Interaction of Integrin $\alpha_v\beta_3$ with Nectin

**IMPLICATION IN CROSS-TALK BETWEEN CELL-MATRIX AND CELL-CELL JUNCTIONS**

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Cell-matrix and cell-cell junctions cross-talk together, and these two junctions cooperatively regulate cell movement, proliferation, adhesion, and polarization. However, the mechanism of this cross-talk remains unknown. An immunoglobulin-like cell-cell adhesion molecule nectin first trans-interacts with each other to form cell-cell adhesion and induces activation of Rap1, Cdc42, and Rac small G proteins through c-Src. Trans-interacting nectin then recruits another cell-cell adhesion molecule cadherin to the nectin-based cell-cell contact sites and forms adherens junctions (AJs). Here, we show that integrin $\alpha_v\beta_3$ functionally and physically associates with nectin. Integrin $\alpha_v\beta_3$ co-localized with nectin at the nectin-based cell-cell adhesion sites. The association of integrin $\alpha_v\beta_3$ with nectin was direct and was mediated through their extracellular regions. This interaction was necessary for the nectin-induced signaling. Focal adhesion kinase, which relays the integrin-initiated outside-in signals to the intracellular signaling molecules, was also involved in the nectin-induced signaling. During the formation of AJs, the high affinity form of integrin $\alpha_v\beta_3$ co-localized with nectin at the primordial cell-cell contact sites, and then after the establishment of AJs, this high affinity form of integrin $\alpha_v\beta_3$ was converted to the low affinity form, which continued to co-localize with nectin. Thus, integrin $\alpha_v\beta_3$ and nectin play pivotal roles in the cross-talk between cell-matrix and cell-cell junctions and the formation of cadherin-based AJs.

There is cross-talk between cell-cell and cell-matrix junctions in epithelial cells and non-epithelial cells (1, 2). The major cell-cell junctions are adherens junctions (AJs) $^2$ and tight junctions (TJs) in epithelial cells and AJs in non-epithelial cells. Cadherin and claudin are key cell-cell adhesion molecules at AJs and TJs, respectively (3, 4). The cell-matrix junctions are formed by the interaction of integrin with extracellular matrix (ECM) proteins, such as collagen, fibronectin, laminin, and vitronectin (5, 6). The integrin-mediated cell-matrix junctions positively or negatively regulate the formation and stability of cell-cell junctions through protein kinases associated with integrins, such as focal adhesion kinase (FAK) and c-Src (5, 7). For instance, this regulation may be important for the epithelial-mesenchymal transition of epithelial cells in both physiological and pathological states (8, 9). During the embryonic development, integrin promotes epithelial cell remodeling, which appears to be related to reduced interaction of cell-cell adhesion molecules at AJs with the cytoskeleton (8). In a colon cancer cell line, the attachment of integrin to ECM proteins induces functional polarization of the cells and reinforces the E-cadherin-based AJs (9). Moreover, the integrin-induced signaling molecules, FAK and paxillin, have been shown to regulate the N-cadherin-based cell-cell adhesion in HeLa cells (10). These data may explain the importance of the cross-talk between cell-cell and cell-matrix junctions for the development of embryo and the metastasis of cancer. However, the molecular mechanisms for the cross-talk between cell-cell and cell-matrix junctions have not fully been understood.

Nectin is an emerging immunoglobulin-like cell-cell adhesion molecule at AJs and constitutes a family that consists of four members (nectin-1, nectin-2, nectin-3, and nectin-4) (11, 12). Nectin is associated with the actin cytoskeleton through afadin, a nectin- and F-actin-binding protein, as cadherin is associated with the actin cytoskeleton through many peripheral membrane proteins, including $\alpha$- and $\beta$-catenins (3, 11, 12). Nectin forms homo-cis-dimers followed by formation of homohetero-trans dimers (trans-interaction) in a Ca$^{2+}$-independent manner, although cadherin forms homo-cis-dimers followed by formation of only homo-trans-dimers (trans-interaction) in a Ca$^{2+}$-dependent manner (3, 11, 12). Nectin first forms cell-cell adhesion and induces activation of Rap1, Cdc42, and Rac small G proteins through c-Src (13–17). Rap1 then binds to afadin, which then binds to p120$^{ctn}$ associated with non-trans-interacting cadherin (18). This Rap1-dependent binding of afadin to p120$^{ctn}$ inhibits the endocytosis of non-trans-interacting cadherin and enhances the accumulation of non-trans-interacting cadherin at the nectin-based cell-cell adhesion sites and the cell-cell adhesion activity of cadherin, eventually resulting in the formation of AJs (18, 19). In addition, Cdc42 activated in this way increases the number of filopodia and cell-cell adhesion sites, whereas activated Rac induces the
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formation of lamellipodia and efficiently seals the cell-cell contacts between filopodia-like a zipper (11, 12). Thus, the trans-interaction of nectin induces the formation of AJs in a complex mechanism.

We have recently found that nectin-like molecule (Necl)-5/Tage-4/PVR/CD155 associates with integrin α3β1 and enhances cell movement and proliferation (20, 21). However, upon cell-cell contacts Necl-5 heterophilically trans-interacts with nectin-3 to initiate the formation of cell-cell junctions (22–24). In addition, this trans-interaction of Necl-5 with nectin-3 induces down-regulation of Necl-5 from the cell surface by clathrin-dependent endocytosis (25). The down-regulation of Necl-5 causes reduction of cell movement and proliferation. Cultured cells continue to move and proliferate until they become confluent. When cultured cells become confluent, they form cell-cell adhesion and gradually reduce and finally stop their movement and proliferation (26, 27). This phenomenon is known for a long time as contact inhibition of cell movement and proliferation. However, the molecular mechanism for this phenomenon has not fully been elucidated. We have proposed that the cell-cell contact-induced trans-interaction of Necl-5 with nectin-3 and the subsequent down-regulation of Necl-5 are at least one mechanism underlying the contact inhibition of cell movement and proliferation (25).

Extending these earlier observations and gaining more insights into the cross-talk between the cell-cell and cell-matrix junctions, we examined here the relationship between nectin and integrin α3β3 and its involvement in both the nectin-induced signaling and the formation of AJs.

EXPERIMENTAL PROCEDURES

Vector Construction, Protein Purification, and Reagents—The following expression vectors were kindly provided: wild-type human integrin α3, integrin α5, integrin β1, and integrin β3 (pcDNA3-α3, pcDNA3-α5, pcDNA3-β1, and pcDNA3-β3, respectively) were from Dr. J. C. Norman (University of Leicester, Leicester, UK), constitutively active and dominant negative mutants of human integrin β3 (pcDNA3.1-Myc-His(+) β3N300T and pcDNA3.1-Myc-His(+)-β3T329C/A347C, respectively) were from Dr. J. Takagi (Osaka University, Suita, Japan), wild-type c-Src (pcDNA3-c-Src-wt) was from Dr. M. Okada (Osaka University, Suita, Japan), HA-FRNK (COOH-terminal 396 amino acids of FAK; pcCX4pur-HA-FRNK) was from Dr. T. Akagi (Osaka Bioscience Institute, Suita, Japan), and FLAG-claudin-1 (pCCL-1F) was from Dr. M. Furuse (Kyoto University, Kyoto, Japan). Expression vectors for FLAG-nectin-1 (amino acids 27–518, pFLAG-CMV1-nectin-1), FLAG-nectin-2 (amino acids 30–467, pFLAG-CMV1-nectin-2), FLAG-nectin-3 (amino acids 56–549, pCAGIPuro-FLAG-nectin-3), Myc-nectin-3 (amino acids 56–549, pCAGIPuro-Myc-nectin-3), FLAG-nectin-4 (amino acids 29–508, pFLAG-CMV1-nectin-4), FLAG-nectin-3ΔCP (amino acids 56–430, pFLAG-CMV1-nectin-3ΔCP), and FLAG-nectin-3ΔEC (amino acids 395–549, pFLAG-CMV1-nectin-3ΔEC) were constructed by inserting each nectin cDNA fragment into pFLAG-CMV1 (Sigma), pCAGIPuro-FLAG, or pCAGIPuro-Myc vector. Expression vectors for GFP-E-cadherin and GFP-N-cadherin were also constructed by inserting full-length mouse E-cadherin and N-cadherin into pCAGGS-GFP and pEGFP-N1 (Clontech) vectors, respectively. Glutathione S-transferase-PAK-Cdc42/Rac interactive binding region (GST-PAK-CRIB) fusion protein and the extracellular regions of nectin-1, nectin-2, and nectin-3 fused to human IgG Fc (Nef-1, Nef-2, and Nef-3, respectively) were also prepared as described (13, 28). Echistatin, flavordin, cyclo-RGDyf, and FITC-phalloidin were purchased from Sigma.

Cell Lines and Transfection—Wild-type NIH3T3 cells and NIH3T3 cells stably expressing nectin-1 (nectin-1-NIH3T3) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum. To obtain nectin-1-NIH3T3 cells, we referred to the same protocol as described (21). Briefly, a full-length mouse nectin-1 cDNA was inserted into an expression vector pMSCVhyg (Clontech). A construct of pMSCVhyg-nectin-1α was co-transfected with a packing vector pCL-Eco (Imgenex) into HEK293 cells and converted to retrovirus. Then NIH3T3 cells were infected with retrovirus and selected by 250 μg/ml hygromycin. For DNA transfection, Lipofectamine 2000 reagent (Invitrogen) or Nucleofection system (Amaxa) was applied following the manufacturer’s instruction. HEK293 cells, MDCK cells, and MDCK cells stably expressing nectin-1 (nectin-1-MDCK cells) were cultured in DMEM supplemented with 10% fetal calf serum.

Antibodies—The rat monoclonal antibody (mAb) against nectin-3 was prepared as described (29), and the mouse mAb and rabbit polyclonal Ab (pAb) against afadin were also prepared as described (30, 31). WOW-1 Fab and a rat anti-E-cadherin mAb (ECD-C-2) were kind gifts from Dr. S. J. Shattil (University of California San Diego, La Jolla, CA) and Dr. M. Takeuchi (RIKEN Center for Developmental Biology, Kobe, Japan), respectively. Hybridoma cells expressing a mouse anti-Myc mAb (9E10) were obtained from American Type Culture Collection and prepared as described (32). The hamster anti-integrin β3 mAb and the non-immunized hamster IgG for flow cytometric analysis were purchased from Pharmingen and Jackson ImmunoResearch, respectively. The following rabbit pAbs were purchased from commercial sources; anti-N-cadherin (Takara), anti-integrin α3 (Chemicon and Pharmingen), anti-integrin β3 (Chemicon and Pharmingen), anti-claudin-1 (Zymed Laboratories Inc.), anti-GFP (MBL), anti-Src (Cell Signaling Technology), and anti-phospho-Src (Tyr416) (Cell Signaling Technology) Abs. The mouse mAbs listed below were also purchased from commercial sources; anti-N-cadherin (Pharmingen), anti-integrin α3β3 (LM609; Chemicon), anti-integrin α3 (Pharmingen), anti-integrin α5 (Pharmingen), anti-integrin β1 (Pierce), anti-Cdc42 (Pharmingen), anti-Rac1 (Upstate), anti-FAK (Pharmingen), anti-phospho-FAK (Tyr397) (Pharmingen), anti-actin (Chemicon), anti-FLAG (Sigma), and anti-HA (Berkeley Antibody Co., Inc.) Abs. The horseradish peroxidase-conjugated and fluorophore-labeled secondary Abs were obtained from Chemicon and Molecular Probes, respectively.

Assays for Cell Spreading and Bead-Cell Contact—The formation of cell spreading was assayed as described (13). For inhibition of the activity of integrin α3β3, the cells were treated with 0.37 μM echistatin or 10 nM flavordin in phosphate-buffered saline (PBS) or PBS alone as a control for 2 h before the trypsin-EDTA treatment and during the culture on the Nef-1- or IgG-
coated coverslips. The bead-cell contact was assayed as described (28). Briefly, NIH3T3 cells were seeded on the laminin-coated coverslips, cultured for 3 h, and then incubated with latex-sulfate microbeads coated with Nef-1 or IgG as a control for 1 h. After the incubation, the cells were fixed and immunostained followed by observation by use of Radian 2100 confocal microscope (Bio-Rad).

**Immunoprecipitation Assay**—HEK293 cells expressing in various combinations of the indicated molecules were lysed with buffer A (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 3 µg/ml leupeptin, 5 µg/ml aprotinin). The cell lysates were centrifuged at 100,000 x g for 15 min, and then the supernatant was incubated with the anti-FLAG mAb or the anti-GFP pAb and the anti-FLAG mAb. To investigate the association of protein G-Sepharose beads at 4 °C for 2 h. After the beads were extensively washed with buffer A, the bound proteins were eluted from the beads by boiling with the SDS sample buffer for 5 min and subjected to SDS-PAGE followed by Western blotting with the indicated Abs. To investigate the association of endogenous integrin αvβ3 with endogenous nectin-3, NIH3T3 cells were lysed with buffer A, and the cell lysates were incubated with the anti-nectin-3 mAb or control rat IgG as a control followed by incubation with protein G-Sepharose precoated with the goat anti-rat IgG. The immunoprecipitated samples were then analyzed by Western blotting. For the treatment with EDTA, which abrogates the ligand binding capability of integrin (33), HEK293 cells were incubated in Ca²⁺/Mg²⁺-free HBSS containing 5 mM EDTA for 30 min before the cell lysis. To quantify the cell surface expression of each nectin, we labeled cell surface proteins including nectins with 0.5 mg/ml sulfo-NHS-ss-biotin (Pierce) for 30 min on ice. After the cell lysis, biotinylated nectins were collected by incubation with streptavidin-coated beads (Amersham Biosciences) and detected by the anti-FLAG mAb.

**In Vitro Binding of Nectin-3 with Integrin αvβ3**—To obtain the recombinant protein of the extracellular region of human integrin αvβ3 heterodimer (integrin αvβ3-EC, αvβ3-EC-CHINESE HAMSTER OVARY LEC 3.2.8.1 cells expressing the extracellular region of human integrin αvβ3 were cultured, and the culture supernatant containing soluble integrin αvβ3-EC was collected (34). The culture supernatant was applied to nickel-Sepharose 6 Fast Flow beads (Amersham Biosciences) equilibrated with buffer B (25 mM Tris-HCl at pH 8.0, 200 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 20 mM imidazole at pH 8.0). After the beads were extensively washed with buffer B and then buffer B containing 0.6 M NaCl, the bound integrin αvβ3-EC was eluted with elution buffer (25 mM Tris-HCl at pH 8.0, 200 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 500 mM imidazole at pH 8.0), and the eluate was dialyzed with buffer B without imidazole. The protein concentration of integrin αvβ3-EC was determined with bovine serum albumin as a reference protein on SDS-PAGE.

To examine the binding of Nef-3 and integrin αvβ3-EC, integrin αvβ3-EC (6 pmol) was immobilized on nickel-Sepharose beads, and Nef-3 (60 pmol) was incubated with the integrin αvβ3-EC-immobilized nickel-Sepharose beads alone in 0.3 ml of buffer B containing 0.5 mg/ml bovine serum albumin. After the beads were extensively washed with buffer B, the bound proteins were eluted with elution buffer. The eluate was subjected to SDS-PAGE followed by Western blotting. The bound Nef-3 was determined by the anti-human IgG Fc pAb conjugated with horseradish peroxidase (American Qualex).

**Assays for c-Src, Cdc42, and Rac Activities**—The assay for c-Src activity was performed as described (15). Briefly, nectin-1-NIH3T3 cells transiently expressing c-Src were cultured in the presence or absence of 0.37 µM echistatin followed by the incubation with Nef-3- or IgG-coated protein A-bound magnetic beads (Dynal Biotech) for 30 min. For the examination of the inhibitory effect of FAK-related non-kinase (FRNK) on c-Src activity, nectin-1-NIH3T3 cells transiently co-expressing c-Src with HA-FRNK or an empty vector as a control were incubated with Nef-3-coated protein A-bound magnetic beads for 30 min. After the incubation the beads were collected and used for analysis of c-Src activity by Western blotting with the anti-phospho-Src (Tyr416) pAb. For the analysis of the activity of Cdc42 and Rac, the pull-down assay was performed as described (16, 17). NIH3T3 cells removed from the dish were separated into two groups; one was replated on a 60-mm dish coated with 25 µg of Nef-1 to attach the cells to the substratum, and the other was kept cultured in suspension during the treatment with 25 µg of Nef-1 pre-clustered using the anti-human IgG (Fc-specific) antiserum (Sigma) to avoid activation of any integrins by contact with the ECM. For the assessment of the effect of integrin αvβ3 on the activation of c-Src, Cdc42, and Rac, the cells were treated with 0.37 µM echistatin or 10 nM flavoridin for 2 h before stimulation of 25 µg of Nef-1 or IgG as a control for 30 min. These cells were then lysed, and the cell lysates were incubated with GST-PAK-CRIB on ice for 30 min followed by incubation with glutathione-agarose beads (Amersham Biosciences) at 2 °C for 1 h. Proteins bound to the beads were used for analysis of the activity of Cdc42 and Rac by Western blotting.

**Wound Healing Assay**—As described previously (20), confluent NIH3T3 cell monolayers were manually scratched with a 26-gauge needle and subjected to the wound healing assay. The cells were fixed at the indicated time points after the wound procedure, immunostained with the indicated Abs, and observed by a confocal microscope.

**Assay for Formation of AJs**—Cell-cell junctions of MDCK cells were assayed as described (35). Briefly, the cells were cultured in DMEM containing 10% fetal calf serum for 24 h. After culture, the cells were washed with 5 ml of phosphate-buffered saline and incubated in DMEM containing 2 mM Ca²⁺ for 1 h. The medium was replaced by DMEM with 5 mM EGTA to reduce the final concentration of Ca²⁺ at 2 mM, and then the cells were incubated for 2 h. The cells were then washed with DMEM and re-cultured in DMEM containing 2 mM Ca²⁺ with or without 0.37 µM echistatin, 10 nM flavoridin, or 10 µg/ml LM609 mAb for 2 h. The cells were fixed, immunostained with the anti-E-cadherin and anti-afadin Abs, and observed with a confocal microscope. The formation of AJ was defined as the cell-cell junction with the well stained for E-cadherin.

**siRNA Experiments**—To knock down integrin β3 and FAK, double-stranded 25-nucleotide RNA duplexes (Stealth™ RNA-mediated interference; Invitrogen) for integrin β3 (5′-AAUCUG-
Co-localization of Integrin $\alpha_\beta_3$ with Nectin at AJs—In moving NIH3T3 cells, which do not contact with other cells, integrin $\alpha_\beta_3$ co-localizes with Necl-5 at the leading edges (20, 25). Neither N-cadherin nor nectin-3 was concentrated there. When these cells become confluent, they form cell-cell adhesion and eventually establish AJs. The immunofluorescence signals for N-cadherin and nectin-3 were concentrated at the cell-cell adhesion sites, which correspond to AJs as described (25) (Fig. 1A). The signal for integrin $\alpha_\nu$ was also concentrated at AJs in addition to the focal adhesion sites. This signal for integrin $\alpha_\nu$ coincided with that for nectin-3 at AJs. The essentially same results were obtained for integrin $\beta_3$ (data not shown). These results indicate that integrin $\alpha_\beta_3$ co-localizes with nectin and N-cadherin at established AJs in NIH3T3 cells.

In MDCK cells, the signals for E-cadherin and afadin, a nectin- and F-actin-binding protein, were concentrated at the cell-cell adhesion sites, which correspond to AJs as described (31) (Fig. 1B). Because the signal for nectin was not detected at the cell-cell adhesion sites in MDCK cells, probably due to the sensitivity of Ab against nectin (28, 36–38), we stained MDCK cells with the anti-afadin Ab. The signal for integrin $\alpha_\beta_3$ was detected by LM609 mAb. The signal for integrin $\alpha_\beta_3$ was observed at the focal adhesion sites on the cell-matrix boundary and was more widely distributed along the lateral plasma membrane than that for afadin. Both signals co-localized at the apical region of the cell-cell adhesion sites. The distribution pattern of integrin $\alpha_\beta_3$ was similar to that for E-cadherin. The signal for integrin $\alpha_\beta_3$ was not overlapped with that for claudin-1, which localizes at TJs (4). These results indicate that integrin $\alpha_\beta_3$ co-localizes with nectin at established AJs not only in fibroblasts but also in epithelial cells but that it does not localize at TJs in epithelial cells.

Physical Association of Integrin $\alpha_\beta_3$ with Nectin-1 and Nectin-3—We then examined by the bead-cell contact and co-immunoprecipitation assays whether integrin $\alpha_\beta_3$ physically associates with nectin-3. In the bead-cell contact assay, NIH3T3 cells were incubated with microbeads coated with Nef-1 (the extracellular region of nectin-1 fused to IgG Fc) or IgG as a control. Nectin-1 has been elucidated to most strongly trans-interact with nectin-3 among the nectin family members (39). The immunofluorescence signal for nectin-3 was concen-

**RESULTS**

**Co-localization of Integrin $\alpha_\beta_3$ with Nectin at AJs**—In moving NIH3T3 cells, which do not contact with other cells, integrin $\alpha_\beta_3$ co-localizes with Necl-5 at the leading edges (20, 25). Neither N-cadherin nor nectin-3 was concentrated there. When these cells become confluent, they form cell-cell adhesion and eventually establish AJs. The immunofluorescence signals for N-cadherin and nectin-3 were concentrated at the cell-cell adhesion sites, which correspond to AJs as described (25) (Fig. 1A). The signal for integrin $\alpha_\nu$ was also concentrated at AJs in addition to the focal adhesion sites. This signal for integrin $\alpha_\nu$ coincided with that for nectin-3 at AJs. The essentially same results were obtained for integrin $\beta_3$ (data not shown). These results indicate that integrin $\alpha_\beta_3$ co-localizes with nectin and N-cadherin at established AJs in NIH3T3 cells.

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**FIGURE 1.** Co-localization of integrin $\alpha_\beta_3$ with nectin at established AJs in NIH3T3 and MDCK cells. A, co-localization of integrin $\alpha_\beta_3$ with nectin-3 at established AJs in NIH3T3 cells. Confluent NIH3T3 cell monolayers were fixed and immunostained with the indicated Abs. Arrowheads, co-localization of each adhesion molecule. B, co-localization of integrin $\alpha_\beta_3$ with nectin at established AJs in NIH3T3 cells. Confluent NIH3T3 cell monolayers were fixed and immunostained with the indicated Abs. The dotted lines in the optical $x\text{-}y$ sectional views correspond to the sites of the $x\text{-}z$ sections. MDCK cells were stained with the anti-afadin Ab to detect the nectin-afadin complex because of the lower sensitivity of Abs against nectin. Integrin $\alpha_\beta_3$ was detected by LM609 mAb. Scale bars, 10 $\mu$m. The results shown in this figure are representative of three independent experiments.
FIGURE 2. Physical association of integrin $\alpha_v\beta_3$ with nectin-1 and nectin-3. A, physical association of integrin $\alpha_v\beta_3$ with nectin-3. $\alpha$, recruitment of integrin $\alpha_v$ and nectin-3 at the contact sites between Nef-1-coated beads and NIH3T3 cells. NIH3T3 cells were incubated with the Nef-1- or IgG-coated microbeads for 1 h, and then they were fixed and immunostained with the anti-nectin-3 mAb and anti-integrin $\alpha_v$ pAb. Positions of the beads are marked with asterisks, and the cell outline is indicated with the dotted line. Insets are higher magnified images of boxed areas. DIC, differential interference contrast. Scale bars, 10 $\mu$m. $b$, co-immunoprecipitation of integrin $\alpha_v\beta_3$ with nectin-3. The cell lysates of HEK293 cells transiently expressing human integrin $\alpha_v$ and integrin $\beta_3$, with or without FLAG-nectin-3 were immunoprecipitated with the anti-FLAG mAb. The immunoprecipitates (IP) were subjected to Western blotting with the anti-integrin $\alpha_v$, anti-integrin $\beta_3$, and anti-FLAG Abs. Five percent of total cell lysates were loaded for the input. $c$, co-immunoprecipitation of endogenous integrin $\alpha_v$ with endogenous nectin-3. NIH3T3 cell lysates were immunoprecipitated with the anti-nectin-3 mAb or control rat IgG, and the immunoprecipitates were then analyzed by Western blotting with the anti-integrin $\alpha_v$ and anti-nectin-3 Abs. $B$, selective association of integrin $\alpha_v\beta_3$ with nectin and no association with cadherin or claudin. $a$, co-immunoprecipitation of integrin $\alpha_v\beta_3$ with nectin-1 and nectin-3. The cell lysates of HEK293 cells transiently expressing human integrin $\alpha_v$, and integrin $\beta_3$, with or without FLAG-nectin-3 were immunoprecipitated with the anti-FLAG mAb followed by the immunoprecipitation assay as described in $Ab$. Right panel, biotinylated proteins were collected with streptavidin-coated beads and were subjected to Western blotting with the anti-integrin $\alpha_v$, anti-integrin $\beta_3$, and anti-FLAG Abs to ensure the equal amount of integrin $\alpha_v\beta_3$ and each FLAG-nectin on the cell surface. Endogenous N-cadherin was also immunoblotted as a loading control. $b$, no co-immunoprecipitation of integrin $\alpha_v\beta_3$ with cadherin. The cell lysates of HEK293 cells transiently expressing human integrin $\alpha_v$, and integrin $\beta_3$, with GFP-N-cadherin or GFP-E-cadherin were immunoprecipitated with the anti-GFP or anti-FLAG Ab followed by the immunoprecipitation assay as described in $Ab$. $c$, no co-immunoprecipitation of integrin $\alpha_v\beta_3$ with claudin-1. The cell lysates of HEK293 cells transiently expressing human integrin $\alpha_v$, and integrin $\beta_3$, with FLAG-claudin-1 were immunoprecipitated with the anti-FLAG mAb followed by the immunoprecipitation assay as described in $Ab$. The results shown in this figure are representative of three independent experiments.
trated at the contact sites between the Nef-1-coated beads and NIH3T3 cells as described (28), and the signal for integrin αv was also concentrated there (Fig. 2Ac). In contrast, the signal for nectin-3 or integrin αv was hardly concentrated at the contact sites between the IgG-coated beads and NIH3T3 cells. The essentially same results were obtained for integrin β3 in these bead-cell contact assays (data not shown). In the co-immunoprecipitation assay, human integrin αv and integrin β3 were transiently co-expressed with FLAG-nectin-3 in HEK293 cells. When FLAG-nectin-3 was immunoprecipitated from the cell lysates with the anti-FLAG mAb, both integrin αv and integrin β3 were co-immunoprecipitated with FLAG-nectin-3 (Fig. 2Ab). About 5% of total integrin αv and integrin β3 were co-immunoprecipitated with nectin-3. In addition, when endogenous nectin-3 was immunoprecipitated from the lysate of NIH3T3 cells with the anti-nectin-3 Ab, endogenous integrin αv was co-immunoprecipitated with endogenous nectin-3 (Fig. 2Ac). Collectively, the results from both the bead-cell contact and co-immunoprecipitation assays indicate that integrin αv/β3 physically associates with nectin-3.

The nectin family consists of four members: nectin-1, nectin-2, nectin-3, and nectin-4 (11, 12). We next examined with which member of nectin integrin αv/β3 physically associates. Human integrin αv and integrin β3 were transiently co-expressed with either FLAG-nectin-1, FLAG-nectin-2, FLAG-nectin-3, or FLAG-nectin-4 in HEK293 cells. When each FLAG-tagged nectin was immunoprecipitated with the anti-FLAG mAb from each cell lysate, both integrin αv and integrin β3 were co-immunoprecipitated with FLAG-nectin-1 and FLAG-nectin-3 but not with FLAG-nectin-2 or FLAG-nectin-4 (Fig. 2Ba), indicating that integrin αv/β3 physically associates with nectin-1 and nectin-3 but not with nectin-2 or nectin-4. Under these conditions all the nectin molecules were expressed on the cell surface to similar extents as estimated by measuring the amount of biotin-labeled nectins.

We then examined whether integrin αv/β3 physically associates with other AJ components, N-cadherin and E-cadherin. Neither human integrin αv nor integrin β3 was co-immunoprecipitated with GFP-N-cadherin or GFP-E-cadherin from the lysate of HEK293 cells transiently co-expressing human integrin αv and integrin β3 with GFP-N-cadherin or GFP-E-cadherin (Fig. 2Bb). The essentially same results were obtained when integrin αv and integrin β3 were transiently co-expressed with FLAG-claudin-1, a major TJ constituent, in HEK293 cells (Fig. 2Bc).

We further examined whether nectin-3 associates with an integrin other than integrin αv/β3. Human integrin α5 and integrin β1 were transiently co-expressed with Myc-nectin-3 in HEK293 cells. When Myc-nectin-3 was immunoprecipitated with the anti-Myc mAb from the lysate, neither integrin α5 nor integrin β1 was co-immunoprecipitated with Myc-nectin-3 (data not shown). Taken together, these results indicate that the physical association of integrin αv/β3 with nectin-1 and nectin-3 is not nonspecific.

**Direct Interaction of Integrin αv/β3 with Nectin through Their Extracellular Regions—**We next examined which region, either the extracellular or intracellular region, of nectin-3 is necessary for the interaction with integrin αv/β3. Human integrin αv, and inte-

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**FIGURE 3. Direct interaction of integrin αv/β3 with nectin through their extracellular regions.** A, co-immunoprecipitation of integrin αv/β3 with nectin-3 through their extracellular regions. The cell lysates of HEK293 cells transiently expressing human integrin αv and integrin β3 with either FLAG-nectin-3-ΔCP or FLAG-nectin-3-ΔEC were immunoprecipitated (IP) with the anti-FLAG mAb. The immunoprecipitates were subjected to Western blotting with the anti-integrin αv, anti-integrin β3, and anti-FLAG Abs. B, direct interaction of integrin αv/β3 with nectin-3. The recombinant samples of soluble, clasped extracellular regions of integrin αv and integrin β3 were immobilized on nickel beads and incubated with Nef-3. After the incubation the eluted samples were subjected to SDS-PAGE followed by Western blotting with the anti-integrin αv/anti-integrin β3 and anti-human IgG Fc Abs. Integrin αv, anti-EC and integrin β3, anti-EC indicate the extracellular regions of integrin αv, and integrin β3, respectively. C, co-immunoprecipitation of both the high and low affinity forms of integrin αv/β3 with nectin-3 in HEK293 cells transiently expressing human integrin αv, human integrin β3, and FLAG-nectin-3 were cultured in suspension in the presence of 1 mM Mn2+ and 60 μM cyclo-RGDfV peptide for 1 h, or HEK293 cells transiently expressing human integrin αv, human integrin β3, and FLAG-nectin-3 were also cultured in suspension for 1 h. These two kinds of cells were then lysed and subjected to the immunoprecipitation with the anti-FLAG mAb followed by Western blotting with the anti-integrin αv, anti-integrin β3, and anti-FLAG mAbs. The results shown in this figure are representative of three independent experiments. WT, wild type.
grin β3 were transiently co-expressed with either FLAG-tagged cytoplasmic region-truncated mutant (ΔCP) or FLAG-tagged extracellular region truncated-mutant (ΔEC) of nectin-3 in HEK293 cells. When each FLAG-tagged protein was immunoprecipitated with the anti-FLAG mAb from each cell lysate, integrin αv and integrin β3 were co-immunoprecipitated with FLAG-nectin-3-ΔCP but not with FLAG-nectin-3-ΔEC (Fig. 3A).

We next examined whether this association of nectin-3 and integrin αvβ3 is direct or not. For this purpose we prepared the recombinant sample of soluble, clapsed extracellular regions of integrin αv and integrin β3, with a hexahistidine tag at the COOH terminus of integrin β3. When Nef-3 (the extracellular region of nectin-3 fused to IgG Fc) was mixed with this soluble, clapsed integrin immobilized on the Ni beads, Nef-3 bound to this integrin (Fig. 3B). These results indicate that integrin αvβ3 directly interacts with nectin-3 through their extracellular regions.

Association of Both the Low and High Affinity Forms of Integrin αvβ3 with Nectin—Integrin has at least two forms, the low and high affinity forms (34). The low affinity form of integrin shows weak adhesion activity for ECM proteins and is inactive, whereas the high-affinity form exhibits increased adhesion activity for its extracellular ligands and is active (40). The low affinity form of integrin is converted to the high affinity form by binding talin (inside-out signaling) (41). Upon binding of ECM proteins, integrin transduces signals inside the cells (outside-in signaling), which induces the reorganization of the actin cytoskeleton, eventually causing the integrin clustering and formation of cell-matrix junctions such as focal complexes and focal adhesions. We then examined which form of integrin αvβ3, the low or high affinity form, physically associates with nectin-3. To explore this issue, HEK293 cells co-expressing human integrin αv and integrin β3 with FLAG-nectin-3 were cultured in suspension in the presence of Mn2⁺ and cyclo-RGDfV peptide, known to induce the high affinity form of integrin even when integrin does not bind to ECM proteins (34), and on the other hand, HEK293 cells expressing human integrin αv, a dominant negative mutant of integrin β3, and FLAG-nectin-3 were cultured in suspension to lock integrin αvβ3 into the low affinity form (42). The cell lysates from both kinds of cells were prepared, and FLAG-nectin-3 was immunoprecipitated with the anti-FLAG mAb. Integrin αv and integrin β3, irrespective of the low or high affinity form, were co-immunoprecipitated with FLAG-nectin-3 (Fig. 3C). These results indicate that both the low and high affinity forms of integrin αvβ3 physically associate with nectin-3.

Cis-Interaction of Integrin αvβ3 with Nectin—Two types of interaction between integrin αvβ3 and nectin are theoretically conceivable, cis- and trans-interactions. Because these two molecules were co-immunoprecipitated from the lysate of the suspended cells, integrin αvβ3 is likely to cis-interact with nectin. To confirm the cis-interaction, we took advantage of the bead-cell contact assay. Nectin-2 as well as nectin-1 trans-interacts with nectin-3 (39), but nectin-2 does not associate with integrin αvβ3 as described above. Therefore, NIH3T3 cells were incubated with microbeads coated with Nef-2 (the extracellular region of nectin-2 fused to IgG Fc) to examine whether the immunofluorescence signal for integrin αvβ3 is observed at the bead-cell contact sites where nectin-3 is recruited. If the signal
FIGURE 5. Requirement of the activation of integrin \( \alpha_v \beta_3 \) for the nectin-induced signaling. A, involvement of integrin in the nectin-induced activation of Cdc42 and Rac. \( \alpha \), inhibition of the nectin-induced activation of Cdc42 and Rac in the suspension cell culture system. After NIH3T3 cells were cultured on the Nef-1-coated dish or in suspension with pre-clustered Nef-1 for the indicated periods of time, the cells were subjected to the pull-down assay using GST-PAK-CRIB followed by Western blotting with the anti-Cdc42 and anti-Rac1 mAbs. ATT, attached on the dish; SUS, in suspension. \( b \), inhibition of the nectin-induced activation of Cdc42 and Rac by echistatin. After NIH3T3 cells were treated with pre-clustered Nef-1 or IgG in the presence or absence of echistatin (0.37 \( \mu \)M) for 30 min, the cells were subjected to the pull-down assay as described in Aa. B, involvement of integrin \( \alpha_v \beta_3 \) in the nectin-induced formation of filopodia and lamellipodia. \( a \), inhibition of the nectin-induced formation of filopodia and lamellipodia by echistatin. NIH3T3 cells were cultured on the Nef-1- or IgG-coated coverslips in the presence or absence of echistatin (0.37 \( \mu \)M) for 1 h and stained for F-actin with FITC-phalloidin. \( b_1 \), knockdown of integrin \( \beta_3 \). siRNA targeted for mouse integrin \( \beta_3 \) or control siRNA was transfected into NIH3T3 cells, and impaired expression of integrin \( \beta_3 \) was determined by flow cytometry. Upper panel, NIH3T3 cells transfected with control siRNA; lower panel, NIH3T3 cells transfected with siRNA for integrin \( \beta_3 \). Dotted lines, the non-immunized hamster IgG; solid lines, the hamster anti-integrin \( \beta_3 \) mAb. \( b_2 \), inhibition of the nectin-induced formation of filopodia and lamellipodia.
for integrin αβ3 is observed at the bead-cell contact sites, it would be the cis-interaction of integrin αβ3 with nectin-3, and contrarily, if not it would not support the cis-interaction of these two molecules. The signals for both integrin αv and nectin-3 were concentrated at the bead-cell contact sites (Fig. 4A), suggesting that integrin αβ3 cis-interacts with nectin-3.

It has been shown that EDTA abrogates the ligand binding capability of integrins (33). If integrin αβ3 trans-interacts with nectin as a ligand, EDTA would dissociate this interaction. Human integrin αv and integrin β3 were transiently co-expressed with FLAG-nectin-3 in HEK293 cells, and the cells were treated with or without EDTA. When FLAG-nectin-3 was immunoprecipitated from the cell lysates with the anti-FLAG mAb, both integrin αv and integrin β3 were co-immunoprecipitated with FLAG-nectin-3 even in the presence of EDTA (Fig. 4B). This result does not support the trans-interaction of integrin αβ3 with nectin-3. Taken together, it could be concluded that integrin αβ3 cis-interacts with nectin-3.

Requirement of the Activation of Integrin αβ3 and FAK for the Nectin-induced Signaling—We have previously shown that the trans-interaction of nectin induces the activation of Rap1, Cdc42, and Rac through c-Src and that this nectin-induced signaling is involved in the recruitment of cadherin to the nectin-based cell-cell adhesion sites to form AJs (15–17). We then examined whether the physical association of integrin αβ3 with nectin is related to this nectin-induced signaling. We first confirmed that Nef-1 indeed induced the activation of Cdc42 and Rac in NIH3T3 cells cultured on the dish (Fig. 5Aa). In contrast, Nef-1 did not induce the activation of these small G proteins in the cells cultured in suspension. In addition, when NIH3T3 cells were incubated with Nef-1 in the presence of an integrin αβ3 inhibitor, echistatin, which blocks the attachment of integrin αβ3 to the substratum and leaves integrin αβ3 inactive (43), the Nef-1-induced activation of Cdc42 and Rac was markedly reduced (Fig. 5Ab). The Nef-1-induced formation of filopodia and lamellipodia was similarly inhibited by echistatin in NIH3T3 cells (Fig. 5Ba). The Nef-1-induced activation of Cdc42 and Rac and subsequent formation of filopodia and lamellipodia were also inhibited by another integrin αβ3 inhibitor, flavoridin (44), to the similar extent to that obtained by echistatin (data not shown). Furthermore, when integrin β3 was knocked down by siRNA in NIH3T3 cells, the amount of cell surface integrin β3 was markedly reduced, and the Nef-1-induced formation of filopodia or lamellipodia was not significantly observed (Fig. 5Bb).

To further substantiate the results that the activation of integrin αβ3 is involved in the activation of Cdc42 and Rac, we examined the effect of a constitutively active mutant of human integrin β3 (β3N305T) on the Nef-1-induced activation of Cdc42

\[ \text{by integrin } \beta_3 \text{ RNA-mediated interference (RNAi), NIH3T3 cells transiently transfected with siRNA for integrin } \beta_3 \text{ or control siRNA were cultured on the Nef-1- or IgG-coated coverslips for 1 h and stained for F-actin with FITC-phalloidin. Scale bars, 10 } \mu \text{m. Bars in } B \text{ and } B_2 \text{ of the quantitative analysis represent the percentage of the spreading cells with filopodia and/or lamellipodia of the total cells counted (n = 50) and are expressed as means } \pm \text{ S.E. of the three independent experiments. C, enhancement of the nectin-induced activation of Cdc42 and Rac by a constitutively active mutant of human integrin } \beta_3. \text{ NIH3T3 cells transiently expressing wild-type (WT) human integrin } \alpha_5 \text{ or wild-type (WT) human integrin } \beta_3 \text{ or NIH3T3 cells transiently transfected with an empty vector were treated with pre-clustered Nef-1 or IgG for 30 min. After the treatment the cells were subjected to the pull-down assay as described in A. D, inhibition of the nectin-induced phosphorylation of c-Src by echistatin. Nectin-1-NIH3T3 cells transiently expressing c-Src were incubated with Nef-3- or IgG-coated magnetic beads for 30 min and then subjected to the co-precipitation assay followed by Western blotting with the anti-Src and anti-phospho-Src (Tyr416) pAbs. The results shown in this figure are representative of three independent experiments.}

\[ \text{and Rac. Integrin } \beta_3\text{N305T was generated by mutationally introducing an } N \text{-glycosylation site into the hybrid-I-like domain of integrin } \beta_3 \text{ to stabilize the integrin headpiece to be the high} \]

FIGURE 6. Requirement of the activation of FAK for the nectin-induced signaling. A, inhibition of the nectin-induced formation of filopodia and lamellipodia by FRNK. NIH3T3 cells transiently expressing HA-FRNK or an empty vector were cultured on the Nef-1-coated coverslips for 1 h and then fixed and immunostained for F-actin and HA-FRNK with FITC-phalloidin (green) and the anti-HA mAb (red), respectively. Scale bars, 10 μm. Bars in the quantitative analysis represent the percentage of the spreading cells with filopodia and/or lamellipodia of the total cells counted (n = 50) and are expressed as the means ± S.E. of the three independent experiments. B, inhibition of the nectin-induced phosphorylation of c-Src by FANK. Nectin-1-NIH3T3 cells transiently co-expressing c-Src with HA-FRNK or an empty vector were incubated with the Nef-3-coated magnetic beads for 30 min and then subjected to the co-precipitation assay followed by Western blotting with the anti-Src and anti-phospho-Src (Tyr416) pAbs. The results shown in this figure are representative of three independent experiments.
affinity form (45). When NIH3T3 cells expressing both wild-type human integrin \(\alpha_v\) and integrin \(\beta_3\) were incubated with Nef-1, the Nef-1-induced activation of Cdc42 and Rac was enhanced as compared with that observed in NIH3T3 cells expressing an empty vector or wild-type human integrin \(\alpha_v\) and integrin \(\beta_3\) (Fig. 5C). Taken together, these results indicate that the activation of integrin \(\alpha_v\beta_3\) is necessary for the nectin-induced activation of Cdc42 and Rac.

We have previously reported that the most upstream molecule in the nectin-induced signaling is c-Src (15–17). We, therefore, examined next whether the activation of integrin \(\alpha_v\beta_3\) is required for the nectin-induced activation of c-Src. For
this purpose we generated nectin-1-NIH3T3 cells (NIH3T3 cells stably expressing nectin-1) and used them for the assay for c-Src activity because the magnetic beads coated with Nef-3 used for this assay more efficiently adhered to nectin-1-NIH3T3 cells than wild-type NIH3T3 cells, and thus, nectin-1-NIH3T3 cells were more useful for the investigation for the nectin-induced activation of c-Src. The cells were incubated with the Nef-3- or IgG-coated magnetic beads in the presence or absence of echistatin. After the incubation, the magnetic beads were collected from the cell lysates. The amount of phosphorylated c-Src at Tyr^416, which indicates the activation of c-Src (46), was increased by Nef-3, and this Nef-3-induced activation of c-Src was abrogated in the presence of echistatin (Fig. 5D). These results indicate that integrin αvβ3 regulates the nectin-induced signaling at the step of c-Src activation.

The integrin-induced signaling is mediated at least through FAK (7, 47–49). We, therefore, examined the requirement of the activation of FAK for the nectin-induced signaling. For this purpose, we used FRNK, which is the COOH-terminal, non-catalytic domain of FAK and functions as a negative regulator of the kinase activity (7). The Nef-1-induced formation of filopodia and lamellipodia was inhibited by HA-FRNK in NIH3T3 cells (Fig. 6A). Moreover, the Nef-3-induced phosphorylation of c-Src was similarly inhibited by HA-FRNK (Fig. 6B). These results indicate that the activation of FAK is also necessary for the nectin-induced signaling at the step of c-Src activation.

Co-localization of the High Affinity Form of Integrin αvβ3 with Nectin at Developing AJs—Because the nectin-induced signaling plays a role during the formation of AJs (15, 16), we next examined which form of integrin αvβ3, the low or high affinity form, associates with nectin-3 during the formation of AJs. Because the anti-integrin αv and anti-integrin β3 Abs used in Fig. 1A do not distinguish the high affinity form of integrin αvβ3 from its low affinity form, we exogenously expressed both human integrin αv and integrin β3 in NIH3T3 cells and used WOW-1 Fab and LM609 mAb. WOW-1 Fab can specifically detect the high affinity form of integrin αvβ3 (50), whereas LM609 mAb recognizes integrin αvβ3 but does not discriminate between the high and low affinity forms of integrin αvβ3 (51).

We performed the wound healing assay using NIH3T3 cells expressing both human integrin αv and integrin β3. After scratching the confluent cell monolayers, the cells on both opposite sides of the wound immediately began to migrate to close the wound. At 2 h after the wounding, the cells, which located at the wound edge, formed cell protrusions with the signal for the high affinity form of integrin αvβ3 detected by WOW-1 Fab (Fig. 7, Aa and Ab). On the other hand, the signal for nectin-3 or N-cadherin was not observed at the free plasma membrane, although these signals were concentrated at the cell-cell adhesion sites apart from the wound. The reason why nectin-3 did not co-localize with the high affinity form of integrin αvβ3 at the leading edge of the cells remains to be clarified, although the high affinity form of integrin αvβ3 was immunoprecipitated with nectin-3 (see Fig. 3C). However, one possible explanation is that at the leading edge, the high affinity form of integrin αvβ3 associates with Necl-5 and that this association might be stronger than that of integrin αvβ3 with nectin-3. When the cells formed the initial cell-cell adhesion at 3 h after the wound-

FIGURE 8. Requirement of the activation of integrin αvβ3 in the formation of AJs in MDCK cells. A, involvement of integrin αvβ3 and FAK in the nectin-induced formation of filopodia and lamellipodia. Aa, inhibition of the nectin-induced formation of filopodia and lamellipodia by echistatin. Nectin-1-MDCK cells were cultured on the Nef-3- or IgG-coated coverslips in the presence or absence of echistatin (0.37 μM) for 2 h and stained for F-actin with FITC-phalloidin. Scale bars, 10 μm. B, knockdown of FAK. The cell lysates from wild-type or siRNA-transfected MDCK cells were separated by SDS-PAGE followed by Western blotting with the anti-FAK mAb. For the loading control the same membrane was immunoblotted with the anti-actin mAb. siRNA #2, of which sequence is indicated under “Experimental Procedures,” remarkably reduced the expression of FAK in MDCK cells and was used for the cell spreading assay. WT, wild type. B, inhibition of the nectin-induced formation of filopodia and lamellipodia by FAK RNA-mediated interference (RNAi). Nectin-1-MDCK cells transfected with siRNA for FAK or luciferase as a control were cultured on the Nef-3- or IgG-coated coverslips for 2 h and stained for F-actin with FITC-phalloidin. Scale bars, 10 μm. Bars in Aa and Ab2 of the quantitative analysis represent the percentage of the spreading cells with filopodia and/or lamellipodia of the total cells counted (n = 50) and are expressed as the means ± S.E. of the three independent experiments. Bars in the quantitative analysis represent the percentage of cell-cell adhesion sites with the signal for E-cadherin at the afadin-positive cell-cell adhesion sites. Bars in the quantitative analysis represent the percentage of cell-cell adhesion sites with the signal for E-cadherin at the afadin-positive cell-cell adhesion sites counted (n = 50) and are expressed as the means ± S.E. of the three independent experiments.
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The signal for the high affinity form of integrin αβ3 was observed at the cell-cell adhesion sites. The signals for nectin-3 and N-cadherin appeared at the initially formed cell-cell adhesion sites and co-localized with that for the high affinity form of integrin αβ3 there. However, when the wound was almost closed and the formation of AJs was completed at 4 h after the wounding, the signal for integrin αβ3 detected by WOW-1 Fab diminished from established AJs, whereas the signals for nectin-3 and N-cadherin were observed there. At 12 h after the wounding the cells became confluent again. The signal for integrin αβ3 detected by WOW-1 Fab was also hardly detected at AJs, but the signal for integrin αβ3 determined by LM609 mAb was observed there together with that for nectin-3 or N-cadherin (Fig. 7, Ba and Bb). These results indicate that the high affinity form of integrin αβ3 co-localizes with nectin and N-cadherin at developing AJs and that the high affinity form of integrin αβ3 is converted to the low affinity form after AJs are established.

Implication of the Activation of Integrin αβ3 in the Formation of AJs in MDCK Cells—In the last set of experiments we examined by the Ca2+ switch assay whether the activation of integrin αβ3 is involved in the formation of AJs in MDCK cells. We first confirmed that the activation of integrin αβ3 and FAK is necessary for the nectin-induced signaling in MDCK cells. When nectin-1-MDCK cells (MDCK cells stably expressing nectin-1) were incubated on the Nef-3- or IgG-coated coverslips in the presence or absence of echistatin, the Nef-3-induced formation of filopodia and lamellipodia was markedly inhibited by echistatin (Fig. 8Aa). Nectin-1-MDCK cells on the IgG-coated coverslips did not form filopodia or lamellipodia irrespective of the presence or absence of echistatin. In addition, when FAK was knocked down by siRNA in nectin-1-MDCK cells, the expression level of FAK was markedly reduced, and the Nef-3-induced formation of filopodia or lamellipodia was not significantly observed (Fig. 8Ab).

We then performed the Ca2+ switch assay. When MDCK cells were cultured at 2 μM Ca2+, the immunofluorescence signal for E-cadherin was concentrated at the cell-cell adhesion sites as described (38, 52). When these cells were cultured at 2 mM Ca2+ for 2 h, the signal for E-cadherin was not observed at any site along the plasma membrane in both wild-type MDCK and nectin-1-MDCK cells as described (38, 52). When these cells were re-cultured at 2 mM Ca2+ in the presence or absence of an integrin inhibitor, echistatin, for 2 h, the signal for E-cadherin was re-concentrated at the cell-cell adhesion sites in the absence of echistatin (control), whereas the re-concentration of E-cadherin was markedly reduced in the presence of echistatin (Fig. 8B). The signal for nectin-afadin complex, which was detected by the anti-afadin mAb, was always concentrated at the cell-cell adhesion sites during the Ca2+ switch experiment. The essentially same results were obtained when flavoridin or LM609 mAb, which also functions as a blocking Ab against integrin αβ3, was used instead of echistatin (data not shown). These results indicate that the activation of integrin αβ3 plays an important role in the formation of AJs in MDCK cells.

DISCUSSION

We have shown here that both the low and high affinity forms of integrin αβ3 have the ability to cis-interact with nectin-3 through their extracellular regions. Of the four nec-
tin family members, integrin αβ3 associates with nectin-1 and nectin-3. This specificity of nectin for integrin αβ3 is similar to that for Par-3, known to be a member of the cell polarity proteins (53); only nectin-1 and nectin-3 directly bind Par-3 (54). The physiological role of the specific interaction of integrin αβ3 with nectin-1 and nectin-3 is currently unknown. Each nectin has two forms; one does not trans-interact and diffusely localizes on the plasma membrane of moving cells, and the other trans-interacts with another nectin molecule to form cell-cell adhesions. It is likely that integrin αβ3 physically associates with both forms of nectin. We have previously shown that integrin αβ3 associates with non-trans-interacting Nect-5 at the leading edges of moving NIH3T3 cells (20, 25). Therefore, integrin αβ3 on the free plasma membrane has at least three pools, which are a pool associating with Nect-5, a pool associating with non-trans-interacting nectin, and a free pool. We have also proposed that when moving cells contact with each other, the transient trans-interaction of Nect-5 with nectin-3 is converted to the stable trans-interaction of nectin-1 with nectin-3 (25). Nect-5 released from nectin-3 is down-regulated from the cell surface, although integrin αβ3 associated with Nect-5 is not down-regulated and remains on the plasma membrane (25). Therefore, integrin αβ3 on the plasma membrane of the contacting cells has at least three pools, which are a major pool associating with trans-interacting nectin, a minor pool associating with non-trans-interacting nectin, and a minor free pool.

It is likely from our present and recent results (25) that Nect-5 pre-associated with integrin αβ3 first trans-interacts with nectin-3 pre-associated with integrin αβ3 at the primordial cell-cell contact sites. This trans-interaction is then converted to the trans-interaction of nectin-3 with nectin-1 pre-associated with integrin αβ3. In these processes, integrin αβ3 released from Nect-5 may re-associate with nectin-1 and nectin-3, which do not pre-associate with integrin αβ3 and then participate in the trans-interaction of nectin. It remains unknown whether nectins, which do not associate with integrin αβ3, trans-interact with each other in intact cells, although it is evident from the cell-free assay that they have the ability to trans-interact without associating with integrin αβ3 (22). We have shown here that cadherin is not co-immunoprecipitated with integrin αβ3, although cadherin apparently co-localizes with integrin αβ3 at AJs in NIH3T3 and MDCK cells. This co-localization is likely to be dependent on nectin. Integrin αβ3 and E-cadherin also co-localize at the basolateral region of the cell-cell adhesion sites in MDCK cells where the immunofluorescence signal for nectin is hardly detected. However, it remains unknown how integrin αβ3 localizes there in a nectin-independent manner.

Integrin regulates many intracellular signaling molecules by adhering to ECM proteins (so-called “outside-in” signaling). Conversely, unlike other receptors, the activity of integrin is altered by
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the activation state of intracellular signaling molecules (so-called “inside-out” signaling). These bidirectional signalings of integrin seem to be crucial for cellular functions such as attachment, migration, and proliferation (5). Among intracellular signaling molecules, FAK directly binds to the cytoplasmic tail of integrin. Under several conditions, c-Src lies downstream of FAK and is phosphorylated by FAK upon the activation of integrin. Then, various kinds of intracellular signaling molecules including Crk, C3G, Rap1, Cdc42, and Rac are activated by FAK and c-Src (7, 55). Thus, the signaling cascade of integrin-FAK-c-Src is likely to be indispensable for the fundamental cellular functions. We have previously demonstrated that the trans-interaction of nectin first induces activation of c-Src, which then induces the Crk-mediated activation of Rap1 through C3G and phosphorlatory FRG, a Cdc42-GEF, and Vav2, a Rac-GEF, to induce activation of Cdc42 and Rac, respectively (15–17). Thus, the signaling molecules activated by the action of nectin and integrin are apparently similar. Extending these lines of evidence, we have first demonstrated here that any integrins including integrin αβ3 are involved in the nectin-induced activation of Cdc42 and Rac in the suspension cell culture system, because in this system, integrins generally become inactive due to the inhibition of their outside-in signals unless they adhere to the substrate. We have then shown here by the RNA-mediated interference experiments as well as the treatment or transfection with specific inhibitors that the activation of integrin αβ3 and FAK is indeed necessary for the nectin-induced signaling. The site of the action of integrin αβ3 mediated by FAK in the nectin-induced signaling is the step of c-Src activation, the most upstream molecule of the nectin-induced signaling. Therefore, the nectin-induced signaling may utilize the c-Src-mediated signaling, which is associated with integrin αβ3 through FAK. These results may provide a new insight into the cross-talk between the cell-cell and cell-matrix junctions.

We have finally shown here that during the formation of AJs, integrin αβ3 is activated at the earlier stage of the formation of cell-cell adhesion and that the inhibition of the integrin αβ3 activation actually suppresses the formation of AJs in MDCK cells. Once AJs are established, integrin αβ3 co-localizing with trans-interacting nectin at AJs appears to be the low affinity form of integrin αβ3 after the establishment of AJs. The molecular mechanism of this conversion of integrin αβ3 after the establishment of AJs remains unknown. However, the inactivation of integrin αβ3, which results in the inhibition of the nectin-induced activation of Cdc42 and Rac at the cell-cell adhesion sites, may play part contribute to the contact inhibition of the cell movement and proliferation, a well known phenomenon for a long time (26, 27). Therefore, it is physiologically and pathologically important to clarify the mechanism of the inactivation of integrin αβ3 after the establishment of AJs in the future.

Acknowledgments—We thank Drs. T. Akagi, M. Furuse, J. C. Norman, M. Okada, and J. Takagi for generous gifts of reagents. We also thank Dr. Akagi for helpful suggestions.
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