Common signaling pathway is used by the *trans*-interaction of Necl-5/Tage4/PVR/CD155 and nectin, and of nectin and nectin during the formation of cell–cell adhesion

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Nectin is a Ca^{2+}-independent Ig-like cell–cell adhesion molecule that forms homo- and hetero-*trans*-dimers (*trans*-interaction). Nectin first forms cell–cell adhesions and then recruits cadherin to the nectin-based cell–cell adhesion sites to form AJ cooperatively with cadherin. In addition, the *trans*-interaction of nectin and nectin induces the activation of Cdc42 and Rac small G proteins, which enhances the formation of AJ. The activation of Cdc42 and Rac by the *trans*-interaction of nectin and nectin is mediated by c-Src, another small G protein, Rap1, a Cdc42-GEF, FRG, and a Rac-GEF, Vav2. Necl-5/Tage4/PVR/CD155 is another Ca^{2+}-independent Ig-like molecule, which does not homophilically *trans*-interact, but heterophilically *trans*-interacts with nectin-3, one member of the nectin family. We show here that the *trans*-interaction of Necl-5 and nectin-3 bidirectionally induces the activation of Cdc42 and Rac. Similarly to the activation of Cdc42 and Rac by the *trans*-interaction of nectin and nectin, the *trans*-interaction of Necl-5 and nectin-3 first recruits and activates c-Src at the Necl-5/nectin-3-based cell–cell contact sites. c-Src then phosphorylates FRG and Vav2, and the tyrosine-phosphorylated FRG and Vav2 are recruited to the Necl-5/nectin-3-based cell–cell contact sites. The *trans*-interaction of Necl-5 and nectin-3 also activates Rap1 through C3G, a Rap-GEF, and this activation of Rap1 is required for the activation of Cdc42 and Rac. These results indicate that the *trans*-interactions of Necl-5 and nectin-3 and of nectin and nectin induce the activation of Cdc42 and Rac through the common signaling molecules c-Src, Rap1, FRG, and Vav2. (*Cancer Sci* 2005; *96*: 578–589)

Necl-5/Tage4/PVR/CD155 is a newly discovered molecule that regulates cell movement and proliferation.[1–3] Human PVR/CD155 was originally identified as the human poliovirus receptor,[4,5] and rodent Tage4 was originally identified as the product of a gene overexpressed in rodent colon carcinoma.[6,7] PVR/CD155 was subsequently shown to be overexpressed in many human cancer cells.[8–10] Necl-5 is one member of the Necl family, which consists of five members, Necl-1, -2, -3, -4, and -5.[1] The Necl family is a group of Ig-like molecules, which have domain structures that are similar to, but not quite identical with, those of nectin. Nectin is a Ca^{2+}-independent Ig-like cell–cell adhesion molecule found at AJ.[1,11] The nectin family contains four members, which have three Ig-like loops in their extracellular regions, one transmembrane segment, and one cytoplasmic region. Each nectin first forms homo-*cis*-dimers and then homo- or hetero-*trans*-dimers (*trans*-interactions) through the extracellular region in a Ca^{2+}-independent manner, causing cell–cell adhesion. Nectin recruits cadherin to the nectin-based cell–cell adhesion sites to cooperatively form AJ in epithelial cells and fibroblasts. In contrast to nectin, Necl-5 does not have homophilic cell–cell adhesion activity.[12,13] The cytoplasmic tail of nectin is associated with the actin cytoskeleton through afadin, an F-actin-binding and nectin-binding protein, whereas the cytoplasmic region of Necl-5 is associated with Tctex-1, a subunit of the dynein motor complex,[14] but not with afadin.[13]

Although the role of Necl-5 as the PVR has been established, its physiological role remained unknown for a long time. We have recently found that it regulates cell movement and proliferation.[2,3] Necl-5 is functionally associated with integrin $\alpha_V\beta_3$ at the leading edges of moving cells, for example in L cells stably expressing Necl-5 and V12Ras-NIH3T3, and also enhances the movement induced by growth factors, for example PDGF and FGF, in an integrin-dependent manner.[2] Necl-5 enhances the growth factor-induced activation of Cdc42 and Rac, causing the formation of filopodia and lamellipodia, respectively, which eventually enhances cell movement. Necl-5 enhances not only cell movement, but also the proliferation induced by growth factors such as PDGF and FGF in many cell lines, for example NIH3T3 cells and V12Ras-NIH3T3 cells.[3] Necl-5 upregulates and downregulates cell cycle regulators, including cyclins D2 and E, and $p27^{kip1}$, through the activation of Ras-Raf-MEK-ERK signaling, and thereby

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Abbreviations: AJ, adherens junctions; CRIB domain, Cdc42/Rac interactive binding domain; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; F-actin, actin filament; FGF, fibroblast growth factors; FRG (ΔDHPH), FRG lacking the DH and PH domains; GEF, GDP/GTP exchange factor; GST, glutathione-S-transferase; Ig, immunoglobulin; Lef-5, the extracellular fragment of Necl-5 fused to the Fc portion of IgG; mAb, monoclonal antibody; Necl, nectin-like molecule; Nef-3, the extracellular fragment of nectin-3 fused to the Fc portion of IgG; pAb, polyclonal antibody; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PVR, poliovirus receptor; RBD, Ras binding domain; SDS, sodium dodecylsulfate; SFK, Src family kinases; V12Ras-NIH3T3, NIH3T3 cells transformed by an oncogenic Ki-Ras; Vav2 (ΔDHPH), Vav2 lacking the DH and PH domains.

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shortens the period of the G_1 phase of the cell cycle. Necl-5 is upregulated in V12Ras-NIH3T3 cells, and this upregulation is mediated by the transcriptional activation of the Necl-5 gene through the V12Ras-Raf-MEK-ERK-AP-1 pathway.[15]

Although Necl-5 does not homophilically *trans*-interact, it has been shown that Necl-5 heterophilically *trans*-interacts with nectin-3.[13,16] We have recently found that this interaction between Necl-5 and nectin-3 causes downregulation of Necl-5 on the cell surface by endocytosis in a cell density-dependent manner in NIH3T3 cells and that this downregulation reduces cell movement and proliferation.[17] As cultured cells become confluent, they form cell–cell adhesions and reduce and finally stop cell movement and proliferation.[18,19] This phenomenon has been known for a long time as the contact inhibition of cell movement and proliferation. We propose that the interaction between Necl-5 and nectin-3 and the subsequent downregulation of Necl-5 may be involved in this contact inhibition. Transformation of cells causes the disruption of cell–cell adhesion, an increase of cell movement, and loss of contact inhibition of cell movement and proliferation, eventually leading transformed cells to uncontrolled cell proliferation, invasion into surrounding tissues, and finally metastasis to other organs.[20] We have shown that upregulated Necl-5 at least partly contributes to the enhancement of cell movement and proliferation and to the loss of contact inhibition in transformed cells.[17]

In addition to the cell–cell adhesion activity of nectin, the *trans*-interaction of nectin and nectin induces the activation of Cdc42 and Rac small G protein, which play an important role in the formation of AJ in cooperation with cadherin.[21–25] Nectin first recruits and activates c-Src at the nectin-based cell–cell adhesion sites. c-Src then tyrosine-phosphorylates FRG, Cdc42-GEF.[23] In addition, c-Src induces the activation of C3G Rap1-GEF through the Crk adaptor protein, resulting in activation of Rap1 small G protein.[24] Rap1 then induces the activation of the tyrosine-phosphorylated FRG locally at the nectin-based cell–cell adhesion sites, eventually causing the activation of Cdc42. Moreover, c-Src tyrosine-phosphorylates Vav2 Rac-GEF.[25] Cdc42 activates tyrosine-phosphorylated Vav2 locally at the nectin-based cell–cell adhesion sites. Cdc42 then increases the number of filopodia and cell–cell contact sites. Rac induces the formation of lamellipodia, which efficiently expands the cell–cell adhesion between filopodia, acting like a 'zipper'. In this way, these small G proteins enhance the formation of AJ.

We recently found that the *trans*-interaction of Necl-5 and nectin-3 is involved in cell–cell adhesion at least at the initial stage of the formation of AJ in L cells expressing Necl-5 and nectin-3.[26] Therefore, we set out to examine whether the *trans*-interaction of Necl-5 and nectin-3 induces the activation of Cdc42 and Rac. Our results indicate that the *trans*-interaction of Necl-5 and nectin-3 bidirectionally induces a signaling pathway similar to that induced by the *trans*-interaction of nectin and nectin.

Materials and Methods

Vector construction and protein purification

The cDNA of FRG/KIAA0793 and the full-length mouse Vav2 cDNA were kindly provided by Dr T. Nagase (Kazusa DNA research Institute, Chiba, Japan) and Dr X.R. Bustelo (State University of New York, Stony Brook, NY, USA), respectively. Expression vectors for GFP-V12Rac1 (pEGFP-V12Rac1), GFP-V12Cdc42 (pEGFP-V12Cdc42), GFP-FRG lacking the DH and PH domains (pEGFP-FRG [ΔDHPH]), Flag-FRG (pEFBOS-Flag-FRG), Myc-FRG (pEFBOS-Myc-FRG), and wild-type c-Src (pcDNA-Src-wt), were prepared as described.[23] Flag-Vav2 (pEFBOS-Flag-Vav2), Myc-Vav2 (pCIneo-myc-Vav2), and Myc-Vav2 lacking the DH and PH domains (pCIneo-myc-Vav2 [ΔDHPH]) were prepared as described previously.[27] Expression vectors for GFP-Rap1GAP (pEGFP-Rap1GAP), Crkl-W169L (pIRM21-Flag-CrkI-W169L), and C3G-dCD (pCAGGS-C3G-dCD) were prepared as described previously.[24] Expression vectors for the GST-RalGDS-Ras-binding domain (pGEX-RalGDS-RBD) and the GST-PAK-Cdc42/Rac interactive binding domain (pGEX-PAK-CRIB) were kindly provided by Dr A. Kikuchi (Hiroshima University, Hiroshima, Japan) and Dr T. Takenawa (Tokyo University, Tokyo, Japan), respectively. All the constructs used here were confirmed by sequencing. The GST-RalGDS-RBD fusion protein (Amersham Biosciences) and GST-PAK-CRIB fusion protein (Amersham Biosciences) were overexpressed and purified from *Escherichia coli* according to the manufacturer's protocol. Nef-3 and Lef-5 were prepared as described previously.[13,28] Protein concentrations were determined by using the BCA protein assay kit (Pierce) with bovine serum albumin as a reference protein.

Cell lines and transfection

L cells stably expressing exogenous Necl-5, Necl-5-ΔCP (Necl-5 of which cytoplasmic region is deleted), or nectin-3 (Necl-5-L, Necl-5-ΔCP-L, or nectin-3-L cells, respectively) were prepared as described previously.[2,28] Transfection and immunofluorescence microscopy of cultured cells with a confocal imaging system (Radiance 2000, Bio-Rad Laboratories) were performed as described previously.[29]

Antibodies

Rat anti-nectin-3 mAb, rat anti-Necl-5 mAb (1A8-8), and rabbit anti-Necl-5 pAb were prepared as described previously.[13,28] Rabbit antiphospho-Src (Tyr416) pAb (Cell Signaling Technology), mouse anti-v-Src mAb (Calbiochem-Novabiochem), mouse anti-Cdc42 mAb (BD Transduction Laboratories), rabbit anti-Rap1/Krev-1 pAb (Santa Cruz Biotechnology), mouse antiphosphotyrosine (pY20) mAb (BD Transduction Laboratories), mouse anti-Flag mAb (Sigma), mouse anti-Rac mAb (Upstate), mouse anti-Myc mAb (Santa Cruz), and secondary Abs (Chemicon) were purchased from commercial sources.

Assays for formation of filopodia and lamellipodia

The formation of filopodia and lamellipodia was assayed as described previously.[21] For inhibition of the activity of SFK, the cells were treated with 0.2% DMSO, 20 μM PP2 (Calbiochem-Novabiochem) or 20 μM PP3 (Calbiochem-Novabiochem) in DMEM for 1 h before the trypsin–EDTA treatment and during the culture on the Nef-3-, Lef-5-, or IgG-coated coverslips. The cells were fixed, immunostained with the rhodamine–phalloidin, and observed by using a confocal microscope with a 60 × oil immersion objective lens.

Pull-down assay

The pull-down assay was performed as described previously.[23] In brief, 26 μg of Nef-3, Lef-5, or human IgG (Sigma) was clustered using 9 μg of antihuman IgG pAb (Fc specific; Sigma) in 50 μL of PBS at room temperature for 1 h. After 16 h serum starvation, the medium was replaced by 1 mL of DMEM containing clustered Nef-3, Lef-5, or IgG, and the cells were incubated for the indicated periods of time. The cells were then washed with 1 mL of ice-cold PBS containing 1 mM sodium vanadate, lysed in buffer A (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate) containing 10 μg GST-fusion proteins, GST-RalGDS-RBD for Rap1, or GST-PAK-CRIB for Cdc42 and Rac, and incubated at 2°C for 30 min. The cell extract was obtained by centrifugation at 20 000 *g* at 0°C for 5 min and incubated with 50 μL of glutathione-agarose beads (Amersham Biosciences) at 2°C for 1 h. After the beads were washed with buffer A, proteins bound to the beads were eluted with Laemmli buffer and subjected to SDS–PAGE, followed by western blotting.

Assay for coprecipitation of c-Src with nectin and Necl-5 and for bead–cell contact

Protein A-bound Dynabeads (1×10^8, 2.8-μm diameter; Dynal Biotech ASA) were washed with PBS and incubated with 45 μg of Nef-3, Lef-5, or IgG in 100 μL of PBS at room temperature for 2 h. Necl-5-L or nectin-3-L cells (5×10^5 cells per 60-mm dish) were transfected with pcDNA3-Src-wt. After 12 h of culture, the cells were moved to another 60-mm dish and further cultured for 12 h. After the culture, Nef-3-, Lef-5-, or IgG-coated magnetic beads (5×10^7 per dish) were added to the medium in the dish and incubated for 15 min. After incubation, the cells were washed with ice-cold PBS containing 1 mM sodium vanadate and harvested with buffer B (20 mM HEPES/KOH, pH 7.3, 100 mM NaCl, 10 μL/mL leupeptin, 10 μg/mL aprotinin, 10 μM [p-amidinophenyl] methanesulfonyl fluoride hydrochloride, 20 mM β-glycerophosphate/Na, 10 mM sodium fluoride, 1 mM sodium vanadate, and phosphatase inhibitor cocktail I [Sigma]). After sonication, the Nef-3-, Lef-5-, or IgG-coated magnetic beads were collected by using a magnetic particle concentrator. The beads were washed with buffer B containing 1% Triton X-100 three times. Proteins bound to the Nef-3-, Lef-5-, or IgG-coated magnetic beads were used for western blotting. The bead–cell contact was assayed as described previously.[22] Briefly, latex-sulfate microbeads coated with Nef-3, Lef-5, or IgG were added onto Necl-5-L or nectin-3-L cells cultured in DMEM containing 10% fetal calf serum. After incubation, the cells were fixed and immunostained.

Assay for tyrosine phosphorylation of FRG and Vav2

The immunoprecipitation assay was performed as described previously,[30] with some modifications. Necl-5-L or nectin-3-L cells (1.5×10^6 cells per 100-mm dish) were transfected with pcDNA-Src-wt and pEFBOS-Flag-FRG or pEFBOS-Flag-Vav2. After 12 h of culture, the cells were serum-starved for 24 h. The medium was then replaced by DMEM containing clustered Nef-3, Lef-5, or IgG, and the cells were incubated for 30 min. The cells were washed with ice-cold PBS containing 1 mM sodium vanadate, lysed in 0.5 mL of buffer C (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μM [p-amidinophenyl] methanesulfonyl fluoride hydrochloride, 20 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium vanadate, and phosphatase inhibitor cocktail I), and incubated on ice for 15 min. The cell extract (750 μg of protein) was obtained by centrifugation at 20 000 *g* at 4°C for 15 min, incubated with the anti-Flag mAb at 4°C for 1 h, and further incubated with protein G-Sepharose beads (Amersham Biosciences) for 1 h. After the incubation, the beads were washed with buffer C. Proteins bound to the beads were used for western blotting with the antiphosphotyrosine, anti-Flag, and anti-v-Src mAbs.

Results

Formation of filopodia and lamellipodia by the *trans*-interaction of Necl-5 and nectin-3

The *trans*-interaction of nectin and nectin induces the activation of Cdc42 and Rac, which then form filopodia and lamellipodia, respectively.[21,23] We first examined whether the *trans*-interaction of Necl-5 and nectin-3 induces the formation of filopodia and lamellipodia. For this purpose, we used nectin-3-L cells, Necl-5-L cells, Nef-3, and Lef-5. When nectin-3-L cells were cultured on the Lef-5-coated coverslips, both filopodia and lamellipodia were markedly formed within 60 min (Fig. 1a). When Necl-5-L cells were cultured on the Nef-3-coated coverslips, both filopodia and lamellipodia were also markedly formed within 60 min. These protrusions were negligibly formed when nectin-3-L or Necl-5-L cells were cultured on the coverslips coated with IgG as a control. When wild-type L cells were cultured on the Lef-5- and Nef-3-coated coverslips, filopodia and lamellipodia were formed to much lesser extents (data not shown). These results indicate that the *trans*-interaction of Necl-5 and nectin-3 bidirectionally induces the formation of filopodia and lamellipodia.

We have previously shown that the activation of Cdc42 and Rac by the *trans*-interaction of nectin and nectin requires their cytoplasmic region, but not their association with afadin.[21] We next examined whether the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3 requires the cytoplasmic tail of Necl-5. When Necl-5-L cells were cultured on the Nef-3-coated coverslips, both filopodia and lamellipodia were also markedly formed (Fig. 1b). However, when L cells expressing Necl-5-ΔCP were cultured on the Nef-3-coated coverslips, these protrusions were negligibly formed (Fig. 1b). This result indicates that the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3 requires the cytoplasmic region of Necl-5.

Involvement of Cdc42 and Rac in the formation of filopodia and lamellipodia, respectively, induced by the *trans*-interaction of Necl-5 and nectin-3

We next examined whether Cdc42 and Rac are involved in the formation of filopodia and lamellipodia, respectively, induced by the *trans*-interaction of Necl-5 and nectin-3. NWASP-CRIB

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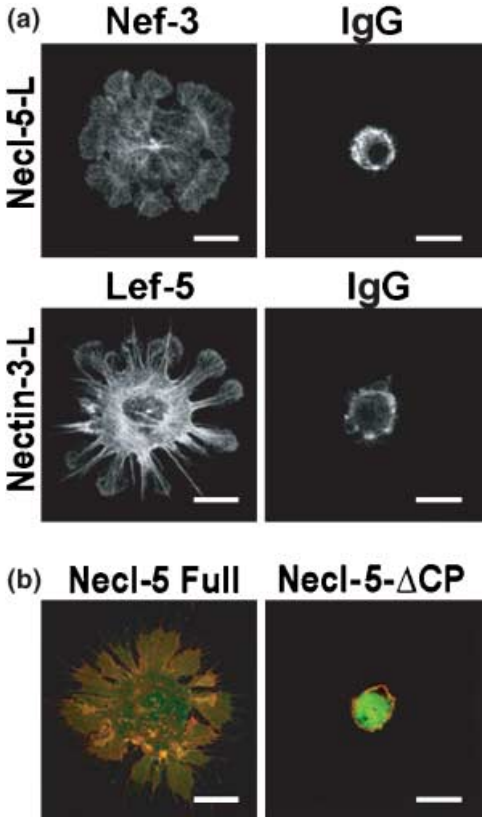

Fig. 1. Formation of filopodia and lamellipodia by the *trans*-interaction of Necl-5 and nectin-3. (a) Necl-5- or nectin3-induced formation of filopodia and lamellipodia. Necl-5-L or nectin-3-L cells were cultured on Nef-3-, Lef-5-, or IgG-coated coverslips for 1 h, and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. (b) Requirement of the cytoplasmic tail of Necl-5 in the formation of filopodia and lamellipodia by the *trans*-interaction of Necl-5 and nectin-3. Necl-5-L or Necl-5-ΔCP-L cells were cultured on Nef-3-coated coverslips and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. Bars = 10 μm.

specifically binds to GTP-Cdc42 and suppresses the action of Cdc42.[31] Overexpression of NWASP-CRIB in nectin-3-L cells cultured on Lef-5-coated coverslips reduced the formation of filopodia and lamellipodia (Fig. 2a). Similarly, overexpression of NWASP-CRIB in Necl-5-L cells cultured on the Nef-3-coated coverslips reduced the formation of filopodia and lamellipodia. Overexpression of N17Rac1, a dominant negative mutant of Rac1, which inhibits the production of GTP-Rac,[32] reduced the formation of lamellipodia, but not that of filopodia, in nectin-3-L cells cultured on Lef-5-coated coverslips or in Necl-5-L cells cultured on Nef-3-coated coverslips (Fig. 2a). These results indicate that the formation of filopodia induced by the *trans*-interaction of Necl-5 and nectin-3 requires Cdc42 and that the formation of lamellipodia requires both Cdc42 and Rac.

We then examined whether Cdc42 and Rac are activated by the *trans*-interaction of Necl-5 and nectin-3 using the pull-down assay with GST-PAK-CRIB. Nectin-3-L cells were incubated with clustered Lef-5 or IgG as a control and then subjected to the pull-down assay. The incubation of nectin-3-L cells with clustered Lef-5 increased the GTP-bound forms of Cdc42 and Rac at 30 min, whereas the incubation of clustered IgG did not increase the GTP-bound form of Cdc42 or Rac (Fig. 2b). Similarly, the incubation of Necl-5-L cells with clustered Nef-3 also increased the GTP-bound forms of Cdc42 and Rac at 30 min. These results indicate that Cdc42 and Rac are activated by the *trans*-interaction of Necl-5 and nectin-3.

Involvement of c-Src in the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3

We have previously shown that SFK, but not PI3K, are involved in the activation of Cdc42 and Rac by the *trans*-interaction of nectin and nectin.[22,23] We next examined the involvement of SFK and PI3K in the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3 using inhibitors of SFK and PI3K, PP2 and wortmannin, respectively. Both filopodia and lamellipodia were formed in the presence of PP3, an inactive analog of PP2 as a control, in nectin-3-L

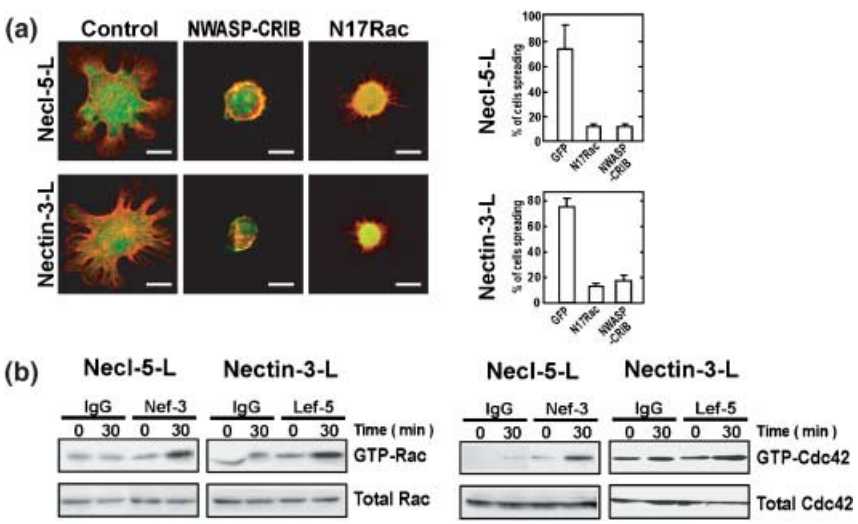

Fig. 2. Involvement of Cdc42 and Rac1 in the formation of filopodia and lamellipodia, respectively, induced by the *trans*-interaction of Necl-5 and nectin-3. (a) Inhibition by NWASP-CRIB or N17Rac1 of the Necl-5- or nectin-3-induced formation of filopodia and lamellipodia in Necl-5-L or nectin-3-L cells. Necl-5-L and nectin-3-L cells expressing GFP-NWASP-CRIB-CAAX or GFP-N17Rac1 were cultured for 24 h, and replated on Nef-3- or Lef-5-coated coverslips for 1 h, respectively, and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. Bars = 10 μm. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted and are expressed as mean ± SE of three independent experiments. (b) Necl-5- or nectin-3-induced activation of Rac1 and Cdc42 in Necl-5-L or nectin-3-L cells. Necl-5-L and nectin-3-L cells were cultured with Nef-3, Lef-5, or human IgG preclustered by the antihuman IgG pAb for the indicated periods of time, respectively. The cells were then subjected to the pull-down assay.

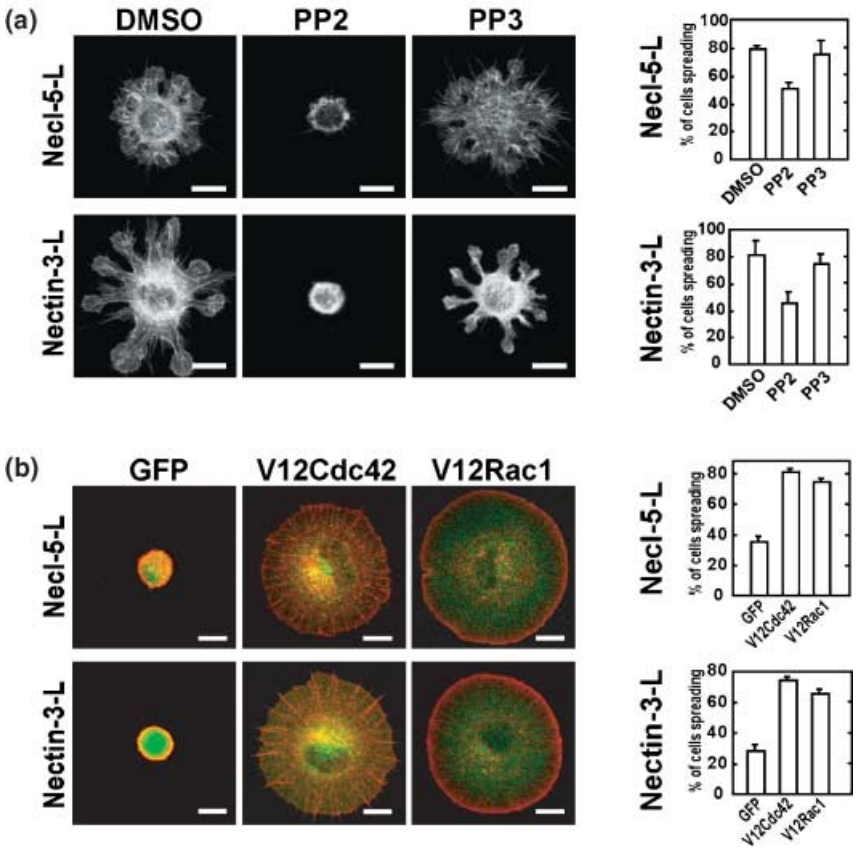

Fig. 3. Involvement of c-Src in the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3. (a) Necl-5-L and nectin-3-L cells were cultured on Nef-3- or Lef-5-coated coverslips, respectively, in the presence of PP2, PP3, or DMSO for 1 h, and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. Bars = 10 μm. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted and are expressed as mean ± SE of three independent experiments. (b) Suppression by V12Cdc42 and V12Rac1 of the inhibitory effect of PP2. Necl-5-L and nectin-3-L cells expressing GFP, GFP-V12Cdc42, or GFP-V12Rac1 were cultured on the Nef-3- or Lef-5-coated coverslips in the presence of PP2 for 1 h, respectively, and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. Bars = 10 μm. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted and are expressed as mean ± SE of the three independent experiments.

cells cultured on the Lef-5-coated coverslips or in Necl-5-L cells cultured on the Nef-3-coated coverslips (Fig. 3a). However, the formation of these protrusions was markedly reduced in the presence of PP2. Both protrusions were formed in the presence of wortmannin (data not shown). These results indicate that the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3 is mediated through the activation of SFK, but not PI3K.

We next examined whether SFK function upstream of Cdc42 and Rac in Necl-5-L and nectin-3-L cells. If SFK function upstream of Cdc42 and Rac, overexpression of V12Cdc42 or V12Rac1, constitutively active mutants of Cdc42 and Rac1, respectively, would suppress the inhibitory effect of PP2 on the formation of filopodia and lamellipodia induced by the *trans*-interaction of Necl-5 and nectin-3. To test this possibility, Necl-5-L cells transiently expressing GFP-V12Cdc42, GFP-V12Rac1, or GFP as a control were cultured on the Nef-3-coated coverslips in the presence of PP2. The formation of filopodia and lamellipodia was markedly reduced in the cells expressing GFP alone in the presence of PP2 (Fig. 3b). Both filopodia and lamellipodia were formed to large and medium extents, respectively, in the cells expressing GFP-V12Cdc42 in the presence of PP2, whereas lamellipodia were mainly formed to a large extent in the cells expressing GFP-V12Rac1 (Fig. 3b). Essentially the same results were obtained for nectin-3-L cells cultured on the Lef-5-coated coverslips (Fig. 3b). Taken together, these results indicate that SFK function downstream of Necl-5 and nectin-3 and upstream of Cdc42 and Rac in the formation of filopodia and lamellipodia induced by the *trans*-interaction of Necl-5 and nectin-3.

Recruitment of active c-Src to the Necl-5/nectin-3-based cell–cell contact sites

We have previously shown that one of the SFK, c-Src, is locally recruited and activated at the nectin-based cell–cell contact sites.[23] We examined whether c-Src is activated and concentrated at the Necl-5/nectin-3-based cell–cell contact sites where Necl-5 heterophilically *trans*-interacts with nectin-3. Necl-5-L and nectin-3-L cells transiently overexpressing c-Src were incubated with Nef-3- or Lef-5-coated magnetic beads, respectively, for 15 min. IgG-coated magnetic beads were used as a control. After incubation, the cells were harvested and lysed, and the magnetic beads were collected from the cell lysate. It has been shown that c-Src is activated by tyrosine phosphorylation.[33] In Necl-5-L cells, tyrosine-phosphorylated c-Src, which was detected with the antiphospho-Src (Tyr416) pAb, bound significantly to the Nef-3-coated beads, but not to the IgG-coated beads (Fig. 4a). Similarly, in nectin-3-L cells, tyrosine-phosphorylated c-Src bound significantly to the Lef-5-coated beads, but not to the IgG-coated beads. The significant binding of c-Src to the Nef-3- or Lef-5-coated beads was not detected in wild-type L cells (data not shown). We then confirmed by immunofluorescence microcopy that tyrosine-phosphorylated c-Src is recruited to the Necl-5/nectin-3-based cell–cell contact sites. Necl-5-L and nectin-3-L cells transiently overexpressing c-Src were incubated with the

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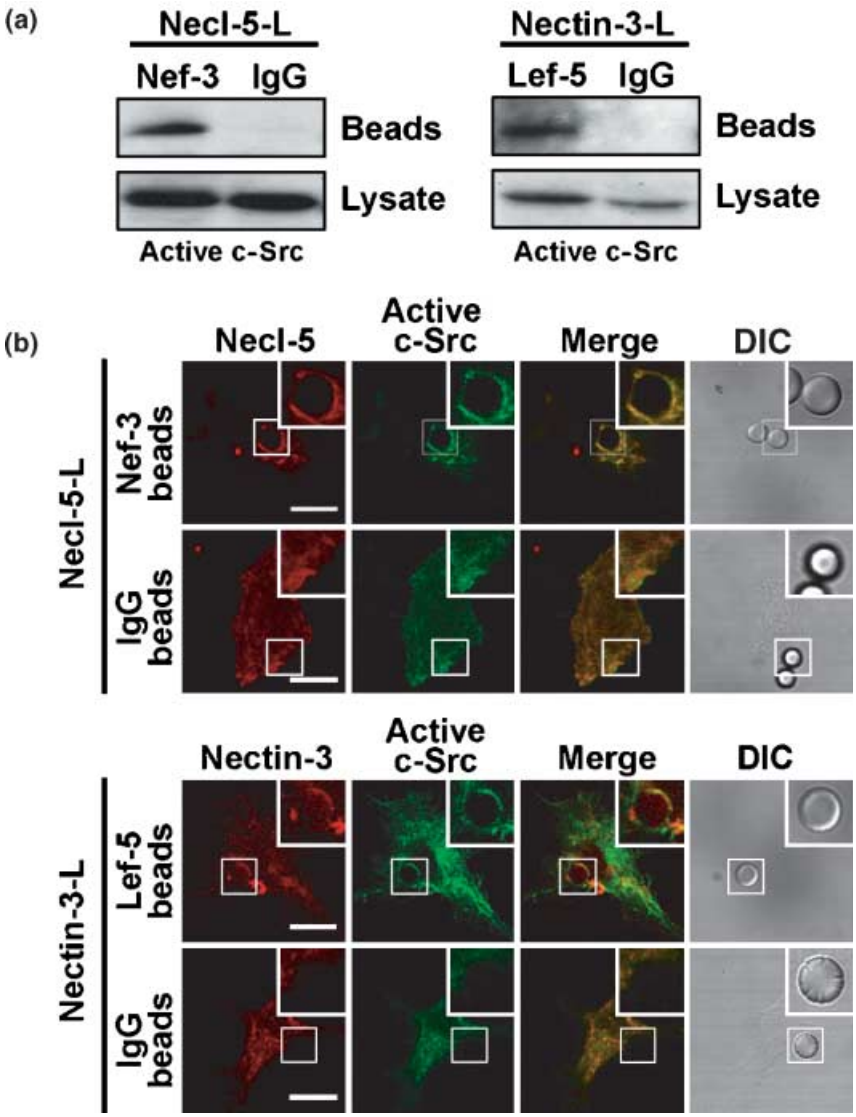

Fig. 4. Recruitment of active c-Src at the Necl-5/nectin-3-based cell–cell contact sites. (a) Association of active c-Src with Necl-5 or nectin-3 in Necl-5-L or nectin-3-L cells. Necl-5-L or nectin-3-L cells transiently overexpressing c-Src were incubated with Nef-3, Lef-5, or IgG-coated magnetic beads for 15 min, and then subjected to the coprecipitation assay, followed by western blotting with the anti-v-Src mAb and antiphospho-Src (Tyr416) pAb. (b) Recruitment of active c-Src to the Necl-5/nectin-3-based cell–cell contact sites. Necl-5-L or nectin-3-L cells transiently overexpressing c-Src were incubated with the Nef-3- or Lef-5-coated beads for 15 min, and immunostained for Necl-5 or nectin-3 and active c-Src with the anti-Necl-5 or the anti-nectin-3 and the antiphospho-Src (Tyr416) pAbs, respectively. The results shown are representative of three independent experiments. Bars = 10 μm.

Nef-3- and Lef-5-coated beads, respectively. The IgG-coated beads were used as a control. The immunofluorescence signal for Necl-5 was concentrated at the contact sites between the Nef-3-coated beads and the Necl-5-L cells overexpressing c-Src, but not between the IgG-coated beads and the Necl-5-L cells overexpressing c-Src (Fig. 4b), consistent with our previous results.[26] The signal for active c-Src was concentrated at the contact sites between the Nef-3-coated beads and the Necl-5-L cells overexpressing c-Src, but not between the IgG-coated beads and the Necl-5 cells overexpressing c-Src. Essentially the same results were obtained for nectin-3-L cells cultured with the Lef-5-coated beads (Fig. 4b). These results indicate that active c-Src is recruited to the Necl-5/nectin-3-based cell–cell contact sites and induces the activation of Cdc42 and Rac.

Involvement of Rap1 in the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3

We have previously shown that Rap1 is activated by the *trans*-interaction of nectin and nectin through c-Src-Crk-C3G signaling and is involved in the nectin-induced, c-Src-mediated activation of Cdc42 and formation of AJ.[24] Rap1GAP has been shown to hydrolyze GTP bound to Rap1 and to inactivate it.[34] We next examined by using Rap1GAP whether Rap1 is involved in the activation of Cdc42 and Rac induced by the *trans*-interaction of Necl-5 and nectin-3. Necl-5-L and nectin-3-L cells were transfected with the GFP-Rap1GAP plasmid or GFP as a control. After 24 h of culture, Necl-5-L and nectin-3-L cells were cultured on the Nef-3- and Lef-5-coated coverslips, respectively. Both filopodia and lamellipodia were markedly formed in Necl-5-L and nectin-3-L cells transiently overexpressing GFP, but these protrusions were negligibly formed in Necl-5-L or nectin-3-L cells transiently overexpressing GFP-Rap1GAP (Fig. 5a). These results indicate that Rap1 is necessary for the activation of Cdc42 and Rac induced by the *trans*-interaction of Necl-5 and nectin-3 and the subsequent formation of filopodia or lamellipodia, respectively.

We then examined whether the *trans*-interaction of Necl-5 and nectin-3 activates Rap1. Necl-5-L and nectin-3-L cells

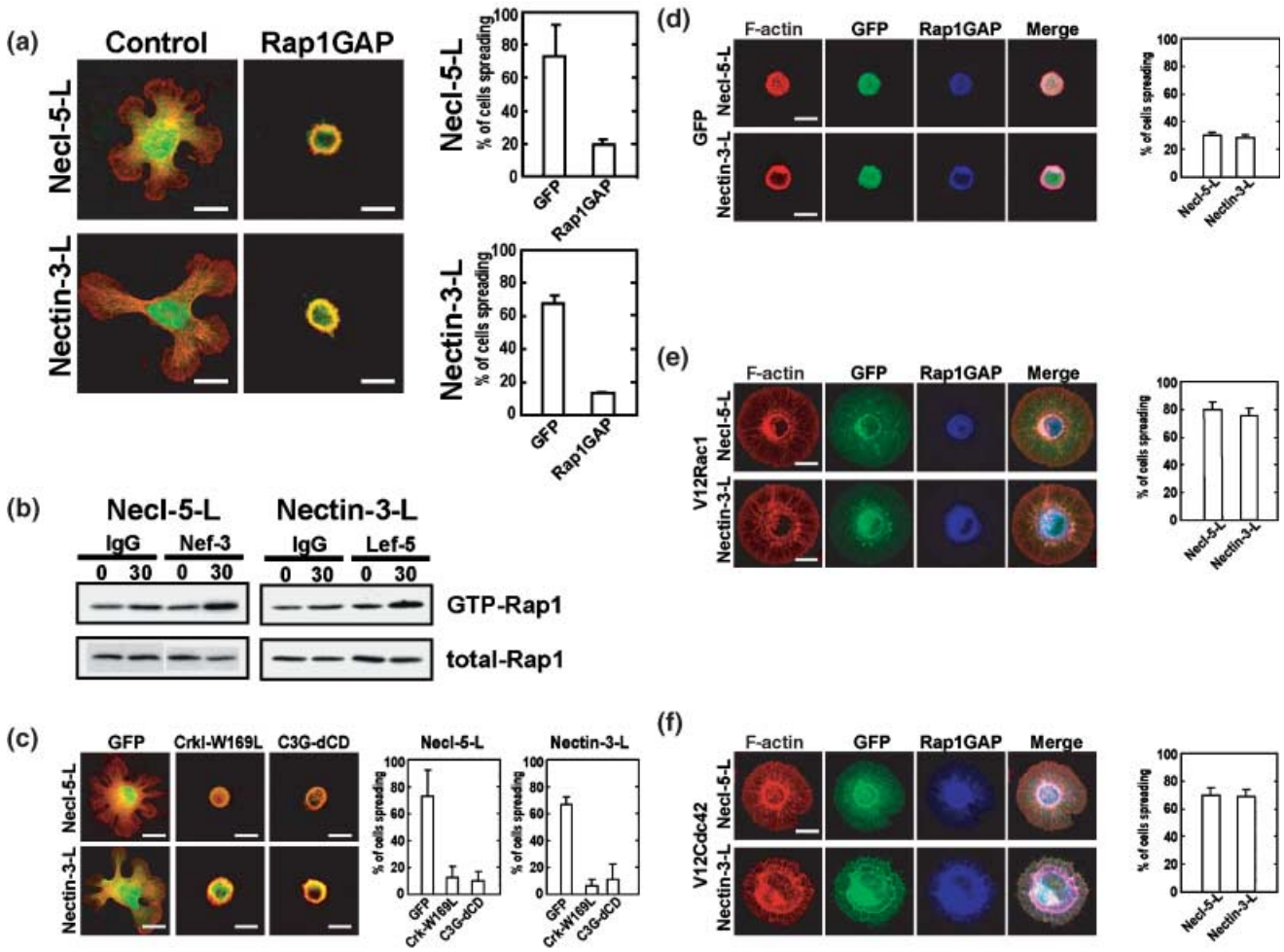

Fig. 5. Involvement of Rap1 in the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3. (a) Inhibition by Rap1GAP of the Necl-5- or nectin-3-induced formation of filopodia and lamellipodia in Necl-5-L or nectin-3-L cells. Necl-5-L and nectin-3-L cells expressing GFP-Rap1GAP were cultured for 24 h, and replated on Nef-3- or Lef-5-coated coverslips, respectively, and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. Bars = 10 μm. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted and are expressed as mean ± SE of the three independent experiments. (b) Necl-5- or nectin-3-induced activation of Rap1 in Necl-5-L or nectin-3-L cells. Necl-5-L and nectin-3-L cells were cultured with Nef-3, Lef-5, or human IgG preclustered by the antihuman IgG pAb for the indicated periods of time, respectively. The cells were then subjected to the pull-down assay. (c) Inhibition by Crkl-W169L and C3G-dCD of the nectin-3- or Necl-5-induced formation of filopodia and lamellipodia in Necl-5-L or nectin-3-L cells. Necl-5-L and nectin-3-L cells expressing GFP-Crkl-W169L or C3G-dCD were cultured for 24 h, and replated on Nef-3- or Lef-5-coated coverslips, respectively, and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. Bars = 10 μm. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted and are expressed as mean ± SE of the three independent experiments. (d–f) Suppression by V12Rac1 and V12Cdc42 of the inhibitory effect of Rap1GAP. Necl-5-L cells expressing Myc-Rap1GAP with GFP, GFP-V12Rac1, or GFP-V12Cdc42 were cultured on Nef-3-coated coverslips for 1 h, respectively, and stained for F-actin with rhodamine–phalloidin and anti-Myc mAb. The results shown are representative of three independent experiments. Bars = 10 μm. Bars in the quantitative analysis show the percentage of cells with filopodia and/or lamellipodia of the total cells counted and are expressed as mean ± SE of three independent experiments.

were incubated with clustered Nef-3 and Lef-5, respectively, and then subjected to the pull-down assay using GST-RalGDS-RBD. The cells incubated with clustered IgG were used as a control. The incubation of clustered Nef-3 with Necl-5-L cells increased the GTP-bound form of Rap1 at 30 min, whereas the incubation of clustered IgG did not increase the GTP-bound form of Rap1 (Fig. 5b). Similarly, the incubation of clustered Lef-5 with nectin-3-L cells increased the GTP-bound form of Rap1. These results indicate that the *trans*-interaction of Necl-5 and nectin-3 indeed activates Rap1.

We have previously shown that Rap1 is activated by the *trans*-interaction of nectin and nectin through the c-Src-Crk-C3G pathway.[24] C3G is a Rap1-GEF, which is activated through the Crk adaptor protein.[35,36] Many types of stimuli are reported to induce the binding of the Crk-C3G complex to phosphotyrosine-containing proteins, including c-Src.[37] We examined whether dominant negative mutants of Crk and C3G, CrkI-W169L and C3G-dCD,[38] inhibit the Cdc42- or Rac-mediated formation of filopodia or lamellipodia, respectively, induced by the *trans*-interaction of Necl-5 and nectin-3. Necl-5-L and nectin-3-L cells

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transiently coexpressing Flag-CrkI-W169L or C3G-dCD were cultured on the Nef-3- and Lef-5-coated coverslips, respectively. Filopodia or lamellipodia were markedly formed in the cells expressing GFP, whereas neither protrusions were formed in Necl-5-L or nectin-3-L cells expressing Flag-CrkI-W169L or C3G-dCD (Fig. 5c). Taken together, these results indicate that Rap1 is activated by the *trans*-interaction of Necl-5 and nectin-3 through the c-Src-Crk-C3G pathway.

We have previously shown that Rap1 functions upstream of Cdc42 and Rac in the nectin-induced formation of filopodia or lamellipodia.[24] To examine whether Rap1 functions upstream of Cdc42 and Rac in the formation of filopodia or lamellipodia induced by the *trans*-interaction of Necl-5 and nectin-3, Necl-5-L and nectin-3-L cells transiently coexpressing GFP-V12Cdc42, GFP-V12Rac1, or GFP as a control with Myc-Rap1GAP were cultured on the Nef-3- and Lef-5-coated coverslips. Both filopodia and lamellipodia were markedly formed in Necl-5-L and nectin-3-L cells expressing GFP alone (data not shown), but these protrusions were negligibly formed in the cells coexpressing GFP with Myc-Rap1GAP (Fig. 5d). Both filopodia and lamellipodia were formed to large and moderate extents, respectively, in the cells coexpressing GFP-V12Cdc42 with Myc-Rap1GAP, whereas lamellipodia were mainly formed to a large extent in the cells coexpressing GFP-V12Rac1 with Myc-Rap1GAP (Fig. 5e,f). These results indicate that Rap1 functions upstream of Cdc42 and Rac in the formation of filopodia and lamellipodia induced by the *trans*-interaction of Necl-5 and nectin-3.

Involvement of FRG and Vav2 in the activation of Cdc42 and Rac, respectively, by the *trans*-interaction of Necl-5 and nectin-3

We have previously shown that FRG and Vav2 are GEF responsible for the nectin-induced, c-Src-mediated activation of Cdc42 and Rac, respectively.[23,25] We then examined whether FRG and Vav2 are also involved in the activation of Cdc42 and Rac, respectively, by the *trans*-interaction of Necl-5 and nectin-3. For this purpose, we used the fragment of FRG (ΔDHPH) and the fragment of Vav2 (ΔDHPH) as dominant negative mutants of FRG and Vav2, respectively. The formation of filopodia and lamellipodia was reduced in Necl-5-L and nectin-3-L cells expressing GFP-FRG (ΔDHPH) (Fig. 6a). The formation of lamellipodia, but not filopodia, was reduced in Necl-5-L and nectin-3-L cells expressing Myc-Vav2 (ΔDHPH) (Fig. 7a). These results suggest that FRG and Vav2 are involved in the formation of filopodia and lamellipodia, respectively, induced by the *trans*-interaction of Necl-5 and nectin-3.

We then examined whether FRG and Vav2 are recruited to the Necl-5/nectin-3-based cell–cell contact sites where Necl-5 heterophilically *trans*-interacts with nectin-3. Necl-5-L and nectin-3-L cells transiently expressing Myc-FRG or Myc-Vav2 were incubated with the Nef-3- and Lef-5-coated beads, respectively. The immunofluorescence signals for Necl-5 and Myc-FRG were concentrated at the contact sites between the Nef-3-coated beads and the Necl-5-L cells expressing Myc-FRG, but not between the IgG-coated beads and the Necl-5-L cells expressing Myc-FRG (Fig. 6b). Similarly, the signals for Necl-5 and Myc-Vav2 were concentrated at the contact sites between the Nef-3-coated beads and the Necl-5-L cells expressing Myc-Vav2, but not between the IgG-coated beads and the Necl-5-L cells expressing Myc-Vav2 (Fig. 7b). Essentially the same results were obtained for nectin-3-L cells cultured on the Lef-5-coated coverslips (Fig. 6b,7b). These results, together with the results discussed earlier, indicate that FRG and Vav2 as well as c-Src are recruited to the Necl-5/nectin-3-based cell–cell contact sites.

We next examined whether FRG and Vav2 are tyrosine-phosphorylated and activated by the *trans*-interaction of Necl-5 and nectin-3 through c-Src. Necl-5-L and nectin-3-L cells transiently overexpressing c-Src and Flag-FRG or Flag-Vav2 were incubated with Nef-3 and Lef-5, respectively, clustered with the antihuman Fc pAb and subjected to the immunoprecipitation assay using the anti-Flag mAb. FRG was tyrosine-phosphorylated by clustered Nef-3 in Necl-5-L cells, whereas it was not tyrosine-phosphorylated by clustered IgG in Necl-5-L cells (Fig. 6c). FRG was tyrosine-phosphorylated by clustered Lef-5 in nectin-3-L cells. The tyrosine-phosphorylation of FRG induced by clustered Nef-3 or Lef-5 was inhibited by PP2. Similarly, Vav2 was tyrosine-phosphorylated by clustered Nef-3 in Necl-5-L cells, whereas it was not tyrosine-phosphorylated by clustered IgG in Necl-5-L cells (Fig. 7c). Vav2 was tyrosine-phosphorylated by clustered Lef-5 in nectin-3-L cells. The tyrosine-phosphorylation of Vav2 induced by clustered Nef-3 or Lef-5 was inhibited by PP2. Taken together, these results indicate that c-Src is recruited and activated at the Necl-5/nectin-3-based cell–cell contact sites, and then tyrosine-phosphorylates and activates FRG and Vav2, eventually causing an increase in the GTP-bound active forms of Cdc42 and Rac, respectively.

Discussion

We have previously shown that Necl-5 colocalizes with integrin $\alpha_v\beta_3$ at the leading edges of moving cells and enhances cell movement and proliferation.[2,3] This localization of Necl-5 may confer on it the ability to first recognize nectin-3, which is diffusely distributed along the free surface of the plasma membrane of other moving cells, and to heterophilically *trans*-interact with it, initiating the formation of the microclusters of the heterophilic *trans*-interaction of Necl-5 and nectin-3. These microclusters may be formed more rapidly than are the microclusters of the *trans*-interaction of nectin and nectin, but may be transient, because the affinity of nectin-3 for Necl-5 is far less than that for nectin-1,[13] and Necl-5 *trans*-interacting with nectin-3 may be replaced by nectin-1, resulting in the formation of the *trans*-interaction of nectin-3 and nectin-1. The *trans*-interaction of nectin-3 and nectin-1 then recruits cadherin there to form AJ. Consistent with this assumption, we have previously demonstrated that there are two roles of the *trans*-interaction of Necl-5 and nectin-3: one is to induce the downregulation of Necl-5, which causes reductions in cell movement and proliferation;[17] and the other is to initiate cell–cell contact.[26] Thus, the *trans*-interaction of Necl-5 and nectin-3 shifts the cells from a moving and proliferating state to an adhering state, which is involved at least partly in the contact inhibition of cell movement and proliferation.

We have previously shown that the *trans*-interaction of nectin and nectin induces the activation of Cdc42 and Rac,

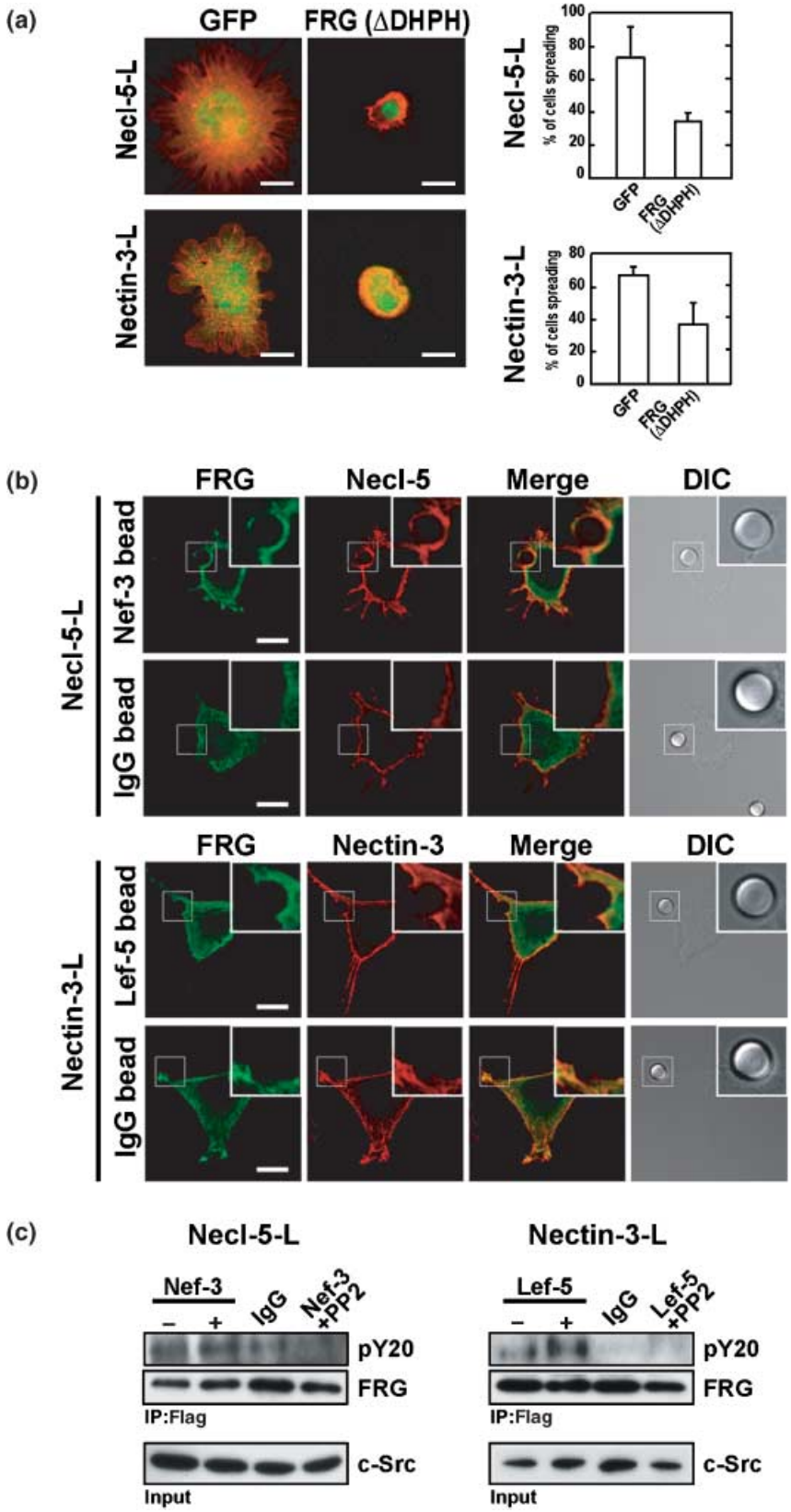

Fig. 6. Involvement of FRG in the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3. (a) Inhibition by FRG (ΔDHPH) of the Necl-5- or nectin-3-induced formation of filopodia and lamellipodia in Necl-5-L or nectin-3-L cells. Necl-5-L and nectin-3-L cells expressing GFP-FRG (ΔDHPH) were cultured for 24 h, and replated on Nef-3- or Lef-5-coated coverslips for 1 h, respectively, and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. Bars = 10 μm. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted and are expressed as mean ± SE of three independent experiments. (b) Recruitment of FRG to the Necl-5/nectin-3-based cell–cell contact sites. Necl-5-L or nectin-3-L cells expressing Myc-FRG were incubated with the Nef-3-, Lef-5-, or IgG-coated beads for 1 h and stained with the anti-Myc mAb and anti-Necl-5 or anti-nectin-3 mAb, respectively. The results shown are representative of three independent experiments. (c) Necl-5- or nectin-3-induced tyrosine phosphorylation of FRG. Necl-5-L or nectin-3-L cells expressing c-Src and Flag-FRG were treated with clustered Nef-3, Lef-5, or IgG in the presence or absence of PP2 for 30 min. Each cell lysate was subjected to the immunoprecipitation assay with the anti-Flag mAb, followed by western blotting with the antiphosphotyrosine (pY20), anti-Flag, and anti-v-Src mAbs.

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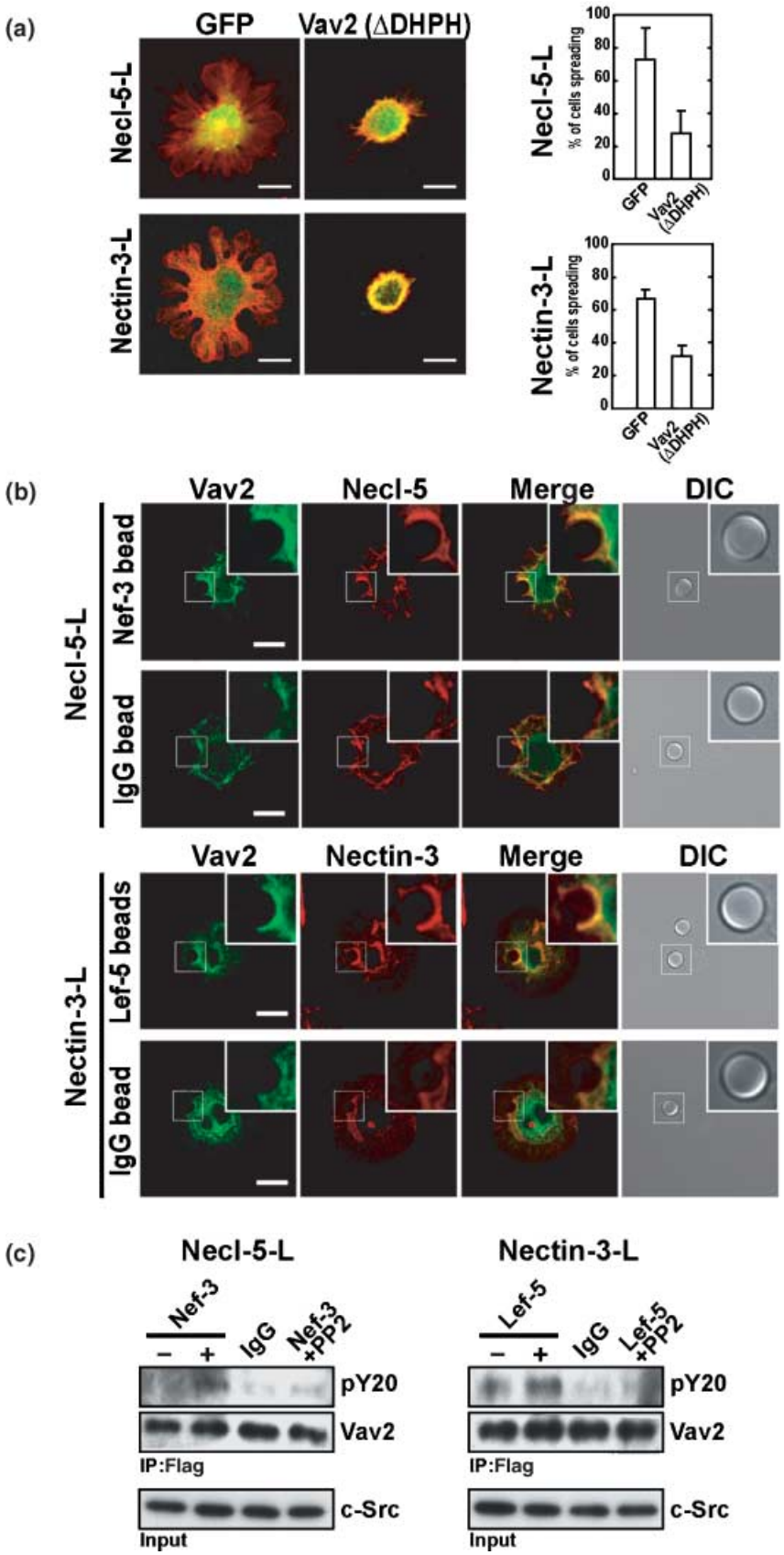

Fig. 7. Involvement of Vav2 in the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3. (a) Inhibition by Vav2 (ΔDHPH) of the Necl-5- or nectin-3-induced formation of filopodia and lamellipodia in Necl-5-L or nectin-3-L cells. Necl-5-L and nectin-3-L cells expressing Myc-Vav2 (ΔDHPH) were cultured for 24 h, replated on the Nef-3- or Lef-5-coated coverslips for 1 h, respectively, and stained for F-actin with rhodamine–phalloidin. The results shown are representative of three independent experiments. Bars = 10 μm. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted and are expressed as mean ± SE of three independent experiments. (b) Recruitment of Vav2 to the Necl-5/nectin-3-based cell–cell contact sites. Necl-5-L or nectin-3-L cells expressing Myc-Vav2 were incubated with Nef-3-, Lef-5-, or IgG-coated beads for 1 h and stained with anti-Myc mAb and anti-Necl-5 or anti-nectin-3 mAb, respectively. (c) Necl-5- or nectin-3-induced tyrosine phosphorylation of Vav2. Necl-5-L or nectin-3-L cells expressing c-Src and Flag-Vav2 were treated with clustered Nef-3, Lef-5, or IgG in the presence or absence of PP2 for 30 min. Each cell lysate was subjected to the immunoprecipitation assay with anti-Flag mAb, followed by western blotting with the antiphosphotyrosine (pY20), anti-Flag, and anti-v-Src mAbs. The results shown are representative of three independent experiments.

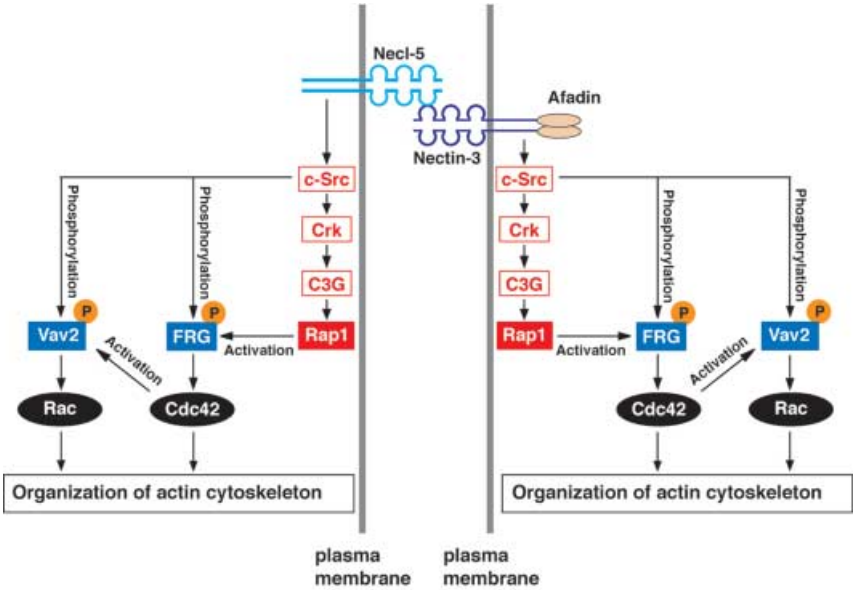

Fig. 8. Schematic model for the intracellular signaling pathway from Necl-5 and nectin-3 to Cdc42 and Rac. Details are given in the Discussion section.

which then facilitate the formation of AJ by forming filopodia and lamellipodia, respectively.[21–25] We have shown here that the *trans*-interaction of Necl-5 and nectin-3 bidirectionally induces the activation of Cdc42 and Rac, which then induce the formation of filopodia and lamellipodia, respectively. The activation of these small G proteins by the *trans*-interaction of Necl-5 and nectin-3 may similarly facilitate the formation of AJ by forming these protrusions as described for the *trans*-interaction of nectin and nectin. In addition, nectin-3 recruited by its *trans*-interaction with Necl-5 may recruit cadherin to the cell–cell contact sites of both sides of protrusions of moving cells, which may also make a contribution at least partly in the formation of AJ.

We then found that the *trans*-interaction of Necl-5 and nectin-3 bidirectionally induces the activation of Cdc42 and Rac through c-Src, Rap1, FRG, and Vav2 (Fig. 8). The *trans*-interaction of Necl-5 and nectin-3 first recruits and activates c-Src at the Necl-5/nectin-3-based cell–cell contact sites. c-Src then phosphorylates FRG and Vav2, and tyrosine-phosphorylated FRG and Vav2 are recruited to the Necl-5/nectin-3-based cell–cell contact sites. The *trans*-interaction of Necl-5 and nectin-3 also activates Rap1 through C3G, a Rap-GEF, and this activation of Rap1 is required for the activation of Cdc42 and Rac. This signaling pathway by the *trans*-interaction of Necl-5 and nectin-3 is similar to that of the previously described *trans*-interaction of nectin and nectin.[21–25] We have previously shown that the activation of Cdc42 and Rac by the *trans*-interaction of nectin and nectin requires its cytoplasmic region, but not an association with afadin.[21] Similarly, we have shown here that the activation of Cdc42 and Rac by the *trans*-interaction of Necl-5 and nectin-3 requires the cytoplasmic tail of Necl-5. It has been reported that the cytoplasmic region of Necl-5 binds Tctex-1, a subunit of the dynein motor complex,[14] although it remains unknown whether the cytoplasmic region of nectin binds Tctex-1. The cytoplasmic tail of Necl-5 is not similar to that of nectin, but the cytoplasmic tails of Necl-5 and nectin seem to be associated with the common signaling molecules, for example c-Src, Rap1, FRG, and Vav2. It remains unknown how these signaling molecules are associated with Necl-5 and nectin-3, but a common protein may associate with them and transduce signaling from them. Because Necl-5 associates with integrin $\alpha_V\beta_3$ at the leading edges of migrating cells,[2] integrin $\alpha_V\beta_3$ may be a candidate for such a molecule.

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