Involvement of the c-Src-Crk-C3G-Rap1 Signaling in the Nectin-induced Activation of Cdc42 and Formation of Adherens Junctions*

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The abbreviations used are: AJ, adherens junction; CRIB, Cdc42/Rac interactive binding region; FRB, fluorescent resonance energy transfer; GAP, GTPase-activating protein; GEF, GDP/GTP exchange factor; GFP, green fluorescent protein; GST, glutathione S-transferase; mAb, monoclonal antibody; pAb, polyclonal antibody; RBD, Rac interactive binding region; FRET, fluorescent resonance energy transfer; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.

Nectins, Ca2+-independent immunoglobulin-like cell-cell adhesion molecules, induce the activation of Cdc42 and Rac small G proteins, enhancing the formation of cadherin-based adherens junctions (AJs) and claudin-based tight junctions. Nectins recruit and activate c-Src at the nectin-based cell-cell contact sites. c-Src then activates Cdc42 through FRG, a Cdc42-GDP/GTP exchange factor. We showed here that Rap1 small G protein was involved in the nectin-induced activation of Cdc42 and formation of AJs. Rap1 was recruited to the nectin-based cell-cell contact sites and locally activated through the c-Src-Crk-C3G signaling and involved in the activation of both molecules was essential for the activation of FRG. The activation of Rap1 was not necessary for the c-Src-mediated phosphorylation or recruitment of FRG. The inhibition of the Crk, C3G, or Rap1 signaling reduced the formation of AJs. These results indicate that Rap1 is activated by nectins through the c-Src-Crk-C3G signaling and involved in the nectin-induced, c-Src- and FRG-mediated activation of Cdc42 and formation of AJs.

Small G proteins comprise a superfamily consisting of the Ras, Rho, Rab, Arf/Sar, and Ran families and act as molecular switches to control a range of cell functions in all eukaryotic cells (1, 2). They cycle between the GDP- and GTP-bound forms by regulators, and the GTP-bound form interacts with downstream effectors (2). Our group and two other groups have independently identified Rap1 small G protein as the closest relative of Ras small G protein and named it Smg p21, Rap1, and Krev-1 (3–5). Rap1/Krev-1 has been shown to antagonize the Ras signaling (5), whereas we have shown that Rap1/Smg p21 stimulates DNA synthesis in Swiss 3T3 cells (6) and activates B-Raf protein kinase (7). Rap1 has subsequently been shown to be involved in many cell functions, including integrin-mediated cell adhesion, exocytosis, neurite outgrowth, and synaptic plasticity (8, 9). In Drosophila, Rap1 has been reported to regulate morphogenesis (10) and formation of cell-cell adhesions (AJs)1 (11). However, it still remains largely unknown how Rap1 is activated and regulates various cell functions including the formation of cell-cell AJs.

A striking feature of small G proteins is the extensive cross-talk and cooperation between small G proteins (1, 12). The physiological significance of cross-talk between the Ras and Rho family members has been most clearly established in the budding process of the yeast: Bud1, a Rap-like small G protein related to mammalian Rap1, is activated at a positional landmark and defines the position of the new bud (1, 9, 13–15). Activated Bud1 recruits and likely activates Cdc24, a GDP/GTP exchange factor (GEF) for Cdc42 small G protein, resulting in the activation of Cdc42 (15). Cdc42 recruits the actin cytoskeleton to the bud site and causes reorganization of the actin cytoskeleton through Rho1 small G protein (15, 16). Although we have previously shown that mammalian Rap1 substitutes for Bud1 (13), it remains unknown whether the small G protein cascade of Rap1 controlling Cdc42 is functionally conserved from yeast to mammals.

Nectins are Ca2+-independent Ig-like cell-cell adhesion molecules that form AJs cooperatively with cadherins (17–20). Nectins comprise a family of four members, nectin-1, -2, -3, and -4. All nectins form homo-cis-dimers and then homo- and hetero-trans-dimers, causing cell-cell adhesion. Nectins first form cell-cell contacts and recruit cadherins to the nectin-based cell-cell contact sites, causing the formation of AJs. In addition, nectins induce activation of Cdc42 and Rac small G proteins. Cdc42 activated in this way is likely to induce the formation of filopodia and to increase the number of cell-cell contact sites at the initial stage of the formation of AJs. On the other hand, Rac activated in this way induces the formation of lamellipodia that efficiently expands the cell-cell adhesion between filopodia, acting like a “zipper.” In epithelial cells, nectins furthermore recruit first junctional adhesion molecules and then claudins to the apical side of AJs, resulting in the formation of tight junctions (TJs). The cell polarity protein complex of Par-3, atypical protein kinase C, and Par-6 is essential for the formation of TJs, and Cdc42 induces the activation of this complex by binding to Par-6 (21). However, it remains unknown how Cdc42 is

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activated in this process. We have recently found that nectins directly bind Par-3, raising the possibility that Cdc42 activated by the action of nectins is involved in the activation of this cell polarity protein complex (22). Thus, Cdc42 and Rac activated by the action of nectins are likely to play important roles in the formation of cell-cell junctions and cell polarity.

Extending these earlier observations, we have recently found that c-Src is recruited and activated by nectins at the nectin-based cell-cell contact sites and that c-Src activated in this way then tyrosine phosphorylates and activates FRG, a Cdc42-GEF, resulting in the activation of Cdc42 (23). Along this line, we examined here whether Rap1 is involved in the nectin-induced, c-Src- and FRG-mediated activation of Cdc42 and formation of AJs.

EXPERIMENTAL PROCEDURES

Plasmid Constructions and Protein Purification—V12Rap1B, in which a glycine residue at amino acid 12 of bovine Rap1B was replaced by a valine (V), was a constitutively active mutant of Rap1 and prepared using the QuikChange site-directed mutagenesis kit (Stratagene). The cDNA of Rap1 GTPase-activating protein (GAP) was a gift from Dr. P. Casey (Duke University, Durham, NC). Expression vectors for GFP-tagged Rap1 (pEGFP-Rap1), GFP-tagged V12Rap1 (pEGFP-V12Rap1), and GFP-tagged Rap1GAP (pEGFP-Rap1GAP) were constructed by inserting the cDNA fragments encoding a wild-type Rap1, V12Rap1, and Rap1GAP into pEGFP, respectively. An expression vector for Myc-tagged Rap1GAP (pEFBOS-Myc-Rap1GAP) was constructed by inserting the cDNA encoding Rap1GAP into pEFBOS-Myc. Expression vectors for FLAG-tagged FRG (pEFBOS-Flag-FRG), Raichu-Rap1 (pRaichu-Rap1), FLAG-tagged dominant negative mutant of CrkI (pIRM21-Flag-CrkI-W169L), a constitutively active mutant of C3G (pcAGGS-C3G-CA), and a dominant negative mutant of C3G (pcAGGS-C3G-dCD) were prepared as described previously (23–25). Expression vectors for GST-tagged constitutively active mutants of Cdc42 (pEGFP-V12Cdc42) and Rac1 (pEGFP-V12Rac1) were kindly supplied by Dr. S. Narumiya (Kyoto University, Kyoto, Japan). Expression vectors for GST-PAK-CRIB fusion protein and GST-RalGDS-RBD fusion protein were overexpressed and purified from Escherichia coli according to the manufacturer’s protocol (Amersham Biosciences). Protein concentrations were determined by using the BCA protein assay kit (Pierce) with bovine serum albumin as a reference protein.

Antibodies—Rabbit anti-nectin-1 and C3G polyclonal antibodies (pAbs) were prepared as described previously (24, 28). A rabbit anti-Rap1/Krev-1 pAb (Santa Cruz Biotechnology), a rabbit anti-phospho-

![Fig. 1. Inhibition by Rap1GAP of the Nef-3-induced activation of Cdc42 and Rac. A, cell spreading assay. Nectin-1-L cells transiently overexpressing GFP-Rap1GAP or GFP were cultured on the Nef-3- or IgG-coated coverslips for 1 h. The cells were fixed and stained for actin filaments (F-actin) with rhodamine-phalloidin. Scale bars, 10 μm. Bars in the graph represent percentage of cells with filopodia (F) or lamellipodia (L) of the total 100 cells counted and are expressed as means ± S.E. of three independent experiments. B and C, pull-down assay. Nectin-1-L cells transiently overexpressing Myc-Rap1GAP or not were cultured with clustered Nef-3 or IgG for 30 min and then subjected to the pull-down assay using GST-PAK-CRIB followed by Western blotting using the anti-Cdc42 and anti-Rac1 mAbs. B, Cdc42; C, Rac1. Bars in the graphs of B and C represent the relative intensity of GTP-Cdc42 or GTP-Rac1 normalized for the total amount of Cdc42 or Rac1, respectively, as compared with a value of clustered IgG stimulation, which is expressed as 1. The results shown are representative of three independent experiments.
Src (Tyr-416) pAb (Cell Signaling Technology), a mouse anti-Cdc42 monoclonal antibody (mAb) (BD Transduction Laboratories), a mouse anti-Rac1 mAb (Upstate Biotechnology), a mouse anti-FLAG mAb (Sigma), a mouse anti-phosphotyrosine (PY20) mAb (BD Transduction Laboratories), a mouse anti-v-Src mAb (Calbiochem-Novabiochem), and secondary antibodies (Chemicon) were purchased from commercial sources. A rat anti-E-cadherin mAb (ECCD-2) was kindly supplied by Dr. M. Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan). Hybridoma cells expressing a mouse anti-Myc mAb (9E10) were obtained from American Type Culture Collection and prepared as described previously (28).

Cell Culture and Fluorescent Resonance Energy Transfer (FRET) Imaging—L or MDCK cells stably expressing exogenous nectin-1 (nectin-I-L or nectin-I-MDCK cells, respectively) were prepared as described previously (28). Transfection and immunofluorescence microscopy of cultured cells with a confocal imaging system (Radian 2000, Bio-Rad) were performed as described previously (28). FRET imaging was also performed as described previously (23, 30). In brief, nectin-I-L cells were transfected with pRAiuchi-Rap1. After a 24-h transfection, the cells were replated on the dishes coated with 50 μg/ml Nef-3 or 50 μg/ml IgG. The cells were then imaged with an Olympus IX71 inverted microscope equipped with a cooled charge-coupled device camera, CoolSNAP HQ (Roper Scientific), controlled by MetaMorph software (Universal Imaging). For dual emission ratio imaging, we used a 440AF21 excitation filter, a 455DRLP dichroic mirror, and two emission filters, versal Imaging). For dual emission ratio imaging, we used a 440AF21

FIG. 2. Suppression by Y12Cd42 and V12Rac1 of the inhibitory effect of Rap1GAP on the formation of filopodia and lamellipodia. A cell spreading assay is shown. Nectin-1-L cells transiently overexpressing Myc-Rap1GAP with GFP-V12Cd42, GFP-V12Rac1, c-Src-CA, or GFP were cultured on the Nef-3-covered coverslips for 1 h. The cells were fixed and stained for F-actin, Myc-Rap1GAP, and c-Src with rhodamine-phalloidin, the anti-Myc mAb, and the anti-phospho-Src (Tyr-416) pAb, respectively. Scale bars, 10 μm. Bars in the graph represent percentage of cells with filopodia (F) or lamellipodia (L) of the total 100 cells counted and are expressed as means ± S.E. of three independent experiments.

Assay for Cell Spreading and Bead-Cell Contact—The cell spreading assay was performed as described previously (23). For inhibition of the activities of Src family kinases, the cells were treated with 20 μM PP2 (Calbiochem-Novabiochem) dissolved in 0.2% Me2SO, 10 μM PP3 (Calbiochem-Novabiochem) dissolved in 0.2% Me2SO, 10 μM SU6656 (Sigma) dissolved in 0.2% Me2SO, or 0.2% Me2SO as a control in ice-cold phosphate-buffered saline containing 1 mM sodium vanadate, 8 h. After a 16-h serum starvation, the medium was replaced by 1 ml of DMEM containing clustered Nef-3 or IgG, and the cells were incubated for the indicated periods of time. The cells were washed twice with 3 ml of phosphate-buffered saline at room temperature for 1 h. Nectin-1-L cells (5 × 105 cells/60-mm dish) were transiently transfected with the plasmid of Rap1GAP and cultured for 8 h. After a 16-h serum starvation, the medium was replaced by 1 ml of DMEM containing clustered Nef-3 or IgG, and the cells were incubated for the indicated periods of time. The cells were then washed with 1 ml of ice-cold phosphate-buffered saline containing 1 mM sodium vanadate, lysed in Buffer A (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-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-PK-CRIB for Cdc42 and Rac, and incubated for 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 Immunoprecipitation and GEF Activity—The immunoprecipitation was performed as described previously (28). In brief, nectin-I-L cells (1.5 × 105 cells/100-mm dish) were transfected with the combination of pEFBS-Flag-FRG, pcDNA-c-Src, pEGFP, pEGFP-V12Rap1, or pEGFP-Rap1GAP. After an 8-h culture, the cells were serum-starved for 16 h. The medium was then replaced by 3 ml of DMEM containing clustered Nef-3 or IgG, and the cells were incubated for the indicated periods of time. The cells were washed twice with 3 ml of ice-cold phosphate-buffered saline containing 1 mM sodium vanadate, lysed in 0.5 ml of Buffer B (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/p-aminophenyl)methanesulfonyl fluoride hydrochloride, 20 mM β-glycerophosphate/sodium, 10 mM sodium fluoride, 1 mM sodium vanadate, and phosphatase inhibitor mixture 1), and incubated on ice for 15 min. The cell extract was obtained by centrifugation at 20,000 × g at 4 °C for 15 min, incubated with 5 μl of the anti-FLAG mAb at 4 °C for 1 h, and further incubated with 50 μl of protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences) for 1 h. After the incubation, the beads were washed with Buffer B. Proteins bound to the beads were eluted with Laemmli buffer and subjected to SDS-PAGE followed by Western blotting. The GEF activity was assayed as described previously (31). The binding of [35S]GTPγS to Cdc42 after incubation for the indicated periods of time in the presence of each immunoprecipitant from the cells treated with clustered Nef-3 or IgG for 30 min.

Assay for Cell-Cell Adhesion—Cell-cell adhesion of nectin-1-MDCK cells transiently overexpressing Flag-CrkI-W169L, C3G-CD, Myc-Rap1GAP, GFP-V12Cd42, GFP, or an empty vector was assayed as described previously (23). Briefly the cells transfected with the plasmid were cultured in DMEM containing 10% fetal calf serum for 24 h. After
the culture, the cells were washed with 5 ml of phosphate-buffered saline and incubated in DMEM (at a final concentration of 2 mM Ca²⁺/H¹¹⁰⁰¹). The medium was replaced by DMEM containing 5 mM EGTA (at a final concentration of 2⁻/H⁹₂⁶² M Ca²⁺/H¹¹⁰⁰¹), and then the cells were incubated for 2 h. After the incubation, the cells were washed with DMEM and further incubated in DMEM (at a final concentration of 2 mM Ca²⁺) for 2 h. The cells were fixed and immunostained with the anti-E-cadherin or anti-Myc mAbs.

RESULTS

Necessity of Rap1 for the Nectin-induced Activation of Cdc42 and Rac—

Rap1GAP has been shown to hydrolyze GTP bound to Rap1 and to inactivate it (32). We first examined by using Rap1GAP whether Rap1 is involved in the nectin-induced activation of Cdc42 and Rac. We have previously shown by using Nef-3 that nectins induce the formation of filopodia and lamellipodia through the respective activation of Cdc42 and Rac in nectin-1-L and nectin-1-MDCK cells (23, 33). Nectin-1-L cells were transfected with the plasmid of GFP-V12Rap1, C3G-CA, or GFP were cultured on the Nef-3-coated coverslips in the presence of PP2, PP3, or SU6656 for 1 h. The cells were fixed and stained for F-actin and C3G-CA with rhodamine-phalloidin and the anti-C3G pAb, respectively. Scale bars, 10 μm. Bars in the graph represent percentage of cells with filopodia (F) and lamellipodia (L) of the total 100 cells counted and are expressed as means ± S.E. of three independent experiments.

Nectin-induced Activation of Cdc42 through Rap1

FIG. 3.

No restoration by a constitutively active mutant of Rap1 or C3G of Src family kinase inhibition on the Nef-3-induced formation of filopodia and lamellipodia and activation of Cdc42 and Rac. A, cell spreading assay. Nectin-1-L cells transiently overexpressing GFP-V12Rap1, C3G-CA, or GFP were cultured on the Nef-3-coated coverslips in the presence of PP2, PP3, or SU6656 for 1 h. The cells were fixed and stained for F-actin and C3G-CA with rhodamine-phalloidin and the anti-C3G pAb, respectively. Scale bars, 10 μm. Bars in the graph represent percentage of cells with filopodia (F) and lamellipodia (L) of the total 100 cells counted and are expressed as means ± S.E. of three independent experiments.

B, pull-down assay. Nectin-1-L cells overexpressing GFP-V12Rap1, Myc-Rap1GAP, c-Src-CA, or not were cultured with clustered Nef-3 or IgG in the presence or absence of PP2, PP3, SU6656, or Me₂SO (DMSO) for 30 min. After the cells were washed, they were lysed and subjected to the pull-down assay using GST-PAR-CRIB followed by Western blotting using the anti-Cdc42 or anti-Rac1 mAbs.
that were transiently overexpressing GFP-Rap1GAP or GFP as a control (data not shown). We then confirmed by the pull-down assay using GST-RalGDS-RBD followed by Western blotting using the anti-Rap1 pAb. a, nectin-1-L cells cultured with clustered Nef-3 or IgG for the indicated periods of time; b, nectin-1-L cells cultured with clustered Nef-3 or IgG for 30 min in the presence of PP2, PP3, or SU6656; c, nectin-1-L cells overexpressing c-Src-CA or an empty vector cultured with clustered Nef-3 or IgG for 30 min. The results shown are representative of three independent experiments. A, FRET imaging. Nectin-1-L cells transiently overexpressing Raichu-Rap1 were cultured on the Nef-3-coated dishes for 15 min and imaged for YFP, CFP, and differential interference contrast (DIC) for the indicated periods of time after plating. YFP/CFP ratio images are shown in the pseudocolor mode with the corresponding differential interference contrast images. In the intensity modulated display (IMD) mode, eight colors from red to blue were used to represent the YFP/CFP ratio with the intensity of each color indicating the mean intensity of YFP and CFP. High YFP/CFP ratio shown in red color indicates high FRET efficiency of the probe, reflecting high GTP/GDP ratio of Rap1. The upper and lower limits of ratio range are shown. Scale bars, 10 μm. The results shown are representative of 10 independent experiments. B, bead-cell assay. Nectin-1-L cells transiently overexpressing GFP-Rap1 were incubated with the Nef-3- or IgG-coated beads for 30 min. The cells were fixed and immunostained for nectin-1 with the anti-nectin-1 pAb. Scale bars, 10 μm. Positions of the beads are marked with asterisks. Bars in the graphs represent percentage of the bead-cell contact sites with the signal for nectin-1 of the total bead-cell contact sites counted (left) or percentage of the bead-cell contact sites with the signal for GFP of the bead-cell contact sites with the signal for nectin-1 counted (right) and are expressed as means ± S.E. of three independent experiments in which a total of 50 bead-cell contact sites were counted.
We have previously shown that c-Src functions downstream of nectins and upstream of Cdc42 and Rac (23). To clarify that Rap1 functions downstream of c-Src, we examined whether the inhibitory effect of Rap1GAP on the Nef-3-induced formation of filopodia and lamellipodia in nectin-1-L cells was not suppressed by co-expression of c-Src-CA. When nectin-1-L cells co-expressing Myc-Rap1GAP with c-Src-CA were cultured on the Nef-3-coated coverslips, neither filopodia nor lamellipodia were formed (Fig. 2). In addition, c-Src-CA enhanced the Nef-3-induced activation of Rap1 in nectin-1-L cells as estimated by the pull-down assay for Rap1 using the GST-RalGDS-RBD (see Fig. 4A, c). These results indicate that Rap1 functions downstream of c-Src.

Insufficiency of the Activation of Rap1 Alone for the Nectin-induced Activation of Cdc42 and Rac—We have previously shown that PP2, an inhibitor of Src family kinases, inhibits the Nef-3-induced formation of filopodia and lamellipodia (23). We examined whether the activation of Rap1 alone is sufficient for the Nef-3-induced formation of filopodia and lamellipodia. Nectin-1-L cells expressing GFP-V12Rap1 or GFP as a control were cultured on the Nef-3-coated coverslips in the presence of PP2 or PP3, an inactive analogue of PP2. In nectin-1-L cells expressing GFP, filopodia and lamellipodia were markedly formed in the presence of PP3, whereas both protrusions were negligibly formed in the presence of PP2 (Fig. 3A). This inhibitory effect of PP2 on the Nef-3-induced formation of filopodia and lamellipodia was confirmed by SU6656, another inhibitor of Src family kinases (34). This inhibitory effect of PP2 was not rescued by GFP-V12Rap1 or C3G-CA, a constitutively active mutant of Rap1-GEF (24). We obtained the essentially same results for nectin-1-MDCK cells (data not shown). These results indicate that the activation of Rap1 is necessary, but not sufficient, for the nectin-induced formation of filopodia and lamellipodia.

We further confirmed that the activation of Rap1 is necessary, but not sufficient, for the Nef-3-induced activation of Cdc42 and Rac. The Nef-3-induced activation of Cdc42 and Rac was inhibited by PP2 and SU6656, but not by PP3, as estimated by the pull-down assay using GST-PAK-CRIB in nectin-1-L cells (Fig. 3B). The inhibition by PP2 of the Nef-3-induced activation of Cdc42 and Rac was not restored by V12Rap1. On the other hand, co-expression of c-Src-CA and Rap1GAP did not induce the activation of Cdc42 or Rac. Taken together, these results indicate that the activation of either c-Src or Rap1 alone is insufficient for and the activation of both is required for the nectin-induced activation of Cdc42 and Rac and formation of filopodia or lamellipodia, respectively.

Nectin-induced, c-Src-mediated Activation of Rap1—We then examined whether Nef-3 indeed activates Rap1. Nectin-1-L cells were incubated with clustered Nef-3 or IgG as a control and then subjected to the pull-down assay using GST-RalGDS-RBD. The incubation of clustered Nef-3 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. 4A, a). To confirm again that c-Src is involved in the nectin-induced activation of Rap1, we co-expressed a dominant negative mutant of CrkI or C3G and Nef-3 as a control with Nectin-1-L cells overexpressing the combination of Flag-CrkI-W169L, C3G-dCD, GFP-V12Rap1, or GFP. The results shown are representative of three independent experiments.
Rap1, nectin-1-L cells treated with PP3, PP2, or SU6656 were cultured with clustered Nef-3 and then subjected to the pull-down assay. The Nef-3-induced activation of Rap1 was inhibited by PP2 and SU6656, but not by PP3, in nectin-1-L cells (Fig. 4A, B). Thus, the Nef-3-induced activation of Rap1 requires c-Src. We next monitored the activation of Rap1 in living nectin-1-L cells cultured on the Nef-3-coated dishes by FRET imaging using the FRET probe Raichu-Rap1 (30). The Nef-3-induced activation of Rap1 was observed at the periphery of the cells where filopodia were formed (Fig. 4B). In addition, Rap1 was also activated at the internal perinuclear region of the cells as described previously (30).

Because Rap1 was activated by Nef-3 near the formation of filopodia, we speculated that Rap1 would be recruited to the nectin-based cell-cell contact sites. To examine this hypothesis, nectin-1-L cells transiently coexpressing Flag-FRG and c-Src with GFP-V12Rap1, GFP-Rap1GAP, or GFP were treated with clustered Nef-3 or IgG in the presence or absence of PP2 for 30 min and then subjected to immunoprecipitation with the anti-FLAG mAb. The immunoprecipitant was incubated with Cdc42 and [35S]GTPγS for the indicated periods of time. The results are expressed as means ± S.E. of three independent experiments.

![Image](https://example.com/image.jpg)

**Fig. 6.** Requirement of both c-Src and Rap1 for the Nef-3-induced activation of FRG. A and B, GEF assay for FRG. Nectin-1-L cells transiently overexpressing Flag-FRG and c-Src with GFP-V12Rap1, GFP-Rap1GAP, or GFP were treated with clustered Nef-3 or IgG in the presence or absence of PP2 for 30 min and then subjected to immunoprecipitation with the anti-FLAG mAb. The immunoprecipitant was incubated with Cdc42 and [35S]GTPγS for the indicated periods of time. The results are expressed as means ± S.E. of three independent experiments.
either Rap1 or c-Src alone is insufficient for and the activation of both is necessary for the nectin-induced activation of FRG.

**No Requirement of Rap1 for the Nectin-induced, c-Src-mediated Recruitment and Phosphorylation of FRG**—We examined whether Rap1 is necessary for the recruitment of FRG to the nectin-based cell-cell adhesion and for the c-Src-mediated tyrosine phosphorylation of FRG. Nectin-1-L cells transiently overexpressing Flag-FRG with GFP-Rap1GAP or GFP-CAAX as a control were incubated with the Nef-3- or IgG-coated beads for 30 min. The cells were fixed and immunostained for nectin-1 and Flag-FRG with the anti-nectin-1 pAb and anti-FLAG mAb, respectively. Positions of the beads are marked with asterisks. Scale bars, 10 μm. A, nectin-1-L cells transiently overexpressing Flag-FRG with GFP-CAAX, B, nectin-1-L cells transiently overexpressing Flag-FRG with GFP-Rap1GAP. Bars in the graphs of A and B represent percentage of the bead-cell contact sites with the signal for FRG of the bead-cell contact sites with the signal for nectin-1 counted and are expressed as means ± S.E. of three independent experiments in which a total of 50 bead-cell contact sites were counted. C, tyrosine phosphorylation assay. Nectin-1-L cells transiently overexpressing Flag-FRG and c-Src with GFP-Rap1GAP, GFP-V12Rap1, or GFP as a control were incubated with clustered Nef-3 or IgG for 30 min and then subjected to the immunoprecipitation assay with the anti-FLAG mAb followed by Western blotting using anti-FLAG, anti-Src, and anti-phosphotyrosine (PY20) mAbs. The results shown are representative of three independent experiments. IB, immunoblot; IP, immunoprecipitation.

**FIG. 7.** No requirement of Rap1 for the Nef-3-induced, c-Src-mediated recruitment and phosphorylation of FRG. A and B, bead-cell assay. Nectin-1-L cells transiently overexpressing Flag-FRG with GFP-Rap1GAP or GFP-CAAX as a control were incubated with the Nef-3- or IgG-coated beads for 30 min. The cells were fixed and immunostained for nectin-1 and Flag-FRG with the anti-nectin-1 pAb and anti-FLAG mAb, respectively. Positions of the beads are marked with asterisks. Scale bars, 10 μm. A, nectin-1-L cells transiently overexpressing Flag-FRG with GFP-CAAX, B, nectin-1-L cells transiently overexpressing Flag-FRG with GFP-Rap1GAP. Bars in the graphs of A and B represent percentage of the bead-cell contact sites with the signal for FRG of the bead-cell contact sites with the signal for nectin-1 counted and are expressed as means ± S.E. of three independent experiments in which a total of 50 bead-cell contact sites were counted. C, tyrosine phosphorylation assay. Nectin-1-L cells transiently overexpressing Flag-FRG and c-Src with GFP-Rap1GAP, GFP-V12Rap1, or GFP as a control were incubated with clustered Nef-3 or IgG for 30 min and then subjected to the immunoprecipitation assay with the anti-FLAG mAb followed by Western blotting using anti-FLAG, anti-Src, and anti-phosphotyrosine (PY20) mAbs. The results shown are representative of three independent experiments. IB, immunoblot; IP, immunoprecipitation.
tured at 2 mM Ca\(^{2+}\), the signal for E-cadherin was not observed at the cell-cell contact sites in the cells expressing CrkI-W169L or C3G-dCD, whereas it was reconstituted there in the cells expressing GFP (Fig. 8A). Moreover, when nectin-1-MDCK cells expressing Myc-Rap1GAP precultured at 2 μM Ca\(^{2+}\) were recultured at 2 mM Ca\(^{2+}\) for 2 h, the signal for E-cadherin was not observed at cell-cell contact sites, and this disappearance of the signal for E-cadherin was rescued by co-expression with GFP-V12Cdc42 (Fig. 8B). These results indicate that Crk, C3G, and Rap1 are required for the nectin-induced, Cdc42-enhanced formation of the E-cadherin-based AJs.

**DISCUSSION**

Evidence is accumulating that Rap1 is involved in many cell functions including the formation of AJs (11, 40), but it remains largely unknown how Rap1 is activated in response to extracellular signals or how Rap1 regulates cell functions. Cdc42 and Rac have also been shown to play important roles in many cell functions including the formation of AJs and TJ (17, 19, 20, 41). It remained unknown how Cdc42 and Rac are activated during the formation of the cell-cell junctions. It has recently been reported that Rac, but not Cdc42, is activated by the action of cadherins (42), and we have found that both Cdc42 and Rac are activated by the action of nectins (23, 33, 43). We have subsequently shown that nectins first recruit and activate c-Src at the nectin-based cell-cell contact sites and that c-Src activated in this way then phosphorylates and activates FRG, inducing the activation of Cdc42 (23).

Extending these earlier observations, the present study showed that Rap1 is additionally involved in the nectin-induced, c-Src- and FRG-mediated activation of Cdc42 and formation of AJs. Nectins first recruit and activate c-Src at the nectin-based cell-cell contact sites. On one hand, c-Src activated in this way then recruits and phosphorylates FRG there, although it does not activate FRG. On the other hand, c-Src induces the activation of Rap1 through the Crk-C3G complex at the same area. Rap1 activated in this way then induces the activation of tyrosine-phosphorylated FRG, eventually inducing the activation of Cdc42. It may be noted that the c-Src-
mediated activation of Rap1 or the phosphorylation of FRG alone is insufficient for and both are essential for the activation of FRG. We have recently revealed that both c-Src and Cdc42 are necessary for the nectin-induced activation of Rac through Vav2, a Rac-GEF (54). We have also shown here that Rap1 is necessary for the activation of Rac presumably through FRG and Cdc42. Taken together, we would like to propose a model for the nectin-induced activation of Cdc42 and Rac as shown in Fig. 9. Cdc42 is likely to induce formation of filopodia and to increase the number of cell-cell contact sites at the initial stage of the formation of AJs. On the other hand, Rac induces formation of lamellipodia that efficiently expands the cell-cell adhesion between filopodia, acting like a zipper (18–20, 44–47). Consistently we demonstrated here that Crk, C3G, and Rap1 are involved in the nectin-induced formation of the E-cadherin-based AJs and that the inhibitory effect of Rap1GAP on the formation of AJs are rescued by a constitutively active mutant of Cdc42. Nectins furthermore recruit first junctional adhesion molecules and then claudins to the apical side of AJs, resulting in the formation of TJs in epithelial cells. Thus, after the formation of AJs, nectins play important roles in both the formation of the cell-cell junctions and the initiation of cell polarization in mammals, and the small G protein pathway of Rap1 controlling Cdc42 is functionally conserved from yeast to mammals and is essential for the formation of cell-cell junctions and cell polarization as described for the budding process in the yeast (15).

Here we have attempted to clarify how Rap1 induces the activation of FRG tyrosine-phosphorylated by c-Src. Several molecules that function downstream of Rap1 have been identified (9, 48). These include phosphatidylinositol 3-kinase, afadin, B-Raf, and RaIGDS. B-Raf activates the MEK-ERK signaling pathway (7), whereas RaIGDS activates Ral as a GEF (49). We have previously shown that wortmannin, a phosphatidylinositol 3-kinase inhibitor, does not inhibit the Nef-3-induced formation of filopodia or lamellipodia in nectin-1-L cells (50) and that afadin is not necessary for the Nef-3-induced activation of Cdc42 or Rac in nectin-1-L cells (33). U0126, an MEK inhibitor, does not inhibit the Nef-3-induced formation of filopodia or lamellipodia in nectin-1-L cells (data not shown). A dominant negative mutant of RalA does not inhibit the Nef-3-induced formation of filopodia or lamellipodia in nectin-1-L cells (data not shown). These results indicate that at least any of these known downstream targets of Rap1 is not involved in the Rap1-induced activation of FRG and suggest that an unidentified molecule(s) may relay the signaling from Rap1 to FRG.

On the basis of the observations thus far available, we finally discuss the physiological role of nectins in the formation of cell-cell junctions and cell polarization. Formation of cell-cell junctions and establishment of cell polarity is a multistep process that is regulated by internal cues or extracellular signals (15, 51). It requires the selection of a specific site at the cell cortex followed by the recruitment and activation of the components involved in actin or microtubule polymerization at that site. These cytoskeletal rearrangements lead to various forms of polarized growth, including asymmetric cell division and the formation of filopodia, lamellipodia, stress fibers, and epithelial cell polarity. However, it remains unclear how epithelial cell polarity is established. During the establishment of epithelial cell polarity, nectins are likely to form initial cell-cell contacts and recruit E-cadherin to the nectin-based cell-cell contact sites (17). Furthermore, at the cell-cell contact sites formed by the trans-interaction of E-cadherin, the nectins, which do not trans-interact with other nectins (non-trans-interacting nectins), inhibit the E-cadherin-induced activation of Rac and the subsequent formation of AJs until eventually the non-trans-interacting nectins trans-interact with other nectin molecules and induce the activation of Cdc42 (43). Therefore, nectins, but not cadherins, are likely a good candidate for the cue to select initial cell-cell contact sites. The trans-interacting nectins at newly formed cell-cell contact sites may organize some signaling microdomains and transduce the positional signal through the c-Src-Crk-C3G-Rap1-FRG-Cdc42-Rac pathway to initiate cell-cell contact, resulting in the formation of cell-cell junctions and establishment of cell polarity in epithelial cells.

Two other groups have recently reported the role of Rap1 in the formation of the E-cadherin-based AJs in epithelial cells (52, 53). One has demonstrated that Ras-transformed MDCK cells, which show the mesenchymal phenotype, form the E-cadherin-based AJs when they are transfected with the plasmid of V12Rap1, and that in contrast overexpression of Rap1GAP disrupts the epithelial cell-cell contacts (52). However, it was not studied in this report how Rap1 is activated. The other one has reported that the trans-interaction of E-cadherin induces the activation of Rap1 through C3G, which binds to the cytoplasmic region of E-cadherin, and that the activation of Rap1 is necessary for the maturation of cell-cell contacts in epithelial cells (53). Our present results are consistent with the results of these two reports in the sense that Rap1 is involved in the formation of AJs.

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Fig. 9. A schematic model for the intracellular signaling pathway from nectin to Cdc42 and Rac. Details are described under “Discussion.”

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Involvement of the c-Src-Crk-C3G-Rap1 Signaling in the Nectin-induced Activation of Cdc42 and Formation of Adherens Junctions
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