Roles of Necl-5/Poliovirus Receptor and Rho-associated Kinase (ROCK) in the Regulation of Transformation of Integrin $\alpha_v\beta_3$-based Focal Complexes into Focal Adhesions*

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Focal complexes are continuously formed and transformed into focal adhesions during cell movement. We previously demonstrated that Necl-5 co-localizes with integrin $\alpha_v\beta_3$ at focal complexes, whereas Necl-5 does not localize at focal adhesions in moving NIH3T3 cells, suggesting that Necl-5 may be dissociated from integrin $\alpha_v\beta_3$ during the transformation of focal complexes into focal adhesions, but the underlying mechanism remains unknown. Here, we explore the roles of Necl-5 and Rho-associated kinase (ROCK) in the regulation of the transformation of focal complexes into focal adhesions. We found that inhibition of Necl-5 expression and expression of a constitutively active mutant of ROCK1 enhanced, whereas treatment with a ROCK inhibitor Y-27632 inhibited the transformation of focal complexes into focal adhesions. In HEK293 cells ectopically expressing Necl-5 and integrin $\alpha_v\beta_3$, treatment of cells with Y-27632 increased the binding of Necl-5 to clustered integrin $\alpha_v\beta_3$. The experiments using inhibitors of myosin ATPase and actin polymerization revealed that actomyosin-driven contractility exerts a similar function as ROCK. The phosphorylation of integrin $\beta_3$ at Tyr747, which is known to be critical for the formation of focal adhesions, plays a pivotal role for the interaction between Necl-5 and integrin $\alpha_v\beta_3$. These results indicate that the transformation of focal complexes into focal adhesions is negatively and positively regulated by Necl-5 and ROCK, respectively, and that ROCK-dependent actomyosin-driven contractility is a critical determinant for the regulation of the interaction between Necl-5 and integrin $\alpha_v\beta_3$.

Cell movement has a pivotal role for physiological processes such as morphogenesis during development and the mobilization of immune cells to sites of infection as well as pathological events including metastasis of cancer cells. While moving in the direction of increasing or decreasing concentrations of external signaling molecules, cells spatiotemporally form special structures at leading edges, which include protrusions such as filopodia and lamellipodia, ruffles, focal complexes, and focal adhesions. Continuous formation and disassembly of these leading edge structures are necessary for facilitating cells to keep moving. Cells adhere to the extracellular matrix via integrins, cell-matrix adhesion molecules which regulate cell movement and exert their regulatory functions in cooperation with cell surface receptors for chemoattractants. Focal complexes are small dot-like integrin adhesion complexes formed at the edges of lamellipodia (1–3). After binding to matrix, integrins recruit cytoskeletal and cytoplasmic proteins, which leads to the cytoskeletal remodeling and the formation of more stable adhesive structures called focal adhesions. Focal adhesions contain proteins such as Src and focal adhesion kinase, integrin $\beta_v$, vinculin, and paxillin, and tyrosine phosphorylation of these proteins is implicated in the formation and turnover of focal adhesions (for review, see Refs. 4 and 5). Reorganization of the actin cytoskeleton, including actin polymerization and stress fiber formation, is required for cell spreading and locomotion and plays a central role in the formation of leading edge structures. During the migration process, the maturation of small focal complexes to larger focal adhesions at the leading edges requires the tension provided by actomyosin-driven contractility (for review, see Ref. 6).

Rho family small G proteins play a central role in the regulation of the formation of leading edge structures, cytoskeletal dynamics, and the maturation of focal complexes to focal adhesions (3, 7, 8). Rac1 triggers the formation of lamellipodia, ruffles, and focal complexes. Cdc42 is critical for the formation of filopodia and focal complexes. RhoA inhibits the formation of these leading edge structures but enhances the formation of focal adhesions and stress fibers. The transformation of focal complexes into focal adhesions depends on the activation of RhoA and its effector Rho-associated kinase (ROCK)2

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* This work was supported by grants-in-aid for Scientific Research and for Cancer Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (2006, 2007). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: ROCK, Rho-associated kinase; CA, constitutively active; DMEM, Dulbecco’s modified Eagle’s medium; PDGF, platelet-derived growth factor; mAb, monoclonal antibody; pAb, polyclonal antibody; BDM, 2,3-butanedione monoxime; Nef-3, a recombinant extracellular fragment of nectin-3 fused to the human IgG Fc; PBS, phosphate-buffered saline; siRNA, small interfering RNA; GFP, green fluorescent protein; EGFP, enhanced GFP.
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and is negatively regulated by Cdc42 and Rac1. It has been shown that ROCK is involved in the stress fiber formation (9) and regulates the contractility required for the cytoskeletal reorganization by two mechanisms, directly by the phosphorylation of myosin light chain (10) and indirectly by the inactivation of myosin phosphatase through the phosphorylation of the myosin binding subunit of myosin phosphatase (11). Although the ROCK-mediated actin-myosin-driven contractility is likely involved in the transformation of focal complexes into focal adhesions, the molecular mechanism by which ROCK regulates this process remains largely unknown.

We previously reported that Necl-5, an immunoglobulin-like adhesion molecule, also termed as poliovirus receptor/CD155 (17). Necl-5 siRNA and a plasmid expressing a GFP-tagged siRNA-resistant mutant of Necl-5 (GFP-Necl-5R) were kindly gifts from Dr. A. Yamada (KAN Research Institute, Kobe, Japan) (17). Necl-5 siRNA and a plasmid expressing a GFP-tagged siRNA-resistant mutant of Necl-5 (GFP-Necl-5R) were prepared as described (16). Mouse anti-phosphotyrosine mAb (4G10, Upstate Biotechnology), hamster anti-integrin αv and anti-integrin β3 mAbs, mouse anti-integrin αv, mAb (BD Biosciences), rabbit anti-integrin αv, and anti-integrin β3 pAbs (Chemicon), rabbit anti-Myc tag pAb (Cell Signaling), rabbit anti-GFP pAb (Medical and Biological Laboratories), mouse anti-vinculin mAb, mouse anti-FLAG mAb (Sigma-Aldrich), and rabbit phospho-integrin β3 (Tyr747) pAb (Santa Cruz Biotechnology) were purchased from commercial sources. Fluorophore (fluorescein isothiocyanate, Cy3, and Cy5)-conjugated secondary Abs were purchased from Jackson ImmunoResearch. Horseradish peroxidase-conjugated secondary Abs were purchased from Amersham Biosciences. Human recombinant PDGF-BB was purchased from PEP-TECH. Vitronectin was purified from human plasma (Kohjindo bio) as described (18). Y-27632, 2,3-butanedione monoxime (BDM) (Calbiochem), calcyclin A (Alexis Biochemicals), and cytochalasin D (Biomol) were obtained from commercial sources.

Down-regulation of Necl-5—A recombinant extracellular fragment of nectin-3 fused to the human IgG Fc (Nef-3) that efficiently interacts with Necl-5 was used to induce down-regulation of Necl-5 from the cell surface (15). Nef-3 was prepared as described previously (19) and cross-linked by the goat anti-human IgG Fc pAb (Jackson Immuno Research) before use. NIH3T3 cells were sparsely plated on dishes, cultured in the serum-containing medium in the absence of Nef-3 for 18 h, washed, re-plated on vitronectin-coated glass coverslips, and then cultured in the serum-containing medium in the absence of Nef-3 for 6 h. In another series of experiments, NIH3T3 cells were sparsely plated on vitronectin-coated glass coverslips, cultured in the serum-containing medium for 18 h, and then cultured in the serum-containing medium in the presence or absence of Nef-3 for 1 h.

Immunofluorescence Microscopy—Cells were fixed with acetone/methanol (1:1), incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS), and then incubated with 20% BlockAce (Dainihon Sumitomo Seiyaku) in PBS (15). The samples were stained with various combinations of the primary Abs and then with appropriate fluorophore-conjugated secondary Abs. Fluorescence signals were visualized by confocal laser scanning microscopes (LSM510 META, Carl Zeiss; Digital Eclipse C1si-ready, Nikon).

Cell Surface Biotinylation—To analyze the cell surface expression of Necl-5 and integrin αvβ3, cells were incubated with 0.2 mg/ml sulfo-succinimidyl 2-(biotinamido) ethyl-dithiopropionate (sulfo-NHS-SS-biotin) (Pierce) in PBS at 4°C for 30 min and washed by sulfo-NHS-SS-biotin blocking reagent (50 mM NH₄Cl in PBS) to quench free sulfo-NHS-SS-biotin followed by several further washes in PBS. Cells were then frozen by liquid N₂, scraped off, and lysed in 500 µl of IP buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 mM EDTA, 10 µg/ml leupeptin, 2 µg/ml aprotonin, and...
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10 μM 4-aminophenylmethanesulfonyl fluoride hydrochloride. Cell extracts were rotated at 4 °C for 30 min and then divided into a detergent-insoluble pellet and a detergent-soluble supernatant by centrifugation. The supernatant was incubated with streptavidin beads (Amersham Biosciences) at 4 °C for 4 h to collect biotinylated proteins. These samples 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.

Directional Stimulation with PDGF—To generate a concentration gradient of PDGF, a μ-Slide V1 flow (uncoated; Ibidi) was used (14). In brief, the μ-Slide VI flow has six parallel channels which were coated with 5 μg/ml vitronectin according to the manufacturer’s protocol. Cells were plated at a density of 5 × 10⁴ cells per square centimeter, cultured for 16 h, and starved of serum with DMEM containing 0.5% bovine serum albumin for 1 h. The concentration gradient of PDGF was applied using DMEM containing 0.5% bovine serum albumin and 30 ng/ml PDGF for 30 min according to the manufacturer’s protocol.

Time-lapse Fluorescence Microscopy—Time-lapse fluorescence microscopy was performed as follows. NIH3T3 cells transfected with pEGFP-N3-integrin β3 or co-transfected with pEGFP-N3-integrin β3 and pBS-H1-Necl-5 were sparsely plated on vitronectin-coated glass-bottomed dishes, cultured in the serum-containing medium for 18 h, and then cultured in DMEM containing 0.5% bovine serum albumin in the presence or absence of Y-27632 for 1 h. An aliquot of PDGF was applied to the area close to the periphery of cells. Fluorescence signals were visualized by a confocal laser scanning microscope (Digital Eclipse C1si-ready, Nikon), and images were recorded in intervals of 1 min.

Co-immunoprecipitation Assay—Co-immunoprecipitation assays were performed as described (14). In brief, HEK293 cells were transfected with various combinations of plasmids, plated on vitronectin-coated dishes, and cultured overnight. After a 1-h serum starvation or treatment with each reagent, cells were washed with ice-cold PBS and lysed with buffer A (20 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM MnCl₂, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM Na₃VO₄, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μg 4-aminophenylmethanesulfonyl fluoride hydrochloride). In some experiments, NIH3T3 cells, instead of HEK293 cells, were sparsely plated vitronectin-coated dishes and cultured overnight. After a 1-h serum starvation or treatment with Y-27632, cells were washed with ice-cold PBS and treated with 2 mM 3,3′-dithiobis-sulfosuccinimidy propionate (DTSSP, Pierce) in PBS for 2 h. After the addition of Tris-HCl at pH 7.4, cells were washed with ice-cold PBS and lysed with buffer A. The lysates were rotated for 30 min and subjected to centrifugation at 12,000 × g for 20 min. In the experiments using HEK293 cells, the supernatant was preincubated with protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) at 4 °C for 1 h and then incubated with protein G-Sepharose beads pre-bound to the anti-FLAG mAb at 4 °C for 4 h. In the experiments using NIH3T3 cells, the supernatant was preincubated with protein A-Sepharose beads (Amersham Biosciences) at 4 °C for 1 h and incubated with protein A-Sepharose beads pre-bound to the anti-integrin αv pAb at 4 °C for 4 h. After the beads were extensively washed with buffer A, bound proteins were eluted by boiling the beads in SDS sample buffer for 5 min and subjected to SDS-PAGE followed by Western blotting.

RESULTS

Inhibition by Necl-5 of the Transformation of Focal Complexes into Focal Adhesions—We first examined whether Necl-5 is needed for the formation of focal complexes as well as whether the formation of focal complexes is required for the formation of focal adhesions. For this purpose, we used Nef-3 (the extracellular fragment of nectin-3 fused to the Fc portion of IgG) that trans-interacts with Necl-5 and induces its down-regulation from the cell surface (15). When NIH3T3 cells, which were sparsely plated on dishes and cultured in the serum-containing medium for 18 h, were re-plated on vitronectin-coated glass coverslips and then cultured in the medium containing serum for 6 h, most cells formed polarized structures with lamellipodia at the leading edges, consistent with our earlier observations (Figs. 1, Aa and 1Ba) (12, 14). The immunofluorescence signal for phosphotyrosine, an excellent marker for focal complexes and focal adhesions (20), was observed as dot-like structures under the peripheral ruffles and at sites to the rear of the leading edges. The dot-like structures under the peripheral ruffles were smaller in size than those at sites to the rear of the leading edges. These smaller dot-like structures under the peripheral ruffles corresponded to focal complexes, whereas larger dot-like structures at sites to the rear of the leading edges corresponded to focal adhesions. The signals for integrin β3 and Necl-5 were concentrated and co-localized with the signal for phosphotyrosine at focal complexes. In addition, the signal for integrin β3, but not that for Necl-5, was concentrated and co-localized with the signal for phosphotyrosine at focal adhesions. Essentially the same results were obtained when integrin αv was stained instead of integrin β3. Indeed, unless otherwise specified, essentially identical results were obtained for both integrin αv and integrin β3 in the experiments that follow. In NIH3T3 cells stably expressing Nef-5 (Nef-5-NIH3T3 cells), most of the signals for phosphotyrosine, integrin β₃, and Nef-5 were seen at focal complexes, and only faint signals were observed at focal adhesions (Fig. 1Ab), consistent with our earlier results (14). Here, we determined that the smaller dot-like structures observed under the peripheral ruffles, which were immunopositive for Nef-5, integrin αv, integrin β₃, and phosphotyrosine, corresponded to focal complexes, whereas the relatively larger dot-like structures, which were immunopositive for integrin αv, integrin β₃, and phosphotyrosine, but not for Nef-5, corresponded to focal adhesions. However, when wild-type NIH3T3 cells, which were sparsely plated on dishes and cultured in the serum-containing medium in the presence of Nef-3 for 18 h, were re-plated on vitronectin-coated glass coverslips and then cultured in the medium containing serum, but not Nef-3, for 6 h, most cells did not form polarized structures with lamellipodia (Fig. 1Bb). The signals

³ Y. Nagamatsu, Y. Rikitake, M. Takahashi, Y. Deki, W. Ikeda, K.-i, Hirata, and Y. Takai, unpublished data.
FIGURE 1. Inhibition of the transformation of focal complexes into focal adhesions by Necl-5. A, immunofluorescence images of wild-type NIH3T3 and Necl-5-NIH3T3 cells. Cells plated on vitronectin-coated glass coverslips were stained with the anti-phosphotyrosine mAb (PY), the anti-integrin β1 mAb, and the anti-Necl-5 mAb. a, wild-type NIH3T3 cells; b, Necl-5-NIH3T3 cells. Yellow lines, focal complexes; arrowheads, focal adhesions; insets, higher magnification images; scale bars, 10 μm. B, immunofluorescence images of NIH3T3 cells. NIH3T3 cells were preincubated with or without Nef-3 and/or the anti-Necl-5 mAb, washed, and plated on vitronectin-coated glass coverslips. Cells were stained with the anti-Necl-5 mAb, the anti-integrin β1 mAb, and the anti-vinculin mAb. a, NIH3T3 cells; b, Nef-3-treated NIH3T3 cells; c, Nef-3- and anti-Necl-5 mAb-treated NIH3T3 cells. Arrowheads, leading edges; scale bars, 10 μm. C, cell surface expression levels of Necl-5 and integrin αβ3. NIH3T3 cells were preincubated with or without Nef-3, washed, and plated on vitronectin-coated dishes. The amounts of cell surface Necl-5 and integrin αβ3 were analyzed by the biotinylation method. Results shown are representative of three independent experiments. D, immunofluorescence images of NIH3T3 cells. NIH3T3 cells were plated on vitronectin-coated glass coverslips and incubated with or without Nef-3 and/or the anti-Necl-5 mAb in the presence of serum. Cells were stained with the anti-Necl-5 mAb and the anti-integrin β3 mAb. a, NIH3T3 cells; b, Nef-3-treated NIH3T3 cells; c, Nef-3- and anti-Necl-5 mAb-treated NIH3T3 cells. Arrowheads, leading edges; scale bars, 10 μm. E, cell surface expression levels of Necl-5 and integrin αβ3. NIH3T3 cells were plated on vitronectin-coated dishes and incubated with or without Nef-3. The amounts of cell surface Necl-5 and integrin αβ3 were analyzed by the biotinylation method. F, expression levels of Necl-5 and integrin αβ3. NIH3T3 cells were transfected with control non-silencing siRNA or Necl-5 siRNA, and expressions of Necl-5 and integrin αβ3 were analyzed. Results shown are representative of three independent experiments. G, immunofluorescence images of NIH3T3 cells. NIH3T3 cells transfected with Necl-5 siRNA or co-transfected with Necl-5 siRNA and GFP-Necl-5β were plated on vitronectin-coated glass coverslips. Cells were stained with the anti-Necl-5 mAb, the anti-integrin β3 mAb, and the anti-vinculin mAb. a, Necl-5 siRNA-transfected NIH3T3 cells; b, NIH3T3 cells co-transfected with Necl-5 siRNA and GFP-Necl-5β. Note that Necl-5-knockdown cells showed reduced immunofluorescence signals of Necl-5 and integrin β3 (⁎). Arrowheads, leading edges; scale bars, 10 μm.
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A Y-27632 (-)

B Y-27632 (+)

FIGURE 2. Inhibition of the transformation of focal complexes into focal adhesions by a ROCK inhibitor. A and B, immunofluorescence and differential interference contrast (DIC) images of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated μ-slide dishes. Cells were stained with the anti-Necl-5 mAb and the anti-integrin β3 mAb. A, wild-type NIH3T3 cells; B, NIH3T3 cells treated with 10 μM Y-27632 for 1 h. Arrowheads, leading edges; insets, higher magnification images of leading edges; scale bars, 20 μm. The results shown are representative of three independent experiments.

for Necl-5 and integrin β3 were hardly detected at any peripheral regions. The signal for integrin β3 was markedly reduced, but the signal for vinculin, another marker for focal adhesions, was clearly observed, indicating that integrin αvβ3-positive focal adhesions were reduced, but vinculin-positive focal adhesions were definitely formed. When cells were co-incubated with the anti-Necl-5 mAb that inhibits the interaction of Nef-3 with Necl-5 (15), the effect of Nef-3 was absent; the signals for Necl-5 and integrin were concentrated and co-localized at focal complexes, which was similar to those in cells untreated with Nef-3 (Fig. 1Bc). A biotinylation assay showed that Nef-3 decreased the amount of cell surface Necl-5 but did not affect the amount of cell surface integrin αv or integrin β3, indicating that integrin αv and integrin β3 remained on the cell surface without being endocytosed (Fig. 1C). These results indicate that when the amount of cell surface Necl-5 is reduced by down-regulation, the formation of both focal complexes and focal adhesions are markedly reduced.

We then examined whether Necl-5 inhibits the transformation of focal complexes into focal adhesions. When NIH3T3 cells were sparsely plated on vitronectin-coated glass coverslips and cultured in the presence of serum for 19 h, many cells formed polarized structures with lamellipodia (Fig. 1Da). However, when cells were sparsely plated on vitronectin-coated glass coverslips and cultured for 18 h and then incubated with Nef-3 for 1 h, they did not form polarized structures with lamellipodia. The signals for Necl-5 and integrin β3 were hardly observed at any peripheral regions (Fig. 1Db). The signal for integrin β3 was concentrated at focal adhesions and markedly increased in comparison with that in cells cultured without Nef-3. The signal for Necl-5 was not observed at focal adhesions. Furthermore, when cells were co-incubated with the anti-Necl-5 mAb, the effect of Nef-3 was cancelled; the signals for Necl-5 and integrin β3 were concentrated and co-localized at focal complexes (Fig. 1Dc). The amounts of integrin αv and integrin β3 remained unchanged by Nef-3 despite the reduction of the amount of Necl-5 (Fig. 1E). These results indicate that when Necl-5 is down-regulated by Nef-3 treatment after both focal complexes and focal adhesions are formed, the Nef-3-induced down-regulation of Necl-5 disassembles the clustering of integrin αvβ3 at focal complexes. This leads to diminish focal complexes but enhances focal adhesion formation. We applied another approach, the RNA interference method, to confirm the results of the experiment using Nef-3. We successfully knocked down Necl-5 by the use of Necl-5 siRNA without silencing integrin αvβ3 expression (Fig. 1F). Necl-5-knockdown cells did not form polarized structures with lamellipodia, and the signal for integrin β3 was reduced (see Fig. 1Bb); however, this phenotype was reversed by an expression of a GFP-tagged siRNA-resistant mutant of Necl-5 (GFP-Necl-5(R)) (Fig. 1G, a and b). Taken together, these results indicate that Necl-5 inhibits the transformation of focal complexes into focal adhesions and suggest that Necl-5 is dissociated from integrin αvβ3 during this transformation.

Inhibition of the Transformation of Focal Complexes into Focal Adhesions by a ROCK Inhibitor—To examine whether ROCK activation is necessary for the transformation of focal complexes into focal adhesions during directional cell movement, cells were sparsely plated on μ-slide VI flow dishes pre-coated with vitronectin, starved of serum, and directionally stimulated by PDGF in the presence or absence of a ROCK inhibitor Y-27632. In the absence of Y-27632, most cells became polarized and formed lamellipodia with peripheral ruffles at the leading edges toward higher concentrations of PDGF. The immunofluorescence signals for Necl-5 and integrin β3 were concentrated and co-localized at peripheral ruffles of the leading edges in the middle section of the cells (results not shown), consistent with our earlier observations (12, 14). In the basal section of the cells, the signals for Necl-5 and integrin β3 were observed at focal complexes under peripheral ruffles (Fig. 2A). In the presence of Y-27632, however, the signal for integrin β3 was enhanced and accumulated at focal complexes with a linear staining pattern (Fig. 2B). The signal for integrin β3 at focal adhesions became weaker than that in the absence of Y-27632 (see Fig. 2A). These results indicate that ROCK activation is necessary for the transformation of focal complexes into focal adhesions.

Enhancement of the Transformation of Focal Complexes into Focal Adhesions by a Constitutively Active Mutant of ROCK1—We then examined whether a constitutively active mutant of ROCK1 (ROCK1-CA) enhances the transformation of focal complexes into focal adhesions. Transfection of cells...
with ROCK1-CA decreased the focal complex formation and conversely increased the focal adhesion formation (Fig. 3A). Identical results were obtained when Necl-5-NIH3T3 cells, where the formation of focal complexes were enhanced (14), were used instead of wild-type NIH3T3 cells (Fig. 3B). These results indicate that ROCK activation enhances the transformation of focal complexes into focal adhesions.

Effects of a ROCK Inhibitor and Knockdown of Necl-5 on Integrin αβ3 Dynamics—To investigate the roles of ROCK and Necl-5 in integrin αβ3 dynamics in living cells, we performed time-lapse fluorescence microscopy in NIH3T3 cells transfected with EGF-tagged integrin β3 (integrin β3-EGFP), which was reported to behave like endogenous integrin αβ3 in association with endogenous integrin αv (7). When cells were sparsely plated on vitronectin-coated glass-bottomed dishes, clusters of integrin β3 were observed. When cells moved forward in response to PDGF, small clusters of integrin β3 appeared at the leading edge (Fig. 4A). These clusters remained stationary but maturated into larger clusters when cells moved, indicating that focal complexes were transformed into focal adhesions. In contrast, in the presence of Y-27632, small clusters continuously appeared at peripheral areas but immediately disappeared, indicating that focal complexes failed to mature into focal adhesions (Fig. 4B). In Necl-5-knockdown-NIH3T3 cells, the formation of integrin clusters was hardly observed (Fig. 4C). This is consistent with the result of the experiment using Nef-3 (see Fig. 1Bb). Taken together, these results indicate that ROCK regulates the transformation of focal complexes into focal adhesions and that Necl-5 is needed for the formation of both focal complexes and focal adhesions.

Involvement of ROCK in the Dissociation of Necl-5 from Clustered Integrin αβ3—We then examined whether ROCK regulates the dissociation of Necl-5 from clustered integrin αβ3 that binds to vitronectin. When NIH3T3 cells were cultured on vitronectin-coated dishes and endogenous integrin αv was immunoprecipitated by an anti-integrin αv pAb from cell lysates, the amount of co-immunoprecipitated Necl-5 was increased in the presence of Y-27632 compared with that in the absence of the ROCK inhibitor (Fig. 5A). When HEK293 cells

FIGURE 3. Enhancement of the transformation of focal complexes into focal adhesions by a constitutively active mutant of ROCK1. A and B, immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated μ-slide dishes. Cells were transfected with Myc or Myc-ROCK1-CA and stained with the anti-Myc tag mAb, the anti-Necl-5 mAb, and the anti-integrin β3 mAb. A, wild-type NIH3T3 cells; B, Necl-5-NIH3T3 cells. Arrowheads, leading edges; insets, higher magnification images of leading edges; scale bars, 20 μm. The results shown are representative of three independent experiments.

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ectopically expressing integrin αv, integrin β3-EGFP, and FLAG-tagged Necl-5 (FLAG-Necl-5) were cultured on vitronectin-coated dishes and FLAG-Necl-5 was immunoprecipitated by an anti-FLAG mAb from cell lysates, integrin αv, integrin β3-EGFP, and FLAG-Necl-5 were co-immunoprecipitated (Fig. 5B). The amounts of co-immunoprecipitated integrin αv and integrin β3-EGFP were increased in the presence of Y-27632 compared with those in the absence of the ROCK inhibitor. These results indicate that ROCK activation may induce the dissociation of Necl-5 from clustered integrin αvβ3.

To examine whether the cytoplasmic region of Necl-5 is necessary for the ROCK-induced dissociation of Necl-5 from the clustered integrin αvβ3, we expressed integrin αv, integrin β3-EGFP, and FLAG-Necl-5 in which the cytoplasmic region was deleted (FLAG-Necl-5-ΔCP) in HEK293 cells and performed similar experiments to those described above. Both integrin αv and integrin β3-EGFP were co-immunoprecipitated with FLAG-Necl-5-ΔCP, and the amounts of co-immunoprecipitated integrin αv and integrin β3-EGFP were increased by treatment with Y-27632 (Fig. 5B). This result suggests that integrin αvβ3, but not Necl-5, is likely a target for the ROCK-induced dissociation of Necl-5 from the clustered integrin αvβ3.

Involvement of Myosin in the Dissociation of Necl-5 from Clustered Integrin αvβ3—Because ROCK induces phosphorylation of myosin light chain through the phosphorylation and inactivation of myosin phosphatase (11), we then examined whether myosin-driven contractility is involved downstream of ROCK in the dissociation of Necl-5 from clustered integrin αvβ3 that binds to vitronectin. We showed that the amounts of co-immunoprecipitated integrin αv and integrin β3-EGFP with FLAG-Necl-5 were increased in the presence of BDM, a myosin ATPase inhibitor (21) (Fig. 5C). In the presence of calyculin A that inhibits myosin phosphatase (22), the amounts of co-immunoprecipitated integrin αv and integrin β3-EGFP were decreased (Fig. 5D).

Taken together, these results indicate that myosin-driven contractility plays a role downstream of ROCK in the dissociation of Necl-5 from clustered integrin αvβ3.

Involvement of Actin Polymerization in the Dissociation of Necl-5 from Clustered Integrin αvβ3—To determine the role of actin polymerization in the dissociation of Necl-5 from clus-
The phosphorylation level of integrin \( \alpha_5 \beta_3 \) was reduced in Necl-5-NIH3T3 cells as compared with that in wild-type NIH3T3 cells (Fig. 6A). Western blotting using the anti-FLAG mAb, the anti-integrin \( \alpha_5 \) mAb, and the anti-GFP pAb. B, Y-27632; C, BDM; D, calyculin A; E, cytochalasin D. The results shown are representative of two independent experiments.

To test the possibility that the phosphorylation of integrin \( \beta_3 \) at Tyr747 is critical for the interaction with Necl-5, we compared the effects of wild-type integrin \( \beta_3 \) and the Y747A mutant of integrin \( \beta_3 \) in which Tyr747 was replaced to Ala (23). When wild-type integrin \( \beta_3 \)-EGFP or EGFP-tagged Y747A mutant of integrin \( \beta_3 \) was co-expressed with integrin \( \alpha_v \) and FLAG-Necl-5 in HEK293 cells and FLAG-Necl-5 was immunoprecipitated by the anti-FLAG mAb from cell lysates, the amounts of co-immunoprecipitated integrin \( \alpha_v \) and integrin \( \beta_3 \) were markedly increased in Y747A mutant-transfected cells (Fig. 6B). Notably, Y-27632 had no effect on the amounts of co-immunoprecipitated integrin \( \alpha_v \) and integrin \( \beta_3 \) in Y747A mutant-transfected cells. Collectively, these results indicate that the phosphorylation of integrin \( \beta_3 \) at Tyr747 is critical for the interaction with Necl-5.
action of Necl-5 with integrin αvβ3. In other words, Necl-5 prefers to bind unphosphorylated integrin β3 and prevents its phosphorylation. Once ROCK is activated, activated ROCK induces the dissociation of Necl-5 from clustered integrin αvβ3.

**DISCUSSION**

We showed here that the down-regulation of Necl-5 by pre-incubation with Nef-3 and knockdown of Necl-5 inhibited the formation of polarized structures, focal complexes, and focal adhesions, confirming that Necl-5 regulates the formation of focal complexes and focal adhesions (14). Although integrin β3-positive focal adhesions were reduced in association with reduced Necl-5 expression, vinculin-positive focal adhesions were formed. This result indicates that, although integrins other than integrin αvβ3 may be involved in the formation of vinculin-positive focal adhesions, Necl-5 definitely regulates the formation of integrin αvβ3-based focal adhesions. We previously showed that Necl-5 enhanced the clustering of integrin αvβ3 by cis-interacting with integrin αvβ3, forming the Necl-5-integrin αvβ3 complex (14). The formation of this complex facilitates the integrin-mediated signals, including Rac and Cdc42, which are required for the focal complex formation. Consistently, the PDGF-induced activation of Rac is enhanced and reduced in Necl-5-NIH3T3 cells and Necl-5-knockdown-NIH3T3 cells, respectively (14). Thus, Necl-5 is indispensable for the focal complex formation by regulating the integrin-mediated “outside-in” signals. In addition, we showed here that the down-regulation of Necl-5 by incubation with Nef-3 after cells formed focal complexes and focal adhesions reduced the focal complex formation but enhanced the focal adhesion formation, indicating the negative regulation of the transformation of focal complexes into focal adhesions by Necl-5. This is consistent with the results of our earlier paper in which the number of focal adhesions was reduced in Necl-5-NIH3T3 cells as compared with that in wild-type NIH3T3 cells (14). The reduced phosphorylation of integrin β3 at Tyr747 in Necl-5-NIH3T3 cells in comparison with that in wild-type NIH3T3 cells indicates that Necl-5 inhibits the focal adhesion formation. Because the inactivation of Rac and the phosphorylation of integrin β3 are required for the formation of focal adhesions, the Necl-5-induced activation of Rac and inhibition of the phosphorylation of integrin β3 are likely involved in the Necl-5-induced inhibition of the formation of focal adhesions. Thus, the dissociation of Necl-5 from the clustered integrin αvβ3 is favorable for the transformation of focal complexes into focal adhesions.

The ROCK-dependent actomyosin-driven contractility plays an important role in the dissociation of Necl-5 from integrin αvβ3. To lead this conclusion, we used two strategies. In the first we performed an immunofluorescence study and revealed that treatment with Y-27632 and an expression of ROCK1-CA increased and decreased co-localization of Necl-5 with integrin β3 at focal complexes, respectively. In agreement with our results, previous reports showed that cells treated with Y-27632 accumulate focal complexes (7, 20). The second study is of co-immunoprecipitation assays, demonstrating that treatment with Y-27632 increases the amount of co-immunoprecipitated integrin αvβ3 with Necl-5. We further studied whether downstream events of ROCK, such as myosin light chain phosphorylation and actin polymerization, are involved in the dissociation of Necl-5 from integrin αvβ3 and found that the actin-myosin-driven contractility functions downstream of ROCK to induce the dissociation of Necl-5 from integrin αvβ3. Integrin αvβ3 rather than Necl-5 is likely a target for the ROCK-mediated signaling to regulate the dissociation of Necl-5 from integrin αvβ3 because the effect of inhibition of ROCK on the amount of co-immunoprecipitated integrin αvβ3 was likewise observed when Necl-5-ΔCP, instead of Necl-5, was transfected. In contrast to the effects on clustered integrin αvβ3, inhibition of ROCK had no effect on the amount of co-immunoprecipitated non-clustered integrin αvβ3 with Necl-5 (data not shown). In addition, the Y747A mutant of integrin β3 in which Tyr747 was replaced to Ala was more difficult to dissociate from clustered integrin αvβ3, by the action of ROCK compared with wild-type integrin β3. These results further provide the evidence to support the hypothesis that integrin αvβ3, rather than Necl-5 is a plausible target for the ROCK-mediated signaling in terms of the regulation of the dissociation of Necl-5 from integrin αvβ3.

The molecular mechanism by which ROCK-dependent actomyosin-driven contractility regulates the dissociation of Necl-5 from integrin αvβ3 is currently unknown. Binding to the extracellular matrix promotes the clustering of integrin and further conformational changes that are transmitted to the cytoplasmic tails, which results in the recruitment and anchoring of integrin-associated proteins that connect the actin cytoskeleton (outside-in signaling). On the other hand, cytoskeleton contractility amplifies integrin binding to the extracellular matrix by a positive-feedback mechanism (“inside-out signaling”). Our results suggest that the ROCK-dependent actomyosin-driven contractility may affect the activity and/or conformation of clustered integrin αvβ3, and result in the alteration of its affinity to Necl-5. The functionally important role of stress fibers for the formation of focal adhesions has been reported as the focal adhesion formation is inhibited by BDM or cytochalasin D (24–26). Actomyosin-driven forces appear to be crucial for the recruitment of integrin-associated molecules to the cytoplasmic tails of integrins, and the assembly of an actin-myosin connection with the intracellular domains of integrin is important for the maturation of focal adhesions. It is, therefore, likely that the assembly and disassembly of the interaction of integrins with the actin cytoskeleton control their activities. However, disruption of integrin clustering is an unlikely mechanism because it was reported that treatment of leukocytes with Y-27632 does not influence integrin clustering (27). It remains to be determined whether competitive binding of integrin-interacting molecules or reorganization of the integrin complex might be involved. Future studies will be needed to clarify the molecular mechanism of how the ROCK-dependent actomyosin-driven contractility changes the affinity between Necl-5 and integrin αvβ3.

Acknowledgments—We thank Dr. J. C. Norman (University of Leicester, Leicester, UK), Dr. S. Narumiya (Kyoto University Graduate School of Medicine, Kyoto, Japan), and Dr. A. Yamada (KAN Research Institute, Kobe, Japan) for providing plasmids and siRNA.
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