Involvement of Nectin in Inactivation of Integrin $\alpha_v\beta_3$ after the Establishment of Cell-Cell Adhesion*

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Integrin plays an essential role in the formation of cell-matrix junctions and is also involved in the fundamental cellular functions. In the process of the formation of cell-cell junctions, an immunoglobulin-like cell-cell adhesion molecule nectin initially trans-interacts together and promotes the formation of adherens junctions (AJs) cooperatively with another cell-cell adhesion molecule cadherin. The activation of integrin $\alpha_v\beta_3$ is critically necessary for this nectin-induced formation of AJs. However, after the establishment of AJs, integrin $\alpha_v\beta_3$ becomes inactive and retains the association with nectin at AJs. The molecular mechanism of this dynamic regulation of integrin $\alpha_v\beta_3$ during the formation of AJs remains unclear. We found here that the expression of phosphatidylinositol-phosphate kinase type Iγ (PIPKIγ), which is involved in the regulation of integrin activation, in Madin-Darby canine kidney cells, preferentially reversed the inactivation of integrin $\alpha_v\beta_3$ at cell-cell adhesion sites and partially disrupted E-cadherin-based AJs. The activation of PIPKIγ is correlated with its phosphorylation state. The tyrosine phosphatase protein-tyrosine phosphatase $\mu$ (PTP$\mu$) effectively dephosphorylated PIPKIγ and thus canceled the PIPKIγ-dependent activation of integrin $\alpha_v\beta_3$ by blocking the interaction of integrin $\alpha_v\beta_3$ with talin. Moreover, PTP$\mu$ associated with nectin, and its phosphatase activity was enhanced by the trans-interaction of nectin, leading to the decrease in PIPKIγ90 phosphorylation. Therefore, the trans-interaction of nectin essentially functions in the inactivation of integrin at AJs through the PTP$\mu$-induced inactivation of PIPKIγ.

Integrin is a key cell-cell adhesion molecule at cell-matrix junctions and comprises heterodimers with $\alpha$ and $\beta$ subunits (1). Integrin exhibits intracellular conformational changes between the low and high affinity forms (2). The low affinity form shows weak adhesion activity for extracellular matrix proteins and is inactive, whereas the high affinity form has increased adhesion activity for its extracellular ligands and is active (3). It was reported that integrin is essential for the formation of specialized subcellular apparatuses, such as focal complexes and focal adhesions, and for cell movement, proliferation, and differentiation (1, 4, 5). We recently demonstrated that integrin $\alpha_v\beta_3$ interacts with Necl-5 at the leading edge of moving cells and that this complex enhances cell movement and proliferation together with platelet-derived growth factor receptor by stimulation of platelet-derived growth factor (6, 7). Necl-5 is an Ig-like cell adhesion molecule and resembles nectin in its structure: three Ig-like loops at the extracellular region, a single transmembrane domain, and one cytoplasmic region.

When moving cells collide with each other, the initial cell-cell contact occurs with the trans-interaction of Necl-5 with nectin-3 (8). Nectin is an emerging Ig-like cell-cell adhesion molecule that localizes at adherens junctions (AJs)2 and is involved in the formation of AJs (9). Nectin exerts its cell-cell adhesion activity in a Ca$^{2+}$-independent manner and consists of four members: nectin-1, nectin-2, nectin-3, and nectin-4 (9). However, the trans-interaction of Necl-5 with nectin-3 is tentative, and Necl-5 is down-regulated from the cell surface by clathrin-dependent endocytosis (10). The down-regulation of Necl-5 impairs the integrin $\alpha_v\beta_3$- and platelet-derived growth factor receptor-dependent intracellular signaling for cell movement and proliferation, resulting in the reduction of cell movement and proliferation. The phenomenon that moving and proliferating normal cultured cells arrest both movement and proliferation after they grow confluent and form cell-cell junctions has been well known for a long time (11, 12), but its molecular mechanism is poorly understood. The down-regulation of Necl-5 is likely to be at least partly one of the underlying mechanisms of contact inhibition of cell movement and proliferation. On the other hand, nectin-3 dissociated from Necl-5 is retained on the cell surface and subsequently trans-interacts with nectin-1, which most feasibly trans-interacts with nectin-3 among the nectin family members (8). This trans-interaction of nectins promotes the recruitment of cadherin, a major cell-cell adhesion molecule at AJs, to the nectin-based cell-cell adhesion sites, eventually establishing AJs (9, 13).

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‡ The abbreviations used are: AJ, adherens junction; Ab, antibody; mAb, monoclonal antibody; pAb, polyclonal antibody; PIPKIγ, phosphatidylinositol-phosphate kinase type Iγ; MDCK, Madin-Darby canine kidney; PTP, protein-tyrosine phosphatase; FAK, focal adhesion kinase; GFP, green fluorescent protein; LMW, low molecular weight; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; PIPES, 1,4-piperazinediethanesulfonic acid.
During the nectin-induced formation of cadherin-based AJs, several intracellular signaling molecules including Rap1, Cdc42, and Rac small G proteins are activated, and actin cytoskeleton is reorganized by the trans-interaction of nectin in cooperation with the high affinity form of integrin $\alpha_v\beta_3$ (14–17). In this process, the activation of protein kinase C and FAK, downstream molecules of integrin $\alpha_v\beta_3$, is also required (17, 18). However, after the establishment of AJs, the high affinity form of integrin $\alpha_v\beta_3$ is converted to the low affinity form that also continues to associate with nectin (17, 18). Although the molecular mechanism by which integrin $\alpha_v\beta_3$ is inactivated after the formation of AJs remains to be elucidated, this inactivation seems to be beneficial for the maintenance of AJs, because the sustained activation of integrin renders cells highly motile, which tends to disrupt cell-cell junctions.

Integrin is activated by binding of talin to the cytoplasmic tail of integrin $\beta$ subunit (3), which causes the structural change of the integrin $\alpha/\beta$ dimer from the bent to the extended conformation. This change allows integrin to gain the higher affinity to the extracellular matrix. The binding of talin to integrin is up-regulated by increasing the amount of phosphatidylinositol 4,5-bisphosphate (19), which is generated by phosphatidylinositol-phosphate kinases such as phosphatidylinositol-phosphate kinase type I (PIPKI) (20) itself (20, 21). Moreover, phosphorylated PIPKI$^{\gamma 90}$ (PIPKI$^{\gamma 90}$) that is activated in this way induces the activation of c-Src and FAK, both of which phosphorylate and activate PIPKI$^{\gamma 90}$ (20, 21). These combined mechanisms result in the enhancement of phosphatidylinositol 4,5-bisphosphate synthesis and thus the further promotion of talin binding to integrin, suggesting the positive feedback loop of integrin activation. Thus, the phosphorylation state of PIPKI$^{\gamma}$ is important for the regulation of integrin activation.

Based on these lines of evidence, we examined in this study how integrin $\alpha_v\beta_3$ is inactivated after the nectin-induced formation of AJs by exploring the phosphatase that suppresses the phosphorylation of PIPKI$^{\gamma}$ and whether nectin actually associates with this phosphatase and regulates its phosphatase activity.

**EXPERIMENTAL PROCEDURES**

**Vector Construction**—The following expression vectors were kindly provided: GFP-tagged full-length human PIPKI$^{\gamma 90}$ (pEFGP-PIPKI$^{\gamma 90}$) from Dr. P. De Camilli (Yale University, New Haven, CT), GFP-tagged protein-tyrosine phosphatase $\mu$ (PTPM$\mu$) (pcDNA-GFP-PTPM$\mu$) and PTPM$\mu$ phosphatase-inactive mutant (pcDNA-GFP-PTP$\mu C/S$) were from Dr. S. Brady-Kalnay (Case Western Reserve University, Cleveland, OH), Myc-tagged low molecular weight protein-tyrosine phosphatase (LMW-PTP) (pcDNA3.1/myc-LMW-PTP) was from Dr. T. Konodo (Nagasaki University, Nagasaki, Japan), HA-tagged SHP-1 (pSR$\alpha$-HA-SHP-1) was from Dr. T. Matozaki (Gunma University, Gunma, Japan), wild-type c-Src (pcDNA3-c-Src-wt) was from Dr. M. Okada (Osaka University, Suita, Japan), and HA-tagged E-cadherin (pCAGGneo-HA-E-cadherin) was from Dr. M. Ozawa (Kagoshima University, Kagoshima, Japan). Expression vectors for FLAG-tagged nectin-1 (amino acids 27–518, pFLAG-CMV1-nectin-1), FLAG-tagged nectin-2 (amino acids 30–467, pFLAG-CMV1-nectin-2), FLAG-tagged nectin-3 (amino acids 56–549, pCAGIPuro-FLAG-nectin-3), FLAG-tagged nectin-4 (amino acids 29–508, pFLAG-CMV1-nectin-4), FLAG-tagged nectin-3 lacking its cytoplasmic region (amino acids 56–430, pFLAG-CMV1-nectin-3-ΔCP), FLAG-tagged nectin-3 lacking its extracellular region (amino acids 395–549, pFLAG-CMV1-nectin-3-ΔEC), and Myc-tagged nectin-3 (amino acids 56–549, pCAGIPuro-myc-nectin-3) were prepared as described (17). FLAG-tagged nectin-3 without the C-terminal last four amino acids that is necessary for its binding to afadin (amino acids 56–545, pFLAG-CMV1-nectin-3-ΔC) was constructed by inserting its cDNA fragment into pFLAG-CMV1 vector (Sigma). FLAG-tagged afadin (pCMVF-afadin) and PIPKI$^{\gamma 90}$ (pCMVF-PIPKI$^{\gamma 90}$) were also constructed by inserting full-length rat afadin and human PIPKI$^{\gamma 90}$ cDNA fragments, respectively, into pCMVF vector. cDNA encoding the extracellular region of PTP$\mu$ with 10 repeats of His tag (His-PTP$\mu$-EC; amino acids 1–740) was amplified by PCR and inserted into pFLAG-CMV-5 vector (Sigma).

**Antibodies**—The rabbit polyclonal antibody (pAb) against afadin was prepared as described (23). Hybridoma cells expressing a mouse anti-Myc mAb (9E10) were obtained from American Type Culture Collection, and the anti-Myc mAb was prepared as described (24). WOW-1 Fab, a rabbit anti-PIPKI$^{\gamma 90}$, and a rat anti-E-cadherin monoclonal Ab (mAb) (ECCD2) were kind gifts form Dr. S. J. Shatill (University of California San Diego, La Jolla, CA), Dr. Y. Kanah (University of Tsukuba, Tsukuba, Japan), and Dr. M. Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan), respectively. The following mouse mAbs were purchased from commercial sources; anti-FLAG M2 mAb (Sigma), anti-HA mAb (Berkeley Antibody), anti-phosphotyrosine mAb (4G10; Upstate Biotechnology, Inc.), anti-PTPM$\mu$ mAb (Chemicon), anti-FAK mAb (Pharmingen), anti-PIPKI$^{\gamma}$ mAb (Pharmingen), antitalin mAb (Sigma), and anti-integrin $\alpha_v\beta_3$ mAb (LM609; Chemicon). The following rabbit pAbs were purchased from commercial sources: anti-FLAG pAb (Sigma), anti-His pAb (Santa Cruz Biotechnology), and anti-integrin $\beta_3$ pAb (Chemicon). The goat anti-nectin-3 pAb was purchased from Santa Cruz Biotechnology.

**Cell Lines and Transfection**—MDCK cells, HEK293 cells, and L cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. For DNA transfection, Lipofectamine 2000 or Lipofectamine Plus (Invitrogen) was applied following the manufacturer’s instructions.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy was performed as described (17). Briefly, the cells were fixed with ice-cold acetone-methanol (1:1) solution for 1 min. After being blocked with 1% bovine serum albumin, the cells were immunostained with the indicated first Abs for 1 h, followed by the incubation with fluorophore-labeled secondary Abs for 30 min. The samples were analyzed by LM510 META confocal microscope (Carl Zeiss).
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Immunoprecipitation Assay—MDCK and HEK293 cells expressing various combinations of indicated molecules were lysed with Buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl$_2$, 1 mM Na$_2$VO$_4$, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 5 μg/ml aprotinin). The cell lysates were centrifuged at 100,000 × g at 4 °C for 15 min, and then the supernatant was incubated with the anti-FLAG mAb at 4 °C for 2 h followed by incubation with 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 Laemmli buffer for 5 min and subjected to SDS-PAGE (25), followed by Western blotting with the indicated Abs. To investigate the association of endogenous PTP$_{\mu}$ with nectin-3, MDCK cells cultured on the 0.4-μm pored transwell plate (Corning) were treated with a membrane-impermeable cross-linker bis(sulfosuccinimidyl) suberate (Pierce) according to the manufacturer’s instructions. After the treatment, the cells were lysed with buffer A, and the cell lysates were incubated with the anti-nectin-3 pAb or the control goat IgG, followed by the incubation with protein G-Sepharose. The immunoprecipitated samples were then analyzed by Western blotting.

In Vitro Binding of PTP$_{\mu}$ and Nectin-3—For the preparation of the purified protein of His-PTP$_{\mu}$-EC, HEK293 cells were transfected with pFLAG-CMV-5-PTP$_{\mu}$-EC. At 48 h after the transfection, the culture supernatant containing soluble His-PTP$_{\mu}$-EC was collected and then was applied to nickel-Sepharose 6 fast flow beads (GE Healthcare) equilibrated with Buffer B (25 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 20 mM imidazole at pH 8.0). After the beads were extensively washed with Buffer B, the bound His-PTP$_{\mu}$-EC was eluted with Buffer C (25 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 500 mM imidazole at pH 8.0). The protein concentration of His-PTP$_{\mu}$-EC was determined with bovine serum albumin as a reference protein on SDS-PAGE. The immunoprecipitated samples were then analyzed by Western blotting with the indicated Abs. To investigate the association of endogenous PTP$_{\mu}$ with nectin-3, MDCK cells cultured on the 0.4-μm pored transwell plate (Corning) were treated with a membrane-impermeable cross-linker bis(sulfosuccinimidyl) suberate (Pierce) according to the manufacturer’s instructions. After the treatment, the cells were lysed with buffer A, and the cell lysates were incubated with the anti-nectin-3 pAb or the control goat IgG, followed by the incubation with protein G-Sepharose. The immunoprecipitated samples were then analyzed by Western blotting with the indicated Abs.

Separation of Cytoplasmic and Cytoskeletal Fractions—Triton X-100-soluble (cytoplasmic) and -insoluble (cytoskeletal) fractions were prepared as described previously (27). Briefly, MDCK cells were lysed with Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM Na$_2$VO$_4$) at 4 °C for 1 h. Triton X-100-insoluble and -soluble extracts were separated by centrifugation at 15,000 × g at 4 °C for 5 min. The cytoskeletal pellet was washed twice with Triton X-100-free lysis buffer, and the proteins were extracted using radioimmune precipitation assay buffer (10 mM Tris-HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM Na$_2$VO$_4$).

Sucrose Density Gradient Centrifugation—The assay for isolation of plasma membrane fraction was performed as described previously (28). Briefly, the MDCK cells were washed with phosphate-buffered saline and then sonicated in Buffer D (10 mM HEPES-NaOH at pH 7.5, 100 mM KCl, 1 mM MgCl$_2$, and 25 mM Na$_2$HCO$_3$) on ice for 15 s six times at 3-min intervals. The homogenate was centrifuged at 1,000 × g at 4 °C for 5 min. The supernatant was diluted with Buffer D into 5 mg/ml of protein, and 0.2 ml was applied on a 48-ml continuous sucrose density gradient (10–50% sucrose in Buffer D), followed by centrifugation at 100,000 × g at 4 °C for 1 h with a swing rotor (P55ST2; Hitachi). After the centrifugation, fractions of 0.3 ml each were collected. Each fraction was subjected to SDS-PAGE, followed by Western blotting with the anti-E-cadherin and anti-talin mAbs.

Assessment for Integrin α$_{3}$β$_{3}$ Activity—MDCK cells cultured on 18-mm coverslips in a 12-well dish were used for the Ca$^{2+}$ switch assay as described previously (18). Briefly, the cells were washed with phosphate-buffered saline and incubated in serum-free DMEM (Normal Ca$^{2+}$ medium) for 1 h. Next, the cells were incubated in serum-free DMEM containing 5 mM EGTA (low Ca$^{2+}$ medium) for 3 h. The cells were then incubated in serum-free DMEM (normal Ca$^{2+}$ medium) for indicated period. To detect the high affinity form of integrin α$_{3}$β$_{3}$, we used His-tagged recombinant WOW-1 Fab as described previously (29). Briefly, the cells were incubated with His-tagged recombinant WOW-1 Fab for 30 min before the end of the Ca$^{2+}$ assay. The cells were then washed twice with DMEM and were lysed with Laemmli buffer. The amount of WOW-1 bound to the high affinity form of integrin α$_{3}$β$_{3}$ was detected by Western blotting with the anti-His pAb.

Determination of phosphatase activity was assessed using a Universal tyrosine phosphatase assay kit (Takara Bio) as previously described (30). Briefly, HEK293 cells transiently expressing Myc-nectin-3 or HA-E-cadherin were cultured in confluent and lysed with Lysis buffer attached to this kit and then centrifuged at 100,000 × g at 4 °C for 15 min. The supernatant was preclared by protein G-Sepharose beads, and the preclared supernatant was incubated with the anti-PTP$_{\mu}$ mAb at 4 °C for 2 h, followed by incubation with protein G-Sepharose beads at 4 °C for 2 h. After the beads were extensively washed with Buffer E (0.5% Tween 20, 50 mM PIPES, pH 7.0) three times, these beads were suspended into PTP buffer attached to this kit, and the samples were subjected to the phosphatase assay according to the manufacturer’s instructions. A paired Student t test was performed for statistical analysis.

Knockdown of PIPK1γ and PTP$_{\mu}$—To knock down PIPK1γ and PTP$_{\mu}$, double-stranded 25-nucleotide RNA duplexes (StealthÒ; Invitrogen) for PIPK1γ (duplex 1, 5′-UCUUGUAG-GUGGUUUCACCCAUGGC-3′; duplex 2, 5′-UGAAACCUG-GAAGUCCUGAAGUGGG-3′; duplex 3, 5′-UUCUG-GUGAGGUUCAUGUAUCCGCGG-3′) and PTP$_{\mu}$ (duplex 1, 5′-AUAAAGAGAAUGUAGAAUCCCGCGG-3′; duplex 2, 5′-UUCACACUAAACAUUGUAUUGGG-3′; and duplex
RESULTS

Activation of Integrin $\alpha_3\beta_3$ by PIPKIγ at Cell-Cell Junctions—We previously showed that after the achievement of AJs, integrin $\alpha_3\beta_3$ becomes inactive and localizes at cell-cell adhesion sites as well as focal adhesions (17, 18). Consistent with this, integrin $\alpha_3\beta_3$ was concentrated at the cell-cell adhesion sites and co-localized with E-cadherin in confluent MDCK cells, whereas talin was distributed throughout the cytoplasm and did not co-localize with E-cadherin (Fig. 1A), indicating the different localization of integrin $\alpha_3\beta_3$ from talin. Because talin is involved in the final step of the activation of integrin by directly binding to the cytoplasmic tail of integrin $\alpha_3\beta_3$ subunit (31), this different localization of integrin $\alpha_3\beta_3$ from talin represents the accumulation of the low affinity form of integrin $\alpha_3\beta_3$ at AJs in confluent MDCK cells.

However, when GFP-PIPKIγ90 was transfected into MDCK cells, talin as well as GFP-PIPKIγ90 was preferentially targeted to the plasma membrane of the cell-cell adhesion sites where the immunofluorescence signal for integrin $\alpha_3\beta_3$ was concentrated (Fig. 1B, arrowheads), leading to the notion that PIPKIγ90 induces the reactivation of integrin $\alpha_3\beta_3$ through talin. Interestingly, E-cadherin-based AJs were partially disrupted, probably because of this reactivation of integrin $\alpha_3\beta_3$ (31), this different localization of integrin $\alpha_3\beta_3$ from talin represents the accumulation of the low affinity form of integrin $\alpha_3\beta_3$ at AJs in confluent MDCK cells.

Conversely, the inactivation of PIPKIγ seems to be at least one of the important underlying mechanisms in the inactivation of integrin $\alpha_3\beta_3$ after the establishment of AJs.

FIGURE 1. Involvement of PIPKIγ in the recruitment of talin to the cell-cell adhesion sites and activation of integrin $\alpha_3\beta_3$ in MDCK cells. A, no recruitment of talin to the cell-cell adhesion sites in GFP-transfected MDCK cells. At 24 h after the transfection of GFP, confluent MDCK cells were immunostained with the indicated Abs. B, recruitment of talin to the cell-cell adhesion sites and disruption of cell-cell junctions in PIPKIγ90-transfected MDCK cells. At 24 h after the transfection of GFP-PIPKIγ90, confluent MDCK cells were immunostained with the indicated Abs. Arrowheads, talin targeted to the plasma membrane of cell-cell junctions; Arrow, disrupted cell-cell junctions. Scale bars, 10 µm. C, knockdown of PIPKIγ. The cell lysates from wild-type or siRNA-transfected MDCK cells were immunoprecipitated and immunoblotted with the anti-PIPKIγ Ab. Actin was also immunoblotted for the loading control. The sequence of each siRNA against PIPKIγ was indicated under “Experimental Procedures.” Because siRNA 2 (#2) most effectively reduced the expression of PIPKIγ in MDCK cells, this siRNA was used in the following experiments. WT, wild type; KD, knockdown. D, inhibition of the interaction of integrin $\beta_3$ with talin by knockdown of PIPKIγ in MDCK cells. At 48 h after the transfection of siRNA, the cells were immunostained with the anti-integrin $\beta_3$ and anti-talin Abs. Scale bars, 10 mm. The results shown in this figure are representative of three independent experiments.
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We further examined by knockdown of PIPKIγ whether endogenous PIPKIγ is indeed involved in the association of integrin with talin. The expression of PIPKIγ was markedly reduced in MDCK cells using siRNA against PIPKIγ (Fig. 1C). Although integrin β3 and talin clustered well and co-localized at focal adhesions of wild-type MDCK cells, this clustering or co-localization was not observed in PIPKIγ knockdown MDCK cells (Fig. 1D), indicating the necessity of PIPKIγ for the association of integrin with talin even at the endogenous level.

Identification of PTPµ as a Phosphatase for PIPKIγ—It was reported that the kinase activity of PIPKIγ90 is enhanced by its tyrosine phosphorylation (20–22), resulting in the increased binding of talin to integrin and consequent integrin activation. Thus, the phosphorylation state of PIPKIγ90 is likely to be closely correlated with the regulation of integrin activation. We then examined the implication of the tyrosine phosphatase in the dephosphorylation and inhibition of PIPKIγ90. To explore which phosphatases most effectively dephosphorylate PIPKIγ90, HEK293 cells were co-transfected with FLAG-PIPKIγ90, c-Src, and several tyrosine phosphatases including SHP-1, PTPµ, and LMW-PTP, and the phosphatase-induced decrease in PIPKIγ90 phosphorylation was monitored. In the presence of SHP-1, the phosphorylation of PIPKIγ90 was slightly reduced (Fig. 2A). As compared with SHP-1, PTPµ more markedly decreased the phosphorylation level of PIPKIγ90. In contrast, LMW-PTP did not attenuate PIPKIγ90 phosphorylation. This result indicates that PTPµ is the most promising candidate for the inhibitor of PIPKIγ. Then we confirmed the association of endogenous PTPµ with PIPKIγ in MDCK cells (Fig. 2B). To further specify the role of PTPµ in the dephosphorylation of PIPKIγ, we knocked down PTPµ in MDCK cells and examined whether the phosphorylation level of PIPKIγ is affected by the expression of PTPµ. The expression of PTPµ was markedly reduced in MDCK cells using siRNA against PTPµ (Fig. 2C). The tyrosine phosphorylation of PIPKIγ was actually enhanced in PTPµ knockdown MDCK cells (Fig. 2D). These results indicate that PTPµ specifically acts as a tyrosine phosphatase for PIPKIγ in MDCK cells.

Inhibitory Effect of PTPµ on the PIPKIγ-dependent Recruitment of Talin and Activation of Integrin αβ3—We next investigated whether PTPµ is involved in the inhibition of the PIPKIγ-dependent assembly of talin and the inactivation of integrin αβ3 at the cell-cell adhesion sites in MDCK cells. Co-transfection of FLAG-PIPKIγ90 and GFP-PTPµ into MDCK cells clearly canceled the PIPKIγ90-dependent translocation of talin to the cell-cell adhesion sites and prevented the disruption of AJs, whereas the phosphatase-inactive mutant of GFP-PTPµ (GFP-PTPµC/S) did not exert such inhibitory effects (Fig. 3A). These results indicate that PTPµ inhibits the PIPKIγ-induced translocation of talin and its binding to integrin αβ3 at the cell-cell adhesion sites.

The involvement of endogenous PTPµ in the localization of talin was further certified biochemically in MDCK cells by the knockdown of PTPµ. The subcellular localization of talin as well as FAK, a binding protein of integrin (32), was changed from the cytoplasmic to the cytoskeletal membrane fraction by knockdown of PTPµ (Fig. 3B). In addition, when the total cell lysates were subjected to sucrose density gradient centrifugation, the shift of talin to high density fractions where the membrane marker E-cadherin exists was observed in PTPµ knockdown MDCK cells (Fig. 3C, lanes 3–6). These results provide another line of evidence that PTPµ suppresses the translocation of talin to the plasma membrane.

To gain the distinct evidence for the involvement of PTPµ in the inactivation of integrin αβ3, the high affinity form of integrin αβ3 was monitored using WOW-1 Fab during the formation of cell-cell junctions induced by the Ca2+ switch assay. WOW-1 Fab specifically detects the high affinity form of integrin αβ3 (33). As compared with wild-type MDCK cells, the high affinity form of integrin αβ3 in PTPµ knockdown MDCK cells was increased at 0.5 and 1.5 h after the reculture with normal Ca2+ medium (Fig. 3D). Taken together, these results indicate that PTPµ perturbs the interaction of talin with integrin αβ3 and inactivates integrin αβ3 by the inhibition of PIPKIγ.

Interaction of PTPµ with Nectin—We next examined the relationship between PTPµ and the nectin-afadin system in the
inactivation of integrin $\alpha_\beta_3$, because nectin and integrin $\alpha_\beta_3$ physically and functionally associate together (17). In MDCK cells, PTP$\mu$ co-localized with nectin-3 and afadin endogenously at cell-cell junctions (Fig. 4A). The association of endogenous PTP$\mu$ with nectin-3 in MDCK cells was also confirmed by co-immunoprecipitation assay by chemically cross-linking cell surface proteins (Fig. 4B). Moreover, PTP$\mu$ was co-immunoprecipitated with not only nectin-3 but also other nectin family members including nectin-1, nectin-2, and nectin-4 in HEK293 cells ectopically expressing GFP-PTP$\mu$ with FLAG-nectin-1, FLAG-nectin-2, FLAG-nectin-3, or FLAG-nectin-4, when each FLAG-nectin molecule was immunoprecipitated with the anti-FLAG mAb (Fig. 4C, panel a). However, another tyrosine phosphatase LMW-PTP was not co-immunoprecipitated with FLAG-nectin-3 (Fig. 4C, panel b), suggesting that the co-immunoprecipitation of PTP$\mu$ with nectin is not nonspecific. In addition, PTP$\mu$ was not co-immunoprecipitated with afadin in HEK293 cells overexpressing these molecules (Fig. 4C, panel c). These results indicate that PTP$\mu$ associates with nectin at the cell-cell adhesion sites.

We further examined the mode of interaction of nectin with PTP$\mu$. To test this, GFP-PTP$\mu$ was co-expressed with FLAG-tagged nectin-3 lacking its extracellular region (FLAG-nectin-3ΔEC), FLAG-tagged nectin-3 lacking its cytoplasmic region (FLAG-nectin-3ΔCP), or FLAG-tagged nectin-3 without the C-terminal tail of four amino acids that is necessary for binding to afadin (FLAG-nectin-3ΔC) in HEK293 cells. When each nectin mutant was immunoprecipitated with the anti-FLAG mAb, GFP-PTP$\mu$ was co-immunoprecipitated with FLAG-nectin-3ΔEC, but not FLAG-nectin-3ΔCP, indicating the interaction of nectin-3 with PTP$\mu$ through their extracellular regions (Fig. 5A). We also found co-immunoprecipitation of FLAG-nectin-3ΔC with GFP-PTP$\mu$. This suggests no requirement of the nectin-afadin binding for the interaction of nectin with PTP$\mu$.

We next prepared the recombinant proteins of the His-tagged extracellular region of PTP$\mu$ (His-PTP$\mu$EC) and the extracellular region of nectin-3 fused to IgG Fc (Nef-3) to investigate the direct binding of PTP$\mu$ and nectin-3. When Nef-3 was incubated with His-PTP$\mu$EC immobilized on nickel beads, the interaction of His-PTP$\mu$EC with Nef-3 was detected (Fig. 5B). These results indicate that PTP$\mu$ and nectin-3 physically interact with each other through their extracellular regions at cell-cell junctions.

To examine whether the interaction of nectin with PTP$\mu$ affects the localization of PTP$\mu$ at the cell-cell adhesion sites, GFP-PTP$\mu$ was transfected into L cells expressing full-length nectin-3 (nectin-3L cells) or nectin-3ΔEC (nectin-3ΔEC-L cells) as well as wild-type L cells. The assembly of GFP-PTP$\mu$ at the cell-cell adhesion was markedly increased in nectin-3-L cells compared with wild-type L cells (Fig. 5C). Such an increase in the assembly of GFP-PTP$\mu$ at the cell-cell adhesion was not observed in nectin-3ΔEC-L cells. Thus, the interaction of nectin with PTP$\mu$ was also immunoblotted (IB) for the loading control. The results shown in this figure are representative of three independent experiments.
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**FIGURE 4. Association of PTPμ with nectin.** A, co-localization of endogenous PTPμ with the nectin-afadin complex. Confluent MDCK cells were immunostained with the indicated Abs. Scale bars, 10 μm. B, co-immunoprecipitation (IP) of endogenous PTPμ with nectin-3. The cell lysates from MDCK cells pretreated with cell surface cross-linker were immunoprecipitated with the anti-nectin-3 pAb, followed by Western blotting with the anti-PTPμ and anti-nectin-3 Abs. C, immunoprecipitation assay with phosphatases and nectin family members or afadin. Panel a, co-immunoprecipitation of PTPμ with the nectin family members. The cell lysates of HEK293 cells transfected with the indicated combinations of GFP-PTPμ and FLAG-nectin molecules were immunoprecipitated with the anti-FLAG mAb. The immunoprecipitants were subjected to Western blotting with the anti-Myc and anti-FLAG mAbs. Panel b, no co-immunoprecipitation of LMW-PTP with nectin-3. The cell lysates of HEK293 cells transfected with Myc-LMW-PTP and FLAG-nectin-3 were immunoprecipitated with the anti-FLAG mAb, followed by Western blotting with the anti-Myc and anti-FLAG mAbs. Panel c, no co-immunoprecipitation of PTPμ with afadin. The cell lysates of HEK293 cells transfected with GFP-PTPμ and FLAG-afadin were immunoprecipitated with the anti-FLAG mAb, followed by Western blotting with the anti-PTPμ and anti-FLAG mAbs. The results shown in this figure are representative of three independent experiments. IB, immunoblotting.

**DISCUSSION**

Nectin and the high affinity form of integrin αβ3 cooperatively play a pivotal role in the formation of AJ by activating signaling molecules that are necessary for the formation of AJ (13). However, after the achievement of AJ, the high affinity form of integrin αβ3 is converted into the low affinity form that continues to localize at AJ. The molecular mechanism for how integrin αβ3 becomes inactive during the formation of AJ has not been elucidated yet to date. In this manuscript, we successfully proposed the molecular mechanism by which trans-interacting nectin also functions to inactivate integrin αβ3 at mature AJ through the PTPμ-mediated dephosphorylation of PIPKI90. The schematic representation of this molecular mechanism is depicted in Fig. 7.

Enhancement of PTPμ Phosphatase Activity by Trans-interacting Nectin—We finally examined whether the trans-interaction of nectin actually enhances the phosphatase activity of PTPμ for PIPKI90. When HEK293 cells ectopically expressing FLAG-PIPKI90 and c-Src with or without GFP-PTPμ and Myc-nectin-3 were cultured in confluence to form the trans-interaction of nectin-3, the c-Src-induced phosphorylation of PIPKI90 was reduced by co-transfection of PTPμ (Fig. 6A). Intriguingly, nectin-3 remarkably increased the dephosphorylation of PIPKI90 in the presence of PTPμ. This nectin-induced dephosphorylation of PIPKI90 was not observed when GFP-PTPμ was not transfected. In contrast, when HEK293 cells expressing FLAG-PIPKI90 and c-Src with or without GFP-PTPμ and HA-E-cadherin were cultured in confluence, the phosphorylation of PIPKI90 was not affected by the trans-interaction of E-cadherin, although there is a report that E-cadherin associates with PTPμ (34). Moreover, we confirmed that the trans-interaction of nectin, but not E-cadherin, significantly raised the phosphatase activity of PTPμ (Fig. 6B). Taken together, these results indicate that the trans-interaction of nectin preferentially reduces the phosphorylation of PIPKI90 mediated by the nectin-induced activation of PTPμ, eventually resulting in the inactivation of integrin αβ3 after the establishment of AJs.

Precipitation (IP) of endogenous PTPμ with nectin-3. The cell lysates from MDCK cells pretreated with cell surface cross-linker were immunoprecipitated with the anti-nectin-3 pAb, followed by Western blotting with the anti-PTPμ and anti-nectin-3 Abs. Immunoprecipitation assay with phosphatases and nectin family members or afadin. Panel a, co-immunoprecipitation of PTPμ with the nectin family members. The cell lysates of HEK293 cells transfected with the indicated combinations of GFP-PTPμ and FLAG-nectin molecules were immunoprecipitated with the anti-FLAG mAb. The immunoprecipitants were subjected to Western blotting with the anti-Myc and anti-FLAG mAbs. Panel b, no co-immunoprecipitation of LMW-PTP with nectin-3. The cell lysates of HEK293 cells transfected with Myc-LMW-PTP and FLAG-nectin-3 were immunoprecipitated with the anti-FLAG mAb, followed by Western blotting with the anti-Myc and anti-FLAG mAbs. Panel c, no co-immunoprecipitation of PTPμ with afadin. The cell lysates of HEK293 cells transfected with GFP-PTPμ and FLAG-afadin were immunoprecipitated with the anti-FLAG mAb, followed by Western blotting with the anti-PTPμ and anti-FLAG mAbs. The results shown in this figure are representative of three independent experiments. IB, immunoblotting.
PTPμ is one of the receptor type protein-tyrosine phosphatases expressing in several epithelial cells and endothelial cells (35). PTPμ contains a MAM (Merpin/A5/PTPμ) domain, an Ig-like domain, four fibronectin type III repeats in its extracellular region, and two phoshatase domains in its cytoplasmic region. PTPμ itself trans-interacts through its MAM and Ig-like domains and localizes at AJs (36–38). It was previously demonstrated that PTPμ interacts with the cytoplasmic region of cadherin, β-catenin, and p120^ctn and reduces the phosphorylation of these molecules (39, 40). In addition to this, we found here that nectin physically associates with PTPμ through their extracellular region and increases the phosphatase activity of PTPμ. This increase is somewhat small, but statistically significant, indicating the essential involvement of nectin in the up-regulation of the PTPμ phosphatase activity. Moreover, the assembly of PTPμ at the cell-cell adhesion sites is dependent on the association of PTPμ with nectin, because the signal intensity of GFP-PTPμ at cell-cell junctions are higher in L fibroblasts expressing full-length nectin-3 than those expressing nectin-3-∆EC that is incapable of interacting with PTPμ. Notably, this nectin-mediated PTPμ assembly does not depend on cadherin, because L fibroblasts do not express any cadherins. However, it remains unknown whether these transmembrane proteins, nectin, cadherin, and PTPμ form a ternary complex and how these proteins communicate with each other to increase the phosphatase activity of PTPμ at

![Image](50x368 to 299x734)

**FIGURE 5.** Physical interaction of PTPμ with nectin-3 through their extracellular regions and its involvement in the recruitment of PTPμ to the nectin-based cell-cell adhesion sites. A, co-immunoprecipitation of PTPμ with the cytoplasmic region-deleted mutant of nectin-3. The cell lysates of HEK293 cells transfected with GFP-PTPμ and the indicated FLAG-nectin-3 mutants (nectin-3-ΔCP, nectin-3 without its cytoplasmic region; nectin-3-ΔEC, nectin-3 lacking C-terminal four amino acids that is necessary for binding of nectin to afadin) were immunoprecipitated (IP) with the anti-FLAG mAb. The immunoprecipitates were subjected to Western blotting with the anti-PTPμ and anti-FLAG mAbs. B, direct interaction of PTPμ with nectin-3. The recombinant protein of His-PTPμ-EC was immobilized on nickel-Sepharose beads and incubated with Nef-3. After the incubation, the eluates were subjected to SDS-PAGE, followed by Western blotting with the anti-human IgG Fc for the detection of Nef-3 and anti-His Abs. C, recruitment of PTPμ to the cell-cell adhesion sites dependent on the association of PTPμ with nectin-3. Wild-type L cells or L cells ectopically expressing nectin-3-ΔEC were transfected with GFP-PTPμ and then stained for F-actin with phalloidin. Scale bars, 10 μm. The results shown in this figure are representative of three independent experiments. IB, immunoblotting.

![Image](226x26 to 253x38)

**FIGURE 6.** Nectin-induced dephosphorylation of PIPKIγ90 and up-regulation of the phosphatase activity of PTPμ. A, increased dephosphorylation of PIPKIγ90 by the trans-interaction of nectin, but not E-cadherin. Confluent HEK293 cells expressing FLAG-PIPKIγ90 and c-Src with or without GFP-PTPμ, Myc-nectin-3, and HA-E-cadherin were lysed and immunoprecipitated (IP) with the anti-FLAG mAb, followed by Western blotting with the anti-FLAG and anti-phosphotyrosine (pY) mAbs. B, enhancement of PTPμ phosphatase activity by nectin. The cell lysates from HEK293 cells expressing Myc-nectin-3 or HA-E-cadherin were immunoprecipitated with the anti-PTPμ mAb. Untransfected HEK293 cells used as a control. The phosphatase activity of PTPμ in the immunoprecipitants were analyzed using tyrosine phosphatase assay kit (Takara Bio). The data shown in this graph are the relative PTPμ phosphatase activity as compared with the value of the control, which is expressed as 1. *, p < 0.05. The results shown in this figure are representative of three independent experiments. IB, immunoblotting.
E-cadherin and the $\mu$ subunit of the clathrin adaptor protein complex and facilitates the E-cadherin transport to the plasma membrane through the adaptor protein complex to efficiently form AJs. Down-regulation of PIPK1y by RNA interference impairs the E-cadherin target to the plasma membrane and inhibits the formation of AJs. Combined with our findings that overexpression of PIPK1y90 in MDCK cells disrupts E-cadherin-based AJs by the reactivation of integrin $\alpha_v\beta_3$, the proper amount of PIPK1y and its controlled kinase activation and inactivation are critical for the formation and maintenance of AJs. Because the detailed mechanisms for the prolonged maintenance of AJs remain to be elucidated, further extensive studies are necessary in the future.

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