Cooperative Role of Nectin-Nectin and Nectin-Afadin Interactions in Formation of Nectin-based Cell-Cell Adhesion*

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The nectin cell adhesion molecules interact in trans with each other through their extracellular regions and with afadin through their cytoplasmic tails, forming adhesions junctions in cooperation with cadherins. In a single cell, Necl-5 (nectin-like molecule-5) localizes at the leading edge and regulates directional cell movement in response to a chemoattractant. In such a single cell, afadin also localizes at the leading edge without interacting with nectins or Necl-5. It remains unknown how the nectin-nectin and nectin-afadin interactions are initiated when moving cells contact each other to initiate the formation of adherens junctions. We show here that the Necl-5-nectin interaction induced by cell-cell contact enhances the nectin-afadin interaction. This interaction then enhances the nectin-nectin interaction, which further enhances the nectin-afadin interaction in a positive feedback manner. Thus, the Necl-5-nectin, nectin-nectin, and nectin-afadin interactions cooperatively increase the clustering of the nectin-afadin complex at the cell-cell contact sites, promoting the formation of the nectin-based cell-cell adhesion.

Nectins are Ig-like cell-cell adhesion molecules that compose a family of four members, nectin-1, -2, -3, and -4 (1–3). All members have one extracellular region with three Ig-like loops, one transmembrane segment, and one cytoplasmic region. The extracellular region of each member of the nectin family homophilically and heterophilically interacts in trans (trans-interacts) with that of the same or another member to form a cell-cell adhesion. Nectins form a variety of cell-cell junctions cooperatively with or independently of other cell adhesion molecules, such as cadherins, claudins, and junctional adhesion molecules. For instance, nectins first form cell-cell adhesions and recruit cadherins to the nectin-based cell adhesion site, resulting in the formation of adherens junctions (AJs)3 in fibroblasts and epithelial cells and synapses in neurons. In epithelial cells, nectins further recruit junctional adhesion molecules and claudins to the apical side of AJs to eventually form tight junctions. In contrast, nectins form junctions between spermatids and Sertoli cells in the testis and junctions between commisural axons and floor plate cells in the developing neural tube in a cadherin-independent manner. In addition to the role of nectins in cell-cell adhesion, the extracellular region interacts in cis with the PDGF receptor and integrin αvβ3 and is involved in the regulation of cell proliferation, differentiation, survival, and movement in cooperation with the PDGF receptor and integrin αvβ3.

The cytoplasmic region of nectins has the consensus motif with four amino acids necessary for the binding of the PDZ domain and binds to l-afadin (hereafter simply referred to as afadin) and its short isoform (s-afadin) through this motif (3). Afadin is an F-actin-binding protein with multiple domains: two Ras association (RA) domains, a forkhead-associated domain, a dilute domain, a PDZ domain, three proline-rich domains, and an F-actin-binding domain. s-afadin lacks the third proline-rich domain and the F-actin-binding domain. Afadin is essential for the nectin-induced recruitment of the cadherin-catenin complex to form AJs and for the nectin-induced recruitment of the junctional adhesion molecule-ZO-1 and claudin-ZO-1 complexes to the apical side of AJs to form tight junctions.

In addition to the role of afadin in cell-cell adhesion, afadin is involved in directional cell movement (4, 5). It was shown previously that the PDGF receptor and integrin αvβ3 cooperatively induce movement of NIH3T3 cells (6). We have found that another factor, Necl-5 (nectin-like molecule-5), forms a complex with the PDGF receptor and integrin αvβ3 and is essential for the PDGF-induced, integrin αvβ3-dependent formation of the leading edge structures, such as lamellipodia and ruffles, which facilitate cell movement (7–9). Necl-5 is a member of the Necl family, which consists of five members, and was originally identified as a polio virus receptor (10, 11). The ternary complex of Necl-5, the PDGF receptor, and integrin αvβ3 localizes at the leading edge and induces the activation of Rap1 by the action of PDGF. Activated Rap1 binds to afadin and recruits it to the leading edge. Afadin is less likely to interact with nectins at the leading edge but is critical for the determination of directionality of cell movement (4). Similar observations have been

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3 The abbreviations used are: AJ, adherens junction; RA, Ras association; pAb, polyclonal antibody; mAb, monoclonal antibody; ConA, concanavalin A.
made in human umbilical vein endothelial cells. In this cell type, the VEGF receptor, integrin αβ3, and Necl-5 are involved in directional cell movement in response to VEGF (5).

In addition, Necl-5 is necessary for the PDGF-induced proliferation of NIH3T3 cells in cooperation with the PDGF receptor and integrin αβ2 (8, 12). Upon contact of moving and proliferating NIH3T3 cells, Necl-5, which is in the complex with the PDGF receptor and integrin αβ2, at the leading edge, first interacts in trans with nectin-3 (13). This interaction then causes the down-regulation of Necl-5 by its endocytosis and eventually stops proliferation and movement. This is one of the mechanisms of so-called contact inhibition of cell movement and proliferation (14, 15). The PDGF receptor and integrin αβ2 remain on the plasma membrane and then may form a complex with nectin-1 and/or nectin-3, finally recruiting N-cadherin to form AJs (13).

These results indicate that in a moving single cell, nectins do not interact with afadin. Upon cell-cell contact, the Necl-5-nectin interaction is first induced, followed by the nectin-nectin interaction, raising the possibility that the Necl-5-nectin interaction or the nectin-nectin interaction induces the interaction of afadin with nectins. In this study, we examined this possibility and found that the Necl-5-nectin and nectin-nectin interactions enhance the nectin-afadin interactions and vice versa. These cooperative protein-protein interactions enhance the formation of the nectin-based cell-cell adhesion.

**EXPERIMENTAL PROCEDURES**

Plasmid Construction and Protein Purification—Expression vectors for GFP-tagged afadin and its truncation mutants were constructed as described (16). Expression vectors for nectin-3 and its mutants were prepared as described (17, 18). A baculoviral transfer vector (pFastBac1-Msp-Fc) for the expression of a fusion protein with the N-terminal honeybee melittin signal peptide (Msp) and the C-terminal human IgG Fc was constructed as described (19). A baculoviral transfer vector encoding the extracellular fragment of mouse nectin-1 (amino acids 24–347) fused to the Fc portion of human IgG (Fc-Nef-1) and that of mouse Necl-5 (amino acids 30–347) fused to the Fc portion of human IgG (Fc-Lef-5) were constructed as described (20, 21). Fc-Nef-1 and Fc-Lef-5 were expressed in SF21 insect cells using the Bac-to-Bac baculovirus expression system (Invitrogen) and purified as described (19–21).

Antibodies and Reagents—A rabbit anti-nectin-3 polyclonal antibody (pAb) and a rat anti-nectin-3 monoclonal antibody (mAb) were prepared as described (18). A rabbit anti-afadin pAb (Sigma-Aldrich), a rabbit anti-l/s-afadin pAb (which recognizes both afadin and s-afadin; Sigma-Aldrich), a rabbit anti-FLAG pAb (Sigma-Aldrich), a rabbit anti-GFP pAb (Medical and Biological Laboratories), a rat anti-GFP mAb (Nacalai Tesque), a goat anti-human IgG (Fc-specific) pAb (Sigma-Aldrich), and an anti-rat IgG (Fc-specific) pAb (Jackson Immunoresearch Laboratories) were purchased from the indicated sources. Concanavalin A (ConA) and a human IgG were purchased from Sigma-Aldrich. A rabbit IgG was purchased from Jackson Immunoresearch Laboratories.

**RESULTS**

Enhancement of the Formation of the Nef-1-Nectin-3-Afadin Complex by Nef-1—We first examined whether the binding of afadin to nectin-3 is affected by the trans-interaction of nectin-3 with nectin-1. For this purpose, we used the extracellular region of nectin-1 fused to the Fc portion of human IgG (Fc-Nef-1) because Fc-Nef-1 trans-interacts with nectin-3 when it is added to cells expressing nectin-3 (23). FLAG-nectin-3 was coexpressed with GFP-afadin or GFP in HEK293 cells, and the cells were cultured in the presence of Fc-Nef-1 or control IgG in the medium. The cell lysate was prepared, and GFP-afadin or GFP was immunoprecipitated by the anti-GFP pAb, which was attached to protein A-Sepharose beads (Fig. 1A). The precipitates were then subjected to SDS-PAGE, followed by Western

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Co-immunoprecipitation Assay—HEK293 cells expressing various combinations of expression plasmids were incubated in the presence of 25 μg/ml control IgG, Fc-Nef-1, or Fc-Lef-5 at 37 °C for 15 min. After being washed with ice-cold phosphate-buffered saline twice, the cells were lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5% glycerol, 1 mM MgCl2, 1 mM CaCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 mM NaF, and 1 mM Na3VO4). The cell lysates were subjected to centrifugation at 20,000 × g for 15 min, and the supernatants were then incubated with the anti-GFP rabbit pAb bound to protein A-Sepharose beads (GE Healthcare) at 4 °C for 3 h to precipitate GFP-afadin and its deletion mutants. For immunoprecipitation of endogenous afadin, cell lysates were incubated with the anti-l/s-afadin pAb or the rabbit IgG as a control, which were bound to protein A-Sepharose beads. After the beads had been extensively washed with lysis buffer, bound proteins were eluted by boiling with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 100 mM dithiothreitol, 8% glycerol, and 0.2% brom phenol blue). The samples were subjected to SDS-PAGE, followed by Western blotting using the indicated Abs.

Bead-Cell Contact Assay—The bead-cell contact assays were performed as described (20, 22) except for using the rat anti-nectin-3 mAb-coated beads. For preparation of the rat anti-nectin-3 mAb-coated beads, the rat anti-nectin-3 mAb was adsorbed onto latex-sulfate beads (5-μm diameter; Interfacial Dynamics) coated with the anti-rat IgG (Fc-specific) pAb. Nectin-3-L cells were seeded on coverslips, cultured for 18 h, and then incubated with latex-sulfate microbeads coated with Fc-Nef-1, the rat anti-nectin-3 mAb, or ConA as a control. After a 30-min incubation, the cells were fixed and immunostained with the anti-nectin-3 or anti-afadin pAb. Fluorescence images were obtained with a laser-scanning confocal microscope (LSM 510; Carl Zeiss, Inc.) equipped with a Plan Apochromat 63× objective lens (numerical aperture, 1.4) using LSM 510 v3.2 software (Carl Zeiss, Inc.).

Enhancement of the Formation of the Nef-1-Nectin-3-Afadin Complex by Nef-1—We first examined whether the binding of afadin to nectin-3 is affected by the trans-interaction of nectin-3 with nectin-1 for this purpose.
blotting using the indicated Abs (Fig. 1B). Fc-Nef-1 was precipitated with the beads because the Fc portion bound to protein A. FLAG-nectin-3 was co-precipitated with GFP-afadin when the cells were incubated with Fc-Nef-1, but FLAG-nectin-3 was hardly co-precipitated with GFP-afadin when the cells were not incubated with Fc-Nef-1. FLAG-nectin-3 was not precipitated with GFP irrespective of the presence or absence of Fc-Nef-1 (Fig. 1B). To exclude the possibility that a GFP tag itself affected the nectin-afadin interaction, we further examined whether Fc-Nef-1 promotes the interaction of endogenous afadin with FLAG-nectin-3. HEK293 cells expressing FLAG-nectin-3 were incubated in the presence of Fc-Nef-1 or control IgG, and endogenous afadin was immunoprecipitated by the anti-l/s-afadin pAb. Endogenous afadin and s-afadin were efficiently immunoprecipitated, and the amount of co-precipitated FLAG-nectin-3 was increased by the addition of Fc-Nef-1 (Fig. 1C). These results indicate that the trans-interaction of nectin-3 with Fc-Nef-1 enhances the binding of afadin to nectin-3.

Enhancement of the Nef-1-Nectin-3 Interaction by the Afadin-Nectin-3 Interaction—We showed previously that the C-terminal four amino acid residues of nectins interact with the PDZ domain of afadin (24). Using an afadin mutant lacking the PDZ domain (GFP-afadin-H9004PDZ), we examined whether the interaction of afadin with nectin-3 enhances the trans-interaction of nectin-3 with nectin-1. HEK293 cells expressing FLAG-nectin-3 and either GFP-afadin or GFP-afadin-H9004PDZ were incubated with control IgG or Fc-Nef-1, and GFP-afadin or GFP-afadin-H9004PDZ was immunoprecipitated by the anti-GFP pAb. FLAG-nectin-3 was co-precipitated with GFP-afadin, but FLAG-nectin-3 was not co-precipitated with GFP-afadin-H9004PDZ even in the presence of Fc-Nef-1 (Fig. 2A). These results are consistent with our previous observation (24) and indicate that the interaction of afadin with nectin-3 is required for the trans-interaction of nectin-3 with Fc-Nef-1.

We also confirmed this conclusion using a nectin-3 mutant lacking the C-terminal four amino acid residues (FLAG-nectin-3).
Nectin-Nectin and Nectin-Afadin Interactions

A

|        | Input | IP: GFP |        | Input | IP: GFP |
|--------|-------|---------|--------|-------|---------|
|        | WT    | ΔPDZ    | WT    | ΔPDZ  |         |
| GFP-Nef-1 | -     | +       | -     | +     |         |
| IB: GFP | +     | +       | +     | +     | +       |
| IB: Fc  | +     | -       | +     | +     | +       |
| IB: FLAG| +     | +       | +     | +     | +       |

B

|        | Input | IP: GFP |        | Input | IP: GFP |
|--------|-------|---------|--------|-------|---------|
|        | WT    | ΔC      | WT    | ΔC    |         |
| FLAG-nectin-3 | -     | +       | -     | +     |         |
| IB: GFP | +     | +       | +     | +     | +       |
| IB: Fc  | +     | -       | +     | +     | +       |
| IB: FLAG| +     | +       | +     | +     | +       |

3-ΔC). HEK293 cells expressing FLAG-nectin-3 or FLAG-nectin-3-ΔC together with GFP-afadin were incubated with control IgG or Fc-Nef-1, and GFP-afadin was immunoprecipitated by the anti-GFP pAb. FLAG-nectin-3 was co-precipitated with GFP-afadin, but FLAG-nectin-3-ΔC was not co-precipitated with GFP-afadin even in the presence of Fc-Nef-1 (Fig. 2B). These results are consistent with our previous observation (24) and indicate that the interaction of afadin with nectin-3 is required for the trans-interaction of nectin-3 with Fc-Nef-1.

Enhancement of the Afadin-Nectin-3 Interaction by the Nef-1-Nectin-3 Interaction—We showed previously that the first Ig-like loop is required for the trans-interaction of nectin-2 and that a point mutation of a phenylalanine residue in the first Ig-like loop of nectin-2 (F136L) abolishes trans-interacting activity but not cis-dimerization activity (25). To examine whether the trans-interacting activity of nectins is required for the enhancement of the interaction of afadin with nectin-3, we used two nectin-3 mutants: a point mutant with phenylalanine at position 153 (equivalent to Phe-136 in nectin-2) changed to leucine (pm-nectin-3) and a deletion mutant lacking the first Ig-like loop (nectin-3-ΔIgl1). GFP-afadin was coexpressed with FLAG-nectin-3, FLAG-pm-nectin-3, or FLAG-nectin-3-ΔIgl1 in HEK293 cells. The cells were incubated with control IgG or

FIGURE 2. Enhancement of the Nef-1-nectin-3 interaction by the afadin-nectin-3 interaction. A, requirement of the PDZ domain of afadin for the formation of the Nef-1-nectin-3-afadin complex. HEK293 cells transiently expressing FLAG-nectin-3 and either GFP-afadin or GFP-afadin-ΔPDZ were cultured in the presence of control IgG or Fc-Nef-1 for 15 min and then subjected to the immunoprecipitation assay using the anti-GFP pAb. The immunoprecipitates were analyzed as described in the legend to Fig. 1. B, requirement of the C-terminal afadin-binding motif of nectin-3 for the formation of the Nef-1-nectin-3-afadin complex. HEK293 cells transiently expressing GFP-afadin, but FLAG-nectin-3-ΔIgl1 was hardly co-precipitated with GFP-afadin when the cells were incubated with control IgG (Fig. 4). These results indicate that the trans-interaction of nectin-3 with Fc-Nef-1 is required for the enhancement of the interaction of afadin with nectin-3.

Enhancement of the Formation of the Lef-5-Nectin-3-Afadin Complex by Lef-5—We showed previously that when moving NIH3T3 cells collide with each other, the first contact between these cells is mediated by the trans-interaction of Necl-5 with nectin-3; this Necl-5 molecule is down-regulated from the cell surface by endocytosis, and nectin-3 then trans-interacts with nectin-1 (13). We examined whether afadin interacts with nectin-3 when nectin-3 trans-interacts with the extracellular fragment of Necl-5 fused to the Fc portion of human IgG (Fc-Lef-5). HEK293 cells expressing FLAG-nectin-3 and GFP-afadin were cultured with control IgG or Fc-Lef-5, and GFP-afadin was immunoprecipitated by the anti-GFP pAb (Fig. 4). Fc-Lef-5 was precipitated with the microbeads similar to Fc-Nef-1. FLAG-nectin-3 was co-precipitated with GFP-afadin when the cells were incubated with Fc-Lef-5, but FLAG-nectin-3 was hardly co-precipitated with GFP-afadin when the cells were incubated with control IgG (Fig. 4). These results indicate that the trans-interaction of nectin-3 with Fc-Lef-5 enhances the interaction of afadin with nectin-3.

No Requirement of the Binding of Rap1 to Afadin for the Formation of the Nef-1-Nectin-3-Afadin Complex—Rap1 binds to afadin through the RA domain (26). We showed previously that the trans-interaction of nectin-1 with Nef-3 induces the activation of Rap1 in Madin-Darby canine kidney cells (27) and that Rap1 activated in this way then binds to afadin, promoting the interaction of afadin with p120<sup>crm1</sup> in HEK293 cells (28). We also showed that deletion of the RA domain of afadin promotes the afadin-p120<sup>crm1</sup> interaction. These results suggest that the RA domain of afadin has an inhibitory role in the afadin-p120<sup>crm1</sup> interaction and that the binding of Rap1 to afadin induces a conformational change in afadin so that it interacts with p120<sup>crm1</sup> (28). We further showed that upon binding of VEGF to
the VEGF receptor, which forms a complex with Necl-5 and integrin αvβ3, Rap1 is activated at the leading edge of moving human umbilical vein endothelial cells, and activated Rap1 then binds to afadin, causing the interaction of afadin with the p85 subunit of phosphatidylinositol 3-kinase (5). On the basis of these observations, we examined whether the binding of Rap1 to afadin affects the Fc-Nef-1-induced formation of the afadin-nectin-3 complex by the use of a GFP-afadin mutant lacking the RA domain (GFP-afadinΔRA). GFP-afadin or GFP-afadinΔRA was coexpressed with FLAG-nectin-3 in HEK293 cells, and the cells were incubated with control IgG or Fc-Nef-1. FLAG-nectin-3 was co-immunoprecipitated with GFP-afadinΔRA when the cells were incubated with Fc-Nef-1, but FLAG-nectin-3 was hardly co-immunoprecipitated with GFP-afadinΔRA when the cells were not incubated with Fc-Nef-1 (Fig. 5). The amount of FLAG-nectin-3 co-immunoprecipitated with GFP-afadinΔRA was similar to that co-immunoprecipitated with wild-type afadin. These results indicate that the binding of Rap1 to afadin is not essential for the formation of the Nef-1-nectin-3-afadin complex.

**Clustering of the Nef-1/Nectin-3/Afadin Complex by the Nef-1/Nectin-3 Interaction**—In the last set of experiments, we used the microbead-cell contact assay to analyze whether the trans-interaction of nectin-3 with nectin-1 enhances the binding of afadin to nectin-3. Microbeads coated with Fc-Nef-1 or an Ab against nectin-3 were placed on the surface of nectin-3-L cells. The immunofluorescence signal for nectin-3 was concentrated at the contact sites between the Fc-Nef-1-coated beads and nectin-3-L cells (Fig. 6A, upper panels). Similar results were observed at the contact sites between the beads coated with the anti-nectin-3 Ab and nectin-3-L cells (Fig. 6A, middle panels). The frequency of the signal for nectin-3 observed at the contact sites between the Fc-Nef-1-coated beads and nectin-3-L cells was similar to that observed at the contact sites between the beads coated with the anti-nectin-3 Ab and nectin-3-L cells (Fig. 6A, lower panels). The signal for afadin was concentrated at the contact sites between the Fc-Nef-1-coated beads and nectin-3-L cells (Fig. 6B, upper panels). In contrast, it was hardly observed at most of the contact sites between the beads coated with the anti-nectin-3 Ab and nectin-3-L cells (Fig. 6B, middle panels). The frequency of the signal for afadin observed at the contact sites between the anti-nectin-3 Ab-coated beads and nectin-3-L cells was less than that observed at the contact sites between the Fc-Nef-1-coated beads and nectin-3-L cells (Fig. 6B, graph). The signal for afadin was hardly detected at the contact sites between the ConA-coated beads and nectin-3-L cells (Fig. 6B, lower panels). These results indicate that the interaction of nectin-3 with Fc-Nef-1 enhances the interaction of afadin with nectin-3.

**DISCUSSION**

Using afadin knock-out mice and afadin knockdown cells, it has been reported that afadin plays a critical role in the formation of AJs and tight junctions (29–33): afadin is essential for the nectin-induced recruitment of the cadherin-catenin complex to the nectin-based cell-cell adhesion site and the nectin-induced recruitment of the junctional adhesion molecule-ZO-1 and the claudin-ZO-1 complexes to the apical side of AJs. These actions of afadin are likely to be mediated by its binding to α-catenin and ZO-1, respectively. Thus, the interaction of afadin with nectins is thought to initiate the formation of AJs and tight junctions at the nectin-based cell-cell adhesion sites. However, the regulatory mechanism underlying the nectin-afadin interaction remains to be elucidated.

We have shown here that the trans-interaction of nectin-3 with nectin-1 or Necl-5 enhances the interaction of nectin-3 with afadin and that the interaction of afadin with nectin-3 enhances the trans-interaction of nectin-3 with nectin-1 or Necl-5, as summarized in Fig. 7. The molecular mechanism of how the trans-interaction of nectin-3 with nectin-1 or Necl-5 promotes the nectin-afadin interaction and vice versa remains unclear. However, it is conceivable that the trans-interaction of nectin-3 with nectin-1 or Necl-5 may induce a conformational change in the intracellular region of nectin-3 to facilitate the binding to afadin and that the interaction of afadin with nectin-3 may induce a conformational change in the extracellular region of nectin-3 to promote the trans-interaction.
In a moving single cell, afadin localizes at the leading edge by binding to Rap1, which is activated there by the actions of PDGF and VEGF (4, 5). Afadin does not likely interact with nectins because nectins are present diffusely on the plasma membrane. In contrast, Necl-5 forms a complex with the PDGF receptor or the VEGF receptor and integrin αvβ3 and localizes at the leading edge (4, 5). Necl-5 does not interact with afadin.

Upon cell collision, Necl-5 at the leading edge of one cell first trans-interacts with nectin-3, which is localized diffusely on the plasma membrane of another moving cell (13). This trans-interaction of nectin-3 with Necl-5 then induces the interaction of nectin-3 with afadin. Necl-5, which is pre-interacted with nectin-3, is down-regulated from the cell surface by endocytosis (13). The nectin-3 molecule, which does not interact with Necl-5 but interacts with afadin, seems to remain on the plasma membrane and to trans-interact with nectin-1 of the cell in which Necl-5 is down-regulated. The results from this study suggest that the nectin-3 molecule interacting with afadin more easily trans-interacts with nectin-1 and that this trans-interaction of nectin-1 with nectin-3 enhances the interaction of nectin-1 with afadin. Thus, both the nectin-nectin and nectin-afadin interactions may play a synergistic effect in the formation of the nectin-based cell-cell adhesion.

Using the bead-cell contact assay, we further confirmed that the trans-interaction of nectin-3 with nectin-1 effectively induces the recruitment of afadin to the contact site. We reported previously that both the trans-interaction of nectin-2 and the interaction of nectin-2 with afadin are essential for the
clustering of the afadin-nectin-2 complex at cell-cell contact sites (25); a nectin-2 mutant lacking the trans-interacting activity (pm-nectin-2) binds to afadin but fails to concentrate to cell-cell contact sites, and a nectin-2 mutant lacking the afadin-binding motif (nectin-2 ΔC) retains the trans-interacting activity but again fails to concentrate to cell-cell contact sites. Together with our results presented in this work, these findings indicate that both the nectin-nectin and nectin-afadin interactions cooperatively enhance the clustering of the nectin-afadin complex to establish AJs.

It was originally believed that upon cell-cell contact, the extracellular region of cadherins trans-interacts with that of the contacting cell to form AJs. We then proposed that upon cell-cell contact, the extracellular region of nectins trans-interacts with that of the contacting cell to form the nectin-based cell-cell adhesion where cadherins are recruited to form AJs. Thus, it was believed that cell-cell adhesion is initiated depending on the random interaction of the extracellular regions of cell adhesion molecules. However, we have shown here that the trans-interaction of nectins is enhanced by the interaction of nectin with afadin, and we showed previously that afadin localizes at the leading edge of a moving cell (4, 5). Taken together, these results indicate that when the leading edge of a moving cell makes a contact with another cell, the nectin-based cell-cell adhesion is initiated at the contact site. This cell-cell adhesion site may be determined by the localization of afadin. Thus, it is conceivable that in addition to the extracellular region of cell adhesion molecules, afadin, the molecule interacting with the cytoplasmic region of cell adhesion molecules, serves as a determinant of the site for the formation of AJs.

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