Regulation of E-cadherin Endocytosis by Nectin through Afadin, Rap1, and p120ctn

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Adherens junctions (AJs) are a major cell-cell adhesion structure in epithelial cells that are formed by two major cell-cell adhesion molecules, E-cadherin and nectin. We have previously shown that nectin first forms cell-cell adhesion and then recruits non-trans-interacting E-cadherin to the nectin-based cell-cell adhesion sites, which gradually trans-interact there, eventually forming AJs. We have examined here the effect of trans-interacting nectin on non-trans-interacting E-cadherin endocytosis. Trans-interacting nectin capable of associating with afadin, but not trans-interacting nectin mutant incapable of associating with afadin, inhibited non-trans-interacting E-cadherin endocytosis in intact cells. Afadin is a nectin- and actin filament-binding protein that connects nectin to the actin cytoskeleton. Studies on the mode of action of the nectin-afadin system using cell-free assay revealed that afadin associated with nectin bound Rap1 activated by trans-interacting nectin, interacted with p120ctn, and strengthened the binding of p120ctn to E-cadherin, eventually reducing non-trans-interacting E-cadherin endocytosis. Afadin, which did not bind Rap1, was inactive in this capacity. These results indicate that trans-interacting nectin inhibits non-trans-interacting E-cadherin endocytosis through afadin, Rap1, and p120ctn and thereby further accumulates non-trans-interacting E-cadherin to the nectin-based cell-cell adhesion sites for the formation of AJs.

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EL cells, nectin-1-EL cells, and nectin-1-/H9004 C-EL cells were incubated in the medium with 0.4 μM human IgG (control IgG) for 60 min. The cell surface was biotinylated on ice and cultured at 18 °C for the indicated periods of time to allow E-cadherin endocytosis. Biotinylated proteins on the plasma membrane were then stripped off by glutathione treatment, and biotinylated proteins inside the cells were recovered on streptavidin beads. The bound proteins were analyzed by immunoblotting with the anti-E-cadherin mAb. A, no inhibition of the constitutive E-cadherin endocytosis by non-trans-interacting nectin in intact cells. EL cells, nectin-1-EL cells, and nectin-1-ΔC-EL cells were incubated in the medium with 0.4 μM Nef-3 for 60 min. The cells were then assayed for E-cadherin endocytosis as described in panel A. In all panels, the relative amounts of endocytosed E-cadherin were expressed as percentage of total biotinylated E-cadherin in the bottom panel. The mean (±S.D.) of duplicate assays is shown. Asterisks indicate statistical significance (Student’s t test; *, p < 0.05). The results shown in all panels are representative of at least three independent experiments.

Because IQGAP1 is an actin filament-cross-linking protein and mainly activated by Rac and Cdc42, IQGAP1 at AJs solely by binding to β-catenin (35). We have shown that Rac and Cdc42 activated by trans-interacting nectin mutant incapable of associating with afadin inhibits the non-trans-interacting E-cadherin endocytosis more potently than trans-interacting nectin mutant incapable of associating with afadin. These results suggest that afadin is additionally involved in E-cadherin endocytosis. We studied here the role and mode of action of afadin in E-cadherin endocytosis.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—Expression vectors were constructed in pEGFP-C1 (Clontech) and pFastBac1-maltose-binding protein (MBP) using standard molecular biology methods. The pFastBac1-MBP was constructed with a baculovirus transfer vector, pFastBac1 (Invitrogen), to express an N-terminal MBP fusion protein. Constructs of afadin contained the following amino acids (aa): pEGFP-afadin, aa 1–1829; pEGFP-afadin-ΔRA, aa 352–1829; pFastBac1-MBP-afadin, aa 1–1829; and pFastBac1-MBP-afadin-ΔRA, aa 352–1829. The pIRM21-FLAG-V12Rap1B was provided by Dr. M. Matsuda (Osaka University, Osaka, Japan).

Antibodies—A rat anti-E-cadherin (extracellular portion) mAb (ECCD-2) was provided by Dr. M. Takeichi (Center for Developmental Biology, RIKEN, Kobe, Japan). A mouse anti-p120ctn mAb, a mouse anti-E-cadherin mAb (BD Transduction Laboratories), a rabbit anti-FLAG pAb (MBL), a mouse anti-FLAG mAb (Sigma), and a rabbit anti-MBP pAb (New England BioLabs) were purchased from commercial sources.

Biotinylation Assay for E-cadherin Endocytosis—The assay was performed as described with minor modifications (36). Briefly, after preincubation with IgG or Nef-3, EL cells, nectin-1-EL cells, and nectin-1-ΔC-EL cells were incubated with 0.5 mg/ml sulfo-NHS-sulfo-biotin on ice,
and the cells were incubated at 18 °C to allow the E-cadherin endocytosis for the indicated periods of time. The cells were incubated in several washes with a glutathione buffer (60 mM glutathione, 83 mM NaCl, 83 mM NaOH, and 10% bovine serum albumin) on ice to remove bound biotinyl groups from remaining cell surface-biotinylated proteins. The cells were then lysed in radioimmune precipitation assay (RIPA) buffer (20 mM Tris-HCl (pH 7.4) with 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA). The cell extracts were incubated with streptavidin beads (Sigma) to collect bound biotinylated proteins. Bound proteins were then analyzed by SDS-PAGE and immunoblotting with the anti-E-cadherin mAb. The blots were developed using the ECL kit and quantitated using a densitometer Fluorchem™ (Alpha Innotech).

Cell-free Assay for E-cadherin Endocytosis—The assay was performed as described (27). Briefly, the AJ-enriched fraction was prepared from rat livers as described (37), washed with 0.5M Tris-HCl (pH 7.5), resuspended in Buffer A (20 mM Hepes-KOH (pH 7.4) and 125 mM KOAc), and stored at −80 °C until use. The thawed AJ membrane fraction (20 µg of protein) was incubated at 30 °C in a reaction mixture (36 mM Hepes-KOH (pH 7.4), 0.25 mM sorbitol, 70 mM KOAc, 5 mM EGTA, 1.8 mM CaCl₂, 2.5 mM Mg(OAc)₂, an ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, and 0.2 IU creatine phosphate kinase), 100 µM GTP, and 2.5 µg/ml rat brain cytosol). The reaction was stopped by chilling the tube on ice. The membrane was collected by centrifugation at 20,000 x g for 10 min. The membrane was resuspended by trituration (20 times pipetting) in 50 µl of 20 mM Hepes-KOH (pH 7.2) and 0.25 mM sorbitol and then supplemented with KOAc and Mg(OAc)₂ to final concentrations of 150 and 2.5 mM, respectively (final volume of 60.6 µl). Immediately after addition of KOAc and Mg(OAc)₂, differential centrifugation was performed at medium speed (16,000 x g) for 2 min. The top 42-µl supernatant fraction was harvested and centrifuged at high speed (100,000 x g) for 20 min. Membrane pellets from the high speed spins were solubilized in an SDS sample buffer at room temperature for 30 min with vigorous shaking, and proteins were separated by 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane sheets and immunoblotted with the anti-E-cadherin mAb. The results shown in all panels are representative of at least three independent experiments.

**FIG. 2.** Inhibition of non-trans-interacting E-cadherin endocytosis by afadin in a Rap1-dependent manner. A, flow diagram of the steps of the cell-free assay and schematic morphologies of the AJ membrane. B, inhibition of E-cadherin endocytosis by afadin-ΔRA, but not by afadin. The AJ membrane fraction was incubated in the presence of MBP-afadin or MBP-afadin-ΔRA at the indicated concentrations and assayed for E-cadherin endocytosis as described in panel A. The amount of E-cadherin in the endocytosed vesicles (70% of total SDS-solubilized membrane) was quantitated by immunoblotting with anti-E-cadherin mAb. Quantification of immunoblots is shown as the mean (± S.D.) of duplicate assays in the lower panel. C, inhibition of E-cadherin endocytosis by afadin in a Rap1-dependent manner. The AJ membrane fraction was incubated in the presence of MBP-afadin (60 nM) and Rap1B-GTP-S or Rap1B-GDP-S at the indicated concentrations and assayed for E-cadherin endocytosis as described in panel A. The amount of E-cadherin in the endocytosed vesicles (70% of total SDS solubilized membrane) was quantitated by immunoblotting with anti-E-cadherin mAb. Quantification of immunoblots is shown as the mean (± S.D.) of duplicate assays in the lower panel. Asterisks indicate statistical significance (Student’s t test; *, p < 0.05). The results shown in all panels are representative of at least three independent experiments.
anti-p120ctn mAb. The immunoprecipitates were analyzed by immunoblotting with the anti-p120ctn mAb (20 ng/ml). The sample was then subjected to SDS-PAGE, followed by immunoblotting with the anti-p120ctn mAb and the anti-MBP pAb, followed by horseradish peroxidase-conjugated secondary Ab (Amersham Biosciences). The blots were developed using an ECL kit and quantitated using a densitometer Fluorchem 

**RESULTS**

**Inhibition of Non-trans-interacting E-cadherin Endocytosis by Trans-interacting Nectin—** To elucidate the mechanism of the non-trans-interacting E-cadherin endocytosis by the nectin-afadin system, we first examined the effect of the trans- or non-trans-interacting full-length nectin-1 and the C-terminal 4-aa deletion mutant of nectin-1, which does not associate with afadin (nectin-1-ΔC), on non-trans-interacting E-cadherin endocytosis in intact cells. For this purpose, we first used L cells stably expressing nectin-1 and E-cadherin (nectin-1-EL cells), L cells stably expressing nectin-1-ΔC and E-cadherin (nectin-1-ΔC-EL cells), and L cells stably expressing E-cadherin (EL cells), because E-cadherin is constitutively endocytosed and recycled when cells do not contact other cells and E-cadherin does not trans-interact (27, 40). We assayed at 18 °C to stop this recycling and to measure only the E-cadherin endocytosis in the absence or presence of an extracellular fragment of nectin-3 fused to the Fc portion of IgG (NeF-3), known to potentially trans-interact with cellular nectin-1 in these cells. When these cells were cultured sparsely and did not contact each other in the absence of NeF-3, the amounts of endocytosed E-cadherin were similar among EL cells, nectin-1-EL cells, and nectin-1-ΔC-EL cells (Fig. 1A). In the presence of NeF-3, the amounts of endocytosed E-cadherin were reduced in nectin-1-EL cells and nectin-1-ΔC-EL cells, but not in EL cells (Fig. 1B). The amount of endocytosed E-cadherin in nectin-1-EL cells was less than that in nectin-1-ΔC-EL cells. Thus, trans-interacting nectin-1 inhibited the non-trans-interacting E-cadherin endocytosis more potently than trans-interacting nectin-1-ΔC. We have previously shown that activation of Rac and Cdc42 by trans-interacting full-length nectin inhibits non-trans-interacting E-cadherin endocytosis through the IQGAP1-dependent reorganization of the actin cytoskeleton in nectin-1-EL cells (27). Association of the C-terminal 4 aa of nectin-1 with afadin is not required for this nectin-induced activation of Rac and Cdc42 in nectin-1-EL cells (41). Taken together, in addition to the Rac- and Cdc42-induced inhibition of the non-trans-interacting E-cadherin endocytosis, these results suggest that association of the C-terminal 4 aa of nectin-1 with afadin is required for full inhibition of the non-trans-interacting E-cadherin endocytosis by trans-interacting full-length nectin-1 in nectin-1-EL cells.

**Inhibition of Non-trans-interacting E-cadherin Endocytosis by Afadin in a Rap1-dependent Manner—** We have previously developed a cell-free assay using an AJ-enriched fraction from rat liver in which non-trans-interacting E-cadherin endocytosis is induced (27). To gain insight into the regulation of the activity of afadin on E-cadherin endocytosis, we examined whether afadin affects the non-trans-interacting E-cadherin endocytosis in this cell-free assay as schematically shown in Fig. 2A. Pure recombinant afadin did not affect the E-cadherin endocytosis, but an RA domain-deleted mutant of afadin, afadin-ΔRA, inhibited the E-cadherin endocytosis, suggesting that the RA domain is the negatively regulatory domain for the activity of afadin on E-cadherin endocytosis (Fig. 2B). Afadin binds the GTP-bound form of Rap1B preferentially to the GDP-
bound form of Rap1B. The GTPγS-bound form of Rap1B enhanced the inhibitory activity of afadin on the E-cadherin endocytosis in a dose-dependent manner, similarly to that induced by afadin-ΔRA, whereas the GDPβS-bound form of Rap1B was less effective (Fig. 2C). These results indicate that afadin inhibits non-trans-interacting E-cadherin endocytosis in a Rap1-dependent manner.

**Enhancement of the Binding of p120ctn to E-cadherin by Trans-interacting Nectin in an Afadin-dependent Manner**—It is well established that p120ctn directly binds to the juxtamembrane region of E-cadherin, and it has been suggested that p120ctn is involved in the stabilization of non-trans-interacting E-cadherin on the cell surface (13, 43). We therefore examined whether the nectin-afadin system inhibits non-trans-interacting E-cadherin endocytosis through p120ctn in nectin-1-EL cells. When p120ctn was immunoprecipitated, E-cadherin was co-immunoprecipitated with p120ctn in EL, nectin-1-EL, and nectin-1-ΔC-EL cells (Fig. 3). However, the amount of E-cadherin co-immunoprecipitated with p120ctn in nectin-1-EL cells was much more than that in EL and nectin-1-ΔC-EL cells. The
Thus, we assumed that the two bands were the 120 and 100 kDa isoforms of p120ctn. For quantification of immunoblot, we measured the intensity of both bands collectively. These results indicate that afadin inhibits the release of p120ctn from the AJ membrane in a Rap1-dependent manner.

Binding of Afadin to p120ctn in a Rap1-dependent Manner—To examine the in vivo binding of afadin to p120ctn, a co-immunoprecipitation assay was performed using the HEK293 cell lysate. Afadin, a mixture of afadin and a constitutively active mutant of Rap1B (V12Rap1B: Rap1B-CA), or afadin-ΔRA was transiently overexpressed in HEK293 cells. When afadin was immunoprecipitated, endogenous p120ctn was co-immunoprecipitated (Fig. 5). The amount of co-immunoprecipitated p120ctn was increased by co-expression with Rap1B-CA. Rap1B-CA indeed bound to afadin. The amount of co-immunoprecipitated p120ctn with afadin-ΔRA was more than that with afadin alone and similar to that with the mixture of afadin and Rap1B-CA. Thus, afadin forms a novel trimeric complex with p120ctn and Rap1. To confirm the direct binding of afadin to p120ctn, we performed affinity chromatography using the pure recombinant proteins of afadin and p120ctn. Afadin did not bind p120ctn directly, and this binding was not affected by binding of Rap1B-CA to afadin (data not shown). A modification or another protein(s) may be required for the efficient binding between afadin and p120ctn. Taken together, these results indicate that afadin binds p120ctn in intact cells and inhibits non-trans-interacting E-cadherin endocytosis through p120ctn in a Rap1-dependent manner.

Inhibition of E-cadherin Endocytosis by Afadin in a Rap1-dependent Manner in MDCK Cells—To further validate the results obtained in our assay systems, we finally examined whether afadin affects E-cadherin endocytosis in intact MDCK cells. Activation of c-Met, the cell surface receptor for HGF, enhances E-cadherin endocytosis (28). We examined the effect of afadin on the HGF-induced E-cadherin endocytosis in MDCK cells. Quantitative analysis showed that overexpression of both afadin and Rap1B-CA or afadin-ΔRA inhibited the HGF-induced E-cadherin endocytosis, consistent with our results in the cell-free assay shown in Fig. 4, A and B. Control GFP or afadin alone did not affect the HGF-induced E-cadherin endocytosis. X-Z optical sectioning showed that afadin co-expressed with Rap1B-CA and afadin-ΔRA was more highly concentrated at the cell-cell adhesion sites than afadin alone, consistent with our results in the cell-free assay shown in Fig. 4, B and C (Fig. 6, A and C). These results indicate that afadin inhibits the HGF-induced E-cadherin endocytosis in a Rap1-dependent manner in intact MDCK cells.

DISCUSSION

We have previously shown that non-trans-interacting E-cadherin, but not the trans-interacting one, undergoes endocytosis and that Rac activated by the action of trans-interacting E-cadherin inhibits E-cadherin endocytosis through the IQGAP1-dependent reorganization of the actin cytoskeleton, which shifts the equilibrium from the cell-cell dissociation state to the cell-cell adhesion state (27). In addition, we have previously shown that Rac and Cdc42 activated by trans-interacting nectin also have a potency to inhibit non-trans-interacting E-cadherin endocytosis through the IQGAP1-dependent reorganization of the actin cytoskeleton (27). For this nectin-induced activation of Rac and Cdc42, the association of the C-terminal tail of nectin with afadin has been shown not to be required (41). On the other hand, for the efficient recruitment of E-cadherin to the nectin-based cell-cell adhesion sites, the association of the C-terminal tail of nectin with afadin has been shown to be required (45). We have shown here that, in addition to the Rac and Cdc42-induced inhibition of non-trans-
interacting E-cadherin endocytosis, the association of the C-terminal tail of nectin with afadin is required for full inhibition of non-trans-interacting E-cadherin endocytosis by trans-interacting full-length nectin. The inhibitory effect of afadin on the endocytosis is Rap1-dependent. Afadin enhances the binding of p120ctn to E-cadherin in a Rap1-dependent manner and thereby inhibits non-trans-interacting E-cadherin endocytosis. Moreover, we have recently shown that trans-interacting nectin induces the activation of Rap1 through e-Src/Crk/C3G signaling pathway (23). Taken together, the trans-interacting nectin inhibits non-trans-interacting E-cadherin endocytosis by two pathways, the Rac and Cdc42-IQGAP1 pathway and the Rap1-afadin pathway.

It has been shown that the $K_d$ value for trans-interaction of E-cadherin is $>100$-fold higher than that for trans-interaction of nectin (46, 47). E-cadherin recruited to the nectin-based cell-cell adhesion sites is, therefore, likely to be non-trans-interacting; as the concentration of non-trans-interacting E-cadherin increases, it gradually trans-interacts, eventually establishing AJs. Trans-interaction of nectin induces the activation of Rap1, which binds to afadin. The afadin-Rap1 complex then forms a novel trimeric complex with p120ctn and thereby enhances the binding of p120ctn to E-cadherin (Fig. 7). This binding prevents non-trans-interacting E-cadherin from endocytosis and stabilizes E-cadherin at the nectin-based cell-cell adhesion sites. The binding of Rap1 to afadin does not affect the association of nectin with afadin. In cooperation with the Rap1-afadin-p120ctn-dependent regulation, trans-interacting nectin induces the activation of Cdc42 and Rac, which

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2 W. Ikeda and Y. Takai, unpublished data.
then inhibit non-trans-interacting E-cadherin endocytosis through IQGAP1-dependent reorganization of the actin cytoskeleton and thereby accumulate non-trans-interacting E-cadherin at the nectin-based cell-cell adhesion sites. The recruited non-trans-interacting E-cadherin gradually starts trans-interacting each other. Rac activated by the action of trans-interacting E-cadherin inhibits non-trans-interacting E-cadherin endocytosis through the IQGAP1-dependent reorganization of the actin cytoskeleton and thereby accumulate non-trans-interacting E-cadherin at the nectin-based cell-cell adhesion sites. The recruited non-

**FIG. 7.** A model for an inhibitory effect of trans-interacting nectin on non-trans-interacting E-cadherin endocytosis. Trans-interaction of nectin induces the activation of Rap1, which binds to afadin. The afadin-Rap1 complex then forms a novel trimeric complex with p120ctn and thereby enhances the binding of p120ctn to E-cadherin. This binding prevents non-trans-interacting E-cadherin from endocytosis and stabilizes E-cadherin at the nectin-based cell-cell adhesion sites. The recruited non-trans-interacting E-cadherin gradually starts trans-interacting each other on the opposite side of the membrane. In these ways, trans-interacting nectin shifts the equilibrium from the non-trans-interaction state of E-cadherin to the trans-interaction state and thereby induces formation of the E-cadherin-based AJs. To simplify the model, we did not describe the involvement of Rac and Cdc42-IQGAP1 pathway in the non-trans-interacting E-cadherin endocytosis.
tin cytoskeleton and thereby enhances the accumulation of non-trans-interacting E-cadherin molecule and more at the nectin-based cell-cell adhesion sites. In these ways, trans-interacting nectin shifts the equilibrium from the non-trans-interaction state of E-cadherin to the trans-interaction state and thereby induces formation of AJ through the action of Cdc42 and Rac activated in a trans mode, but Cdc42 and Rac activated in these ways inhibit non-trans-interacting E-cadherin, may dissociate to produce non-interacting E-cadherin, thereby facilitating the formation of filopodia and increases the cell-cell contact sites, whereas Rac activated by the action of trans-interacting nectin induces the formation of lamellipodia, which efficiently expands the cell-cell adhesion between filopodia, acting like a "zipper." Even after the formation of AJ by trans-interaction of E-cadherin, trans-interacting E-cadherin may dissociate to produce non-trans-interacting E-cadherin, but Cdc42 and Rac activated in these ways inhibit non-trans-interacting E-cadherin endocytosis. Thus, nectin plays important roles in the stabilization of E-cadherin on the plasma membrane and the formation and maintenance of AJ through afadin, the small G proteins, and p120ctn.

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