Vav2 as a Rac-GDP/GTP Exchange Factor Responsible for the Nectin-induced, c-Src- and Cdc42-mediated Activation of Rac*

Tomomi Kawakatsu, Hisakazu Ogita, Tatsuro Fukuhara, Taihei Fukuyama, Yukiko Minami, Kazuya Shimizu, and Yoshimi Takai‡

From the Department of Molecular Biology and Biochemistry, Osaka University Graduate School of Medicine/Faculty of Medicine, Osaka 565-0871, Japan

Nectins are Ca\textsuperscript{2+}-independent immunoglobulin-like cell-cell adhesion molecules that form homo- and hetero-trans-dimers (trans-interactions). Nectins first form cell-cell contact and then recruit cadherins to the nectin-based cell-cell contact sites to form adherens junctions cooperatively with cadherins. In addition, the trans-interactions of nectins induce the activation of Cdc42 and Rac small G proteins, which enhances the formation of adherens junctions by forming filopodia and lamellipodia, respectively. The trans-interactions of nectins first recruit and activate c-Src at the nectin-based cell-cell contact sites. c-Src then phosphorylates and activates FRG, a Cdc42-GDP/GTP exchange factor (GEF) for Cdc42. The activation of both c-Src and Cdc42 by FRG is necessary for the activation of Rac, but the Rac-GEF responsible for this activation of Rac remains unknown. We showed here that the nectin-induced activation of Rac was inhibited by a dominant negative mutant of Vav2, a Rac-GEF. Nectins recruited and tyrosine-phosphorylated Vav2 through c-Src at the nectin-based cell-cell contact sites, whereas Cdc42 was not necessary for the nectin-induced recruitment of Vav2 or the nectin-induced, c-Src-mediated tyrosine phosphorylation of Vav2. Cdc42 activated through c-Src then enhanced the GEF activity of tyrosine-phosphorylated Vav2 on Rac1. These results indicate that Vav2 is a GEF responsible for the nectin-induced, c-Src-, and Cdc42-mediated activation of Rac.

In multicellular organisms, cell adhesion is critical for many events, including morphogenesis and maintenance of normal tissues (for reviews, see Refs. 1–3). Adherens junctions (AJs)\textsuperscript{1} play key roles in cell adhesion and also in the concentration of many biologically active molecules, such as membrane receptors and signaling molecules (for reviews, see Refs. 4–8). At AJs in epithelial cells, E-cadherin functions as a major cell-cell adhesion molecule (for reviews, see Refs. 2, 9, and 10). E-cadherin is a member of the cadherin superfamily consisting of over 80 members, each of which is expressed in a wide variety of cells not only in epithelial cells but also in non-epithelial cells (for reviews, see Refs. 11–13). E-cadherin first forms homo-cis-dimers and then homo-trans-dimers (trans-interactions) through the extracellular region in a Ca\textsuperscript{2+}-dependent manner, inducing cell-cell adhesion (9). The cytoplasmic region is linked to the actin cytoskeleton through α- and β-catenins, which strengthens its cell-cell adhesion activity (5, 7, 8). Nectins are Ca\textsuperscript{2+}-independent Ig-like cell-cell adhesion molecules at AJs (for reviews, see Refs. 14 and 15). Nectins comprise a family of four members, nectin-1, -2, -3, and -4. Each member first forms homo-cis-dimers and then homo- or hetero-trans-dimers (trans-interactions) through the extracellular region in a Ca\textsuperscript{2+}-independent manner, causing cell-cell contact. The cytoplasmic region is associated with the actin cytoskeleton through afadin, a nectin- and F-actin-binding protein. Nectins are involved in the formation of AJs cooperatively with E-cadherin in epithelial cells; the trans-interactions of nectins first form cell-cell contact and then recruit E-cadherin to the nectin-based cell-cell contact sites, inducing the formation of AJs. In addition, nectins play a role in the formation of AJs in fibroblasts, synapses in neurons, contacts between commissural axons and floor plate cells in developing mice, and spermatid-Sertoli cell junctions in the testis cooperatively with, or independently of, cadherins (14).

On the other hand, the formation of AJs is enhanced through the Cdc42- and Rac-mediated formation of filopodia and lamellipodia, respectively (16–20). The Cdc42-formed filopodia increase the cell-cell contact sites, whereas the Rac-formed lamellipodia efficiently zip the cell-cell contact between filopodia, acting like a “zipper,” eventually enhancing the formation of AJs. Cdc42 is activated by the trans-interactions of nectins (15, 20–22), whereas Rac is activated by the trans-activations of E-cadherin and nectins (23–27). The trans-interactions of nectins first recruit and activate c-Src. c-Src then tyrosine-phosphorylates FRG, a specific GDP/GTP exchange factor (GEF) for Cdc42, and induces the activation of Rap1 small G protein through C3G, a Rap-GEF, at the nectin-based cell-cell contact sites (28, 53). Activated Rap1 then enhances the GEF activity of tyrosine-phosphorylated FRG on Cdc42. The activation of both c-Src and Cdc42 is necessary for the activation of Rac, but the Rac-GEF responsible for this activation of Rac remains unknown. Many GEFs for Rac, including Vav, Tiam, Grnp, Abr, Bcr, Pix, P-Rex1, Asef, and Trio, have been identified (for a review, see Ref. 29). Among them, the Vav family members, consisting of three members, Vav1, -2, and -3, are strictly

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† To whom correspondence should be addressed: Dept. of Molecular Biology and Biochemistry, Osaka University Graduate School of Medicine/Faculty of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-3410; Fax: 81-6-6879-3419; E-mail: ytkai@molbio.med.osaka-u.ac.jp.

The abbreviations used are: AJ, adherens junction; GEF, GDP/GTP exchange factor; Vav2-DN, a dominant negative mutant of Vav2; Vav2-CA, a constitutively active mutant of Vav2; CRIB, Cdc42 and Rac-interacting binding; pAb, polyclonal antibody; mAb, monoclonal antibody; ConA, concanavalin A; Nef-3, the extracellular fragment of nectin-3 fused to the Fc portion of IgG; SFK, Src family kinase; GFP, green fluorescent protein; EGF, enhanced GFP, MDCK, Madin-Darby canine kidney cells; GTP*S, guanosine 5′-3′-O-(thio)triphosphate.
regulated by tyrosine phosphorylation on their GEF activity (30, 31). In addition, it has recently been reported that Vav2 is tyrosine-phosphorylated in cells transfected with activated c-Src and induces the activation of Rac1 and the formation of lamellipodia (32, 33). Therefore, we have examined here whether Vav2 is involved in the nectin-induced, c-Src-, and Cdc42-mediated activation of Rac.

EXPERIMENTAL PROCEDURES

Expression Plasmids—The full-length mouse Vav2 cDNA was kindly provided by Dr. X. R. Bustelo (State University of New York, Stony Brook, NY). A Vav2 mutant changing Leu212 to Gln was used as a dominant negative mutant of Vav2 (Vav2-DN), and an N-terminal region (amino acids: 1–183)-truncated mutant of Vav2 was used as a constitutively active mutant of Vav2 (Vav2-CA) as described (34). Expression vectors for Myc-tagged and FLAG-tagged wild-type Vav2 (pCIneo-Myc-Vav2 and pEF-BOS-FLAG-Vav2) were constructed by inserting a full-length of Vav2 cDNA into pCIneo-Myc and pEF-BOS-FLAG, respectively. An expression vector for Myc-tagged Vav2-CA (pCIneo-Myc-Vav2-CA) was constructed by inserting amino acids 184–868 of Vav2 cDNA into pCIneo-Myc. An expression vector for Myc-tagged Vav2-DN (pCIneo-Myc-Vav2-DN) was point-mutated at Leu212 to Gln, and the expression plasmid for pEF-BOS-NWASP-CRIB-CAXAAX was described (35). The full-length human Tiam1 cDNA was kindly supplied by Dr. A. M. Crompton (Onyx Pharmaceuticals Inc., Richmond, CA). An expression vector for the Myc-tagged dominant negative form of Tiam1 (pCMV-Myc-Tiam1-DN) was prepared as described (36). pEGFP-CAXAAX and pEGFP-NWASP-CRIB-CAXAAX were also prepared as described (20). NWASP-CRIB-CAXAAX, consisting of the NWASP-Cdc42 and Rac-interactive binding (CRIB) domain and the C-terminal polybasic sequence of amino acids (CAXAAX, C is Cys, A is aliphatic, and X is a variety of amino acids), is preferentially targeted to the plasma membrane and inhibits the Cdc42 activity by specifically binding to the GTP-bound form of Cdc42 (20, 27). Expression vectors for GFP-V12Cdc42 (pEGFP-V12Cdc42) and GFP-V12Rac1 (pEGFP-V12Rac1) were kindly supplied from Prof. S. Narumiya (Kyoto University, Kyoto, Japan). V12Cdc42 and V12Rac1 were constitutively active Rac1, respectively, changing Gly12 to Val. An expression vector for FLAG-V12Cdc42 (pEF-BOS-FLAG-V12Cdc42) was constructed by inserting V12Cdc42 cDNA into pEF-BOS-FLAG. An expression vector for wild-type c-Src (USC-Src) (pUSE-Src) was purchased from Upstate Biotechnology. All the constructs used here were confirmed by sequencing. Protein concentrations were determined by using the BCA protein assay kit (Pierce).

Cell Lines, Cell Culture, and Transfection—Mouse fibroblast L cells and MDCK cells were kindly supplied by Dr. T. Tsukita (Kyoto University, Kyoto, Japan) and Dr. W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany), respectively. L cells and MDCK cells stably expressing nectin-1a (nectin-1-L cells) and nectin-1-MDCK cells were prepared as described (37). These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Transfection and immunofluorescence microscopy of cultured cells with a confocal imaging system (Radian 2100, Bio-Rad Laboratories) were performed as described (37).

Antibodies—Rabbit anti-nectin-1 and anti-nectin-2 polyclonal antibodies (pAbs) were prepared as described (34, 37). A rabbit anti-phospho-Src (Tyr416) pAb (Cell Signaling Technology), a mouse anti-Src monoclonal antibody (mAb) (Calbiochem-Novabiochem), a mouse anti-phosphotyrosine mAb (BD Transduction Laboratories), a mouse anti-FLAG mAb and a rabbit anti-FLAG pAb (Sigma), and secondary antibodies (Chemicon) for immunofluorescence microscopy and Western blotting were purchased from commercial sources. Hybridoma cells expressing a mouse anti-Myc mAb (9E10) were obtained from the American Type Culture Collection, and the anti-Myc mAb was prepared as described (38).

Assays for the Formation of Filopodia and Lamellipodia and the Bead-Cell Contact—The formation of filopodia and lamellipodia was assayed as described (21, 27). The bead-cell contact was also assayed as described (22, 28). Briefly, nectin-1-L cells were seeded on each well of a 24-well plate and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum for 12 h. Latex-sulfate microbeads coated with the extracellular fragment of nectin-3 fused to the Fc portion of IgG (Nef-3) or concanavalin A (ConA) as a control were added to each well. After a 60-min incubation, the cells were fixed and immunostained.

Assays for the Immunoprecipitation and the Vav2 GEF Activity—The immunoprecipitation assay was performed as described (28). In brief, nectin-1-L cells (1.5 × 10⁶ cells/100-mm dish) were transfected in the combination with 6 μg of pEF-BOS-FLAG-Vav2, pcDNA-c-Src, pEGFP-NWASP-CRIB-CAXAAX, pEGFP-V12Cdc42, and pEGFP. After an 8-h culture, the cells were serum-starved for 16 h. The medium was then replaced by 3 ml of Dulbecco’s modified Eagle’s medium containing clustered Nef-3 or IgG with or without 20 μM P2 (Calbiochem-Novabiochem), an inhibitor for Src family kinases, and the cells were incubated for 30 min. The cells were washed with 1 ml of ice-cold phosphate-buffered saline containing 1 mM sodium vanadate, lysed in 0.5 ml of Buffer A (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 μg/ml phenylmethylsulfonyl 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 (750 μg of protein) 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 A. 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 (39). The binding of [32P]GTP[S] to Rac (10 pmol) was assayed by measuring the radioactivity of [32P]GTP[S] bound to Rac after incubation for indicated periods of time in the presence of each immunoprecipitant from the cells treated with clustered Nef-3 or IgG for 30 min.

RESULTS

Inhibition of the Nectin-induced Formation of Lamellipodia by a Dominant Negative Mutant of Vav2—Nectins induce the activation of Cdc42 and Rac, which then form filopodia and lamellipodia, respectively, in L fibroblasts (21, 28). To determine whether nectin-1-L cells and Nef-3, we first examined whether Vav2 is involved in the nectin-induced formation of lamellipodia. When nectin-1-L cells were cultured on the Nef-3-coated coverslips, both filopodia and lamellipodia were formed as described (21, 28). However, lamellipodia were hardly formed in nectin-1-L cells overexpressing Myc-Vav2-DN. The formation of filopodia was also reduced by Myc-Vav2-DN, although the effect of Myc-Vav2-DN on the formation of filopodia was less than that on the formation of lamellipodia. This result is different from our previous observation on the effect of a dominant negative mutant of Rac, which inhibits the Nef-3-induced formation of lamellipodia but not that of filopodia (21). The exact reason for this difference is not known, but Myc-Vav2-DN may interfere with FRG and thereby inhibit the FRG-induced activation of Cdc42. Moreover, when the cells were cultured on the IgG-coated coverslips, the formation of lamellipodia in nectin-1-L cells overexpressing Myc-Vav2-DN appeared to be further inhibited than that in nectin-1-L cells overexpressing an empty vector. Although the exact reason for this further inhibition of the formation of lamellipodia by Myc-Vav2-DN remains unclear, Vav2 may play a role other than a Rac-GEF. The Nef-3-induced formation of filopodia and lamellipodia was not inhibited by overexpression of a dominant negative mutant of Tiam1, another Rac-GEF, in nectin-1-L cells, suggesting that Vav2 functions as a specific Rac-GEF in the nectin-induced formation of lamellipodia, although the involvement of another Rac-GEF(s) could not be excluded. When the similar experiments were performed with nectin-1-MDCK cells, the essentially same results were obtained (data not shown). These results, together with the earlier observation that the Nef-3-induced formation of lamellipodia is mediated by Rac (21, 28), indicate that Vav2 is necessary for the nectin-induced activation of Rac in nectin-1-L and nectin-1-MDCK cells.

If Vav2 functions upstream of Rac, overexpression of V12Rac1, a constitutively active mutant of Rac1, in nectin-1-L cells would suppress the inhibitory effect of Vav2-DN. Consistently, overexpression of GFP-V12Rac1 restored the formation of lamellipodia, but not filopodia, in nectin-1-L cells expressing Myc-Vav2-DN when the cells were cultured on the Nef-3-coated coverslips.
Fig. 1. Inhibitory effect of a dominant negative mutant of Vav2 on the nectin-induced formation of lamellipodia in nectin-1-L cells. A, inhibitory effect of a dominant negative mutant of Vav2 on the nectin-induced formation of lamellipodia in nectin-1-L cells. Nectin-1-L cells transfected with the plasmid of Myc-Vav2-DN, Myc-Tiam1-DN, or an empty vector were cultured on the Nef-3- or IgG-coated coverslips for 1 h and
A analogue of PP2 (Fig. 3 inhibitor of SFKs, but not in the presence of PP3, an inactive dia was inhibited in nectin-1-L cells in the presence of PP2, an SFKs. The nectin-induced formation of filopodia and lamellipodia (28). We first confirmed that Vav2 functions downstream of (SFKs) are necessary for the nectin-induced activation of Rac. We have previously shown that Src family kinases—

Involvement of c-Src in the Nectin-induced, Vav2-mediated Formation of Lamellipodia and the Tyrosine Phosphorylation of Vav2—We have previously shown that Src family kinases (SFKs) are necessary for the nectin-induced activation of Rac (28). We first confirmed that Vav2 functions downstream of SFKs. The nectin-induced formation of filopodia and lamellipodia was inhibited in nectin-1-L cells in the presence of PP2, an inhibitor of SFKs, but not in the presence of PP3, an inactive analogue of PP2 (Fig. 3A). The inhibitory effects of PP2 were suppressed in nectin-1-L cells expressing Myc-Vav2-CA regarding the formation of lamellipodia. It may be noted that filopodia were not formed in nectin-1-L cells expressing Myc-Vav2-CA in the presence of PP2. This may be due to the fact that the inhibition by PP2 of the c-Src-induced, FRG-mediated activation of Cdc42 was not rescued by Vav2-CA. These results suggest that Vav2 functions downstream of SFKs and induces the activation of Rac, but not Cdc42, in nectin-1-L cells. Although Vav2-CA significantly restored the formation of lamellipodia in the presence of PP2, there was a slight difference from the control on the extent of the formation of lamellipodia and the cell morphology. When nectin-1-L cells overexpressing both Vav2-CA and V12Cdc42 were cultured on the Nef-3-coated coverslips, lamellipodia as well as filopodia were formed to similar extents to the control levels, and the cell morphology of these cells was also similar to that of the control cells. Taken together, these results suggest that the activation of Cdc42, which is induced by the trans-interactions of nectins through FRG, a Cdc42-GEF, might be additionally necessary to induce the maximal activation of Vav2 on Rac.

We then examined whether Vav2 is tyrosine-phosphorylated by c-Src. Nectin-1-L cells transiently overexpressing FLAG-tagged Vav2 and c-Src were incubated with Nef-3 clustered with an anti-human Fc pAb and subjected to the immunoprecipitation assay using an anti-FLAG mAb. IgG clustered with an anti-human Fc pAb was used as a control. Vav2 was tyrosine-phosphorylated by clustered Nef-3, whereas it was not tyrosine-phosphorylated by clustered IgG (Fig. 3B). Moreover, the tyrosine phosphorylation of Vav2 induced by clustered Nef-3 was inhibited by PP2. These results indicate that nectins induce the c-Src-mediated tyrosine phosphorylation of Vav2 in nectin-1-L cells.

coverslips (Fig. 1B). Essentially the same results were obtained for nectin-1-MDCK cells (data not shown). Taken together, these results indicate that Vav2 functions upstream of Rac in the nectin-induced formation of lamellipodia in nectin-1-L cells and nectin-1-MDCK cells.

Recruitment of Vav2 to the Contact Sites between the Nef-3-coated Beads and Nectin-1-L Cells—We have previously shown that c-Src and FRG are recruited to the nectin-based cell-cell contact sites (28). We next examined whether Vav2 is recruited to the nectin-based cell-cell contact sites. Nectin-1-L cells transiently expressing FLAG-Vav2 were incubated with the Nef-3-coated beads. The ConA-coated beads were used as a control. The immunofluorescence signals for nectin-1 and FLAG-Vav2 were concentrated at the contact sites between the Nef-3-coated beads and the nectin-1-L cells expressing FLAG-Vav2 but not between the ConA-coated beads and the nectin-1-L cells expressing FLAG-Vav2 (Fig. 2A). In contrast, when nectin-1-L cells expressing Myc-Tiam1 were incubated with the Nef-3- or ConA-coated beads, the signal for Myc-Tiam1 was not concentrated at either of the bead-cell contact sites (Fig. 2B). These results indicate that Vav2 is likely to be specifically recruited by nectins to the nectin-based cell-cell contact sites.

stained for F-actin and Myc-Vav2-DN with rhodamine-phalloidin and the anti-Myc mAb, respectively. Scale bars, 10 μm. B, suppression by a constitutively active Rac1 of the inhibitory effect of a dominant negative mutant of Vav2. Nectin-1-L cells transfected with the combination of plasmids for Myc-Vav2-DN, GFP-V12Rac1, and GFP were cultured on the Nef-3-coated coverslips and stained with rhodamine-phalloidin and the anti-Myc mAb. Scale bars, 10 μm. The bars in the graphs of A and B represent the percentage of cells with filopodia (F) or lamellipodia (L) of the total cells counted (n = 50) and are expressed as means ± S.E. of three independent experiments.
No Requirement of Cdc42 for the Nectin-induced Recruitment of Vav2—We have previously shown that Rap1 is necessary for the Nef-3-induced activation of tyrosine-phosphorylated FRG but not necessary for the Nef-3-induced recruitment of FRG or the Nef-3-induced, c-Src-mediated tyrosine phosphorylation of FRG (28, 53). We next examined whether Cdc42 is necessary for the Nef-3-induced recruitment of Vav2 and the c-Src-mediated tyrosine phosphorylation of Vav2. When nectin-1-L cells expressing Vav2 were incubated with the Nef-3-coated beads or the ConA-coated beads, the immunofluorescence signals for nectin-1 and Vav2 were observed at the contact sites between the Nef-3-coated beads and nectin-1-L cells but not between the ConA-coated beads and nectin-1-L cells (Fig. 4A). The localization of nectin-1 and Vav2 was not affected by GFP-NWASP-CRIB-C AAX, a specific inhibitor of activated Cdc42 (40) (Fig. 4B). In Fig. 4A, when GFP-C AAX as a control for GFP-NWASP-CRIB-C AAX was co-expressed with Vav2, we observed that the recruitment of GFP-C AAX to the bead-cell contact sites might not be dependent on the trans-interactions of nectins. Nectin-1-L cells transiently overexpressing FLAG-tagged Vav2 and c-Src were incubated with Nef-3 clustered with an anti-human Fc pAb and subjected to the immunoprecipitation assay using an anti-FLAG mAb. Vav2 was tyrosine-phosphorylated by Nef-3 clustered with an anti-human Fc pAb, but this tyrosine phosphorylation was not inhibited by GFP-NWASP-CRIB-C AAX (Fig. 3B). These results indicate that Cdc42 is not necessary for the Nef-3-induced recruitment of Vav2 or the Nef-3-induced, c-Src-mediated tyrosine phosphorylation of Vav2.

c-Src- and Cdc42-mediated Activation of Vav2—In the final set of experiments, we examined in a cell-free assay system whether Nef-3 enhanced the GEF activity of Vav2 on Rac. The GEF activity on Rac of FLAG-Vav2 immunoprecipitated by the anti-FLAG mAb from the Nef-3-treated nectin-1-L cells was more active than that from the IgG-treated cells, indicating that Nef-3 induces the activation of Vav2 (Fig. 5). This Nef-3-induced activation of Vav2 was inhibited by PP2 and GFP-NWASP-CRIB-C AAX (20). Moreover, the inhibition of the GEF activity of Vav2 in the presence of PP2 was not restored by V12Cdc42, a constitutively active form of Cdc42. Taken together, these results indicate that the activation of only Cdc42 is insufficient and that both the activation of Cdc42 and the nectin-induced, c-Src-mediated tyrosine phosphorylation of Vav2 are necessary for the activation of Vav2.

DISCUSSION

We have previously shown that the trans-interactions of nectins induce the activation of Cdc42 and Rac (21, 28). We have then studied how they induce the activation of these small G proteins and have found that the trans-interactions of nectins first recruit and activate c-Src at the nectin-based cell-cell contact sites. Nectin-1-L cells transiently overexpressing active Vav2 and c-Src were incubated with Nef-3 clustered with an anti-human Fc pAb and subjected to the immunoprecipitation assay using an anti-FLAG mAb. Vav2 was tyrosine-phosphorylated by Nef-3 clustered with an anti-human Fc pAb, but this tyrosine phosphorylation was not inhibited by GFP-NWASP-CRIB-C AAX (Fig. 3B). These results indicate that Cdc42 is not necessary for the Nef-3-induced recruitment of Vav2 or the Nef-3-induced, c-Src-mediated tyrosine phosphorylation of Vav2.

V12Cdc42, or an empty vector were cultured on the Nef-3-coated coverslips for 1 h and stained for F-actin, Myc-Vav2-CA, and FLAG-V12Cdc42 with rhodamine-phalloidin, the anti-Myc mAb, and the anti-FLAG pAb, respectively. Scale bars, 10 µm. The bars in the graph represent the percentage of cells with filopodia (F) or lamellipodia (L) of the total cells counted (n = 100) and are expressed as means ± S.E. of three independent experiments. PP3, protein phosphatase 3. B, nectin-induced tyrosine phosphorylation of Vav2. Nectin-1-L cells transiently expressing FLAG-Vav2 and c-Src with or without GFP-NWASP-CRIB-C AAX were treated with clustered Nef-3 or IgG in the presence or absence of PP2 for 30 min. Cell lysates were subjected to the immunoprecipitation (IP) assay with the anti-FLAG mAb (α-Flag) followed by Western blotting with the anti-phosphotyrosine (α-PY), anti-FLAG, and anti-Src mAbs (α-Src). The results shown are representative of three independent experiments.
contact sites (28). Activated c-Src then tyrosine-phosphorylates FRG, a Cdc42-GEF, and activates C3G, a Rap1-GEF, through Crk, an adaptor of c-Src, at the nectin-based cell-cell contact sites. Rap1 then enhances the GEF activity on Cdc42 of tyrosine-phosphorylated FRG (53). At that time, we have found that the activation of both c-Src and Cdc42 is necessary for the activation of Rac, but it remained unknown which Rac-GEF is responsible for this activation of Rac. We have clarified in this study that at least Vav2 is a GEF responsible for this activation of Rac; Vav2 is recruited, tyrosine-phosphorylated by c-Src, and activated at the nectin-based cell-cell contact sites. The c-Src-mediated tyrosine phosphorylation of Vav2 alone is insufficient for the activation of Vav2, and the activation of Cdc42 is additionally necessary for it, consistent with the earlier observations (28, 33, 41). It may be noted that Cdc42 is not necessary for the nectin-induced recruitment of Vav2 to the nectin-based cell-cell contact sites or the nectin-induced, c-Src-mediated tyrosine phosphorylation of Vav2.

The Vav gene was originally isolated as an oncogene (42). In mammals, Vav1 expression is highly restricted in hematopoietic cells, whereas Vav2 and Vav3 are ubiquitously expressed (for reviews, see Refs. 43 and 44). All Vav proteins share several characteristic structural domains; they have a calponin homology domain, an acidic-rich domain, a Dbl homology domain, a pleckstrin homology domain, an Src homology 2 domain, and two Src homology 3 domains from the N terminus to the C terminus in this order. Overexpression of wild-type or N-terminal truncated Vav proteins induce cellular transformation, which is generally related to tumor metastasis (45, 46). There are several reports showing that Vav2 activates RhoA and Cdc42 as well as Rac1 (33, 47, 48). However, the extent of the activation of each small G protein is dependent on the in vitro and in vivo experiments or cell lines used. In our assay system, Vav2 specifically induces the activation of Rac.

The structural analysis of Vav proteins has revealed that at the resting state, the GEF activity of Vav is inhibited by its N-terminal arm, which occludes the Dbl homology domain essential for interacting with small G proteins and changing from the GDP form to the GTP form of these proteins (49). When the tyrosine residue, Tyr174, in the N-terminal arm of Vav is phosphorylated by activated c-Src, the N-terminal arm is released from the Dbl homology domain, resulting in the exposure of the site with GEF activity and the activation of Rac. Thus, the tyrosine phosphorylation of Vav2 is required for its activation. However, the tyrosine phosphorylation of Vav2 alone is not sufficient at least for the nectin-induced activation of Rac, and activated Cdc42 is additionally required. It remains unknown how Cdc42 activates tyrosine-phosphorylated Vav2. In this study, when we used the N-terminal truncated form of Vav2 (amino acids: 184–868) as Vav2-CA (34), which lacks the N-terminal inhibitory domain and constitutively activates Rac, the restoration by Vav2-CA of the formation of lamellipodia in the presence of PP2 was almost complete, but there is a slight difference from the control. Nectin-1-L cells overexpressing both Vav2-CA and V12Cdc42 formed lamellipodia as well as filopodia to similar extents to the control levels. This raises the

![Fig. 4. No requirement of Cdc42 for the nectin-induced recruitment of Vav2 in nectin-1-L cells.](http://www.jbc.org/)

A and B, nectin-1-L cells transiently overexpressing FLAG-Vav2 with GFP-CAAX or GFP-NWASP-CRIB-CAAX were incubated with the Nef3- or ConA-coated beads for 1 h. The cells were fixed and immunostained for nectin-1 and FLAG-Vav2 with the anti-nectin-1 pAb and the anti-FLAG mAb, respectively. Scale bars, 10 μm. The positions of the beads are marked with asterisks. The bars in the graphs of A and B represent the percentage of the bead-cell contact sites with the signal for FLAG-Vav2 of the bead-cell contact sites with the signal for nectin-1 (n = 50) and are expressed as means ± S.E. of three independent experiments. DIC, differential interference contrast.
The present results are consistent with the recent report that Vav2 is tyrosine-phosphorylated in cells transfected with activated c-Src and induces the activation of Rac1 and the formation of lamellipodia (32, 33). However, there is a recent report that the cell spreading is actually observed in T cells expressing GEF activity-impaired Vav1 (50). The reason why these discrepancies exist remains unknown, but it may be due to the difference of cell types or the difference of the function between Vav1 and Vav2. Moreover, platelets from Vav1−/− or Vav1−/−/Vav2−/− mice do not show any inhibitory effects on cell spreading when platelets are plated on the fibronogen-coated coverslips (51). In Vav1−/−/Vav2−/− mice, Vav3 may compensate for the lack of Vav1 and Vav2 on the integrin-induced cell spreading. A further investigation using all Vav proteins-null (Vav1/2/3 knock-out) mice, which have been recently generated, could contribute to resolving this issue (52).

Taken together, we propose here a current model for the nectin-induced activation of Cdc42 and Rac in Fig. 6. Nectins first recruit and activate c-Src at the nectin-based cell-cell contact sites. c-Src then recruits and tyrosine-phosphorylates FRG there, whereas c-Src induces the activation of Rap1 through the Crk-C3G complex at the same sites. Rap1 then induces the activation of tyrosine-phosphorylated FRG, eventually inducing the activation of Cdc42. Cdc42 then induces the formation of filopodia through the reorganization of the actin cytoskeleton, which enhances the formation of AJs (16). On the other hand, c-Src recruits and tyrosine-phosphorylates Vav2. Cdc42 activates tyrosine-phosphorylated Vav2, eventually inducing the activation of Rac. Rac then induces the formation of lamellipodia, which also enhances the formation of AJs (19).

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Vav2 as a Rac-GDP/GTP Exchange Factor Responsible for the Nectin-induced, c-Src- and Cdc42-mediated Activation of Rac
Tomomi Kawakatsu, Hisakazu Ogita, Tatsuro Fukuhara, Taihei Fukuyama, Yukiko Minami, Kazuya Shimizu and Yoshimi Takai

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