-JAM-1 pAb was supplied by Dr. T. Kita (Kyoto University, Kyoto, Japan). A mouse anti-FLAG M1 mAb,
FIGURE 2. Involvement of afadin in the formation of AJs and subsequent formation of TJs. Aa, reduction in the immunofluorescence signal for E-cadherin at cell-cell adhesion sites in afadin-knockdown MDCK cells. After the Ca\(^{2+}\) switch assay, the wild-type MDCK cells co-transfected with pBS-H1-afadin and pEGFP were fixed and stained with various combinations of the indicated Abs. The GFP-positive cells were monitored as a marker of transfection. Bars, 10 μm. The results shown are representative of three independent experiments. Ab, quantitative analysis of Aa. Open bars represent the percentage of cell-cell adhesion sites with the signals for the components of AJs among the total cell-cell adhesion sites between GFP-negative cells (n = 30). Filled bars represent the percentage of cell-cell adhesion sites with the signals for the components of AJs among the total cell-cell...
rabbit anti-α-catenin pAb, rat anti-DEMA-1 mAb, and goat anti-human IgG (Fc-specific) Ab were purchased from Sigma. Mouse anti-p120ctn and anti-E-cadherin (clone 36) mAbs were purchased from BD Transduction Laboratories. A mouse anti-β-catenin mAb was purchased from Santa Cruz Biotechnology. A rabbit anti-claudin-1 pAb and mouse anti-occludin mAb were purchased from Zymed Laboratories Inc.. A rat anti-occludin mAb and mouse anti-ZO-1 mAb were purchased from SANKO JUNYAKU. Horseradish peroxidase-conjugated and fluorophore-conjugated secondary Abs were purchased from Amersham Biosciences and Chemicon, respectively. A fragment of glycoprotein D fused to human IgG Fc (gD) and an extracellular fragment of nectin-3 fused to human IgG Fc (Nef-3) were prepared as described (37, 44). Expression vectors for GFP-Rap1GAP (pEGFP-Rap1GAP) and GFP-NWASP-Cdc42/Rac interactive binding domain (CRIB) were prepared as described (16, 45). pEGFP-p120ctn was supplied by Dr. M. Takeichi. A mammalian expression vector pCMV-HA was designed to express NH2-terminal hemagglutinin (HA)-tagged proteins (46). A mammalian expression vector expressing an NH2-terminal truncated form of p120ctn (amino acids 347–912) was constructed using pCMV-HA (HA-p120ctnΔN). An expression vector for pEGFP-afadin (GFP-afadin) was constructed as described (41). To obtain RNAi-resistant afadin, pEGFP-afadin was generated by mutagenesis of 5′-GACAATCTCGTGTCTACC-3′ to create 5′-AACAATACTACTGTCAACT-3′ using the QuikChange site-directed mutagenesis kit (Stratagene).

**Knockdown Experiment**—For knockdown using a small interfering RNA (siRNA) method, a double-stranded 19-nucleotide RNA duplex to afadin (5′-GACAATCTCGTGTCTACC-3′) and a similar duplex to luciferase (5′-CGTACCGGGAATCATTCCA-3′) with 3′-overhang sequences as a control were purchased from Greiner Bio-One. The duplexes were transfected using an Amaxa Nucleofector kit according to the manufacturer’s protocol. For knockdown of afadin, Rac, and IQGAP1 by a short hairpin RNA method, pBS-H1 containing the H1 promoter was used for expression of the short hairpin RNA. To generate vectors for knockdown of afadin (pBS-H1-afadin), Rac (pBS-H1-Rac), and IQGAP1 (pBS-H1-IQGAP1), a specific insert for afadin, Rac, or IQGAP1 was subcloned into pBS-H1 as described (47). The inserts for afadin, Rac, and IQGAP1 were 5′-GAACAATCTCGTGTCTACC-3′, 5′-GAGAAGAAGAAAATTCTGTG-3′, and 5′-CGTATCCGATGTTGAAT-3′, respectively. The cells were co-transfected with pBS-H1 and pEGFP or pDsRed (Clontech) using the Lipofectamine 2000 reagent. The GFP- or DsRed-positive cells were monitored as a marker of the co-transfection.

Ca2+ Switch Assay—Ca2+ switch assay using nectin-1-MDCK, wild-type MDCK, or GFP-E-cadherin-MDCK cells was carried out as described (48). Briefly, the cells (1 × 10⁵) were plated on 18-mm glass coverslips in 12-well culture dishes. At 72 h after plating, the cells were washed with phosphate-buffered saline (PBS) and cultured at 2 mM Ca2+ in DMEM without serum for 1 h. Next, the cells were pre-cultured at 2 mM Ca2+ in DMEM with 5 mM EGTA for 3 h and then re-cultured at 2 mM Ca2+ for 4 h. When the cells were treated with a mixture of gD and Nef-3, the cells were washed with PBS and cultured at 2 mM Ca2+ in DMEM without serum for 1 h. Subsequently, the cells were pre-cultured at 2 mM Ca2+ in DMEM with 5 mM EGTA for 3 h and then re-cultured at 2 mM Ca2+ in the presence or absence of 60 μg/ml gD and 60 μg/ml Nef-3 for 4 h. When the cells were treated with latrunculin A (WAKO), PP2, or PP3 (Calbiochem-Novabiochem), the cells were washed with PBS and cultured at 2 mM Ca2+ in DMEM without serum for 1 h. Subsequently, the cells were pre-cultured at 2 mM Ca2+ in DMEM with 5 mM EGTA for 3 h and then re-cultured at 2 mM Ca2+ in the presence or absence of 0.2 μM latrunculin A, 10 μM PP2, or 10 μM PP3 for 4 h.

**Cell Surface Biotinylation**—After the Ca2+ switch assay, the cells were grown on filters and then incubated with 0.5 mg/ml sulfo-succinimidyl 2-(biotinamido)ethylthiopropionate (sulfo-NHS-SS-biotin; Pierce), which was applied to both the apical and basal sides of the filter. Next, the filters were washed with PBS containing 50 mM NH4Cl to quench free sulfo-NHS-SS-biotin followed by several further washes in PBS. The cells were then scraped off the filters and suspended in a radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). The cell lysates were centrifuged, and the supernatants were incubated with streptavidin beads (Sigma) to collect biotinylated proteins. The samples were then subjected to SDS-PAGE followed by Western blotting with the anti-E-cadherin mAb.

**Immunoprecipitation Assay**—After the Ca2+ switch assay, the cells were extracted with buffer A (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM MgCl2, 0.25 mM dithiothreitol, 5 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). The cell extracts obtained after centrifugation at 100,000 × g for 15 min were incubated with the anti-E-cadherin mAb (5 μg) at 4 °C for 18 h. Next, the immunocomplexes were precipitated with protein G-Sepharose 4FF beads (Amersham Biosciences). After extensive washing of the beads with buffer A, the bound proteins were eluted by boiling in SDS sample buffer. The samples were then subjected to SDS-PAGE followed by Western blotting with the anti-E-cadherin mAb, anti-p120ctn mAb, or anti-β-catenin mAb.

**Bead-Cell Contact Assay**—Nectin-1-MDCK cells (5 × 10⁵) were plated on 14-mm glass coverslips in 24-well culture dishes. 16 h after plating, the cells were co-transfected with pBS-H1-afadin and pEGFP or pDsRed using the Lipofectamine 2000 reagent according to the manufacturer’s protocol. 72 h after the transfection, the cells were washed with PBS and cultured at 2 mM Ca2+ in DMEM without serum for 1 h. Next, the cells were pre-cultured at 2 mM Ca2+ in DMEM with 5 mM EGTA containing latex-sulfate microbeads coated with Nef-3 or ConA for 3 h and then re-cultured at 2 mM Ca2+ in DMEM for 1 h. The cells were fixed and immunostained as described (49).

adhesion sites between GFP-positive cells (n = 30). Data are expressed as the means ± S.D. of three independent experiments. B. Reduction in the immunofluorescence signals for the TJs components at cell-cell adhesion sites in afadin-knockdown MDCK cells. After the Ca2+ switch assay, the wild-type MDCK cells co-transfected with pBS-H1-afadin and pEGFP were fixed and stained with various combinations of the indicated Abs. The GFP-positive cells were monitored as a marker of transfection. Bars, 10 μm. The results shown are representative of three independent experiments. Bb, quantitative analysis of B. Open bars represent the percentage of cell-cell adhesion sites with the signals for the components of TJs among the total cell-cell adhesion sites between GFP-negative cells (n = 30). Filled bars represent the percentage of cell-cell adhesion sites with the signals for the components of TJs among the total cell-cell adhesion sites in GFP-positive cells (n = 30). Data are expressed as the means ± S.D. of three independent experiments. Ca, concentration of the immunofluorescence signals for E-cadherin and claudin-1 at cell-cell adhesion sites in the afadin-knockdown cells re-expressing GFP-afadin. After the Ca2+ switch assay, the wild-type MDCK cells co-transfected with either pBS-H1-afadin and pEGFP or pBS-H1-afadin and pEGFP-afadin, which is resistant to the RNAi, were fixed and stained with various combinations of the indicated Abs. The GFP- or GFP-afadin-positive cells were monitored as a marker of transfection. Asterisks indicate the afadin-knockdown MDCK cells expressing pEGFP-afadin or GFP-afadin. Cb, quantitative analysis of Ca. Filled bars represent the percentage of cell-cell adhesion sites with the signals for E-cadherin and claudin-1 among the total cell-cell adhesion sites between GFP-positive cells (n = 30). Filled bars represent the percentage of cell-cell adhesion sites with the signals for E-cadherin and claudin-1 among the total cell-cell adhesion sites between GFP-afadin-positive cells (n = 30). Data are expressed as the means ± S.D. of three independent experiments.
FIGURE 3. Assembly of E-cadherin with weak cell-cell adhesion activity at nectin-based cell-cell adhesion sites in an afadin-independent manner. Aa, amount of E-cadherin on the plasma membrane. After the Ca²⁺ switch assay, the wild-type MDCK cells transfected with either an siRNA duplex to afadin or an siRNA duplex to luciferase as a control were surface-biotinylated. Detergent-soluble surface-biotinylated proteins on the plasma membrane were recovered on streptavidin beads and analyzed by SDS-PAGE followed by Western blotting with the anti-E-cadherin mAb. The results shown are representative of three independent experiments. Surface, surface-biotinylated proteins; Total, total cell extracts. Ab, quantitative analysis of the amounts of surface-biotinylated E-cadherin in Aa. Open and filled bars represent the intensity of Western blotting in control and afadin-knockdown MDCK cells, respectively. The intensity of Western blotting in control MDCK cells is normalized to 1.0. Data are expressed as the means ± S.D. of three independent experiments. Ac, co-immunoprecipitation of p120ctn and β-catenin with E-cadherin. After the Ca²⁺ switch assay, the wild-type MDCK cells transfected with either the siRNA duplex to afadin or the siRNA duplex to luciferase as a control were lysed and immunoprecipitated (IP) with the anti-E-cadherin mAb. The samples were subjected to SDS-PAGE followed by Western blotting with the indicated Abs. The results shown are representative of three independent experiments. Ad, quantitative analysis of the amounts of E-cadherin, p120ctn, and β-catenin immunoprecipitated with the anti-E-cadherin mAb in Ac. Open and filled bars represent the intensities of Western blotting in control and afadin-knockdown MDCK cells, respectively. The intensity of Western blotting in control MDCK cells is normalized to 1.0. Data are expressed as the means ± S.D. of three independent experiments. Bb, assembly of GFP-E-cadherin at cell-cell adhesion sites in afadin-knockdown MDCK cells. After the Ca²⁺ switch assay, the GFP-E-cadherin-MDCK cells co-transfected with pBS-H1-afadin and pDsRed were fixed and stained with the anti-E-cadherin (ECCD2) and anti-afadin Abs. The DsRed-positive cells were monitored as a marker of transfection. Bars, 10 μm. The results shown are representative of three independent experiments. Bb, quantitative analysis of Bb. Open bars represent the percentage of cell-cell adhesion sites with the signals for afadin.
Cell Dissociation Assay—Cell dissociation assay was carried out as described (50). Briefly, cells (1 × 10^6) were transfected using an Amaxa Nucleofector kit. After the Ca^{2+} switch assay, the cells were washed with HEPES-buffered saline (HBS; pH 7.4) treated with 0.1% trypsin supplemented with 1 mM Ca^{2+} in HBS (TC treatment) or 0.1% trypsin supplemented with 1 mM EDTA in HBS (TE treatment) at 37 °C for 1 h, and dissociated through pipetting 10 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.

Immunofluorescence Microscopy—Cells were fixed in a mixture of 50% acetone and 50% methanol at −20 °C for 1 min or in PBS containing 1% formaldehyde for 15 min followed by PBS containing 0.2% Triton X-100 for 15 min at room temperature. After being blocked in Tris-buffered saline containing 1% bovine serum albumin and 1 mM Ca^{2+} for 1 h, the cells were incubated in the same buffer containing various combinations of Abs for 1 h. The samples were washed three times with Tris-buffered saline (TBS) containing 1 mM Ca^{2+} for 5 min and incubated for 30 min in TBS containing 1% bovine serum albumin and 1 mM Ca^{2+} with the secondary pAbs. The samples were then washed three times with Tris-buffered saline containing 1 mM Ca^{2+} for 5 min and mounted in GEL/MOUNT (Biomeda). The samples were analyzed using a Radiance 2100 confocal laser scanning microscope (Bio-Rad) and an LSM 510 META confocal microscope (Carl Zeiss). The intensities of the immunofluorescence signals for the components of AJs and TJs at cell-cell adhesion sites or bead-cell contact sites were measured using the NIH Image software. The recruitment index was defined as positive when the intensity was above 150/256 of the gradation of the total pixels on the cell-cell adhesion sites or bead-cell contact sites.

RESULTS

Involvement of Afadin in the Formation of AJs and Subsequent Formation of TJs—We first attempted to knock down afadin in MDCK cells using RNAi. Wild-type MDCK cells were transfected with pBS-H1-afadin or a control mock vector. Western blotting showed that the amount of afadin was markedly reduced in afadin-knockdown MDCK cells (Fig. 1A, a and b). The amounts of other components of AJs or TJs, including nectin-2, E-cadherin, p120ctn, β-catenin, α-catenin, claudin-1, occludin, JAM-1, ZO-1, IQGAP1, and actin, remained unchanged. Immunofluorescence microscopy revealed that the signal for afadin was undetectable at cell-cell adhesion sites in afadin-knockdown MDCK cells (Fig. 1B, a and b). Using the afadin-knockdown MDCK cells co-transfected with pEGFP, we examined the signals for the components of AJs and TJs. When the cells were pre-cultured at 2 μM Ca^{2+} were re-cultured at 2 mM Ca^{2+}, the signals for all the components of AJs, including afadin, E-cadherin, p120ctn, β-catenin, and α-catenin, were concentrated at the cell-cell adhesion sites in the wild-type MDCK cells that did not express GFP (Fig. 2A, a and b). Similarly, all the components of TJs, including claudin-1, occludin, JAM-1, and ZO-1, were concentrated at the cell-cell adhesion sites in wild-type MDCK cells (Fig. 2B, a and b). In contrast, the signals for claudin-1, occludin, JAM-1, and ZO-1 were undetectable at the cell-cell adhesion sites, similar to the case for afadin and E-cadherin, in the afadin-knockdown MDCK cells that expressed GFP, although the signals for p120ctn, β-catenin, and α-catenin were concentrated at these sites. The signals for nectin-1 and -3 in both wild-type MDCK and afadin-knockdown MDCK cells were hardly detected because of their low expression levels and the low sensitivity of our Abs against these molecules as described (39, 49, 51–53). p120ctn binds directly to the juxtanuclear region of E-cadherin, whereas β-catenin binds directly to the cytoplasmic region of E-cadherin (7, 54, 55). In addition, α-catenin is known to bind directly to β-catenin (56). The finding that the signals for these E-cadherin-binding proteins were concentrated at nectin-based cell-cell adhesion sites in afadin-knockdown MDCK cells after the Ca^{2+} switch assay suggests that the absence of the signal for E-cadherin at the cell-cell adhesion sites may be because of the inability of the anti-E-cadherin mAb, ECCD2, to detect its presence. However, we were also unable to detect any signals for E-cadherin at the nectin-based cell-cell adhesion sites using other anti-E-cadherin Abs, clone 36 and DECMA-1, in afadin-knockdown MDCK cells (data not shown).

To confirm that afadin plays a role in mediating the formation of cell-cell adhesion, wild-type MDCK cells were co-transfected with either pBS-H1-afadin and pEGFP or pBS-H1-afadin and pEGFP-afadin (GFP-afadin), and the Ca^{2+} switch assay was performed. This GFP-afadin was resistant to the RNAi of afadin. The immunofluorescence signals for E-cadherin and claudin-1 were re-concentrated at cell-cell adhesion sites in the afadin-knockdown MDCK cells that re-expressed GFP-afadin, whereas the signal for E-cadherin or claudin-1 was not re-concentrated at the cell-cell adhesion sites in the afadin-knockdown MDCK cells that expressed GFP (Fig. 2C, a and b). These results indicate that afadin is essential for the formation of TJs and is involved in the assembly of E-cadherin, but not p120ctn, β-catenin, or α-catenin, at nectin-based cell-cell adhesion sites.

Assembly of E-cadherin with Weak Cell-Cell Adhesion Activity at Nectin-based Cell-Cell Adhesion Sites in an Afadin-independent Manner—Next we investigated why the immunofluorescence signal for E-cadherin was not concentrated at nectin-based cell-cell adhesion sites in afadin-knockdown MDCK cells. We first examined whether E-cadherin was present on the plasma membrane of afadin-knockdown MDCK cells using a cell surface biotinylation method. The amount of E-cadherin on the plasma membrane of afadin-knockdown MDCK cells was similar to that of wild-type MDCK cells (Fig. 3A, a and b). We then examined whether this E-cadherin was associated with p120ctn and β-catenin. When E-cadherin was immunoprecipitated from an extract of afadin-knockdown MDCK cells, p120ctn and β-catenin were co-immunoprecipitated with E-cadherin. The amounts of these proteins were similar to the amounts co-immunoprecipitated with E-cadherin in wild-type MDCK cells (Fig. 3A, c and d). Next, we knocked down afadin in the GFP-E-cadherin-MDCK cells and examined by use of them whether the signal for GFP was concentrated at the nectin-based cell-cell adhesion sites between DsRed-negative cells (n = 30). Filled bars represent the percentage of cell-cell adhesion sites with the signals for afadin and GFP-E-cadherin among the total cell-cell adhesion sites between DsRed-positive cells (n = 30). Data are expressed as the means ± S.D. of three independent experiments. Ca, weak cell-cell adhesion activity of E-cadherin in afadin-knockdown MDCK cells. After the Ca^{2+} switch assay, the cell-cell adhesion activity of E-cadherin in the wild-type (wt) MDCK or GFP-E-cadherin MDCK cells transfected with either the siRNA duplex to afadin or the siRNA duplex to luciferase as a control was compared by cell dissociation assay. The microscopic images shown are representative of three independent experiments. Cb, cell dissociation index, N_{TC}/N_{TE}, of Ca. Values are the means ± S.D. of three independent experiments. Dc, recruitment of the immunofluorescence signals for p120ctn, β-catenin, and α-catenin to nectin-based bead-cell contact sites in afadin-knockdown MDCK cells. Nef-3- or ConA-coated beads were added to the nectin-1-MDCK cells co-transfected with pBS-H1-afadin and pDsRed or pEGFP followed by the Ca^{2+} switch assay. The cells were then fixed and stained with various combinations of the indicated Abs. The DsRed- or GFP-positive cells were monitored as a marker of transfection. Dic, differential interference contrast image. Bars, 10 μm. The results shown are representative of three independent experiments. Db, quantitative analysis of Dc. Open bars represent the percentage of Nef-3-coated bead-cell contact sites with the signals for nectin-1, afadin, and the components of AJs among the total Nef-3-coated bead-cell contact sites in DsRed- or GFP-positive cells (n = 30). Filled bars represent the percentage of ConA-coated bead-cell contact sites with the signals for nectin-1, afadin, and the components of AJs among the total ConA-coated bead-cell contact sites in DsRed- or GFP-positive cells (n = 30). Data are expressed as the means ± S.D. of three independent experiments.
cell adhesion sites. The signals for p120\textsuperscript{Catenin}, β-catenin, and α-catenin were concentrated at the nectin-based cell-cell adhesion sites in afadin-knockdown GFP-E-cadherin-MDCK cells (data not shown). Similarly, the signal for GFP was concentrated there (Fig. 3B, a and b). These results strongly suggest that E-cadherin, which is associated with catenins but hardly detected by the anti-E-cadherin Abs used in this study, localized at the nectin-based cell-cell adhesion sites in afadin-knockdown MDCK cells. The reason why the signal for endogenous E-cadherin was not detected at the nectin-based cell-cell adhesion sites in afadin-knockdown MDCK cells may be because of decreases in the sensitivity of the E-cadherin Abs used, resulting from the conformational change of E-cadherin.

Theoretically, there may be at least two forms of E-cadherin on the cell surface plasma membrane; one is a trans-interacting form that shows strong cell-cell adhesion activity, whereas the other is a non-trans-interacting form that shows no cell-cell adhesion activity. We next examined whether the conformational change of E-cadherin, which decreased the sensitivity of the anti-E-cadherin Abs used in this study, was derived from the non-trans-interacting form. For this purpose we compared the cell-cell adhesion activities of wild-type MDCK, afadin-knockdown MDCK, GFP-E-cadherin-MDCK, and afadin-knockdown GFP-E-cadherin-MDCK cells using the cell dissociation assay. Afadin-knockdown MDCK and afadin-knockdown GFP-E-cadherin-MDCK cells formed similar sizes of aggregates, which were smaller than those formed by wild-type MDCK and GFP-E-cadherin-MDCK cells (Fig. 3C, a and b). These results indicate that the cell-cell adhesion activities of E-cadherin in afadin-knockdown MDCK and afadin-knockdown GFP-E-cadherin-MDCK cells are weaker than those of wild-type MDCK and GFP-E-cadherin-MDCK cells. Taken together, it is likely that E-cadherin, which is associated with p120\textsuperscript{Catenin}, β-catenin, and α-catenin, is assembled at the nectin-based cell-cell adhesion sites in an afadin-independent manner and that this E-cadherin is most likely to be the non-trans-interacting form. Although we cannot completely exclude the possibility that the GFP moiety in the overexpressed GFP-E-cadherin non-specifically interacts with other known or unknown junctional proteins and causes the decrease in the cell-cell adhesion activity of GFP-E-cadherin in afadin-knockdown GFP-E-cadherin-MDCK cells, we consider that this possibility is unlikely because only the cadherin molecule has thus far been identified to interact directly with the cytoskeleton is necessary for the association of Cdc42 (61), was expressed or Rac was knocked down nectin-1-MDCK cells (Fig. 5, A and E). In the afadin-knockdown nectin-1-MDCK cells expressing Rap1GAP, which hydrolyzes GTP and inactivates Rap1 (60), the signal for α-catenin concentrated at the nectin-based cell-cell adhesion sites was markedly reduced (Fig. 5, B and E). When NWASP-CRIB, which specifically binds to GTP-Cdc42 and suppresses the actions of Cdc42 (61), was expressed or Rac was knocked down in afadin-knockdown nectin-1-MDCK cells, the signal for α-catenin concentrated at the nectin-based cell-cell adhesion sites was also markedly reduced (Fig. 5, C–E). Similar results were obtained for p120\textsuperscript{Catenin} and β-catenin (data not shown). These results indicate that the nectin-independent activation of Cdc42 and Rac through c-Src and Rap1 is involved in the assembly of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites.

Involvement of the IQGAP1-dependent Actin Cytoskeleton in the Assembly of Non-trans-interacting E-cadherin—Next, we investigated how nectin assembles non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites in an afadin-independent manner. We have previously shown that afadin is not necessary for the activation of Cdc42 or Rac and that the actin cytoskeleton is necessary for the association of trans-interacting E-cadherin with trans-interacting nectin in epithelial cells (45). The nectin-induced activation of Cdc42 and Rac requires the activation of c-Src and Rap1 (16, 58). In the presence of P22, an inhibitor of c-Src (59), the immunofluorescence signal for α-catenin concentrated at the nectin-based cell-cell adhesion sites was markedly reduced in afadin-knockdown nectin-1-MDCK cells (Fig. 5, A and E). In the afadin-knockdown nectin-1-MDCK cells expressing Rap1GAP, which hydrolyzes GTP and inactivates Rap1 (60), the signal for α-catenin concentrated at the nectin-based cell-cell adhesion sites was markedly reduced (Fig. 5, B and E). When NWASP-CRIB, which specifically binds to GTP-Cdc42 and suppresses the actions of Cdc42 (61), was expressed or Rac was knocked down in afadin-knockdown nectin-1-MDCK cells, the signal for α-catenin concentrated at the nectin-based cell-cell adhesion sites was also markedly reduced (Fig. 5, C–E). Similar results were obtained for p120\textsuperscript{Catenin} and β-catenin (data not shown). These results indicate that the nectin-independent activation of Cdc42 and Rac through c-Src and Rap1 is involved in the assembly of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites.
MDCK cells, the immunofluorescence signal for α-catenin concentrated at the nectin-based cell-cell adhesion sites was markedly reduced (Fig. 6b, a and b). Similar results were obtained for p120ctn and β-catenin (data not shown). These results indicate that at least the IQGAP1-dependent actin cytoskeleton participates in the assembly of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites.

** Enhancement of the Adhesion Activity of E-cadherin by Overexpression of p120ctn — It has been shown that the p120ctn-binding juxtamembrane region of E-cadherin negatively regulates the cell-cell adhesion activity of E-cadherin and that an NH2-terminal truncated mutant of p120ctn, but not full-length p120ctn, enhances the cell-cell adhesion activity of E-cadherin (71, 72). Furthermore, we recently found that afadin associates indirectly with p120ctn and stabilizes its association with E-cadherin in a Rap1-dependent manner (41). Rap1 is activated by the action of the trans-interaction of nectin (16). These earlier observations and the above result that E-cadherin shows weak cell-cell adhesion activity in afadin-knockdown MDCK cells suggest that the enhancement of the trans-interaction of E-cadherin by afadin is mediated by p120ctn and that this action of afadin enhances the trans-interaction of the non-trans-interacting E-cadherin assembled at nectin-based cell-cell adhesion sites. To address this issue, we overexpressed HA-tagged full-length p120ctn (HA-p120ctn full) or the NH2-terminal truncated mutant of p120ctn (HA-p120ctn ΔN) in afadin-knockdown MDCK cells. In the afadin-knockdown MDCK cells overexpressing HA-p120ctn full, the immunofluorescence signals for β-catenin and α-catenin, but not for E-cadherin, were concentrated at the nectin-based cell-cell adhesion sites. In contrast, the signal for E-cadherin was concentrated at these sites in the afadin-knockdown MDCK cells overexpressing HA-p120ctn ΔN (Fig. 7A, a and b). In addition, the cell dissociation assay revealed that the afadin-knockdown MDCK cells overexpressing HA-p120ctn ΔN formed aggregates as large as those formed by wild-type MDCK cells, whereas the afadin-knockdown MDCK cells overexpressing HA-p120ctn full formed aggregates as small as those formed by the afadin-knockdown MDCK cells harboring a control mock vector (HA) (Fig. 7B, a and b). Taken together, these results suggest that afadin enhances the cell-cell adhesion activity of E-cadherin through p120ctn.

** DISCUSSION **

Our previous series of studies suggested that afadin, which binds to the cytoplasmic region of nectin and connects nectin to the actin cytoskeleton, is involved in the assembly of E-cadherin and its binding proteins at nectin-based cell-cell adhesion sites (20, 21). In addition, afadin recruits claudin-1, occludin, and JAM-1 to the apical side of AJs. However, definitive evidence for these roles and the modes of action of afadin has not yet been obtained. Here, we used afadin-knockdown MDCK cells to demonstrate that afadin is involved in the formation of E-cadherin-based AJs as well as claudin- and occludin-based TJs. Our detailed analyses have revealed that afadin is not essential for the assembly of E-cadherin with weak cell-cell adhesion activity, presumably the non-trans-interacting form, at nectin-based cell-cell adhesion sites. This form is associated with p120ctn, β-catenin, and α-catenin. Although afadin is not essential for this assembly, nectin-based cell-cell adhesion is essential for this process. We have previously shown that the trans-interaction of nectin induces the activation of Cdc42 and Rac

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**FIGURE 4. Necessity of the trans-interaction of nectin for the afadin-independent assembly of non-trans-interacting E-cadherin. A and B, reduction in the immunofluorescence signals for p120ctn, β-catenin, and α-catenin together with nectin-1 at nectin-based cell-cell adhesion sites mediated by the nectin inhibitors gD and Nef-3. After the Ca2+ switch assay in the presence or absence of the nectin inhibitors, the nectin-1-MDCK cells co-transfected with pBS-H1-afadin and pEGFP were fixed and stained with various combinations of the indicated Abs. The GFP-positive cells were monitored as a marker of cell-cell adhesion sites between GFP-negative cells (n = 30). Open bars represent the percentage of cell-cell adhesion sites with the signals for the components of AJs among the total cell-cell adhesion sites between GFP-negative cells (n = 30). Data are expressed as the means ± S.D. of three independent experiments.**
AJ Formation by Nectin and Afadin

Through c-Src and Rap1 in an afadin-independent manner (16, 17, 45, 58). Here, we have shown that this nectin signaling is involved in the afadin-independent assembly of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites. We, as well as other groups, have previously shown that the actin cytoskeleton is involved in the formation of AJs (62–68). Here, we have shown that at least the latrunculin A-sensitive actin cytoskeleton is involved in the afadin-independent assembly of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites. We have also shown that at least IQGAP1, an F-actin-binding protein that is one of the downstream effectors of Cdc42 and Rac (73–75), links the actions of Cdc42 and Rac to the actin cytoskeleton. The actin cytoskeleton reorganized in this way then assembles E-cadherin, which is associated with p120ctn, b-catenin, and a-catenin. E-Cadherin assembled at nectin-based cell-cell adhesion sites in this way shows weak cell-cell adhesion activity and could not be recognized by the conventional anti-E-cadherin Abs, ECCD2, clone 36, and DECMA-1.

It has been reported that the $K_d$ value for the trans-interaction of E-cadherin is about $8 \times 10^{-5}$ M (76), whereas for the trans-interaction of nectin-1 and -3 is about $2 \times 10^{-8}$ M (77). These results suggest that the non-trans-interacting E-cadherin molecules recruited to nectin-based cell-cell adhesion sites do not trans-interact with each other until their concentrations become sufficiently high. On the other hand, Rac has been shown to be activated by the action of trans-interacting E-cadherin (78–82). In addition, Rac is activated by trans-interacting nectin (45). Rac activated in these ways inhibits the endocytosis of non-trans-interacting E-cadherin (41, 83). This inhibition of endocytosis is mediated by the IQGAP1-dependent reorganization of the actin cytoskeleton. Therefore, Rac continually enhances the accumulation of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites. Non-trans-interacting E-cadherin molecules, which are highly accumulated at these sites, then gradually trans-interact with each other, resulting in the formation of AJs. In these ways presented here and in previous studies, trans-interacting nectin shifts the equilibrium from the non-trans-interacting form of E-cadherin to the trans-interacting form.

In the present study we have demonstrated that afadin increases the trans-interaction activity of the non-trans-interacting E-cadherin assembled at nectin-based cell-cell adhesion sites. The p120ctn-binding juxtamembrane region of E-cadherin and p120ctn iso- forms 1, 2 (71, 72); 2) overexpression of p120ctn isoform 4, which is similar to the NH2-terminal truncated mutant of p120ctn (45), Rac activated in these ways inhibits the endocytosis of non-trans-interacting E-cadherin (41, 83). This inhibition of endocytosis is mediated by the IQGAP1-dependent reorganization of the actin cytoskeleton. Therefore, Rac continually enhances the accumulation of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites. Non-trans-interacting E-cadherin molecules, which are highly accumulated at these sites, then gradually trans-interact with each other, resulting in the formation of AJs. In these ways presented here and in previous studies, trans-interacting nectin shifts the equilibrium from the non-trans-interacting form of E-cadherin to the trans-interacting form.
the major p120\(^{\text{NH2}}\) isoforms, which retain the NH\(_2\) terminus (84); 3) the NH\(_2\) terminus of p120\(^{\text{NH2}}\) is required for cell motility of MDCK cells rather than cell-cell adhesion in the presence of growth factors (85). We have previously shown that afadin interacts with p120\(^{\text{NH2}}\) in a Rap1-dependent manner (41). Specifically, Rap1 activated by the action of trans-interacting nectin through the c-Src-Crk-C3G pathway binds directly to afadin, which then interacts with p120\(^{\text{NH2}}\). This Rap1-dependent binding of afadin to p120\(^{\text{NH2}}\) inhibits the endocytosis of non-trans-interacting E-cadherin and eventually results in the accumulation of non-trans-interacting E-cadherin (41). Consistent with these earlier observations, we have shown here that overexpression of the NH\(_2\)-terminal truncated form of p120\(^{\text{NH2}}\), but not full-length p120\(^{\text{NH2}}\), enhances the cell-cell adhesion activity of E-cadherin in afadin-knockdown MDCK cells. The Rap1-dependent binding of afadin to p120\(^{\text{NH2}}\) may induce its activation, which then enhances the cell-cell adhesion activity of E-cadherin. Thus, the assembly of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites is mediated by nectin signaling and reorganization of the actin cytoskeleton. In addition, the cell-cell adhesion activity of E-cadherin is regulated by the nectin-induced signaling, afadin, and p120\(^{\text{NH2}}\).

Taken together, the mechanisms for the formation of nectin- and E-cadherin-based AJs are as follows: nectin initially forms cell-cell adhesions and then induces the activation of Rap1, Cdc42, and Rac through c-Src. Subsequently, Cdc42 and Rac bind to IQGAP1 and induce the reorganization of the actin cytoskeleton, thereby causing the accumulation of non-trans-interacting E-cadherin at nectin-based cell-cell adhesion sites. On the other hand, Rap1 binds to afadin, which then interacts with p120\(^{\text{NH2}}\), thereby inhibiting the endocytosis of the accumulated non-trans-interacting E-cadherin and also enhancing the trans-interaction of the accumulated non-trans-interacting E-cadherin. In parallel with these processes, Cdc42 increases the number of filopodia through the WASP family and cell-cell contact sites. Rac induces the formation of lamellipodia, which efficiently zip the cell-cell adhesion between the filopodia, acting like a “zipper.”

We have previously shown that 1) nectin recruits claudin and occludin to the apical sides of nectin-based cell-cell adhesion sites (51), 2) overexpression of nectin increases the velocity of the formation of TJs (57), and 3) inhibition of nectin-based cell-cell adhesion by the nectin inhibitors gD and Nef-3 reduces the velocity of TJ formation (39, 51, 52). In this study we have shown that afadin is essential for the recruitment of claudin and occludin to the apical side of nectin-based cell-cell adhesion sites.

![FIGURE 6. Involvement of the IQGAP1-dependent actin cytoskeleton in the assembly of non-trans-interacting E-cadherin. Aa, involvement of the actin cytoskeleton. After the Ca\(^{2+}\) switch assay in the presence or absence of latrunculin A, the nectin-1-MDCK cells co-transfected with pBS-H1-afadin and pEGFP were fixed and stained with various combinations of the indicated Abs. The GFP-positive cells were monitored as a marker of transfection. Lat A, in the presence of latrunculin A; Control, in the absence of latrunculin A. Bars, 10 \(\mu\)m. The results shown are representative of three independent experiments. Ab, quantitative analysis of Aa. Open bars represent the percentage of cell-cell adhesion sites with the signals for the components of AJs among the total cell-cell adhesion sites between GFP-positive cells (n = 30). Filled bars represent the percentage of cell-cell adhesion sites with the signals for the components of AJs among the total cell-cell adhesion sites between GFP-negative cells (n = 30). Data are expressed as the means \pm S.D. of three independent experiments. Bb, involvement of IQGAP1. After the Ca\(^{2+}\) switch assay, the nectin-1-MDCK cells co-transfected with pBS-H1-afadin, pBS-H1-IQGAP1, and pEGFP were fixed and stained with various combinations of the indicated Abs. The GFP-positive cells were monitored as a marker of transfection. Bars, 10 \(\mu\)m. The results shown are representative of three independent experiments. shRNA, short hairpin RNA. Bb, quantitative analysis of Bb. Open bars represent the percentage of cell-cell adhesion sites with the signal for \(\alpha\)-catenin among the total cell-cell adhesion sites between GFP-positive cells (n = 30). Filled bars represent the percentage of cell-cell adhesion sites with the signal for \(\alpha\)-catenin among the total cell-cell adhesion sites between GFP-negative cells (n = 30). Data are expressed as the means \pm S.D. of three independent experiments.]


sion sites. This result is consistent with these earlier observations, although the mechanism for how the nectin-afadin system recruits claudin and occludin to the apical side of such nectin-based cell-cell adhesion sites remains unknown.

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FIGURE 7. Enhancement of the adhesion activity of E-cadherin by overexpression of p120ctn. Aa, re-concentration of the immunofluorescence signal for E-cadherin by overexpression of HA-p120ctnΔN, but not HA-p120ctnfull, at cell-cell adhesion sites in afadin-knockdown MDCK cells. After the Ca2+ switch assay, the wild-type MDCK cells co-transfected with either pBS-H1-afadin and pEGFP, pBS-H1-afadin and pCMV-HA-p120ctnfull, or pBS-H1-afadin and pCMV-HA-p120ctnΔN were fixed and stained with various combinations of the indicated Abs. The GFP-, HA-p120ctnfull-, or HA-p120ctnΔN-positive cells were monitored as a marker of transfection. Asterisks indicate the afadin-knockdown MDCK cells expressing HA-p120ctnfull or HA-p120ctnΔN. Bars, 10 μm. The results shown are representative of three independent experiments. Ab, quantitative analysis of Aa. Open bars represent the percentage of cell-cell adhesion sites with the signal for E-cadherin among the total cell-cell adhesion sites between GFP-negative cells (n = 30) or between HA-negative cells (n = 30). Filled bars represent the percentage of cell-cell adhesion sites with the signal for E-cadherin among the total cell-cell adhesion sites between GFP-positive cells (n = 30) or between HA-positive cells (n = 30). Data are expressed as the means ± S.D. of three independent experiments. Ba, enhancement of the cell-cell adhesion activity of E-cadherin by overexpression of HA-p120ctnΔN, but not HA-p120ctnfull, in afadin-knockdown MDCK cells. After the Ca2+ switch assay, the cell-cell adhesion activities of E-cadherin in the wild-type MDCK cells co-transfected with either the siRNA duplex to afadin and pCMV-HA vector, the siRNA duplex to afadin and pCMV-HA-p120ctnfull, or the siRNA duplex to luciferase alone as a control were compared by cell dissociation assay. The microscopic images shown are representative of three independent experiments. Bb, cell dissociation index, NTC/NTE of Ba. Values are the means ± S.D. of three independent experiments.

REFERENCES

1. Farquhar, M. G., and Palade, G. E. (1963) J. Cell Biol. 17, 375–412
2. Yap, A. S., Brieher, W. M., and Gumbiner, B. M. (1997) Annu. Rev. Cell Dev. Biol. 13, 119–146
3. Tsukita, S., and Furuse, M. (1999) Trends Cell Biol. 9, 268–273
4. Tsukita, S., Furuse, M., and Itoh, M. (2001) Nat. Rev. Mol. Cell. Biol. 2, 285–293
5. Dejana, E. (2004) Nat. Rev. Mol. Cell. Biol. 5, 261–270
6. Itoh, M., Sasaki, H., Furuse, M., Ozaki, H., Kita, T., and Tsukita, S. (2001) J. Cell Biol. 154, 491–497
7. Daniel, J. M., and Reynolds, A. B. (1995) Mol. Cell. Biol. 15, 4819–4824
8. Gumbiner, B. M. (1996) Cell 84, 345–357
9. Gumbiner, B. M. (2000) J. Cell Biol. 148, 399–404
