Necl-5/Poliovirus Receptor Interacts in cis with Integrin αvβ3 and Regulates Its Clustering and Focal Complex Formation

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Integrin αvβ3, which forms focal complexes at leading edges in moving cells, is up-regulated in cancer cells and so is implicated in their invasiveness. Necl-5, originally identified as a poliovirus receptor and also up-regulated in cancer cells, co-localizes with integrin αvβ3 at leading edges in moving cells and enhances growth factor-induced cell movement. Here, we show that Necl-5 interacts directly, in cis, with integrin αvβ3, and enhances integrin αvβ3 clustering and focal complex formation at leading edges in NIH3T3 cells. The extracellular region of Necl-5, but not the cytoplasmic region, is necessary for its interaction with integrin αvβ3; however, both regions are necessary for its action. An interaction between integrin αvβ3 and vitronectin and PDGF-induced activation of Rac are also necessary for integrin αvβ3 clustering. The interaction between Necl-5 and integrin αvβ3 enhances PDGF-induced Rac activation, facilitating integrin αvβ3 clustering presumably in a feedback amplification manner. Thus, Necl-5 has a critical role in integrin αvβ3 clustering and focal complex formation.

Integrins are key molecules for adhesion between cells and extracellular matrix (ECM) proteins (1). They are heterodimers of α and β subunits, both of which have one transmembrane segment. The extracellular region of integrins binds to ECM proteins, whereas the cytoplasmic region is directly and indirectly associated with many F-actin-binding proteins, such as talin, vinculin, and α-actinin, and intracellular signaling molecules, such as FAK and c-Src (1, 2). Integrins have at least two forms: conformations with either low or high affinity for their ECM binding partners (1, 3, 4). When talin binds to the low affinity form, this is converted to the high affinity form (4).

Upon binding to ECM proteins, integrins transduce signals inside cells that then cause the reorganization of the actin cytoskeleton, eventually resulting in integrin clustering and the formation of focal complexes and focal adhesions (4). Focal complexes are formed at leading edges in moving cells, whereas focal adhesions, also called focal contacts, are formed at sites to the rear of the leading edges (5, 6). Focal complexes are generally smaller than mature focal adhesions, being less than 0.5 μm in diameter. Protrusions, such as filopodia and lamellipodia, are also formed at leading edges, while ruffles are formed on the dorsal surfaces of lamellipodia. Focal complexes are formed under these protrusions. Focal complexes, protrusions, and ruffles are formed by the actions of the small G proteins Rac and Cdc42; their formation is inhibited by the action of the small G protein RhoA (5, 7). By contrast, focal adhesions are formed by the action of RhoA. Inactivation of Rac and Cdc42 and activation of RhoA lead to the transformation of focal complexes into focal adhesions. The dynamic formation of all of these structures is necessary for efficient cell movement. The dynamic activation and inactivation of Rac, Cdc42, and RhoA are regulated not only by the outside-in signaling of integrins but also by growth factor-induced signaling (8). Of the many integrins, integrin αvβ3 has a particularly important role in focal complex formation (9). This integrin is expressed in many cell types, such as fibroblasts and vascular endothelial cells (10, 11), and is often up-regulated in many cancer cells, such as colon carcinoma, melanoma, and glioblastoma cells (12–14).

We found that an Ig-like molecule Necl-5/poliovirus receptor (PVR)/CD155/Tage4 co-localizes with integrin αvβ3 at leading edges in moving cells and enhances growth factor-induced cell movement (15). Human PVR/CD155 was originally identified as a poliovirus receptor (16, 17), whereas rodent Tage4 was originally identified as the product of a gene that is overexpressed in rodent colon carcinoma (18). PVR/CD155 is also overexpressed in many human cancer cells, such as colon carcinoma, melanoma, and glioblastoma cells, in which integrin αvβ3 is up-regulated (19–21). This molecule, with four nomenclatures, is also named nectin-like molecule-5, Necl-5 (22). Necl-5 is expressed in many cell types, such as fibroblasts and vascular endothelial cells, in which integrin αvβ3 is expressed (23, 24). Necl-5 does not show homophilic cell-cell adhesion activity, but it heterophilically interacts in trans (i.e. present on a different cell) with nectin-3, a member of the Ig-like nectin family that is a Ca²⁺-independent cell-cell adhesion molecule and forms adherens junctions cooperatively with cad...
herins (22, 25). When cells come into contact with other cells, Necl-5 is down-regulated from the cell surface by trans-interacting withnectin-3 (26); however, when there is no contact with other cells, Necl-5 is up-regulated at the transcriptional level by growth factor-induced signaling (27). Up-regulation of Necl-5 enhances growth factor-induced cell movement,

A Role of Necl-5 in Integrin $\alpha_\beta_3$ Clustering

![Image](image-url)

**FIGURE 1. Co-localization of Necl-5 with integrin $\alpha_\beta_3$, at peripheral ruffles and focal complexes.** A, immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on vimentin-coated µ-slide dishes. Cells were double- or triple-stained with various combinations of the anti-Necl-5 mAb, the anti-integrin $\beta_3$ mAb, and the anti-talin mAb. a, wild-type NIH3T3 cells; b, Necl-5-NIH3T3 cells; c, wild-type NIH3T3 cells co-transfected with siRNA vector and pEGFP-tub; c1, Necl-5-knockdown-NIH3T3 cells; c2, control-NIH3T3 cells. Arrowheads, leading edges; asterisks, siRNA vector-transfected cells; scale bars, 10 μm. Inset boxes show the area of high magnification images. The high magnification images are indicated as intensity ratio images. Analysis of the co-localization of Necl-5 and integrin $\beta_3$, and generation of the intensity ratio images were performed by ImageJ 1.34S software with RG2B colocalization plugin. B, quantitative analysis of various structures in NIH3T3 and Necl-5-NIH3T3 cells. a, quantification of peripheral ruffles at leading edges. To quantify peripheral ruffles, the signal for F-actin, which is a major component of peripheral ruffles, was observed. Peripheral ruffles at leading edges were measured and classified into two categories: small ruffles (less than 10 μm in width) and large ruffles (10 μm or more in width). The results are the means ± S.E. of the three independent experiments. b, strength of peripheral ruffles at leading edges. To analyze the strength of peripheral ruffles, the maximum intensity of F-actin signal at the peripheral ruffles was measured using ImageJ 1.34S software and averaged ($n = 27$). *, $p < 0.0002$. The results are the means ± S.E. of the twenty-seven cells. c, quantification of focal complexes at leading edges. The patterns of the signal for integrin $\beta_3$ at focal complexes of leading edges were measured and classified into three categories: scattered focal complexes (Scattered FX), line-like distributed focal complexes (Line-like FX), and belt-like distributed focal complexes, which were assembled more than two line-like FX (Belt-like FX). The results are the means ± S.E. of the three independent experiments. Immunofluorescence images are the staining of integrin $\beta_3$, and examples of three categories of focal complexes. d, quantification of focal adhesions per cell. The signal for integrin $\beta_3$ at focal adhesions was counted and averaged per cell ($n = 27$). **, $p < 0.0001$. The results are the means ± S.E. of the 27 cells.
whereas down-regulation of Necl-5 reduces it (26). Cultured cells continue to move until they contact other cells and become confluent. They then undergo cell-cell adhesion and stop moving. This phenomenon has been known for more than fifty years as contact inhibition of cell movement (28, 29). We have proposed that Necl-5 has a role, at least in part, in this contact inhibition of cell movement (26). However, we did not previously examine which structure of leading edges Necl-5 localizes to or whether, and if so how, Necl-5 regulates integrin $\alpha_v\beta_3$-based focal complex formation. We attempt to address these issues here using NIH3T3 cells as a model cell type, which express both integrin $\alpha_v\beta_3$ and Necl-5.

**EXPERIMENTAL PROCEDURES**

Construction—pCAGIpuro-FLAG-Necl-5, pCAGIzeo-FLAG-Necl-5-ΔCP, and pCAGIzeo-FLAG-Necl-5-ΔEC were prepared as described (15, 25). A small interfering RNA (siRNA) vector against Necl-5 (pBS-H1-Necl-5) and a control siRNA vector against luciferase (pBS-H1-control) were prepared as described (26). To obtain siRNA-resistant mutants, FLAG-Necl-5R and FLAG-Necl-5-ΔCPR were generated by mutagenesis of 5'-GGTATGTTGGCCTCACTAA-3' to create 5'-GGTACGTAAATGATTAACGAA-3' using the QuikChange site-directed mutagenesis kit (Stratagene) from pFLAG-CMV1-Necl-5 and pFLAG-CMV1-Necl-5-ΔCP (15, 25) and cloned into pCMV5 vector (pCMV5-FLAG-Necl-5R and pCMV5-FLAG-Necl-5-ΔCPR, respectively). Necl-5-ΔCP fused to a Myc tag at the C terminus was inserted into the vector pCAGIpuro (pCAGIpuro-Necl-5-ΔCP-myc). Expression vectors were kindly provided as follows: wild-type human integrin $\alpha_v$ and human integrin $\beta_3$ (pcDNA3-$\alpha_v$ and -$\beta_3$) were from Dr. J. C. Norman (University of Leicester, Leicester, UK), a dominant-negative
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**Aa**

| FLAG IP | Control | Necl-5 | Necl-5-$\Delta$CP | Necl-5-$\Delta$EC |
|---------|---------|--------|-------------------|-----------------|
| WB:     |         |        |                   |                 |
| FLAG    |          |        |                   |                 |

| Cell lysate | Control | Necl-5 | Necl-5-$\Delta$CP | Necl-5-$\Delta$EC |
|-------------|---------|--------|--------------------|-------------------|
|             |         |        |                   |                  |

**Ab**

| FLAG IP | Cell lysate |
|---------|-------------|
| WB:     |             |
| FLAG    |             |

**Ac**

| Control IP | Integrin $\alpha_\nu$ IP | Cell lysate |
|------------|--------------------------|-------------|
|            |                          |             |

| Integrin $\alpha_\nu$ |
|-----------------------|
| Integrin $\beta_3$    |
| Necl-5                 |
| PDGF                   |

| Input (1%) | Eluate |
|------------|--------|

**B**

| WB: | Necl-5 |
|-----|--------|

| Integrin $\alpha_\nu$ |
|-----------------------|
| Integrin $\beta_3$    |

| Necl-5 EC | Integrin $\alpha_\nu\beta_3$ EC |
|-----------|---------------------------------|
mutant of human integrin $\beta_3$ (pcDNA3.1-Myc-His($+)\beta_3$T329C/A347C) was from Dr. J. Takagi (Osaka University, Suita, Japan). GST-PAK-Cdc42/Rac-interactive binding region (pGEX-PAK-CRIB) was a kind gift from Dr. S. Narumiya (Kyoto University, Kyoto, Japan). pEFBOS-myc-Rac1CA and -Rac1DN were prepared as described (30).

Cell Culture, Transfection, and siRNA Experiments—NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum. HEK293 cells were maintained in DMEM supplemented with 10% fetal calf serum. NIH3T3 cells stably expressing FLAG-tagged Necl-5 (Necl-5-NIH3T3 cells) were obtained by transfection with pcAGIPuro-FLAG-Necl-5 using Lipofectamine 2000 reagent (Invitrogen) and by selection with puromycin. Necl-5-ΔCp-NIH3T3 cells were prepared as described (15). For transient expression experiments, cells were transfected with various expression vectors using Lipofectamine 2000 reagent. Knockdown of Necl-5 was performed as described (26). Briefly, NIH3T3 cells were co-transfected with siRNA vector against Necl-5, pBS-H1-necl-5, and pEGFP-tub (Clontech), because EGFP-tagged tubulin, but not EGFP, is resistant to acetone/methanol fixation. As a control, siRNA vector against luciferase, pBS-H1-control, was used. pBS-H1-vector was a gift from Dr. H. Shibuya (Tokyo Medical and Dental University, Tokyo, Japan). Alternatively, cells were transfected with a double-stranded 19-nt RNA duplex to Necl-5 (5’-GGTATGTGGC-CTCATAA-3’) or a similar duplex to luciferase (5’-CTGTA-CGGGAATACCTTCGA-3’) as a control using Hyperfect (Qiagen). To confirm knockdown of Necl-5 by FACS, pBS-EGFP-H1-necl-5 and pBS-EGFP-H1-control were used. These vectors were constructed by subcloning the insert encoding the EGFP expression unit from pEGFP-C1 (Clontech) into a region not related to siRNA expressions of pBS-H1-necl-5 and pBS-H1-control, respectively.

Antibodies and Reagents—A rat monoclonal antibody (mAb) against the extracellular region of Necl-5 (mAb-i, 1A8-8) was prepared as described (25). Hamster anti-integrin $\alpha_v$ and $\beta_3$ mAbs (H9.2B8 and 2C9.G2, respectively; for immunostaining) were purchased from BD Biosciences. The following mouse mAbs were purchased from commercial sources: anti-integrin $\alpha_v$, anti-integrin $\beta_3$, anti-integrin $\alpha_5$, and anti-integrin $\beta_1$ (for Western blotting; BD Biosciences), anti-talin (8D4) and anti-FLAG M2 (for immunoprecipitation, Western blotting and FACS) (Sigma), and anti-actin mAbs (Chemicon). The following rabbit polyclonal antibodies (pAbs) were purchased from commercial sources: anti-N-cadherin (TAKARA), anti-integrin $\alpha_v$ (for immunoprecipitation), and anti-integrin $\beta_3$ pAbs (for Western blotting) (Chemicon). Hybridoma cells (9E10) expressing a mouse anti-Myc mAb were purchased from the American Type Culture Collection. Fatty acid-free BSA, fibronectin, laminin, ConA, and cyclo-RGDfV were purchased from Sigma. Horseradish peroxidase-conjugated secondary Abs was purchased from Amersham Biosciences. Fluorophore (FITC, Cy3, Cy5, and R-PE)-conjugated secondary Abs were purchased from Jackson ImmunoResearch. Human recombinant platelet-derived growth factor (PDGF)-BB was purchased from PEPROTECH. Vitronecin was purified from human plasma (Kohjinbio) as described (31). Chemical cross-linker, 3’,3’-dithiobis [sulfo succinimidylpropionate] (DTSSP), was purchased from Pierce.

Directional Stimulation by PDGF—To generate a concentration gradient of PDGF, a $\mu$-Slide VI flow (uncoated; Ibidi) was used. The $\mu$-Slide VI1 flow has six parallel channels, which were coated with 5 $\mu$g/ml vitronectin, 25 $\mu$g/ml fibronectin, or 80 $\mu$g/ml laminin. Cells were seeded at a density of $5 \times 10^3$ cells per square centimeter, cultured for 16 h, and starved of serum with DMEM containing 0.5% BSA for 1 h. The concentration gradient of PDGF was made using DMEM containing 0.5% BSA and 30 ng/ml PDGF according to manufacturer’s protocol. After 30 min, cells were fixed with acetone/methanol (1:1), incubated with 1% BSA in PBS, and then incubated with 20% BlockAce in PBS, prior to immunofluorescence microscopy (26). The samples were analyzed by confocal laser-scanning microscope systems, digital eclipse C1si-ready (Nikon), and LSM510 META (Carl Zeiss MicroImaging).

Co-immunoprecipitation Assay—HEK293 cells were co-transfected with various combinations of plasmids, cultured for 24 h, detached with 0.05% trypsin and 0.53 mM EDTA, and treated with a trypsin inhibitor. To make predominantly high affinity integrin $\alpha_v\beta_3$, cells were cultured in suspension with Ca$^{2+}$-free DMEM (Invitrogen) containing 0.5% BSA, 1 mM MnCl$_2$, and 50 $\mu$g/ml cyclo-RGDfV for 1 h, collected by centrifugation, washed with Wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM Na$_3$VO$_4$), and lysed with Buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM MnCl$_2$, 10% glycerol, 1% Nonidet P-40, 10 mM Na$_3$VO$_4$, 10 $\mu$g/ml leupeptin, 2 $\mu$g/ml aprotinin, and 10 $\mu$M APMSF). To make predominantly low affinity integrin $\alpha_v\beta_3$, cells were cultured in suspension with DMEM containing 0.5% BSA for 1 h, collected by centrifugation, washed with Wash buffer and lysed with Buffer B (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10% glycerol, 1% Nonidet P-40, 10 mM Na$_3$VO$_4$, 10 $\mu$g/ml leupeptin, 2 $\mu$g/ml aprotinin, and 10 $\mu$M APMSF). The lysates were rotated for 30 min and subjected to centrifugation at 12,000 $\times$ g for 20 min. The supernatant was preclarified with protein G-Sepharose 4 Fast Flow beads (American Biosciences) at 4 °C for 1 h, incubated with the anti-FLAG
mAb at 4 °C for 4 h, and then incubated with protein G-Sepharose beads at 4 °C for 2 h. After the beads were extensively washed with Buffer A or B, bound proteins were eluted by boiling the beads in SDS Sample Buffer (60 mM Tris-HCl, pH 6.7, 3% SDS, 2% 2-mercaptoethanol, and 5% glycerol) for 5 min and subjected to SDS-PAGE followed by Western blotting.

To examine the interaction between endogenous Necl-5 and endogenous integrin αvβ3, a co-immunoprecipitation assay was performed using NIH3T3 cells. Cells were plated at a density of 5 × 10^3 cells per square centimeter on vitronectin-coated dish, cultured for 16 h, starved of serum with DMEM containing 0.5% BSA for 1 h, and then stimulated with DMEM containing 0.5% BSA and/or 3 ng/ml PDGF. After 30 min, cells were washed with ice-cold PBS and incubated with 2 mM DTSSP in PBS at 4 °C for 2 h. To quench the cross-linking reaction, 1 M Tris-HCl (pH 7.5) was added at a final concentration of 50 mM. Cells were washed with PBS and lysed with Buffer B. The lysates were rotated for 30 min and subjected to centrifugation at 12,000 × g for 20 min. The supernatant was precleared with protein A-Sepharose (Amersham Biosciences) at 4 °C for 1 h, incubated with the anti-integrin αv pAb at 4 °C for 16 h, and then incubated with protein A-Sepharose beads at 4 °C for 4 h. After the beads were extensively washed with Buffer B, bound proteins were eluted by boiling the beads in SDS sample buffer for 5 min and subjected to SDS-PAGE followed by Western blotting.

In Vitro Binding of Necl-5 to Integrin αvβ3—Necl-5 EC was prepared as described (25). To obtain integrin αvβ3 EC, CHO-lec 3.2.8.1 cells expressing integrin αvβ3 EC were cultured, and the culture supernatant containing soluble integrin αvβ3 EC was collected (3). This cell line was kindly supplied by Dr. J. Takagi (Osaka University, Suita, Japan). The culture supernatant was
applied to Ni-Sepharose 6 Fast Flow beads (Amersham Biosciences) and equilibrated with Buffer C (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 C and then Buffer C containing 0.6 M NaCl, bound integrin \( \alpha_v \beta_3 \) 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 C. The protein concentration of integrin \( \alpha_v \beta_3 \) EC was determined with BSA as a reference protein by SDS-PAGE.

To examine the binding of Necl-5 to integrin \( \alpha_v \beta_3 \) EC, integrin \( \alpha_v \beta_3 \) EC (6 pmol) was immobilized on Ni-Sepharose beads and soluble Necl-5 EC (60 pmol) was incubated with integrin \( \alpha_v \beta_3 \) EC-immobilized Ni-Sepharose beads or Ni-Sepharose beads alone in 0.3 ml of Buffer C containing 1% BSA. After the beads were extensively washed with Buffer C, bound proteins were eluted with Elution buffer. The eluate was then subjected to SDS-PAGE, followed by Western blotting.

**Assay for Bead-Cell Contact**—Latex-sulfate microbeads (4.55 \( \times \) 10⁶; 10-µm diameter; Polyscience Inc.) were washed, resuspended in 0.2 ml of PBS, and incubated with 10 µg of vitronectin, 50 µg of fibronectin, or 160 µg of laminin with gentle mixing at room temperature. After the incubation, the beads were washed three times with 0.5 ml of PBS and resuspended in 0.1 ml of PBS containing 1% BSA. ConA-coated beads were prepared as described (32) and used as control beads. Cells were starved of serum with DMEM containing 0.5% BSA for 18 h. After serum starvation, cells were detached with 0.05% trypsin and 0.53 mM EDTA and then treated with a trypsin inhibitor (Sigma). Cells were plated at a density of 1 \( \times \) 10⁴ cells per square centimeter on laminin-coated coverglass, cultured for 3 h, and incubated with vitronectin-, fibronectin-, laminin-, or ConA-coated beads for 1 h. Cells were fixed with acetone/methanol (1:1), incubated with 1% BSA in PBS, and then incubated with 20% BlockAce in PBS, followed by immunofluorescence microscopy (26).

**Assay for Rac Activation**—The assay for Rac activation using GST-PAK CRIB was performed as described (33). Briefly, NIH3T3, Necl-5-NIH3T3, or Necl-5-ΔCP-NIH3T3 cells were cultured on vitronectin-coated dishes for 16 h, starved of serum with DMEM containing 0.5% BSA for 1 h, stimulated with 3 ng/ml PDGF, and then subjected to the assay for Rac activation. For analysis of Necl-5-knockdown cells, NIH3T3 cells were twice transfected with siRNA oligo every 24 h, cultured for 24 h, and subjected to the assay.

**RESULTS**

Co-localization of Necl-5 with Integrin \( \alpha_v \beta_3 \) at Peripheral Ruffles and Focal Complexes—NIH3T3 cells were sparsely plated on \( \mu \)-slide VI flow dishes precoated with vitronectin, an ECM protein that binds to integrin \( \alpha_v \beta_3 \) (1), starved of serum, and directionally stimulated by PDGF. Most cells became polarized and formed lamellipodia with peripheral ruffles at leading edges, in the direction of higher concentrations of PDGF. The immunofluorescence signals for Necl-5 and integrin \( \beta_3 \) were concentrated and co-localized at the peripheral ruffles of the leading edges in the middle section of the cells (Fig. 1Aa, consistent with earlier observations (15). In the basal section of the cells, the signal for integrin \( \beta_3 \) was observed as dot-like structures under the peripheral ruffles. The signal for Necl-5 was also observed as fuzzy dot-like structures and mostly overlapped with the signal for integrin \( \beta_3 \). Additionally, the signal for integrin \( \beta_3 \), but not that for Necl-5, was observed as dot-like structures at sites to the rear of the leading edges. Essentially the same results were obtained when integrin \( \alpha_v \) was stained instead of integrin \( \beta_3 \). Indeed, unless otherwise specified, essentially the same results were obtained for both integrin \( \alpha_v \) and integrin \( \beta_3 \) in the experiments that follow. The dot-like structures under the peripheral ruffles (which were immunopositive for Necl-5, integrin \( \alpha_v \), and integrin \( \beta_3 \)) were smaller in size than those at sites to the rear of the leading edges (which were immunopositive for integrin \( \alpha_v \) and integrin \( \beta_3 \), but not for Necl-5). The signal for talin co-localized with both types of dot-like structure, consistent with earlier observations (6). In contrast to these signals, the signal for N-cadherin was not concentrated at any site on the entire plasma membrane (supplemental Fig. S1A). Here we determine that the dot-like structures, which are immunopositive for Necl-5, integrin \( \alpha_v \), integrin \( \beta_3 \), and talin and locate under the peripheral ruffles, correspond to focal complexes, whereas the dot-like structures, which are immunopositive for integrin \( \alpha_v \), integrin \( \beta_3 \), and talin, but not for Necl-5, correspond to focal adhesions. These results indicate that Necl-5 co-localizes with integrin \( \alpha_v \beta_3 \) both at peripheral ruffles over the lamellipodia and at focal complexes under the peripheral ruffles of leading edges, but not at focal adhesions, in NIH3T3 cells. Integrin \( \alpha_v \beta_3 \) at these structures includes at least the high affinity form, because talin co-localizes with it (4).

Regulation by Necl-5 of Integrin \( \alpha_v \beta_3 \) Clustering at Peripheral Ruffles and Focal Complexes—We previously showed that the amount of cell surface Necl-5 is regulated by cell density (26). Therefore, we next examined the effect of the amount of cell surface Necl-5 on integrin \( \alpha_v \beta_3 \) clustering at peripheral ruffles and focal complexes in NIH3T3 cells. When NIH3T3 cells stably overexpressing Necl-5 (Necl-5-NIH3T3 cells) were cultured on \( \mu \)-slide dishes as described above and directionally stimulated by PDGF, most cells showed morphologies similar

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**FIGURE 3. Co-localization of Necl-5 with integrin \( \alpha_v \beta_3 \) at contact sites between vitronectin-coated beads and cells.** A, immunofluorescence images of the contact sites between the vitronectin- or ConA-coated beads and NIH3T3 cells. Cells were double- or triple-stained with various combinations of the anti-Necl-5 mAb, the anti-integrin \( \beta_3 \) mAb, and the anti-talin mAb. a, wild-type NIH3T3 cells; b, Necl-5-NIH3T3 cells; c, wild-type NIH3T3 cells co-transfected with siRNA vector and pEGFP-Tub; c1, Necl-5-knockdown-NIH3T3 cells; c2, control-NIH3T3 cells; c1, b1, c1, and c2, vitronectin-coated beads; a2 and b2, ConA-coated beads. N, nucleus; DIC, differential interference contrast; asterisks, positions of the beads; scale bars, 10 µm. The results shown are representative of three independent experiments. B, quantification of the accumulation of Necl-5 and integrin \( \beta_3 \) at the bead-cell contact sites in A. The ordinate indicates the percentage of beads positive for immunofluorescence signals for Necl-5 and integrin \( \beta_3 \). a, wild-type NIH3T3 and Necl-5-NIH3T3 cells; b, Necl-5-knockdown-NIH3T3 and control-NIH3T3 cells.* p < 0.001. The results shown are the means ± S.E. of three independent experiments.

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3 Y. Takai, unpublished data.
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A

| WB: FLAG | Cell lysate |
|----------|-------------|
| FLAG IP | kDa |
| 84.7 | 114 |

B

Necl-5-\( \triangle \)CP-NIH3T3

FLAG-Necl-5

Integrin \( \alpha_\nu \)

Integrin \( \beta_3 \)

Necl-5-\( \triangle \)CP-myc

Integrin \( \alpha_\nu \)

Integrin \( \beta_3 \)

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FIGURE 4. Regulation of integrin \( \alpha_\nu \beta_3 \) clustering by Necl-5-integrin \( \alpha_\nu \beta_3 \) interaction. A, inhibition of Necl-5-integrin \( \alpha_\nu \beta_3 \) interaction by Necl-5-\( \triangle \)CP. HEK293 cells were transfected with various combinations of the indicated vectors and cultured in suspension in the presence of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \). FLAG-tagged Necl-5 was immunoprecipitated using the anti-FLAG mAb, and samples were assessed by Western blotting using the anti-FLAG mAb, the anti-integrin \( \alpha_\nu \) mAb, the anti-integrin \( \beta_3 \) mAb, and the anti-Myc mAb, \( \beta_3 \) immunofluorescence images of PDGF-stimulated Necl-5-\( \triangle \)CP-NIH3T3 cells cultured on vitronectin-coated \( \mu \)-slide dishes. Cells were double-stained with the anti-FLAG mAb and the anti-integrin \( \beta_3 \) mAb. Scale bars, 10 \( \mu \)m. The results shown are representative of three independent experiments.

to those of wild-type NIH3T3 cells, except that they formed more marked peripheral ruffles than wild-type cells (Fig. 1, \( Ab, \ Ba, \) and \( Bb, \) see also \( Aa \)). The immunofluorescence signals for Necl-5, integrin \( \beta_3 \), and talin, but not that for N-cadherin, were highly concentrated at peripheral ruffles and focal complexes (Fig. 1\( Ab \) and supplemental Fig. S1B). The number of focal complexes in the basal sections of these cells was increased compared with that in wild-type cells, but their diameters were unchanged (Fig. 1, \( Ab \) and \( Bc, \) see also \( Aa \)). In addition, the immunofluorescence signals for integrin \( \beta_3 \) and talin were markedly decreased at focal adhesions in Necl-5-NIH3T3 cells compared with those in wild-type cells (Fig. 1, \( Ab \) and \( Bd, \) see also \( Aa \)). The signal for Necl-5 was observed as belt-like structures at leading edges and partly overlapped with the signals for integrin \( \beta_3 \) and talin at focal complexes. The Necl-5-positive belt-like structures might be due to the increased number of dot-like structures caused by overexpression of Necl-5. When similar experiments were performed using NIH3T3 cells in which Necl-5 was knocked down (Necl-5-knockdown-NIH3T3 cells) by siRNA vector (about 80–90% decrease of Necl-5; supplemental Fig. S2A), most cells did not form definitely leading edges, but randomly formed lamellipodia at various sites; the signal for Necl-5 was negligible (Fig. 1Ac, 1 and 2). The signal for integrin \( \beta_3 \) was not concentrated at any region. The amounts of cell surface integrin \( \alpha_\nu \) and integrin \( \beta_3 \) were indistinguishable among wild-type NIH3T3, Necl-5-NIH3T3, Necl-5-knockdown-NIH3T3, and control-NIH3T3 cells as estimated by FACS analysis (supplemental Fig. S3, A and B).

These results indicate that Necl-5 enhances integrin \( \alpha_\nu \beta_3 \) clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells, and suggest that Necl-5 physically interacts with integrin \( \alpha_\nu \beta_3 \).

Physical Interaction of Necl-5 with Integrin \( \alpha_\nu \beta_3 \)—We therefore examined the physical interaction between Necl-5 and integrin \( \alpha_\nu \beta_3 \) by three methods: immunoprecipitation, in vitro binding, and bead-cell contact assays. In the immunoprecipitation assay, we prepared low and high affinity forms of integrin \( \alpha_\nu \beta_3 \) to determine with which form Necl-5 interacts. To make predominantly high affinity integrin \( \alpha_\nu \beta_3 \), human integrin \( \alpha_\nu \), human integrin \( \beta_3 \), and FLAG-Necl-5 were co-expressed in HEK293 cells, and the cells were cultured in suspension in the presence of \( \text{Mn}^{2+} \) and cyclo-

RGDFV (3). Alternatively, to make predominantly low affinity integrin \( \alpha_\nu \beta_3 \), human integrin \( \alpha_\nu \), human integrin \( \beta_3^{T329C/A347C} \), and FLAG-Necl-5 were co-expressed in HEK293 cells, and the cells were cultured in suspension in the presence of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \). Integrin \( \beta_3^{T329C/A347C} \) is stabilized in the low affinity form and cannot convert to the high affinity form with integrin \( \alpha_\nu \) (34). When FLAG-Necl-5 was immunoprecipitated by an anti-FLAG mAb from cell lysates, both human integrin \( \alpha_\nu \) and human integrin \( \beta_3 \) or human integrin \( \beta_3^{T329C/A347C} \) were co-immunoprecipitated (Fig. 2Aa). These results indicate that Necl-5 has the ability to physically interact with both the low and high affinity forms of integrin \( \alpha_\nu \beta_3 \). The interaction of Necl-5 with integrin \( \alpha_\nu \beta_3 \) was not as specific, because integrin \( \alpha_\nu \beta_3 \) was not co-immunoprecipitated with FLAG-Necl-5 under comparable conditions (Fig. 2Ab).

The interaction of endogenous Necl-5 with endogenous integrin \( \alpha_\nu \beta_3 \) was then confirmed by a co-immunoprecipitation assay using lysates of NIH3T3 cells. NIH3T3 cells were cultured on vitronectin-coated dishes in the presence or absence of PDGF, and then treated with or without a chemical cross-linker DTSSP, which is membrane insoluble and has a disulfide bond.
When endogenous integrin αv was immunoprecipitated from lysates of cells cultured in the presence or absence of PDGF and treated with DTSSP, integrin β3 and Necl-5 were co-immunoprecipitated with it from both cell lysates irrespective of the presence and absence of PDGF, although the amount of co-immunoprecipitated Necl-5 in cells cultured in the presence of PDGF was slightly less than that in cells cultured in the absence of PDGF (Fig. 2Ac). The amount of integrin β3 co-immunoprecipitated with integrin αv was not affected by PDGF stimulation. Integrin β3, but not Necl-5, was co-immunoprecipitated with integrin αv from lysates of cells which were not pretreated with DTSSP. The exact reason why Necl-5 was not co-immunoprecipitated with integrin αv in the absence of the cross-linker is currently unknown, but may be due to low amounts of these proteins in NIH3T3 cells and low affinities of these proteins.

We next examined whether the extracellular and/or cytoplasmic regions of Necl-5 are necessary for the interaction with integrin αvβ3. We expressed human integrin αv, human integrin β3, or integrin β3T329C/A347C and FLAG-Necl-5 with the cytoplasmic or extracellular region deleted (FLAG-Necl-5-EC or FLAG-Necl-5-CP, respectively), in HEK293 cells, and performed similar experiments to those described above. Both human integrin αv and human integrin β3 or integrin β3T329C/A347C were co-immunoprecipitated with FLAG-Necl-5-CP, whereas neither of them was co-immunoprecipitated with FLAG-Necl-5-EC (Fig. 2Aa). These results indicate that the extracellular region of Necl-5, but not the cytoplasmic region, is essential for the interaction with both low and high affinity forms of integrin αvβ3.

We then confirmed this result by use of purified recombinant protein samples. We prepared recombinant proteins of the extracellular region of Necl-5 fused to human IgG Fc portion (Necl-5 EC) and a heterodimer containing the extracellular regions of human integrin αv and human integrin β3 with a hexa-histidine-tag fused to the integrin β3 COOH terminus (integrin αvβ3 EC) (3). When Necl-5 EC was applied to a column of integrin αvβ3 EC-immobilized Ni-Sepharose beads, Necl-5 EC bound to integrin αvβ3 EC (Fig. 2B).

We furthermore confirmed the physical interaction between Necl-5 and integrin αvβ3 by the bead-cell contact assay. We first prepared microbeads coated with vitronectin and placed them on the surface of NIH3T3 cells that were starved of serum and sparsely plated on glass coverslips precoated with laminin. Consistently, the immunofluorescence signals for integrin β3 and talin were concentrated at the bead-cell contact sites (Fig. 3Aa1). The signal for Necl-5 was also concentrated there. These
signals were markedly enhanced by overexpression of Necl-5, and reduced by its knockdown (Fig. 3A, b1, c1, and c2). These results were statistically significant (Fig. 3B, a and b). When ConA-coated beads were used as a control, none of these proteins were concentrated at the bead-cell contact sites (Fig. 3A, a2 and b2). These results support the cis-interaction between Necl-5 and integrin αvβ3.

**Regulation of Integrin αvβ3 Clustering by Necl-5-Integrin αvβ3 Interaction**—We then examined whether the interaction between Necl-5 and integrin αvβ3 indeed regulates integrin αvβ3 clustering and the formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells. For this purpose, we took advantage of a Necl-5 construct in which the cytoplasmic region was deleted (Necl-5-ΔCP), because we previously showed that this deletion mutant reduces the stimulatory effect of full-length Necl-5 on movement of L cells stably overexpressing Necl-5 and NIH3T3 and V12-Ki-Ras-NIH3T3 cells (15) and showed above that the extracellular region of Necl-5 interacts in cis (i.e. in the same cell membrane) with the extracellular region of integrin αvβ3. Consistently, Necl-5-ΔCP inhibited the interaction between Necl-5 and integrin αvβ3 as detected by the immunoprecipitation assay (Fig. 4A). When Necl-5-ΔCP was stably expressed in NIH3T3 cells that were cultured on μ-slide dishes as described above and directionally stimulated by PDGF, most cells did not form leading edges but randomly formed lamellipodia at various sites (Fig. 4B). The immunofluorescence signal for Necl-5-ΔCP was diffusely distributed. The signal for integrin β3 was markedly decreased at focal complexes compared with that in wild-type cells. These results indicate that Necl-5-ΔCP shows a dominant-negative effect on intact Necl-5 through the inhibition of the interaction between Necl-5 and integrin αvβ3 and that the interac-
tion between Necl-5 and integrin \(\alpha_\gamma\beta_3\) regulates integrin \(\alpha_\gamma\beta_3\) clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells.

**Necessity of Both the Extracellular and Cytoplasmic Regions of Necl-5 for Integrin \(\alpha_\gamma\beta_3\) Clustering**—We examined whether integrin \(\alpha_\gamma\beta_3\) clustering needs both the extracellular and cytoplasmic regions of Necl-5. For this purpose, various Necl-5 mutants were expressed in Necl-5-knockdown-NIH3T3 cells. When full-length FLAG-Necl-5R, which is resistant to Necl-5 siRNA, was expressed in Necl-5-knockdown-NIH3T3 cells (supplemental Fig. S2B, a and b), and the cells were cultured on \(\beta_3\)-slide dishes pre-coated with vitronectin and directionally stimulated by PDGF, they showed morphologies similar to those of Necl-5-NIH3T3 cells (Fig. 5A, see also Fig. 1Ab). They had a polarized structure with lamellipodia and peripheral ruffles and the immunofluorescence signals for Necl-5 and integrin \(\beta_3\) were concentrated at peripheral ruffles over the lamellipodia and focal complexes under the peripheral ruffles. When FLAG-Necl-5-\(\Delta\)CP, which is also resistant to Necl-5 siRNA, or FLAG-Necl-5-\(\Delta\)EC was expressed in Necl-5-knockdown-NIH3T3 cells (supplemental Fig. S2B, a and b), the phenotypes of most Necl-5-NIH3T3 cells were not restored (Fig. 5, B and C). Morphologically, most cells did not form leading edges but randomly formed lamellipodia at various sites. The signals for FLAG-Necl-5-\(\Delta\)CP and FLAG-Necl-5-\(\Delta\)EC were diffusely distributed. The signal for integrin \(\beta_3\) was not concentrated at any region, although the intensity of its signal was higher in the

**FIGURE 6.** Necessity of binding of integrin \(\alpha_\gamma\beta_3\) to specific ECM proteins for its clustering. A, immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on fibronectin- or laminin-coated dishes. Cells were triple-stained with the anti-Necl-5 mAb, the anti-integrin \(\beta_3\) mAb, and the anti-talin mAb. a, fibronectin-coated dishes; b, laminin-coated dishes; 1, wild-type NIH3T3 cells; 2, Necl-5-NIH3T3 cells. White arrowheads, leading edges; red arrowheads, fibrillar adhesions; scale bars, 10 \(\mu\)m. Inset boxes show the area of high magnification images. The high magnification images are indicated as intensity ratio images. Analysis of the co-localization of Necl-5 and integrin \(\beta_3\) and generation of the intensity ratio images were performed by ImageJ 1.34S software with RG2B colocalization plugin. B, immunofluorescence images of the contact sites between fibronectin- or laminin-coated beads and NIH3T3 cells. Cells were triple-stained with the anti-Necl-5 mAb, the anti-integrin \(\beta_3\) mAb, and the anti-talin mAb. a, fibronectin-coated beads; b, laminin-coated beads; 1, wild-type NIH3T3 cells; 2, Necl-5-NIH3T3 cells; N, nuclei; DIC, differential interference contrast; asterisks, positions of the beads; scale bars, 10 \(\mu\)m. The results shown are representative of three independent experiments. c, quantification of the accumulation of Necl-5 and integrin \(\beta_3\) at bead-cell contact sites in a and b. The ordinate indicates the percentage of beads positive for immunofluorescence signals for Necl-5 and integrin \(\beta_3\). *, \(p < 0.001\). The results shown are the means \(\pm\) S.E. of three independent experiments.
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cells expressing FLAG-Necl-5-$\Delta$CP than in the cells expressing FLAG-Necl-5-$\Delta$EC. These results indicate that both the extracellular and cytoplasmic regions of Necl-5 are necessary for integrin $\alpha_v\beta_3$ clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells.

Necessity of Binding of Integrin $\alpha_v\beta_3$ to Specific ECM Proteins for Necl-5-enhanced Integrin $\alpha_v\beta_3$ Clustering—It has been shown that the clustering of integrin requires its binding to ECM proteins and that integrin $\alpha_v\beta_3$ binds both vitronectin and fibronectin, but does not bind laminin (1). We examined whether the binding of integrin $\alpha_v\beta_3$ to its ECM proteins is necessary for PDGF-induced, Necl-5-enhanced integrin $\alpha_v\beta_3$ clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells.

Necessity of PDGF Stimulation for Necl-5-enhanced Integrin $\alpha_v\beta_3$ Clustering—It has been shown that the synergistic activity of PDGF- and integrin-induced signaling (10). We therefore examined the effect of PDGF on Necl-5-enhanced integrin $\alpha_v\beta_3$ clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells. When NIH3T3 cells were cultured on $\mu$-slide dishes precoated with vitronectin and not stimulated by PDGF, most cells failed to form leading edges (Fig. 7A). The immunofluorescence signals for Necl-5, integrin $\beta_v$, and talin were negligible at peripheral regions. However, the large dot-like signals for integrin $\beta_3$ and talin were concentrated at focal adhesions and the number of focal adhesions was increased compared with those observed in the presence of PDGF (see also Fig. 1Aa). When similar experiments were performed using Necl-5-NIH3T3 cells, most cells did not form leading edges (Fig. 7B). The signal for Necl-5 was diffusely observed on the plasma membrane but the signals for integrin $\beta_3$ and talin were again negligible at peripheral regions. However, the signals for integrin $\beta_3$ and talin were observed at focal adhesions, although they were not observed in Necl-5-NIH3T3 cells stimulated by PDGF (see also Fig. 1Ab). When similar experiments were performed using Necl-5-knockdown-NIH3T3 cells, most cells did not form leading edges (Fig. 7C). The signal for Necl-5 was essentially nil. The signal for integrin $\beta_3$ was not concentrated anywhere, and the phenotype was different to those observed in NIH3T3 and Necl-5-NIH3T3 cells (see also Fig. 7, A and B). These results indicate that PDGF stimulation is necessary for Necl-5-enhanced integrin $\alpha_v\beta_3$ clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells.

Involvement of Rac Activation in PDGF-induced, Necl-5-enhanced Integrin $\alpha_v\beta_3$ Clustering—It has been shown that Rac, which is activated by the action of PDGF, regulates these morphological changes (6, 8, 38). We previously showed that overexpression of Necl-5 enhances the serum-induced activation of Rac in an integrin $\alpha_v\beta_3$-dependent manner in L fibroblasts (15). We therefore examined whether Necl-5-integrin $\alpha_v\beta_3$ interaction enhances PDGF-induced Rac activation in NIH3T3 cells. PDGF-induced Rac activation was estimated by a pull-down assay. This form of Rac activation was enhanced and sustained by overexpression of Necl-5 and reduced by its knockdown (about 70% decrease of Necl-5) (Fig. 8A, a and b). It was also reduced by overexpression of Necl-5-$\Delta$CP (Fig. 8Aa). These results indicate that Necl-5-integrin $\alpha_v\beta_3$ interaction enhances PDGF-induced Rac activation in NIH3T3 cells.

We then examined whether Rac is necessary for PDGF-induced, Necl-5-enhanced integrin $\alpha_v\beta_3$ clustering in NIH3T3 cells. Transient expression of a dominant-negative mutant of Rac1 (Rac1DN) in NIH3T3 cells or Necl-5-NIH3T3 cells cultured on $\mu$-slide dishes precoated with vitronectin and directionally stimulated by PDGF, inhibited the formation of lamellipodia and ruffles at the peripheral regions of both cell lines (Fig. 8B, a and b). The immunofluorescence signals for Necl-5, integrin $\beta_3$, and Rac1DN were not concentrated at peripheral
regions, but were concentrated at currently unknown structures on the plasma membrane and inside the cells. These results indicate that Rac is necessary for PDGF-induced, Necl-5-enhanced integrin αvβ3 clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells.

**Necessity of Both Rac Activation and Necl-5-Integrin αvβ3 Interaction for PDGF-induced Integrin αvβ3 Clustering**—We next examined whether Rac activation alone is sufficient for integrin αvβ3 clustering and focal complex formation in NIH3T3 cells. For this purpose, we expressed a constitutively active mutant of Rac1 (Rac1CA) in the absence of PDGF stimulation. When Rac1CA was transiently expressed in NIH3T3 cells cultured on μ-slide dishes precoated with vitronectin in the absence of PDGF, most cells showed round structures with lamellipodia and peripheral ruffles over all peripheral regions (Fig. 9Aa). The immunofluorescence signals for Necl-5, integrin β3, and Rac1CA were concentrated at peripheral ruffles and focal complexes. In addition, the signal for integrin β3, but not that for Necl-5 or Rac1CA, was concentrated at focal adhesions. The number of focal complexes was markedly increased by Rac1CA compared with that in wild-type cells in the absence of PDGF (see also Fig. 7A). The reason why lamellipodia, peripheral ruffles, and focal complexes were formed randomly over all peripheral regions in the cells expressing Rac1CA might be due to the random localization of exogenously expressed Rac1CA to the plasma membrane. When NIH3T3 cells overexpressing Rac1CA were cultured on μ-slide dishes precoated with laminin instead of vitronectin, the cells showed morphologies different from those observed in the wild-type cells cultured on vitronectin, and formed lamellipodia unevenly along the plasma membrane, but did not show round shapes (Fig. 9Ab). In these cells, the immunofluorescence signal for Necl-5 or integrin β3 was not concentrated at any region.

Necl-5-NIH3T3 cells overexpressing Rac1CA showed morphologies and staining patterns of Necl-5, integrin β3, and Rac1CA similar to those of wild-type cells, except that they formed more marked peripheral ruffles and showed more marked staining of these markers than wild-type cells (Fig. 9Ba, see also Aa). When Necl-5-NIH3T3 cells overexpressing Rac1CA were cultured on μ-slide dishes precoated with laminin instead of vitronectin, the cells showed morphologies and staining patterns of Necl-5, integrin β3, and Rac1CA similar to those of wild-type cells cultured on laminin (Fig. 9Bb, see also Ab). When NIH3T3 or Necl-5-NIH3T3 cells overexpressing Rac1CA were cultured on μ-slide dishes precoated with vitronectin and directionally stimulated by PDGF, PDGF showed no additional effect.3 In contrast to wild-type and Necl-5-NIH3T3 cells, Necl-5-knockdown-NIH3T3 cells overexpressing Rac1CA showed morphologies different from those observed in wild-type cells; they formed lamellipodia unevenly along the plasma membrane, but did not show round shapes (Fig. 9C, see also Aa). The signal for Necl-5 was negligible. The signal for integrin β3 was not concentrated at any region, similar to the observation of Necl-5-knockdown-NIH3T3 cells in the absence of PDGF (see also Fig. 7C). Necl-5-DCP-NIH3T3 cells overexpressing Rac1CA showed phenotypes similar to those of Necl-5-knockdown-NIH3T3 cells overexpressing Rac1CA (Fig. 9D). Taken together, these results indicate that Rac activation alone or Necl-5-integrin αvβ3 interaction alone is not sufficient for integrin αvβ3 clustering and formation of lamellipodia, peripheral ruffles, and focal complexes in NIH3T3 cells. Rather, Rac activation, Necl-5-integrin αvβ3 interaction, and integrin αvβ3-vitronectin interaction are all necessary for integrin αvβ3 clustering and morphological changes; however, Rac activation and integrin αvβ3-vitronectin interaction are sufficient, without Necl-5-integrin αvβ3 interaction, for lamellipodium formation.

**DISCUSSION**

We first showed here that Necl-5 and integrin αvβ3 localized at leading edges in moving NIH3T3 cells predominantly where...
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FIGURE 8. Involvement of Rac activation in PDGF-induced, Necl-5-enhanced integrin αβ3 clustering. A, effect of Necl-5 on PDGF-induced Rac activation. Cells were cultured on vitronectin-coated dishes, starved of serum, stimulated with PDGF, and then subjected to the assay for Rac activation. a, wild-type NIH3T3, Necl-5-NIH3T3, and Necl-5-ΔCP-NIH3T3 cells. b, control- and Necl-5-knockdown-NIH3T3 cells. Wild-type NIH3T3 cells were transfected with siRNA oligo. Expression level of endogenous Necl-5 in Necl-5-knockdown-NIH3T3 cells was less than 30% of that in control-NIH3T3 cells. B, immunofluorescence images of PDGF-stimulated NIH3T3 cells expressing Rac1DN, cultured on vitronectin-coated μ-slide dishes. Cells were transfected with Myc-tagged Rac1DN and triple-stained with the anti-Necl-5 mAb, the anti-integrin β3 mAb, and the anti-Myc mAb. a, wild-type NIH3T3 cells; b, Necl-5-NIH3T3 cells. Scale bars, 10 μm. The results shown are representative of three independent experiments.

It was previously shown that cell movement requires the synergistic activity of PDGF- and integrin-induced signaling (10). It was also shown that the PDGF receptor and integrin αβ3 interact with each other, and that PDGF-induced signaling requires integrin αβ3 activation by binding to its ECM proteins (10). The binding of talin to integrin increases the affinity of integrin for its specific ECM protein, and the binding of integrin to its specific ECM protein transduces signals inside cells leading to the reorganization of the actin cytoskeleton as necessary for integrin clustering (1, 4). These inside-out and outside-in signaling pathways from the growth factor receptor and integrin bring about the formation of focal complexes and focal adhesions. Consistently, we confirmed that both the binding of PDGF to its receptor and the binding of integrin αβ3 to its ECM proteins, vitronectin and fibronectin, were necessary for integrin αβ3 clustering and the formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells. We also showed that Necl-5-integrin αβ3 inter-

lamellipodia and peripheral ruffles were formed, and that they colocalized at peripheral ruffles and focal complexes that were formed over and under lamellipodia, respectively. We showed that Necl-5 did not localize at focal adhesions where integrin αβ3 also localized. The immunofluorescence signal for Necl-5 was observed as fuzzy dot-like structures, which mostly, but not perfectly, overlapped with the dot-like signal for integrin αβ3 at focal complexes. Because Necl-5 did not localize at focal adhesions and it was reported that focal complexes are transformed into focal adhesions by inactivation of Rac and Cdc42 and activation of RhoA (5, 7), Necl-5 might dissociate from integrin αβ3 during this transformation, causing the formation of the fuzzy dot-like structures. The mechanism of dissociation of Necl-5 from integrin αβ3 at focal complexes remains unknown.

We also showed that Necl-5 enhanced integrin αβ3 clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells. Studies on the mode of action of Necl-5 revealed that Necl-5 directly inter-
action is additionally necessary for integrin \(\alpha_v\beta_3\) clustering and morphological changes. Thus, three transmembrane proteins, the PDGF receptor, integrin \(\alpha_v\beta_3\), and Necl-5, physically and functionally associate to regulate integrin \(\alpha_v\beta_3\) clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells. We further showed that the cytoplasmic region of Necl-5 was not necessary for Necl-5-integrin \(\alpha_v\beta_3\) interaction, but that both the extracellular and cytoplasmic regions of Necl-5 were necessary for integrin \(\alpha_v\beta_3\) clustering and formation of these structures.

Rac was previously shown to have a critical role in the dynamic reorganization of the actin cytoskeleton necessary for the formation of focal complexes (6, 8, 38). We previously showed that Necl-5 enhances the serum-induced, integrin \(\alpha_v\beta_3\)-dependent activation of Rac (15). Consistently, we showed here that PDGF-induced Rac activation was necessary for PDGF-induced integrin \(\alpha_v\beta_3\) clustering and morphological changes in NIH3T3 cells. Rac activation alone or Necl-5-integrin \(\alpha_v\beta_3\) interaction alone was insufficient to induce these events; indeed, Rac activation, Necl-5-integrin \(\alpha_v\beta_3\) interaction, and integrin \(\alpha_v\beta_3\)-vitronectin interaction were all necessary for integrin \(\alpha_v\beta_3\) clustering and changes in morphology, although the Rac activation and integrin \(\alpha_v\beta_3\)-vitronectin interaction were sufficient, without Necl-5-integrin \(\alpha_v\beta_3\) interaction, for lamellipodium formation. We noted that these structures were randomly formed at peripheral regions when Rac1CA was expressed instead of being directionally stimulated by PDGF. These results indicate that PDGF-induced Rac activation is necessary for cell polarization and specific localization of lamellipodia, peripheral ruffles, and focal complexes in NIH3T3 cells.

Considering all the evidence thus far available, we propose the following mechanism of integrin \(\alpha_v\beta_3\)

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**FIGURE 9. Necessity of both Rac activation and Necl-5-integrin \(\alpha_v\beta_3\) interaction for PDGF-induced integrin \(\alpha_v\beta_3\) clustering.** Cells were transfected with Myc-tagged Rac1CA and/or siRNA vector, cultured on vitronectin- or laminin-coated \(\mu\)-slide dishes in the absence of PDGF, and triple-stained with the anti-Necl-5 mAb, the anti-integrin \(\beta_3\) mAb, and the anti-Myc mAb. A, wild-type NIH3T3 cells; B, Necl-5-NIH3T3 cells; C, Necl-5-knockdown-NIH3T3 cells; D, Necl-5-\(\Delta\)CP-NIH3T3 cells; Aa, Ba, C, and D, cells cultured on vitronectin; Ab and Bb, cells cultured on laminin. Insets, high magnification of peripheral regions; asterisks, siRNA vector-transfected cells; scale bars, 10 \(\mu\)m. The results shown are representative of three independent experiments.
clustering and formation of lamellipodia, peripheral ruffles, and focal complexes at leading edges in NIH3T3 cells: PDGF reaches and binds to its receptor and induces Rac activation; Rac activated in this way induces reorganization of the actin cytoskeleton, which induces clustering of the Necl-5-integrin αβ₃ complex; the clustered complex then enhances PDGF-induced Rac activation in a positive feedback manner. Such a feedback amplification mechanism might efficiently form leading edges in the direction of higher concentrations of PDGF. We used here only NIH3T3 cells as a model cell, but both Necl-5 and integrin αβ₃ are expressed in the same types of cells, such as other fibroblasts, vascular endothelial cells, colon carcinoma cells, melanoma cells, and glioblastoma cells, this mechanism may be applied to these cell types. Further studies are necessary for generalization of this mechanism.

The exact mechanism of how Necl-5 enhances integrin αβ₃ clustering remains unknown, but one possible explanation is that the Necl-5-integrin αβ₃ interaction induces a conformational change in the cytoplasmic region of integrin αβ₃, making it sensitive to binding talin, which converts the low affinity integrin αβ₃ to the high affinity form. This process and/or the subsequent clustering process might be facilitated by Rac. Another possible explanation is that the cytoplasmic region of Necl-5 makes talin sensitive to binding integrin αβ₃ by an unknown mechanism. It remains unknown how the clustered Necl-5-integrin αβ₃ complex enhances PDGF-induced Rac activation, but signals downstream of the complex might enhance the signaling pathway of the PDGF receptor at a step upstream of Rac. Further studies are necessary to elucidate these unresolved issues.

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