Activation of Cdc42 by trans interactions of the cell adhesion molecules nectins through c-Src and Cdc42-GEF FRG

Tatsuro Fukuhara,[1] Kazuya Shimizu,[1] Tomomi Kawakatsu,[1] Taihei Fukuyama,[1] Yukiko Minami,[1] Tomoyuki Honda,[1] Takashi Hoshino,[1] Tomohiro Yamada,[1] Hisakazu Ogita,[1] Masato Okada,[2] and Yoshimi Takai[1]

[1]Department of Molecular Biology and Biochemistry, Osaka University Graduate School of Medicine/Faculty of Medicine, and [2]Department of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Suita 565-0871, Osaka, Japan

Nectins, Ca^{2+}-independent immunoglobulin-like cell–cell adhesion molecules, initiate cell–cell adhesion by their trans interactions and recruit cadherins to cooperatively form adherens junctions (AJs). In addition, the trans interactions of nectins induce the activation of Cdc42 and Rac small G proteins, which increases the velocity of the formation of AJs. We examined here how nectins induce the activation of Cdc42 in MDCK epithelial cells and L fibroblasts. Nectins recruited and activated c-Src at the nectin-based cell–cell adhesion sites. FRG, a GDP/GTP exchange factor specific for Cdc42, was then recruited there, tyrosine phosphorylated by c-Src, and activated, causing an increase in the GTP-bound active form of Cdc42. Inhibition of the nectin-induced activation of c-Src suppressed the nectin-induced activation of FRG and Cdc42. Inhibition of the nectin-induced activation of FRG or depletion of FRG by RNA interference suppressed the nectin-induced activation of Cdc42. These results indicate that nectins induce the activation of Cdc42 through c-Src and FRG locally at the nectin-based cell–cell adhesion sites.

Introduction

Cell–cell adhesion is an essential feature of epithelial cells that ensures their polarized status and therefore their differentiation and physiological functions. In polarized epithelial cells, cell–cell adhesion is mediated through a junctional complex comprised of tight junctions (TJs), cell–cell adherens junctions (AJs), and desmosomes (DSs; Farquhar and Palade, 1963). These junctional structures are typically aligned from the apical to the basal side, although DSs are independently distributed in other areas (Farquhar and Palade, 1963). AJs are the sites of cell recognition and adhesion (Tsukita et al., 1991), and the formation and maintenance of TJs and DSs are dependent on the formation and maintenance of AJs (Tsukita and Furuse, 1999). At TJs, claudins are key Ca^{2+}-independent cell–cell adhesion molecules (Tsukita and Furuse, 1999). At AJs, E-cadherin is a key Ca^{2+}-dependent cell–cell adhesion molecule (Takeichi, 1988). E-cadherin forms cis-dimers and then trans-dimers through the extracellular region, causing cell–cell adhesion. The cytoplasmic tail of E-cadherin is linked to the actin cytoskeleton through many peripheral membrane proteins, including α-catenin, β-catenin, vinculin, and α-actinin, which strengthen the cell–cell adhesion activity of E-cadherin (Gumbiner, 2000). Nectins are Ca^{2+}-independent Ig-like cell–cell adhesion molecules at AJs (Takai and Nakanishi, 2003; Takai et al., 2003). Nectins comprise a family of four members, nectin-1, -2, -3, and -4. All nectins form homo-cis-dimers and then homophilically or heterophilically trans interact, causing cell–cell adhesion. Nectins are associated with the actin cytoskeleton through afadin, a nectin- and actin filament (F-actin)–binding protein. Nectins initiate cell–cell adhesion and recruit cadherins to cooperatively form AJs in epithelial cells and fibroblasts and synapses in neurons (Takai and Nakanishi, 2003; Takai et al., 2003). Furthermore, nectins recruit first junctional adhesion molecules and then claudins

to the apical side of AJs in cooperation with cadherins, resulting in the formation of TJs in epithelial cells. Junctional adhesion molecules are Ca^{2+}-independent Ig-like cell–cell adhesion molecules that recruit the cell polarity protein complex, consisting of Par-3, atypical PKC, and Par-6, by directly binding Par-3 (Ohno, 2001). Nectin-1 and -3, but not nectin-2, also play a role in cell polarization by directly binding Par-3 (Takekuni et al., 2003).

The Rho family small G proteins, Rho, Rac, and Cdc42, affect the formation and/or maintenance of AJs (Van Aelst and Symons, 2002). Rho is necessary for the formation of AJs in keratinocytes (Braga et al., 1997). In MDCK cells, a constitutively active mutant of Rho does not affect the formation of the E-cadherin–based AJs (Takaishi et al., 1997), but Rho activity is reduced by the formation of the E-cadherin–based AJs (Noren et al., 2003). Rac is necessary for the formation and maintenance of AJs and TJs in epithelial cells (Braga et al., 1997; Takaishi et al., 1997; Jou and Nelson, 1998; Ehrlich et al., 2002). Rac is activated by the trans interactions of nectins and/or E-cadherin (Kawakatsu et al., 2002; Yap and Kovacs, 2003). Moreover, not only Rac but also Cdc42 is activated by the trans interactions of nectins (Kawakatsu et al., 2002). Thus, evidence is accumulating that Rho and Rac are necessary for the formation and maintenance of AJs. In contrast, there was no direct evidence that Cdc42 regulates the formation and/or maintenance of AJs except for our report that Cdc42 is involved in the maintenance of AJs in MDCK cells (Kodama et al., 1999). Recently, we have shown that Cdc42 activated by nectins enhances the velocities of the formation of the E-cadherin–based AJs and the claudin-based TJs in MDCK cells (Fukuhara et al., 2003). In addition, the nectin-induced activation of Cdc42 is required for the E-cadherin–induced activation of Rac and the formation of AJs in L and MDCK cells (Hoshino et al., 2004). Thus, these three Rho family members play crucial roles in the formation and maintenance of AJs in epithelial cells. However, it remains unknown how Cdc42 and Rac are activated by nectins and E-cadherin. We have investigated here the mode of action of nectins in the activation of Cdc42 and Rac, particularly focusing on Cdc42.

Results

Involvement of Src family kinases (SFKs) in the nectin-induced formation of filopodia and lamellipodia

We have previously shown that when MDCK and L cells were plated on the coverslips precoated with Nef-3, the recombinant extracellular fragment of nectin-3 fused to the Fc portion of human IgG, filopodia and lamellipodia were formed to small extents through the activation of Cdc42 and Rac, respectively (Kawakatsu et al., 2002). We first examined by use of MDCK cells stably expressing exogenous nectin-1 (nectin-1-MDCK cells) and L cells stably expressing nectin-1 (nectin-1-L cells) that these protrusions are formed by the specific interaction of Nef-3 with nectin-1. Both filopodia and lamellipodia were more markedly formed in nectin-1-MDCK cells than in wild-type MDCK cells when they were cultured on the Nef-3–coated coverslips (Fig. 1 A). These protrusions
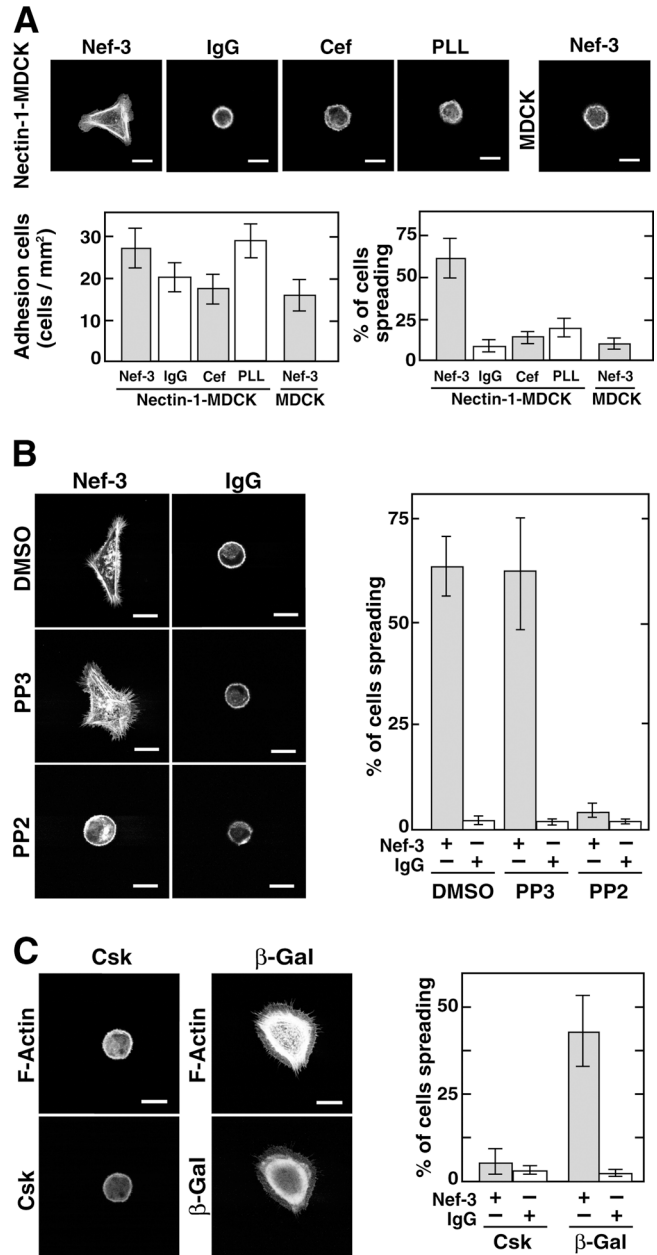
Figure 1.   **Inhibition by PP2 and Csk of the nectin-induced formation of filopodia and lamellipodia in nectin-1-MDCK cells.** (A) Nectin-induced formation of filopodia and lamellipodia. Nectin-1-MDCK or wild-type MDCK cells were cultured on the Nef-3–, IgG-, Cef-, or PLL-coated coverslips for 2 h and stained for F-actin with rhodamine-phalloidin. Bars in the quantitative analysis represent number of the cells attached on the coverslips per millimeter squared and percentage of the cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means ± SEMs of the three independent experiments. (B) Inhibitory effect of PP2. Nectin-1-MDCK cells were cultured on the Nef-3– or IgG-coated coverslips in the presence of PP2, PP3, or DMSO for 2 h and stained with rhodamine-phalloidin. (C) Inhibitory effect of Csk. Nectin-1-MDCK cells infected with Av1CATcsk or Av1CATlacZ were cultured on the Nef-3–coated coverslips for 2 h and stained with rhodamine-phalloidin and the anti-Csk or the anti–β-galactosidase mAb, respectively. β-Gal, β-galactosidase. Bars in the quantitative analysis of B and C represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means ± SEMs of the three independent experiments. Bars, 10 μm.

were negligibly formed when nectin-1-MDCK cells were cultured on the coverslips coated with Cef, the extracellular fragment of E-cadherin fused to the Fc portion of human IgG, poly-L-lysine (PLL), or IgG as controls. The essentially same results were obtained for nectin-1-L cells (Fig. 2 A). These results indicate that the formation of filopodia and lamellipodia is caused by the specific interaction of Nef-3 with nectin-1 and not by a nonspecific response to adhesion of the cells to the substratum.

We examined by use of PP2, a specific inhibitor of SFKs, and PP3, an inactive analogue of PP2 as a control (Inoue et al., 2003), whether or not SFKs are involved in the nectin-induced formation of filopodia and lamellipodia in nectin-1-MDCK and nectin-1-L cells. Neither filopodia nor lamellipodia were formed in the presence of PP2 in nectin-1-MDCK cells cultured on the Nef-3–coated coverslips, whereas both protrusions were formed in the presence of PP3 (Fig. 1 B). Neither protrusions were formed irrespective of the presence of PP2 or PP3 in nectin-1-MDCK cells cultured on the IgG-coated coverslips. The essentially same results were obtained for nectin-1-L cells (Fig. 2 B). COOH-terminal Src kinase (Csk) phosphorylates the tyrosine residue located near the carboxyl terminus of SFKs and inactivates them (Okada et al., 1991). Neither filopodia nor lamellipodia were formed in nectin-1-MDCK cells overexpressing Csk cultured on the Nef-3–coated coverslips, whereas both of them were formed in nectin-1-MDCK cells overexpressing β-galactosidase as a control (Fig. 1 C). The essentially same results were obtained for nectin-1-L cells (Fig. 2 C). These results suggest that SFKs are necessary for the nectin-induced formation of filopodia and lamellipodia in MDCK and L cells.

Involvement of SFKs in the nectin-induced activation of Cdc42 and Rac

We have previously shown that nectins induce activation of Cdc42 and Rac as estimated by the fluorescent resonance energy transfer (FRET) imaging using the FRET probes Raichu-Cdc42 and Raichu-Rac1 (Itoh et al., 2002; Honda et al., 2003b) and by the pull-down assay using GST-PAK-Cdc42 and Rac interactive binding domain (CRIB; Kawakatsu et al., 2002). We examined by this method if SFKs are indeed involved in the nectin-induced activation of Cdc42 and Rac in nectin-1-L cells. Nectins induced the activation of Cdc42 in nectin-1-L cells as described previously (Kawakatsu et al., 2002; Honda et al., 2003b), and this nectin-induced activation of Cdc42 was inhibited by PP2, but not by PP3 (Fig. 3, A–C). Nectins induced the activation of Rac in nectin-1-L cells as described previously (Kawakatsu et al., 2002; Honda et al., 2003b), and this nectin-induced activation of Rac was inhibited by PP2, but not by PP3 (unpublished data).

We next examined if SFKs function upstream of Cdc42 and Rac in nectin-1-L cells. If SFKs 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 nectin-induced formation of filopodia and lamellipodia. To test this possibility, nectin-1-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 or PP3. Neither filopodia nor lamellipodia were formed
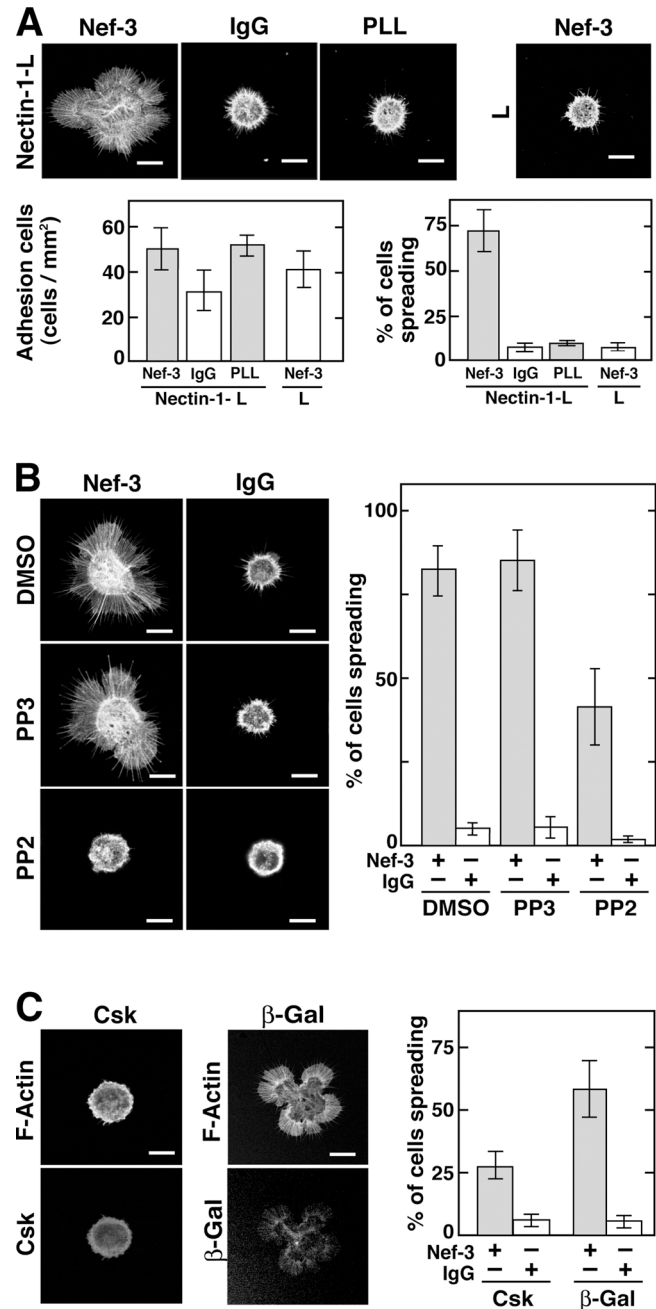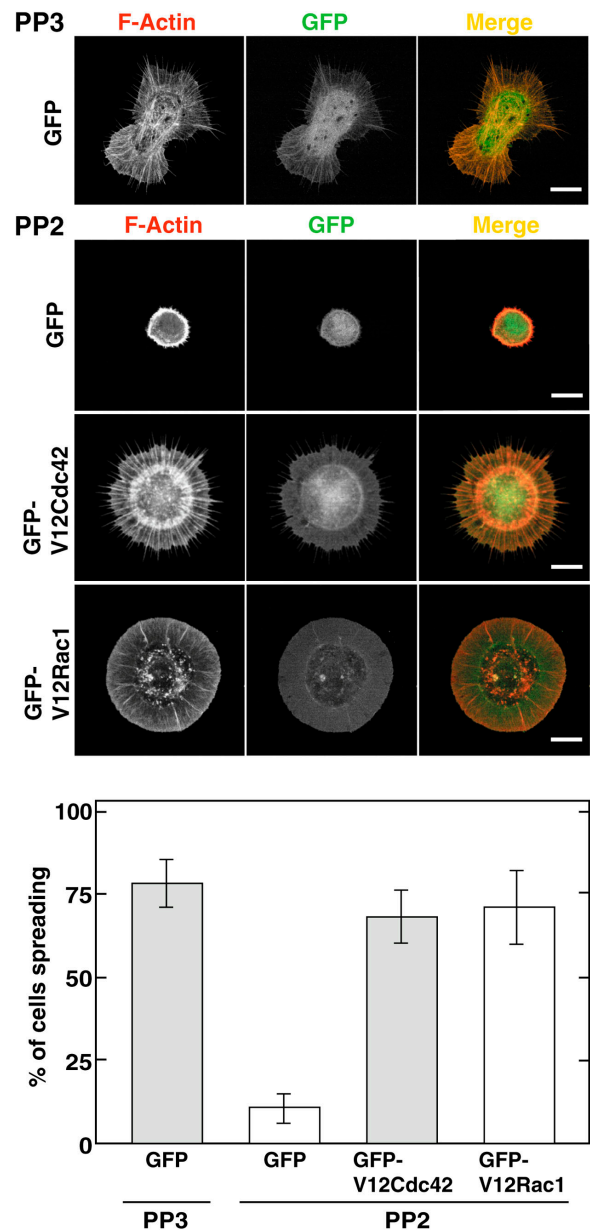

Figure 2. **Inhibition by PP2 and Csk of the nectin-induced formation of filopodia and lamellipodia in nectin-1-L cells.** (A) Nectin-induced formation of filopodia and lamellipodia. Nectin-1-L or wild-type L cells were cultured on the Nef-3–, IgG-, or PLL-coated coverslips for 30 min and stained for F-actin with rhodamine-phalloidin. Bars in the quantitative analysis represent the number of cells attached on the coverslips per millimeter squared and the percentage of cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means $\pm$ SEMs of the three independent experiments. (B) Inhibitory effect of PP2. Nectin-1-L cells were cultured on the Nef-3– or IgG-coated coverslips in the presence of PP2, PP3, or DMSO for 30 min and stained with rhodamine-phalloidin. (C) Inhibitory effect of Csk. Nectin-1-L cells infected with Av1CATcsk or Av1CATlacZ were cultured on the Nef-3–coated coverslips for 30 min and stained with rhodamine-phalloidin and the anti-Csk or the anti–β-galactosidase mAbs, respectively. β-Gal, β-galactosidase. Bars in the quantitative analysis of B and C represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means $\pm$ SEMs of three independent experiments. Bars, 10 μm.

A PP2

B PP3

C
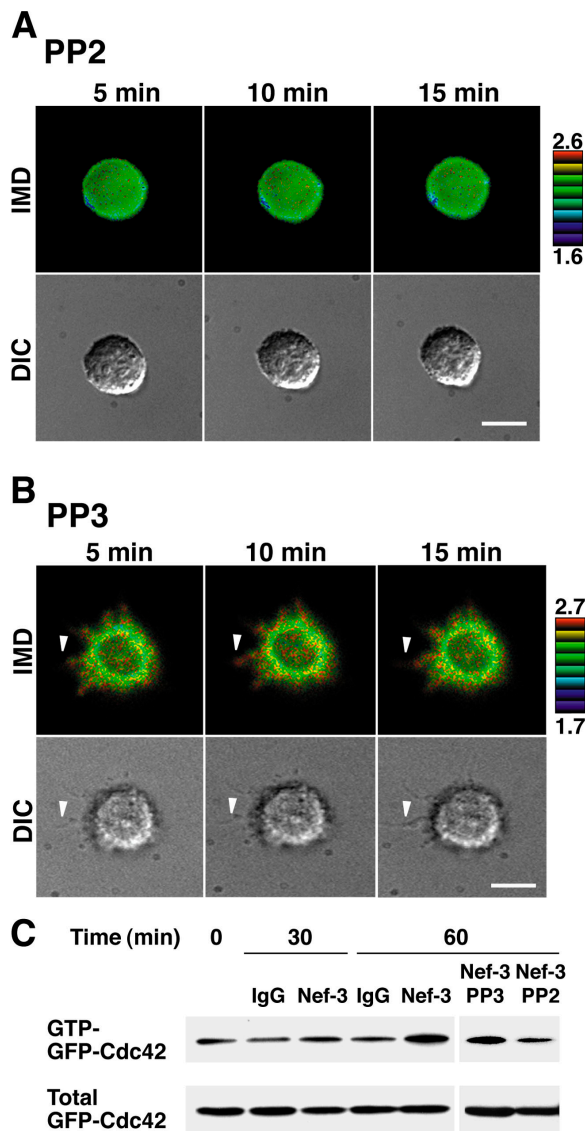
Figure 3. **Inhibition by PP2 of the nectin-induced activation of Cdc42 in nectin-1-L cells.** (A and B) FRET imaging. Nectin-1-L cells expressing Raichu-Cdc42 were cultured on the Nef-3–coated dishes in the presence of PP2 (A) or PP3 (B) for 1 h, and FRET imaging was obtained at indicated periods of time. The upper and lower limits of the YFP/CFP ratio range are shown. Arrowheads indicate the position of filopodia. Bars, 10 μm. The results shown are representative of ten independent experiments. (C) Pull-down assay. Nectin-1-L cells expressing c-Src and GFP-Cdc42 were cultured with clustered Nef-3 or IgG in the presence or absence of PP2 or PP3 for indicated periods of time and then subjected to the pull-down assay. The results shown are representative of three independent experiments.

Figure 4. **Suppression by V12Cdc42 and V12Rac1 of the inhibitory effect of PP2 in nectin-1-L cells.** Nectin-1-L cells transiently over-expressing GFP-V12Cdc42, GFP-V12Rac1, or GFP were cultured on the Nef-3–coated coverslips in the presence of PP2 or PP3 for 1 h, and stained for F-actin with rhodamine-phalloidin. Bars, 10 μm. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means ± SEMs of the three independent experiments.

in the cells expressing GFP alone in the presence of PP2, whereas both protrusions were formed in the presence of PP3 (Fig. 4). In the presence of PP2, filopodia and lamellipodia were formed to large and medium extents, respectively, in the cells expressing GFP-V12Cdc42, whereas lamellipodia were mainly formed to a large extent in the cells expressing GFP-V12Rac1. We have previously shown in nectin-1-L cells that the activation of Cdc42 is necessary, but not sufficient, for the activation of Rac, whereas the activation of Rac is not necessary for the activation of Cdc42 (Ono et al., 2000; Kawakatsu et al., 2002). Together, these

results indicate that SFKs function downstream of nectins and upstream of Cdc42 and Rac in the nectin-induced formation of filopodia and lamellipodia in nectin-1-L cells and presumably also in nectin-1-MDCK cells.

Involvement of c-Src in the nectin-induced formation of filopodia and lamellipodia

SFKs comprise a family of eight members, and c-Src, Fyn, and c-Yes are ubiquitously expressed (Brown and Cooper, 1996). We selected c-Src because it is involved in the organi-
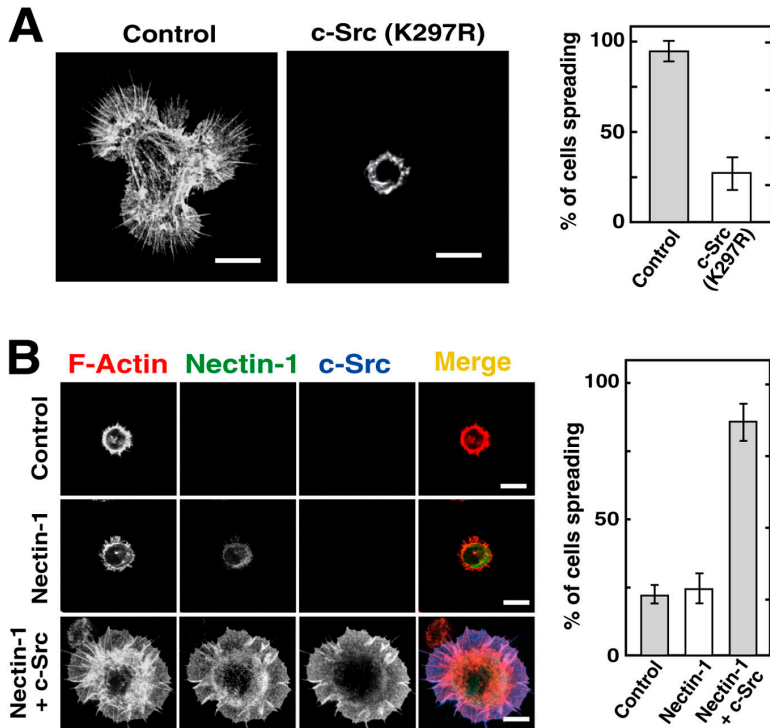
Figure 5. **Involvement of c-Src in the nectin-induced formation of filopodia and lamellipodia.** (A) Inhibitory effect of a kinase-inactive c-Src in nectin-1-L cells. Nectin-1-L cells transiently expressing c-Src (K297R) were cultured on the Nef-3–coated coverslips for 1 h and stained for F-actin and c-Src with rhodamine-phalloidin and the anti–phospho-Src (Tyr416) pAb, respectively. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted (n 5 50) and are expressed as means ± SEMs of the three independent experiments. (B) Inability of nectins to induce the formation of filopodia and lamellipodia and restoration by expression of exogenous c-Src in SYF cells. SYF cells transiently expressing exogenous nectin-1 with or without c-Src were cultured on the Nef-3–coated coverslips for 15 min and stained with rhodamine-phalloidin, the anti-nectin pAb, and the anti–phospho-Src (Tyr416) pAb. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means ± SEMs of the three independent experiments. Bars, 10 μm.

zation of cell–cell adhesion in keratinocytes (Calautti et al., 1998). We first examined by use of a kinase-inactive mutant of c-Src, c-Src (K297R) (Tu et al., 2003), if c-Src is involved in the nectin-induced formation of filopodia and lamellipodia in nectin-1-L cells. Neither filopodia nor lamellipodia were formed in nectin-1-L cells transiently expressing c-Src (K297R) cultured on the Nef-3–coated coverslips, whereas both protrusions were formed in the nontransfected control nectin-1-L cells (Fig. 5 A), suggesting that at least c-Src is involved in the formation of filopodia and lamellipodia.

To obtain more definitive evidence for the involvement of c-Src in the nectin-induced formation of these protrusions, we used SYF cells (deficient for c-Src, Yes, and Fyn; Klinghoffer et al., 1999). Neither filopodia nor lamellipodia were formed in SYF cells cultured on the Nef-3–coated coverslips (Fig. 5 B). SYF cells transiently expressing nectin-1 showed the same results. However, both protrusions were formed in SYF cells transiently expressing both nectin-1 and c-Src cultured on the Nef-3–coated coverslips. These results provide another line of evidence that at least c-Src is involved in the nectin-induced formation of filopodia and lamellipodia.

Recruitment and activation of c-Src at the nectin-based cell–cell adhesion sites

We examined whether or not c-Src is activated by nectins. Wild-type L or nectin-1-L cells transiently overexpressing c-Src or Fyn as a control were incubated with the Nef-3–coated magnetic beads for 15 min. The IgG- or concanavalin A (ConA)–coated magnetic beads were used as controls. After the incubation, the cells were harvested and lysed, and the magnetic beads were collected from the cell lysate. In nectin-1-L cells, c-Src bound significantly to the Nef-3–coated beads, but not to the IgG- or ConA-coated beads, whereas Fyn did not bind to the Nef-3–coated beads as well

as the IgG- or ConA-coated beads (Fig. 6 Aa). The significant binding of c-Src to the Nef-3–coated beads was not detected in L cells. It has been shown that c-Src is activated by tyrosine phosphorylation (Hunter, 1987). The level of tyrosine-phosphorylated c-Src in total c-Src increased at 15 min in nectin-1– and Nef-3–dependent manners (Fig. 6 Ab). These results indicate that c-Src is locally concentrated and activated at the nectin-based cell–cell adhesion sites.

We next confirmed by immunofluorescence microscopy that c-Src is recruited to the nectin-based cell–cell adhesion sites. Nectin-1-L cells transiently overexpressing c-Src were incubated with the Nef-3–coated beads. The IgG-, ConA-, or PLL-coated beads were used as controls. The immunofluorescence signal for nectin-1 was concentrated at the contact sites between the Nef-3–coated beads and the nectin-1-L cells overexpressing c-Src, but not between the IgG- or ConA-coated beads and the nectin-1-L cells overexpressing c-Src (Fig. 6 Ba), which is consistent with our previous results (Honda et al., 2003c). The signal for activated c-Src was concentrated at the contact sites between the Nef-3–coated beads and the nectin-1-L cells overexpressing c-Src, but not between the IgG- or ConA-coated beads and the nectin-1-L cells overexpressing c-Src (Fig. 6 Bb). The signal for activated c-Src was not concentrated at the contact sites between the PLL-coated beads and the nectin-1-L cells overexpressing c-Src (unpublished data). The essentially same results obtained for the Nef-3–coated beads were obtained for the beads coated with both Nef-3 and ConA (unpublished data). The signal for activated c-Src was not concentrated at the contact sites between the Nef-3–coated beads and the nontransfected control nectin-1-L cells (unpublished data). These results indicate that c-Src is recruited and activated at the nectin-based cell–cell adhesion sites and induces the formation of filopodia and lamellipodia.
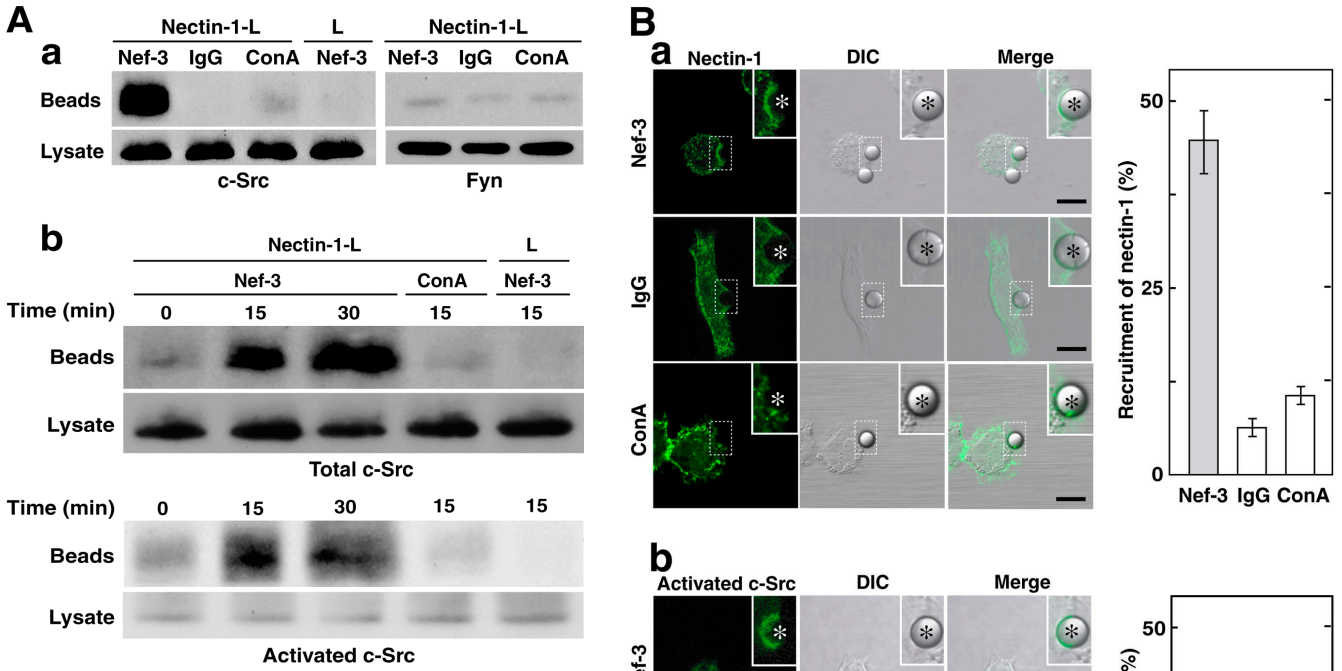
Figure 6. Recruitment and activation of c-Src at the nectin-based cell–cell adhesion sites. (A) Association of activated c-Src with nectins in nectin-1-L cells. (a) Specific binding of c-Src to the Nef-3–coated beads. Nectin-1-L or wild-type L cells transiently overexpressing c-Src or Fyn were incubated with the Nef-3–, ConA-, or IgG-coated magnetic beads for 15 min, and then subjected to the coprecipitation assay, followed by Western blotting with the anti–v-Src and the anti-Fyn mAbs. (b) Binding of activated c-Src to the Nef-3–coated beads. Nectin-1-L or wild-type L cells transiently overexpressing c-Src were incubated with Nef-3– or ConA-coated magnetic beads for indicated periods of time, and then subjected to the coprecipitation assay, followed by Western blotting for total c-Src and activated c-Src with the anti–v-Src mAb and the anti-Src pY418 pAb, respectively. The results shown are representative of three independent experiments. (B) Recruitment of activated c-Src to the contact sites between the Nef-3–coated beads and nectin-1-L cells. Nectin-1-L cells transiently overexpressing c-Src were incubated with the Nef-3–, ConA-, or IgG-coated beads for 15 min, and immunostained for nectin-1 and activated c-Src with the anti–nectin-1 and the anti–phospho-Src (Tyr416) pAbs, respectively. Bars, 10 μm. Positions of the beads are marked with asterisks. The inset indicates the magnified image of the boxed area. (a) Anti–nectin-1 pAb; (b) anti–phospho-Src (Tyr416) pAb. Bars in the quantitative analysis of panel a and panel b represent the percentage of the bead–cell contact sites with the signal for nectin-1 or activated c-Src of the total bead–cell contact sites counted ($n = 50$) and are expressed as means ± SEMs of the three independent experiments.

Involvement of FRG in the nectin-induced, c-Src–mediated activation of Cdc42

It has been reported that FRG/KIAA0793 is a GDP/GTP exchange factor (GEF) specific for Cdc42 that is ubiquitously expressed and directly tyrosine phosphorylated by activated c-Src in the endothelin-A signaling pathway (Miyamoto et al., 2003). Thus, we reasoned that FRG would be a Cdc42-GEF that might function downstream of nectins and c-Src. We first examined by use of the fragment of FRG lacking the DH and PH domains (FRG (ΔDHPH)) as a dominant-negative FRG (Miyamoto et al., 2003) whether or not FRG is involved in the nectin-induced formation of filopodia and lamellipodia. The nectin-induced formation of filopodia and lamellipodia was reduced in nectin-1-L cells expressing GFP-FRG (ΔDHPH), but not by wild-type GFP-FRG or GFP alone (Fig. 7 A), suggesting that FRG is involved in the nectin-induced formation of filopodia and lamellipodia. We then examined whether or not FRG functions downstream of c-Src. The nectin-induced formation of filopodia and lamellipodia was inhibited in nectin-1-L cells coexpressing c-Src (K297R) and wild-type FRG, but the inhibitory effects of c-Src (K297R) were suppressed in nectin-1-L cells coexpressing FRG (DHPH), a constitutively active form of FRG (Miyamoto et al., 2003), with c-Src (K297R) (Fig. 7 B), suggesting that FRG functions downstream of c-Src. We confirmed that FRG was involved in the nectin-induced, c-Src–mediated formation of filopodia and lamellipodia also in nectin-1-MDCK cells. Expression of Flag-FRG (ΔDHPH) inhibited the nectin-induced formation of filopodia and lamellipodia, whereas coexpression with GFP-V12Cdc42 or GFP-V12Rac1 suppressed this inhibitory effect in nectin-1-MDCK cells (Fig. 7 C).

To obtain more definitive evidence for the involvement of FRG in the nectin-induced formation of filopodia and lamellipodia, we performed RNA interference experi-
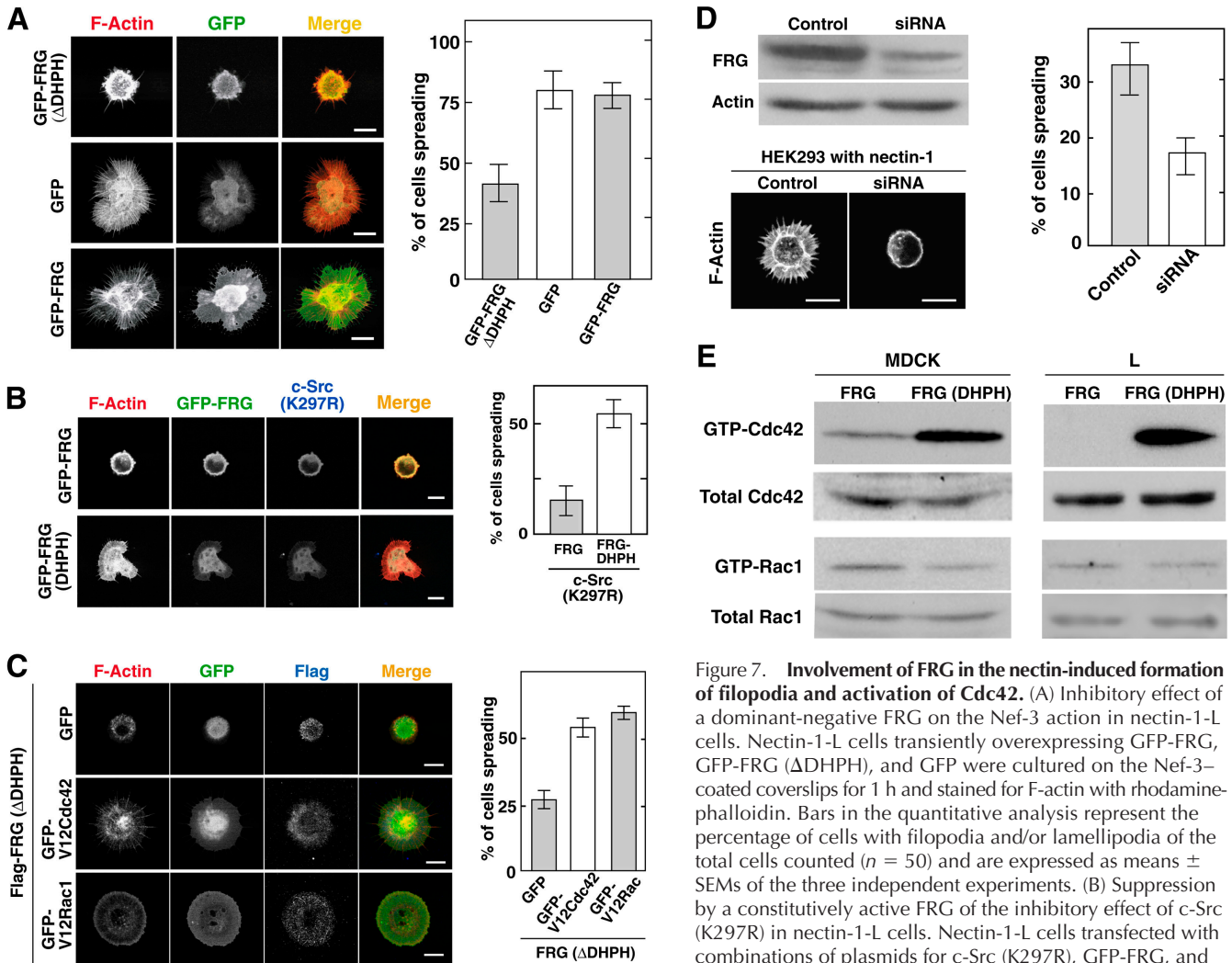
Figure 7. **Involvement of FRG in the nectin-induced formation of filopodia and activation of Cdc42.** (A) Inhibitory effect of a dominant-negative FRG on the Nef-3 action in nectin-1-L cells. Nectin-1-L cells transiently overexpressing GFP-FRG, GFP-FRG (ΔDHPH), and GFP were cultured on the Nef-3–coated coverslips for 1 h and stained for F-actin with rhodamine-phalloidin. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means $\pm$ SEMs of the three independent experiments. (B) Suppression by a constitutively active FRG of the inhibitory effect of c-Src (K297R) in nectin-1-L cells. Nectin-1-L cells transfected with combinations of plasmids for c-Src (K297R), GFP-FRG, and GFP-FRG (DHPH) were cultured on the Nef-3–coated coverslips and stained with rhodamine-phalloidin and anti–phospho-Src (Tyr416) pAb. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means $\pm$ SEMs of the three independent experiments. (C) Suppression by V12Cdc42 and V12Rac1 of the inhibitory effect of a dominant-negative form of FRG in nectin-1-MDCK cells. Nectin-1-MDCK cells coexpressing Flag-FRG (ΔDHPH) with GFP-V12Cdc42, GFP-V12Rac1, or GFP were cultured on the Nef-3–coated coverslips and stained with rhodamine-phalloidin and the anti-Flag mAb. The results are representative of three independent experiments. (D) Loss of function of FRG in HEK293 cells. The siRNA targeting FRG were transfected in HEK293 cells to confirm that the siRNA inhibits the expression of FRG. 48 h after transfection, HEK293 cells transfected with or without siRNA were subjected to SDS-PAGE, followed by Western blotting with the anti-FRG pAb. HEK293 cells cotransfected with the plasmid of nectin-1 and siRNA targeting FRG or transfected with the plasmid of nectin-1 alone were subjected to the assay for the formation of filopodia and lamellipodia. The cells were stained with rhodamine-phalloidin and anti–nectin-1 pAb, and the figures represent the rhodamine-phalloidin staining. The results are representative of three independent experiments. Bars in the quantitative analysis represent the percentage of cells with filopodia and/or lamellipodia of the total cells counted ($n = 50$) and are expressed as means $\pm$ SEMs of the three independent experiments. (E) Activation of Cdc42 by FRG (DHPH) in MDCK and L cells. MDCK or L cells transfected with plasmids for GFP-Cdc42 or GFP-Rac1 with wild-type FRG or FRG (DHPH) were subjected to the pull-down assay, followed by Western blotting with anti-Cdc42 or anti-Rac mAbs. The results are representative of three independent experiments. Bars, 10 μm.

ments. The expression level of FRG was reduced in HEK293 cells transfected with small interfering RNA (siRNA) oligonucleotides against FRG (Fig. 7 D). Filopodia and lamellipodia were hardly formed in HEK293 cells cotransfected with the plasmid of nectin-1 and siRNA targeting FRG cultured on the Nef-3–coated coverslips, whereas both protrusions were markedly formed in HEK293 cells transfected with the plasmid of nectin-1 alone (Fig. 7 D). These results provide another line of evidence that FRG is involved in the nectin-induced formation of filopodia and lamellipodia.

It has been shown that FRG is specific for Cdc42, but not for Rac (Miyamoto et al., 2003). Consistently, a constitutively active mutant of FRG activated Cdc42, but not Rac1, in both L and MDCK cells as estimated by the pull-down assay using the GST-PAK-CRIB domain (Fig. 7 E). We have previously shown that the activation of Cdc42 is necessary, but not sufficient, for the activation of Rac in nectin-1-L cells (Ono et al., 2000; Kawakatsu et al., 2002). Together, FRG is a GEF responsible for the nectin-induced, c-Src–mediated activation of Cdc42, but not that of Rac, although FRG is necessary for the activation of Rac through the activation of Cdc42.

Figure 8. **Nectin-induced, c-Src–mediated activation of FRG.** (A) Recruitment of FRG to the contact sites between the Nef-3–coated beads and nectin-1-L cells. Nectin-1-L or EL cells transiently overexpressing GFP-FRG were incubated with the Nef-3–, ConA-, or Cef-coated beads for 1 h and stained with the anti–nectin-1 pAb. The signal for E-cadherin–β-catenin complex was detected by the anti–β-catenin mAb. (a) Nectin-1-L cells; (b) EL cells. Bars, 10 μm. Positions of the beads are marked with asterisks. The inset indicates the magnified image of the boxed area. Bars in the quantitative analysis of panels a and b represent the percentage of the bead–cell contact sites with the signal for nectin-1 or E-cadherin of the total bead–cell contact sites counted ($n = 50$) or the percentage of the bead–cell contact sites with the signal for GFP of the bead–cell contact sites with the signal for nectin-1 or E-cadherin counted ($n = 50$) and are expressed as means ± SEMs of the three independent experiments. (B) Nectin-induced tyrosine phosphorylation of FRG. (a) Wild-type L, nectin-1-L, or EL cells transiently overexpressing Flag-FRG and c-Src were treated with clustered Nef-3 or IgG in the presence or absence of PP2 for indicated periods of time. (b) Nectin-1-L cells transiently overexpressing c-Src and Flag-FRG or Flag-frabin were treated with clustered Nef-3 or IgG for 30 min. Each cell lysate was subjected to the immunoprecipitation assay with the anti-Flag mAb, followed by Western blotting with the anti-phosphotyrosine, anti-Flag, and anti–v-Src mAbs. (C) GEF activity of FRG or frabin on Cdc42. The binding of [^{35}S]GTPγS to Cdc42 was assayed by incubation with the immunoprecipitant prepared in B for indicated periods of time. The results shown are representative of three independent experiments.
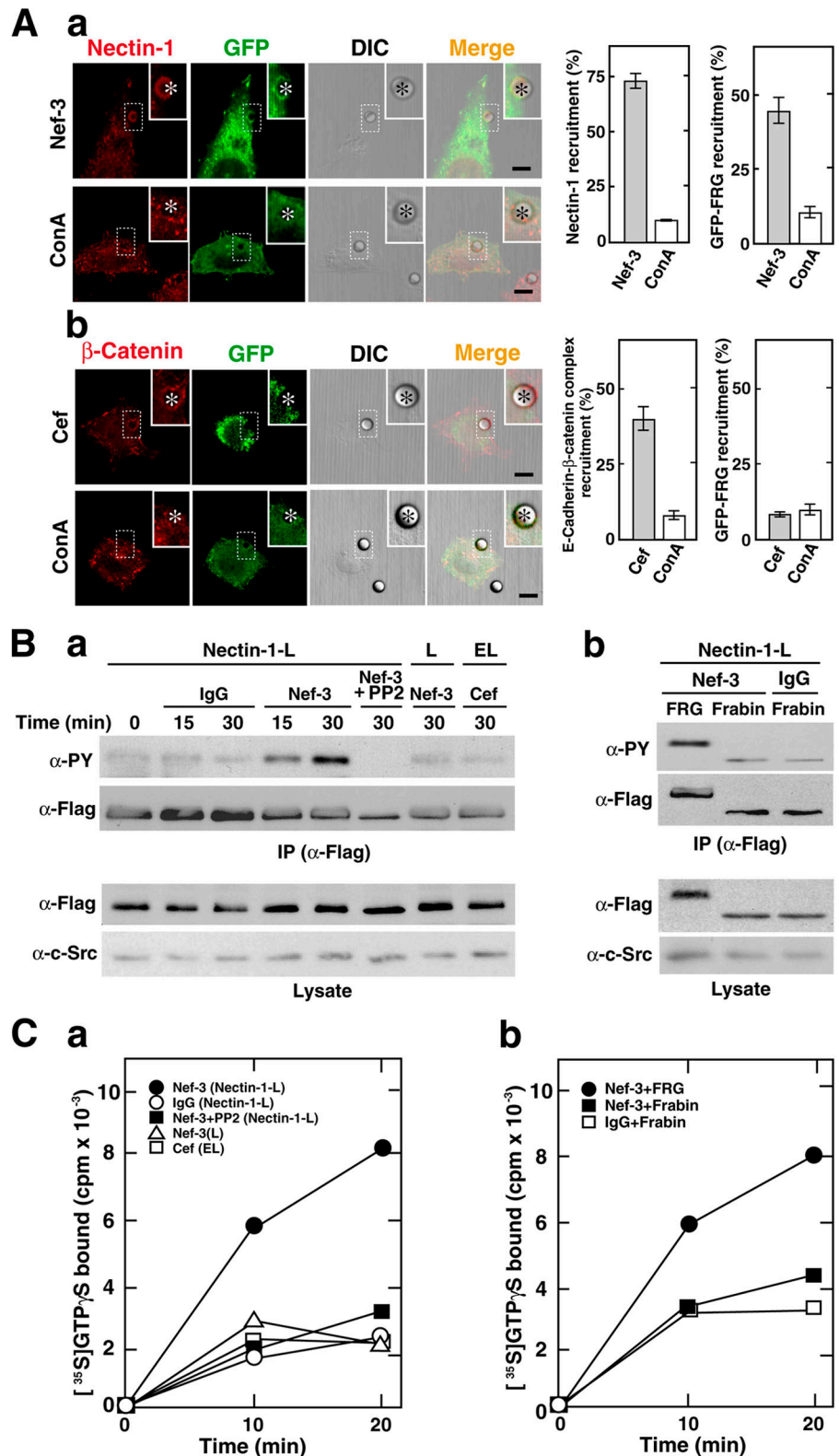
Recruitment of FRG to the nectin-based cell–cell adhesion sites and its phosphorylation and activation by c-Src there

We examined whether or not FRG is recruited to the nectin-based cell–cell adhesion sites. Nectin-1-L cells transiently expressing GFP-FRG were incubated with the Nef-3–coated beads. EL cells were used as a control for nectin-1-L cells, and the Cef- or ConA-coated beads were used as controls for the Nef-3–coated beads. The immunofluorescence signals for nectin-1 and GFP-FRG were concentrated at the contact sites between the Nef-3–coated beads and the nectin-1-L cells expressing GFP-FRG, but not between the ConA-coated

beads and the nectin-1-L cells expressing GFP-FRG (Fig. 8 Aa). The signal for E-cadherin–β-catenin complex, which was detected by the anti–β-catenin mAb, was concentrated at the contact sites between the Cef-coated beads and the EL cells expressing GFP-FRG, but not between the ConA-coated beads and the EL cells expressing GFP-FRG, whereas the signal for GFP-FRG was not concentrated at these bead–cell contact sites (Fig. 8 Ab). These results, together with the aforementioned results, indicate that FRG as well as c-Src is recruited to the nectin-based cell–cell adhesion sites.

We next examined whether FRG is tyrosine phosphory-lated and activated by nectins through c-Src. Nectin-1-L cells transiently overexpressing Flag-FRG and c-Src were incubated with Nef-3 clustered with the anti-human Fc poly-clonal antibody (pAb) and subjected to the immunoprecipi-tation assay using the anti-Flag mAb. Wild-type L and EL cells were used as controls for nectin-1-L cells, whereas IgG and Cef clustered with an anti-human Fc pAb were used as controls for clustered Nef-3. FRG was tyrosine-phosphory-lated by clustered Nef-3 in nectin-1-L cells, but not in wild-type L cells, whereas it was not tyrosine phosphorylated by clustered IgG in nectin-1-L cells and clustered Cef in EL cells (Fig. 8 Ba). Moreover, the tyrosine phosphorylation of FRG induced by clustered Nef-3 was inhibited by PP2. To confirm the specificity of the nectin-induced, c-Src–medi-ated tyrosine phosphorylation of FRG, frabin, another Cdc42-GEF (Obaishi et al., 1998), was used. Frabin was not tyrosine phosphorylated by clustered Nef-3 as well as clus-tered IgG in nectin-1-L cells expressing c-Src (Fig. 8 Bb).

We finally measured the GEF activity on Cdc42 of FRG immunoprecipitated from the Nef-3– or IgG-treated nectin-1-L cells. The activity of FRG from the Nef-3–treated cells was much stronger than that from the IgG-treated cells (Fig. 8 Ca), indicating that nectins induce the activation of FRG on Cdc42. The nectin-induced activation of FRG on Cdc42 was reduced by PP2. FRG was not activated in L cells treated with clustered Nef-3 and in EL cells treated with clustered Cef. Frabin was not activated in nectin-1-L cells treated with clustered Nef-3 (Fig. 8 Cb). Together, these re-sults indicate that c-Src is recruited and activated at the nec-tin-based cell–cell adhesion sites, and then tyrosine phos-phorylates and activates FRG, eventually causing an increase of the GTP-bound active form of Cdc42.

Involvement of SFKs in the nectin-induced formation of the E-cadherin–based AJs in MDCK cells

We have previously demonstrated that nectins increase the velocity of the formation of the E-cadherin–based AJs through the activation of Cdc42 in MDCK cells (Fukuhara et al., 2003). In the last set of experiments, we examined if SFKs are involved in the formation of AJs in MDCK cells. Because SFKs function downstream of nectins and upstream of Cdc42, PP2 would decrease the nectin-enhanced velocity of the formation of the E-cadherin–based AJs, and this effect of PP2 would be suppressed by V12Cdc42, a constitutively active mutant of Cdc42. When wild-type MDCK and nec-tin-1-MDCK cells were cultured at 2 mM Ca^{2+}, the immu-nofluorescence signal for E-cadherin was concentrated at the cell–cell adhesion sites as described previously (Takaishi et al., 1997; Honda et al., 2003a; unpublished data). When

these cells were cultured at 2 μM Ca^{2+} for 2 h, the signal for E-cadherin was not observed at any site along the plasma membrane in both wild-type MDCK and nectin-1-MDCK cells as described previously (Takaishi et al., 1997; Honda et al., 2003a; unpublished data). Reculture of these cells at 2 mM Ca^{2+} in the presence of PP2 or PP3 for 2 h caused re-concentration of the signal for E-cadherin at the cell–cell ad-hesion sites in the presence of PP3, but did not reconcen-trate it there in the presence of PP2 (Fig. 9 A). However, when nectin-1-MDCK cells transiently expressing GFP-V12Cdc42 precultured at 2 μM Ca^{2+} for 2 h were recul-tured at 2 mM Ca^{2+} in the presence of PP2 for 2 h, the sig-nal for E-cadherin was reconcentrated at cell–cell adhesion sites (Fig. 9 B). Nectin-1-MDCK cells transiently expressing GFP as a control did not reconcentrate it there. These re-sults indicate that SFKs are involved in the nectin-induced, Cdc42-enhanced formation of the E-cadherin–based AJs in MDCK cells.

Discussion

We have first shown here using nectin-1-MDCK and nec-tin-1-L cells that SFKs are involved in the nectin-induced formation of filopodia and lamellipodia. We have previously shown that the nectin-induced formation of these protru-sions is mediated by the nectin-induced activation of Cdc42 and Rac in these cell lines (Kawakatsu et al., 2002). We have confirmed here using nectin-1-L cells that SFKs are involved in the nectin-induced activation of Cdc42 and Rac. SFKs comprise a family of eight members (Brown and Cooper, 1996). We have shown here that at least c-Src is involved in these activities of nectins in nectin-1-L cells. We have not examined here whether c-Src is involved in these activities of nectins in MDCK cells, but c-Src is likely to mediate these activities of nectins also in MDCK cells. Studies on the mode of action of nectins in the activation of c-Src in nec-tin-1-L cells have revealed that c-Src is recruited, tyrosine phosphorylated, and activated at the nectin-based cell–cell adhesion sites. It may be noted that any cadherins are not necessary for the nectin-induced recruitment or activation of c-Src because nectin-1-L cells do not express any cadherins (Nagafuchi et al., 1987).

We have next shown here that FRG is at least one of the GEFs responsible for the nectin-induced, c-Src–mediated activation of Cdc42. Studies on the mode of action of nec-tins in the activation of FRG have revealed that FRG is re-cruited to the nectin-based cell–cell adhesion sites and tyro-sine phosphorylated and activated by c-Src there, eventually causing an increase in the GTP-bound active form of Cdc42. FRG has not only DH and PH domains but also a FERM (band 4.1, ezrin, radixin, and moesin) domain that links cell surface glycoproteins to the actin cytoskeleton (Sun et al., 2002). ERM (ezrin, radixin, and moesin) proteins form an intramolecular association that is regulated by pro-tein phosphorylation or lipid interactions. The NH_2-termi-nal FERM domain of ERM proteins tightly associates with the COOH-terminal residues. In a hypophosphorylated state, ERM proteins adopt a "closed" conformation that masks the binding sites for F-actin and CD44. Phosphoryla-tion separates the NH_2 and COOH termini to result in an
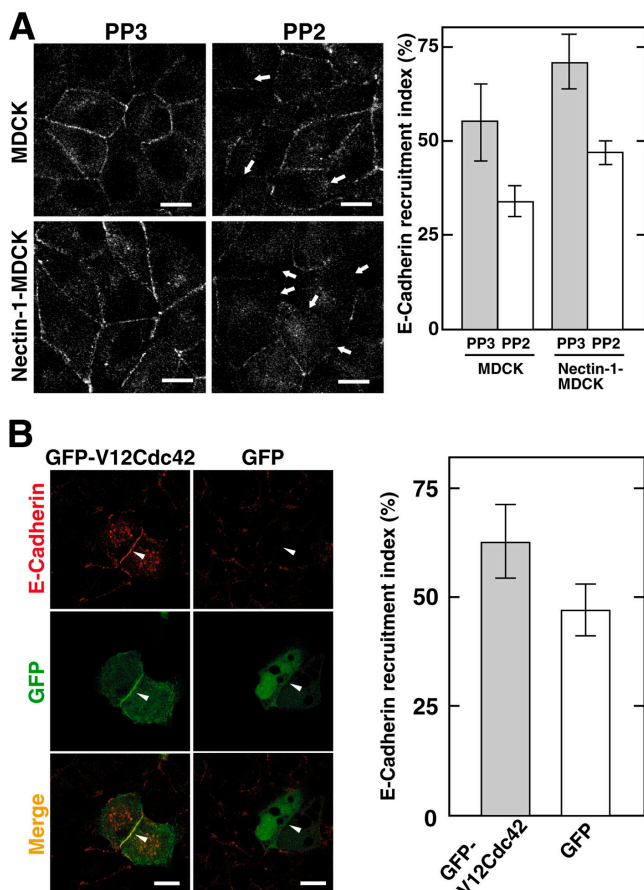
Figure 9. **Involvement of SFKs in the nectin-induced formation of the E-cadherin–based AJs in MDCK cells.** (A and B) Inhibitory effects of PP2 on the nectin-induced, E-cadherin–based formation of AJs in MDCK cells. Wild-type MDCK, nectin-1-MDCK, and nectin-1-MDCK cells overexpressing GFP-V12Cdc42 or GFP were incubated at 2 μM Ca^{2+} for 2 h and then incubated at 2 mM Ca^{2+} in the presence of PP2 or PP3 for 2 h. After the incubation, the cells were immunostained with the anti–E-cadherin mAb. Bars, 10 μm. (A) Wild-type MDCK and nectin-1-MDCK cells in the presence of PP2 or PP3. Arrows show the absence of the signal for E-cadherin at the cell–cell adhesion sites. (B) Nectin-1-MDCK cells expressing GFP-V12Cdc42 or GFP in the presence of PP2. Arrowheads show the presence or absence of the signal for E-cadherin at the cell–cell adhesion site. Bars in the quantitative analysis of A and B represent the percentage of cell–cell adhesions with the signal for E-cadherin of the total cell–cell adhesions counted ($n = 50$) and are expressed as means $\pm$ SEMs of the three independent experiments.

"open" form, which permits interactions with the actin cytoskeleton and other proteins. Thus, activated c-Src recruited to the nectin-based cell–cell adhesion sites may phosphorylate FRG there, which switches from a close to open form and exerts its GEF activity on Cdc42.

It remains unknown how c-Src and FRG are recruited and activated at the nectin-based cell–cell adhesion sites, but afadin is likely to be unnecessary for this recruitment or activation because we have previously shown that afadin is not necessary for the nectin-induced, Cdc42- and Rac-mediated formation of filopodia and lamellipodia, respectively, in nectin-1-L cells (Kawakatsu et al., 2002). It is also unknown either if c-Src is first recruited to the nectin-based cell–cell adhesion sites and then activated there, or if c-Src is first ac-

tivated and activated c-Src is then recruited there. Available evidence suggests that nectins form new cell–cell adhesion sites, organize some signaling microdomains there, and transduce the positional signal to initiate cell–cell adhesion, resulting in establishment of cell adhesion and polarity in epithelial cells (Takai et al., 2003). FRG shows sequence similarity to a human faciogenital dysplasia gene product (FGD1), which is a specific activator of Cdc42 and appears to be a family of related Cdc42 GEFs including Fgd2, Fgd3, and frabin (Schmidt and Hall, 2002). Frabin has an F-actin–binding domain as well as DH and PH domains (Obaishi et al., 1998). The association of frabin with the actin cytoskeleton through its F-actin–binding domain is essential for the formation of filopodia through the activation of Cdc42 (Umikawa et al., 1999). FRG might recognize some specific actin structure mediated by the nectin-based microdomains and might be recruited to the nectin-based cell–cell adhesion sites where it is tyrosine phosphorylated and activated by c-Src. Further studies are necessary to understand how c-Src and FRG are locally activated at the nectin-based cell–cell adhesion sites.

Rac and the Rac-induced formation of lamellipodia are involved in the formation of cell–cell junctions (Ehrlich et al., 2002). Filopodia are observed at developing cell–cell contacts in many cell types (Yonemura et al., 1995; Adams et al., 1998; Vasioukhin et al., 2000). However, it has not been examined if Cdc42 and the Cdc42-induced formation of filopodia are involved in the formation of cell–cell junctions. We have previously shown that the nectin-induced activation of Cdc42 increases the velocity of the formation of AJs and the subsequent formation of TJs in MDCK cells (Fukuhara et al., 2003). We have shown here that inhibition of SFKs by PP2 reduces the velocity of the formation of AJs and that this reduction is rescued by a constitutively active mutant of Cdc42. These results suggest that the nectin-induced, SFKs- and Cdc42-mediated formation of filopodia are also involved in the formation of AJs. It has been reported that c-Src and c-Yes are enriched at cell–cell AJs in various types of rat adult tissues and cultured cells including Madin-Darby bovine kidney cells and keratinocytes (Tsukita et al., 1991). Formation of AJs is impaired in keratinocytes treated with tyrosine kinase inhibitors and in keratinocytes lacking Fyn and/or Src (Calautti et al., 1998). Our present results are consistent with these earlier observations that SFKs show a positive effect on the formation of AJs. Further studies are necessary to establish the roles and modes of action of the nectin-induced formation of filopodia and lamellipodia through the c-Src-FRG-Cdc42-Rac signaling pathways in the organization of cell–cell junctions.

Materials and methods

Vector construction and protein purification
An expression vector for Fyn (pME18S-Fyn-wt) was prepared as described previously (Takeuchi et al., 1993). The cDNA of FRG/KIAA0793 was supplied by T. Nagase (Kazusa DNA research Institute, Chiba, Japan). Expression vectors for GFP-tagged V12Rac1 (pEGFP-V12Rac1), GFP-tagged V12Cdc42 (pEGFP-V12Cdc42), GFP-FRG (pEGFP-FRG), GFP-FRG (ΔDHPH) (pEGFP-FRG (ΔDHPH)), GFP-FRG (DHPH) (pEGFP-FRG (DHPH)), Flag-tagged FRG (pEFBOS-Flag-FRG), Flag-FRG (ΔDHPH) (pEF-BOS-Flag-FRG (ΔDHPH)), Flag-frabin (pFLAG-CMV-2-frabin), and wild-type c-Src (pcDNA-Src-wt) were constructed by inserting the cDNA frag-

ments encoding V12Rac1, V12Cdc42, full-length FRG, FRG encoding 539–856 aa, full-length FRG, FRG lacking 539–929 aa, full-length frabin, and full-length c-Src, respectively. pRaichu-Rac1 and pRaichu-Cdc42 were prepared as described previously (Itoh et al., 2002). Expression vectors for GFP-Cdc42 (pEGFP-Cdc42), GFP-V12Cdc42 (pEGFP-V12Cdc42), and GST-PAK-CRIB (pGEX-PAK-CRIB) were supplied by S. Narumiya (Kyoto University, Kyoto, Japan). An expression vector for a kinase-inactive c-Src (pUSE-Src (K297R)) was purchased from Upstate Biotechnology. Lipid-modified GST-PAK-CRIB fusion protein was prepared as described previously (Kawakatsu et al., 2002). Protein concentrations were determined with BSA as a reference protein using the DC protein assay kit (Bio-Rad Laboratories).

Cell lines and transfection
Wild-type MDCK cells were supplied by W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany), and wild-type L and EL cells were supplied from S. Tsukita (Kyoto University, Kyoto, Japan). Nectin-1-MDCK and nectin-1-L cells were prepared as described previously (Takahashi et al., 1999). SYF cells were purchased from American Type Culture Collection. Transfection and immunofluorescence microscopy of cultured cells with a confocal imaging system (model Radian 2000; Bio-Rad Laboratories) were performed as described previously (Takahashi et al., 1999).

Antibodies
A rabbit anti–nectin-1 pAb was prepared as described previously (Takahashi et al., 1999). A rat anti–E-cadherin mAb (ECCD-2) and a rabbit anti-FRG pAb were supplied by M. Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan) and H. Itoh (Nara Institute of Science and Technology, Ikoma, Japan), respectively. A rabbit anti-Src pY418 pAb (Biosource International), a rabbit anti–phospho-Src (Tyr416) pAb (Cell Signaling Technology), a mouse anti–v-Src mAb (Calbiochem-Novabiochem), a mouse anti-Cdc42 mAb, a mouse anti-Fyn mAb, a mouse anti-Csk mAb, a mouse anti-phosphotyrosine (PY20) mAb (BD Biosciences), a mouse anti-Flag mAb (Sigma-Aldrich), a mouse anti-Rac mAb (Upstate Biotechnology), a mouse anti-actin mAb (Chemicon), a mouse anti–β-catenin mAb (Santa Cruz Biotechnology, Inc.), and secondary Abs (Chemicon) were purchased from commercial sources.

Assays for formation of filopodia and lamellipodia
Formation of filopodia and lamellipodia was assayed as described previously (Kawakatsu et al., 2002). For inhibition of the activity of SFKs, the cells were treated with 0.2% DMSO, 20 μM PP2 (Calbiochem-Novabiochem) containing 0.2% DMSO, or 20 μM PP3 (Calbiochem-Novabiochem) containing 0.2% DMSO in DME for 1 h before the trypsin-EDTA treatment and during the culture on the Nef-3– or IgG-coated coverslips. Cell–cell junctions of wild-type MDCK and nectin-1-MDCK were assayed as described previously (Fukuhara et al., 2003). In brief, the cells were cultured in DME containing 10% FCS for 24 h. After the culture, the cells were washed with 5 ml PBS and incubated in DME at a final concentration of 2 mM Ca^{2+} for 1 h. The medium was replaced by DME containing 5 mM EGTA at a final concentration of 2 μM Ca^{2+}, and then the cells were incubated for 2 h. The cells were washed with DME and incubated in DME at a final concentration of 2 mM Ca^{2+} containing 1 μM PP2 or 1 μM PP3 for 2 h. The cells were fixed, immunostained with the anti–E-cadherin mAb, and observed by use of a confocal microscope with a 60× oil immersion objective lens.

FRET imaging
The FRET imaging was performed as described previously (Honda et al., 2003b). In brief, 24 h after the transfection, nectin-1-L cells transfected with pRaichu-Cdc42 were replated on the dishes coated with 50 μg/ml of Nef-3. The cells were then imaged with an inverted microscope (model IX71; Olympus) equipped with a cooled charge-coupled device camera (model CoolSNAP HQ; Roper Scientific). For dual-emission ratio imaging, we used a 440AF21 excitation filter, a 455DRLP dichroic mirror, and two emission filters, 480AF30 for CFP and 535AF25 for YFP (Omega Optical Inc.). The cells were illuminated with a 75-W xenon lamp through a 6% neutral density filter (Omega Optical Inc.) and a 60× oil immersion objective lens, and images of CFP, YFP, and a differential interference contrast were obtained by 200 ms of exposure times. After background subtraction, the ratio image of YFP/CFP was created with the MetaMorph software (Universal Imaging Corp) and shown in the intensity modulated display mode to indicate FRET efficiency. In the intensity modulated display 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 Cdc42.

Pull-down assay for Cdc42 and Rac
The pull-down assay was performed as described previously (Kawakatsu et al., 2002) with some modifications. 26 μg of Nef-3 or human IgG (Fc specific; Sigma-Aldrich) was clustered using 9 μg of the anti-human IgG pAb (Sigma-Aldrich) in 50 μl PBS at RT for 1 h. Nectin-1-L cells were transfected with pcDNA3-Src-wt and pEGFP-Cdc42 or pEGFP-Rac1 and cultured for 12 h. After 24-h serum starvation, the medium was replaced by 1 ml DME containing clustered Nef-3 or IgG, and the cells were incubated for indicated periods of time. The cells were washed with 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 PMSF, and 1 mM sodium vanadate) containing 30 μg GST-PAK-CRIB, and incubated on ice for 30 min. The cell extract (600 μg of protein) 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 used for Western blotting.

Assays for coprecipitation of SFKs with nectins and for bead–cell adhesion
Protein A- or tosyl reactive groups-bound Dynabeads (10^8, 2.8-μm diameter; Dynal) were washed with PBS and incubated with 45 μg of Nef-3, 45 μg of ConA, or 45 μg of IgG in 100 μl PBS at RT for 2 h. Nectin-1-L cells (5×10^5 cells/60-mm dish) were transfected with pcDNA3-Src-wt or pME18S-Fyn-wt. After a 12-h culture, the cells were replaced on a 60-mm dish and further cultured for 12 h. After the culture, Nef-3–, ConA-, or IgG-coated magnetic beads (5×10^7/dish) were added to the medium in the dish and incubated for indicated periods of time. After the 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 1; Sigma-Aldrich). After sonication, the Nef-3–, ConA-, or IgG-coated magnetic beads were collected by a magnetic particle concentrator. The beads were washed with buffer B containing 1% Triton X-100 three times. Proteins bound to Nef-3–, ConA-, or IgG-coated magnetic beads were used for Western blotting. The bead–cell adhesion was assayed as described previously (Honda et al., 2003c). In brief, latex-sulfate microbeads coated with Nef-3, Cef, ConA, or IgG were added onto nectin-1-L or EL cells cultured in DME containing 10% FCS. After a 30-min incubation, the cells were fixed and immunostained.

Assay for FRG activity
The immunoprecipitation assay was performed as described previously (Miyamoto et al., 2003) with some modifications. Nectin-1-L or L cells (1.5×10^6 cells/100-mm dish) were transfected with pcDNA3-Src-wt and pEFBOS-Flag-FRG or pFLAG-CMV-2-frabin. After a 12-h culture, the cells were serum starved for 24 h. The medium was then replaced by DME containing clustered Nef-3 or IgG and the cells were incubated for indicated periods of time. 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/Na, 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 or the FRG activity assay as described previously (Yamamoto et al., 1990).

Other procedures
Adenovirus infection was performed as described previously (Hoshino et al., 2004). A recombinant adenovirus carrying the *csk* gene (Av1CATcsk) or the *lacZ* gene (Av1CATlacZ) was prepared as described previously (Takayama et al., 1999) or purchased from Takara, respectively. After a 36-h culture, the cells were subjected to the cell adhesion assay. RNA interference experiments were performed as described previously (Krichevsky and Kosik, 2002). The siRNA duplexes with a 2-nucleotide (2′-deoxy thymidine) 3′ overhang were designed. The following *FRG* gene-specific se-

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