Inhibition of cell movement and proliferation by cell–cell contact-induced interaction of Necl-5 with nectin-3

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Immunoglobulin-like Necl-5/Tage4/poliovirus receptor (PVR)/CD155, originally identified as the PVR, has been shown to be up-regulated in cancer cells and to enhance growth factor–induced cell movement and proliferation. In addition, Necl-5 heterophilically trans-interacts with nectin-3, a cell–cell adhesion molecule known to form adherens junctions in cooperation with cadherin. We show here that Necl-5 was down-regulated from cell surface upon cell–cell contacts in NIH3T3 cells. This down-regulation of Necl-5 was initiated by its interaction with nectin-3 and was mainly mediated by clathrin-dependent endocytosis. Then, the down-regulation of Necl-5 induced in this way reduced movement and proliferation of NIH3T3 cells. These results indicate that the down-regulation of Necl-5 induced by its interaction with nectin-3 upon cell–cell contacts may be at least one mechanism underlying contact inhibition of cell movement and proliferation.

Introduction

Necl-5/Tage4/poliovirus receptor (PVR)/CD155 is an emerging molecule that regulates cell movement and proliferation (Ikeda et al., 2004; Kakunaga et al., 2004). Human PVR/CD155 was originally identified as human PVR (Mendelsohn et al., 1989; Koike et al., 1990), whereas rodent Tage4 was originally identified as the product of a gene overexpressed in rodent colon carcinoma (Chadeneau et al., 1994, 1996). PVR/CD155 was subsequently shown to be overexpressed in many human cancer cells (Gromeier et al., 2000; Masson et al., 2001; Sloan et al., 2004). Necl-5 is one member of the nectin-like molecule family, which consists of five members—Necl-1, -2, -3, -4, and -5 (Takai et al., 2003). Nectin-like molecules have been named for a group of Ig-like molecules with domain structures that are similar to, but slightly different from, those of nectins. Nectins are Ca2+-independent Ig-like cell–cell adhesion molecules that constitute a family consisting of four members—nectin-1, -2, -3, and -4 (Takai and Nakanishi, 2003). Necl-5 is functionally associated with integrin αβ3 at leading edges of moving cells, such as L cells stably expressing Necl-5 and NIH3T3 cells transformed by an oncogenic Ki-Ras (V12Ras-NIH3T3 cells), and enhances the movement induced by growth factors, such as PDGF, in an integrin-dependent manner in NIH3T3 cells (Ikeda et al., 2004). Necl-5 enhances the growth factor–induced activation of Cdc42 and Rac, causing the formation of filopodia and lamellipodia, respectively, which eventually enhances cell movement. The cytoplasmic region of Necl-5 binds to the nectin-based cell–cell adhesion sites to cooperatively form adherens junctions in epithelial cells and fibroblasts. In contrast to nectins, Necl-5 does not show homophilic cell–cell adhesion activity (Aoki et al., 1997; Ikeda et al., 2003). Nectins are associated with the actin cytoskeleton through afadin, a nectin- and actin-filament–binding protein, but Necl-5 does not bind afadin (Ikeda et al., 2003; Takai and Nakanishi, 2003; Takai et al., 2003).

Although the role of Necl-5 as PVR has been established, its physiological role remained unknown for a long time. We recently found that Necl-5 is functionally associated with integrin αβ3 at leading edges of moving cells, such as L cells stably expressing Necl-5 and NIH3T3 cells transformed by an oncogenic Ki-Ras (V12Ras-NIH3T3 cells), and enhances the movement induced by growth factors, such as PDGF, in an integrin-dependent manner in NIH3T3 cells (Ikeda et al., 2004). Necl-5 enhances the growth factor–induced activation of Cdc42 and Rac, causing the formation of filopodia and lamellipodia, respectively, which eventually enhances cell movement. The cytoplasmic region of Necl-5 binds to the nectin-based cell–cell adhesion sites to cooperatively form adherens junctions in epithelial cells and fibroblasts. In contrast to nectins, Necl-5 does not show homophilic cell–cell adhesion activity (Aoki et al., 1997; Ikeda et al., 2003). Nectins are associated with the actin cytoskeleton through afadin, a nectin- and actin-filament–binding protein, but Necl-5 does not bind afadin (Ikeda et al., 2003; Takai and Nakanishi, 2003; Takai et al., 2003).

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Abbreviations used in this paper: Ab, antibody; DN, dominant-negative; MEF, mouse embryonic fibroblast; pAb, polyclonal Ab; PVR, poliovirus receptor; siRNA, small interfering RNA.

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factors, such as PDGF and FGF, in NIH3T3 cells (Kakunaga et al., 2004). Necl-5 enhances the activation of the Ras–Raf–MEK–ERK signaling and causes up- and down-regulation of the cell cycle regulators, including cyclins D2 and E and p27kip1, thereby shortening the period of the G1 phase of cell cycle. Necl-5 is up-regulated in V12Ras-NIH3T3 cells, and this up-regulation is mediated by the transcriptional activation of the Necl-5 gene through the V12Ras–Raf–MEK–ERK–AP-1 pathway (Hirota et al., 2005). On the other hand, it has been shown that Necl-5 heterophilically trans-interacts with nectin-3 (Ikeda et al., 2003; Mueller and Wimmer, 2003), but the physiological function of the interaction of Necl-5 with nectin-3 remains unknown.

We describe here that the cell–cell contact-induced interaction of Necl-5 with nectin-3 causes the endocytosis-mediated down-regulation of Necl-5 from the cell surface, resulting in reduction of cell movement and proliferation.

Results
Cell density-dependent down-regulation of Necl-5

NIH3T3 cells were starved for 24 h and cultured in the presence of serum. The cells continued to proliferate until they became confluent (Fig. 1 A, a, closed blue squares). At various periods of time, the cell surface proteins containing Necl-5 were labeled with biotin and the amount of cell surface Necl-5 was measured. The amount of cell surface Necl-5 gradually decreased as cell density increased (Fig. 1 A, a [closed red circles] and b), whereas the amount of cell surface nectin-1, nectin-3, or N-cadherin did not change. Expression of nectin-2, Necl-1, or Necl-2 was not detected by Western blotting in NIH3T3 cells (Shingai et al., 2003; unpublished data). When NIH3T3 cells were cultured at two different cell densities for 24 h, the amount of cell surface Necl-5 decreased in the cells cultured at...
a higher density as compared with that in the cells cultured at a lower density (Fig. 1 B), whereas the amount of cell surface nectin-1, nectin-3, or N-cadherin did not change at these different cell densities. Similar results were obtained by FACS analysis (Fig. 1 C). The immunofluorescence signal for Necl-5 was concentrated presumably at the leading edges of the cells cultured at the lower density (Fig. 1 D, a1–a3). The signal for nectin-3 did not concentrate anywhere (Fig. 1 D, b1–b3). The signal for N-cadherin was mainly detected at the cytoplasmic and perinuclear regions and slightly detected at the entire plasma membrane (Fig. 1 D, c1–c3). When the cells were cultured at the higher density, the signals for nectin-3 and N-cadherin were concentrated at the cell–cell contact sites (Fig. 1 D, d2, d3, and e1–e3), but the signal for Necl-5 was hardly detected there (Fig. 1 D, d1 and d3). These results indicate that Necl-5 is down-regulated in a cell density–dependent manner. We observed the similar down-regulation of Necl-5 in other cell lines, including Swiss3T3 cells (unpublished data) and mouse embryonic fibroblasts (MEFs; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200501090/DC1).

**Down-regulation of Necl-5 by its interaction with nectin-3**

We next examined how this cell density–dependent down-regulation of Necl-5 is induced in NIH3T3 cells. Necl-5 does not homophilically trans-interact, but heterophilically trans-interacts with nectin-3 (Aoki et al., 1997; Ikeda et al., 2003; Mueller and Wimmer, 2003). Therefore, one possible mechanism is that the cell density–dependent interaction of Necl-5 with nectin-3 triggers the down-regulation of Necl-5. To examine this possibility, we used an mAb against the extracellular region of Necl-5 (mAb-i) known to inhibit the trans-interaction of Necl-5 with nectin-3 (Ikeda et al., 2003). NIH3T3 cells were starved for 24 h and cultured in the presence of serum and this mAb-i. The signal for N-cadherin was mainly detected at the cytoplasmic and perinuclear regions and slightly detected at the entire plasma membrane (Fig. 1 D, c1–c3). When the cells were cultured at the higher density, the signals for nectin-3 and N-cadherin were concentrated at the cell–cell contact sites (Fig. 1 D, d2, d3, and e1–e3), but the signal for Necl-5 was hardly detected there (Fig. 1 D, d1 and d3). These results indicate that Necl-5 is down-regulated in a cell density–dependent manner. We observed the similar down-regulation of Necl-5 in other cell lines, including Swiss3T3 cells (unpublished data) and mouse embryonic fibroblasts (MEFs; Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200501090/DC1).

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We then performed knockdown of nectin-3 using the small interfering RNA (siRNA) method. Transfection of the synthetic siRNA against nectin-3 reduced the total amount of nectin-3 as estimated by Western blotting (Fig. 2 A, a). The total amount of nectin-1 or N-cadherin did not change (unpublished data). The amount of cell surface nectin-3, but not that of nectin-1 or N-cadherin, similarly decreased (Fig. 2 B, a). Immunofluorescence microscopy also showed that the signal for nectin-3 markedly decreased (Fig. 2 A, b1–c2). The signal for N-cadherin did not change, which might be attributable to the presence of nectin-1. When the nectin-3 knockdown cells were cultured at the lower or higher density, the down-regulation of cell surface Necl-5 at the higher density markedly decreased as estimated by Western blotting (Fig. 2 B, a). Immunofluorescence microscopy showed that the cell surface signal for Necl-5 did not decrease in the nectin-3 knockdown cells cultured at the higher density (Fig. 2 B, b1–c2). These results indicate that nectin-3 is a major molecule trans-interacting with Necl-5 and induces its down-regulation.

We further confirmed this conclusion by another method using the recombinant protein of the extracellular fragment of nectin-3 fused to human IgG Fc portion (Nef-3), which interacts with cellular Necl-5 as well as nectin-1 and -3 (Honda et al., 2003; Sato et al., 2004). When NIH3T3 cells were incubated with Nef-3, Nef-3 reduced the cell surface signal for Necl-5 at the leading edges of the cells (Fig. 3 A, a [arrowheads] and b1), whereas Nef-1 did not do that for Necl-5 (Fig. 3 A, C, arrowheads). Nef-1 is the recombinant protein of the extracellular fragment of nectin-1, fused to human IgG Fc portion and inter-
acts with nectin-1 and -3, but not with Necl-5 (Honda et al., 2003; Ikeda et al., 2003). The activity of Nef-3 was blocked by the anti–Necl-5 mAb-i (Fig. 3 A, d, arrowheads). The activity of Nef-3 was also blocked by Nef-1, which is known to interact with not only cellular nectin-1 and -3 but also Nef-3 (Honda et al., 2003; Ikeda et al., 2003; Fig. 3 A, e, arrowheads). The Nef-3–induced down-regulation of cell surface Necl-5 was confirmed by Western blotting and FACS analysis (Fig. 3, B and C). This effect of Nef-3 was dose dependent, and the minimum concentration of Nef-3 necessary for this effect was ~7 nM. Together, these three lines of evidence indicate that the cell density–dependent down-regulation of Necl-5 is induced by the interaction of Necl-5 mainly with nectin-3.

**Down-regulation of Necl-5 through endocytosis**

The down-regulation of Necl-5 from the cell surface might be the result of its endocytosis, reduction of de novo synthesis, or both. When NIH3T3 cells were incubated with Nef-3, Nef-3 not only reduced the cell surface immunofluorescence signal for Necl-5 but also increased the spotlike signal for Necl-5 inside the cells (Fig. 3 A, b1). This Nef-3–induced spotlike intracellular signal for Necl-5 was inhibited by the anti–Necl-5 mAb-i and Nef-1 (Fig. 3 A, d and e). The spotlike signal for Necl-5 was not observed in the presence of Nef-1 (Fig. 3 A, c). When Necl-5 was stained without permeabilization of the cell to detect only cell surface Necl-5, the intracellular signal for Necl-5 was not detected (Fig. 3 A, f). The spotlike intracellular signal for Necl-5 mostly coincided with the signal for Rab7, a marker for the late endosome (Fig. 3 A, g1–g3; Feng et al., 1995), suggesting that endocytosed Necl-5 is transported to the late endosome. The signal for Necl-5 colocalized with the spotlike signal for Necl-5 (Fig. 3 A, b1–b3). This Nef-3 was likely to interact with Necl-5 and to be coendocytosed with Necl-5. Western blotting and FACS analyses showed that Nef-3 not only reduced the amount of cell surface Necl-5 (Fig. 3, B and C) but also increased the amount of endocytosed Necl-5 (Fig. 3 D). These results suggest that the down-regulation of Necl-5 is at least partly attributable to its endocytosis.

We then measured Necl-5 mRNA levels. Although the Necl-5 mRNA levels showed no obvious difference between the cells cultured at the lower and higher densities for 24 h (Fig. 1 E), they gradually decreased in a cell density–dependent manner when they were cultured for longer periods of time (Fig. 1 A, a, closed orange triangles). These results suggest that the cell density–dependent down-regulation of Necl-5 at the early stage is mainly mediated by endocytosis, although its down-regulation at the late stage is mediated by both endocytosis and transcriptional inactivation of the Necl-5 gene.

**Clathrin-dependent endocytosis of Necl-5**

There are at least two types of endocytosis: clathrin-dependent and -independent ones (Conner and Schmid, 2003). Epsin and Eps15 are regulatory components of the formation of clathrin-coated vesicles (Conner and Schmid, 2003), and the ENTH (epsin NH2-terminal homology domain) and the DIII domain of Eps15 act as dominant-negative (DN) mutants for the clathrin-dependent endocytosis (Benmerah et al., 1998; Nakashima et al., 1999). Dynamin is an important regulator for the formation of both clathrin-dependent and -independent endocytic vesicles, and its DN mutant, dynamin1 K44A, inhibits various types of endocytosis (Conner and Schmid, 2003). Caveolin regulates the formation of caveolae and its endocytosis, and an NH2-terminal–truncated mutant of caveolin, DGV-caveolin, serves as a DN mutant and inhibits the formation of caveolae by perturbing intracellular cholesterol trafficking (Conner and Schmid, 2003). To test whether or not the endocytosis of Necl-5 depends on clathrin, various DN mutants were expressed in...
NIH3T3 cells, and the cells were plated and cultured at the higher density. The nectin-3–induced decrease of the cell surface signal for Necl-5 was not affected by the expression of the EGFP-tagged caveolin DN mutant (Fig. 4 A, d1–d4, asterisks). These results indicate that Necl-5 is down-regulated by the clathrin-dependent endocytosis.

We next examined how the interaction of Necl-5 with nectin-3 induces the clathrin-dependent endocytosis of Necl-5. Microbeads coated with Nef-3 were put on the surface of NIH3T3 cells cultured at the lower density and incubated for 1 h. The beads coated with IgG were used as a control. The signals for Necl-5 and clathrin heavy chain colocalized at the contact sites between the Nef-3–coated beads and NIH3T3 cells (Fig. 4 B, a1–a3) but not between the IgG-coated beads and the cells (Fig. 4 B, b1–b3). The essentially similar results were obtained when adaptin β, a component of adaptor protein complex AP2, which is involved in the formation of endocytotic clathrin-coated vesicles (Conner and Schmid, 2003), was stained instead of clathrin heavy chain (unpublished data). In the absence of the beads, the signal for Necl-5 was concentrated presumably at the leading edges, whereas the signal for clathrin heavy chain was detected as the spot-like signals at the cytoplasmic and perinuclear regions and did not colocalize with that for Necl-5 (Fig. 4 B, c1–c3). These results suggest that the interaction of Necl-5 with nectin-3 induces the assembly of the components necessary for the formation of the endocytotic clathrin-coated vesicles at the cytoplasmic region of Necl-5, eventually leading to the endocytosis of Necl-5.

Reduction of cell movement and proliferation by the down-regulation of Necl-5

We next examined the effect of the down-regulation of Necl-5 on cell movement and proliferation. NIH3T3 cells, which were cultured at the lower or higher density in the presence or absence of the anti–Necl-5 mAb-i, were replated, and cell movement after 9 h was assayed by the Boyden chamber method. In the absence of this mAb-i, the amount of cell surface Necl-5 and the degree of movement of NIH3T3 cells pre-cultured at the higher density decreased in a roughly parallel manner as compared with those of the cells precultured at the lower density (Fig. 5 A, a and b). The decreases of these parameters were blocked by the mAb-i (Fig. 5 A, a and b). The level of Necl-5 down-regulated in the cells cultured at the higher density did not return to the original level at 9 h after replating at the lower density, but returned to the original level at 24 h (Fig. 5 A, b; and not depicted). The cells were cultured at the lower or higher density in the presence or absence of the mAb-i, and DNA synthesis was assayed by measuring the incorporation of BrdU. In the absence of this mAb-i, the amount of cell surface Necl-5 and the degree of DNA synthesis of NIH3T3 cells cultured at the higher density decreased in a roughly parallel manner as compared with those of the cells precultured at the lower density (Fig. 5, A [b] and B [a, c, and e]). The decreases of these parameters were inhibited by the mAb-i (Fig. 5 A, a and b). The level of Necl-5 down-regulated in the cells cultured at the higher density did not return to the original level at 9 h after replating at the lower density, but returned to the original level at 24 h (Fig. 5 A, b; and not depicted). The cells were cultured at the lower or higher density in the presence or absence of the mAb-i, and DNA synthesis was assayed by measuring the incorporation of BrdU. In the absence of this mAb-i, the amount of cell surface Necl-5 and the degree of DNA synthesis of NIH3T3 cells cultured at the higher density decreased in a roughly parallel manner (Fig. 5, A [b] and B [a, c, and e]). The decreases of these parameters were inhibited by the mAb-i (Fig. 5, A [b] and B [b, d, and e]). In the nectin-3 knockdown cells, the cell density–dependent decreases of cell movement and DNA synthesis were similarly inhibited (Fig. 6, A and B). The levels of this inhibition were roughly similar to

Figure 4.

Clathrin-dependent endocytosis of Necl-5. (A) Inhibition of down-regulation of Necl-5 by a DN mutant of epsin, Eps15, or dynamin, but not by a DN mutant of caveolin. Immunofluorescence images of the cells transfected with various DN mutants. The cells were transfected with myc-epsin DN mutant (a), EGFP-Eps15 DN mutant (b), HA-dynamin DN mutant (c), or EGFP-caveolin DN mutant (d); cultured at the higher density; and double or triple stained with various combinations of the anti–Necl-5 mAb-i, the anti-myc mAb, the anti-HA mAb, and the anti–nectin-3 pAb. Asterisks show the transfected cells. Bars, 10 μm. (B) Assembly of clathrin heavy chain at the cytoplasmic region of Necl-5, which interacts with Nef-3. NIH3T3 cells were cultured at the lower density and then cultured in the presence or absence of Nef-3– or IgG-coated beads for 1 h. The cells were double stained with the anti–Necl-5 mAb-i and the anti-clathrin heavy chain mAb. (a) In the presence of the Nef-3–coated beads; (b) in the presence of the IgG-coated beads; (c) in the absence of the beads. Asterisks, the positions of the beads; insets, magnified images of the boxed areas. Bars, 10 μm. The results shown are representative of three independent experiments.
Bars, 20. presence of the anti–Necl-5 mAb-i; (e) quantification of BrdU-positive cells.

DAPI. (a and c) In the absence of the anti–Necl-5 mAb-i; (b and d) in the presence or absence of the anti–Necl-5 mAb-i. (B) Measurement of DNA synthesis by the down-regulation of Necl-5.

Figure 5. Cell density–dependent reduction of cell movement and DNA synthesis by the down-regulation of Necl-5. (A) Measurement of cell movement by Boyden chamber assay. (a) Quantification of migrated cells. NIH3T3 cells were cultured at the lower or higher density in the presence or absence of the anti–Necl-5 mAb-i for 24 h, replated in culture insert, and cultured for 9 h. The migrated cells were counted. *, \(P < 0.001\). (b) Reversion of the level of cell surface Necl-5. The cells were cultured at the lower or higher density in the presence or absence of the anti–Necl-5 mAb-i, replated at the lower density, and cultured for 9 h. Biotinylated cell surface Necl-5 was collected before (0 h) or after (9 h) replating and subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti–Necl-5 mAb-i. (B) Measurement of DNA synthesis by BrdU incorporation. The cells were cultured at the lower or higher density in the presence or absence of the anti–Necl-5 mAb-i for 24 h, incubated with BrdU for 2 h, and then double stained with the anti-BrdU mAb and DAPI. (a and c) in the absence of the anti–Necl-5 mAb-i; (b and d) in the presence of the anti–Necl-5 mAb-i; (e) quantification of BrdU-positive cells. Bars, 20 μm. *, \(P < 0.001\). The results are the mean ± SEM of the three independent experiments.

those of the inhibition of the cell density–dependent down-regulation of cell surface Necl-5 (Fig. 2). In addition, when the cells were starved for 24 h and cultured in the presence of serum and the mAb-i, the growth rate did not markedly change, but the cell density rose to slightly higher than that of the cells cultured in the absence of the mAb-i after they became confluent (Fig. 1 A, a, open blue squares). These results indicate that the interaction of Necl-5 with nectin-3 causes the down-regulation of Necl-5, which subsequently reduces cell movement and proliferation.

We furthermore confirmed the inhibitory effects of the down-regulation of Necl-5 on cell movement and proliferation by knocking down Necl-5 using the siRNA method. Transfection of the siRNA vector against Necl-5 reduced the amount of cell surface Necl-5 as estimated by FACS analysis (Fig. 7 A, a). It also reduced the total amount of Necl-5 as estimated by Western blotting and immunofluorescence microscopy (Fig. 7 A, b–c2). The total amount of Necl-5 decreased to ~20%. Movement and DNA synthesis of the Necl-5 knockdown cells decreased as compared with those of the control cells as estimated by the methods just mentioned (Fig. 7, B and C). These results indicate that the down-regulation of Necl-5 reduces cell movement and proliferation.

Discussion

We have shown here that cell surface Necl-5 is down-regulated in a cell density–dependent manner in cultured NIH3T3 cells. As cell density increases, incidence of cell–cell contacts increases. We previously showed that Necl-5 localizes at the leading edges (Ikeda et al., 2004). Therefore, when moving cells collide, Necl-5 may be one of the molecules that first interact with a molecule of another moving cell. At present, three molecules that trans-interact with Necl-5 have been identified: nectin-3, CD226/DNAM-1, and CD96/Tactile (Bottino et al., 2003; Ikeda et al., 2003; Mueller and Wimmer, 2004). CD226 and CD96 are also Ig-like molecules that are exclusively expressed in blood cells, including NK cells and T cells (Bottino et al., 2003; Fuchs et al., 2004). Thus, of these molecules, only nectin-3 is expressed in NIH3T3 cells. Moreover, our series of studies has revealed that when moving cells collide, nectin first forms cell–cell contacts where cadherin is recruited, resulting in the formation of adherens junctions (Takai and Nakanishi, 2003; Takai et al., 2003). Therefore, it is most likely that when moving cells collide, Necl-5 first interacts with nectin-3, which then induces the down-regulation of Necl-5. We have presented three lines of evidence for this conclusion: (1) the anti–Necl-5 mAb-i, which is able to inhibit the interaction of Necl-5 with nectin-3, inhibits the cell density–dependent down-regulation of Necl-5; (2) the knockdown of nectin-3 inhibits the cell density–dependent down-regulation of Necl-5; and (3) Nef-3 induces the down-regulation of Necl-5 of the cells that do not contact other cells. These results do not exclude the possibility that Necl-5 interacts with a still unidentified molecule in NIH3T3 cells in addition to nectin-3 and induces the down-regulation of Necl-5, but the present results indicate that nectin-3 is at least a major molecule that interacts with Necl-5 upon cell–cell contacts and induces the down-regulation of Necl-5 in NIH3T3 cells.

The cell–cell contact-induced down-regulation of Necl-5 from the cell surface might be the result of its endocytosis, reduction of de novo synthesis, or both. We have shown here that the cell density–dependent down-regulation of Necl-5, at least at the early stage, is mainly mediated by clathrin-dependent endocytosis. Therefore, the interaction of Necl-5 with nectin-3 down-regulates Necl-5 through clathrin-dependent endocytosis. The detailed molecular mechanism of the endocytosis induced by the interaction of Necl-5 with nectin-3 is not known, but we have shown here that this interaction recruits the components necessary for the clathrin-dependent endocytosis, including clathrin heavy chain and adaptin β, to the cytoplasmic region of Necl-5, which interacts with nectin-3. Therefore, the mechanism of endocytosis of Necl-5 is likely to be analogous...
to that of the endocytosis of cell surface receptors for hormones and cytokines, which are soluble molecules and coendocytosed with their respective membrane receptors upon their activation by hormones and cytokines. When Nef-3 is used as a ligand for Necl-5, it also serves as a soluble ligand for Necl-5 and is similarly coendocytosed with Necl-5. In contrast, nectin-3 is a transmembrane protein that trans-interacts with Necl-5 and is resistant to coendocytosis with Necl-5. One possible mechanism of the nectin-3–induced endocytosis of Necl-5 is that the interaction of Necl-5 with nectin-3 causes the clustering of Necl-5, where the components of the clathrin-dependent endocytosis assemble and eventually form coated pits, followed by budding and pinching off of vesicles. In these processes, Necl-5, which interacts with nectin-3, may be replaced by nectin-1, resulting in the formation of free Necl-5, which is finally endocytosed, because the affinity of nectin-3 for nectin-1 is 10-fold higher than that of nectin-3 for Necl-5 (Ikeda et al., 2003). The interaction of nectin-3 with nectin-1 then induces the recruitment of N-cadherin there, resulting in the formation of adherens junctions. Another possible mechanism is that nectin-3, which interacts with Necl-5, is cleaved at the extracellular region and that its extracellular fragment is coendocytosed with Necl-5. Conversely, Necl-5, which interacts with nectin-3, may be cleaved at the extracellular region, and its extracellular fragment may be coendocytosed with nectin-3. However, the former possibility is less likely because cell surface nectin-3 did not decrease in a cell density–dependent manner.

When the cells were treated with Nef-3, the spotlike intracellular signal for Necl-5 increased and mostly colocalized with the signal for Rab7, which is a late endosomal marker.

Figure 6. Inhibition of cell density-dependent reduction of cell movement and DNA synthesis by the knockdown of nectin-3. NIH3T3 cells were transfected with the synthetic siRNA against nectin-3 or the control synthetic siRNA and cultured for 48 h. The knockdown of nectin-3 inhibited the cell density–dependent down-regulation of Necl-5 (see Fig. 2). (A) Measurement of cell movement by Boyden chamber assay. The transfected cells were cultured at the lower or higher density for 24 h, replated in culture insert, and cultured for 9 h. The migrated cells were counted. *, *P < 0.001. (B) Measurement of DNA synthesis by BrdU incorporation. The transfected cells were cultured at the lower or higher density for 24 h, incubated with BrdU for 2 h, and then double stained with the anti-BrdU mAb and DAPI. (a and c) Control; (b and d) nectin-3 siRNA; (e) quantification of BrdU-positive cells. Bars, 20 μm. *, *P < 0.001. The results are the mean ± SEM of the three independent experiments.

Figure 7. Reduction of cell movement and DNA synthesis by the knockdown of Necl-5. NIH3T3 cells were transfected either with the siRNA vector against Necl-5 and the pEGFP-tub vector or with the control siRNA vector and the pEGFP-tub vector and cultured at the lower density for 48 h. The transfection efficiency was ~20%. (A) Confirmation of the knockdown of Necl-5. (a) FACS analysis. The cells were stained with the anti–Necl-5 mAb and the pEGFP-tub–positive cells were monitored. (b) Western blotting. The pEGFP-tub–positive cells were sorted by FACS and then the lysate of the sorted cells was subjected to SDS-PAGE (10% polyacrylamide gel), followed by Western blotting with the anti–Necl-5 mAb. (c) Immunofluorescence images. The cells were single stained with the anti–Necl-5 mAb. (c1) Necl-5; (c2) Necl-5 and pEGFP-tub. Asterisks show the Necl-5 knockdown cells. Bars, 10 μm. (B) Measurement of cell movement by Boyden chamber assay. The transfected cells (~20% transfection efficiency) were cultured at the lower density, directly replated in culture insert, and cultured for 9 h. The pEGFP-tub–positive migrated cells were counted. The number of control EGFP-tub–positive migrated cells was consistent with that of control migrated cells precultured at lower density in Fig. 5 A (a) and Fig. 6 A, considering the transfection efficiency. *, *P < 0.001. (C) Measurement of DNA synthesis by BrdU incorporation. The transfected cells (~20% transfection efficiency) were directly cultured at the lower density for 18 h and incubated with BrdU for 2 h. The cells were double stained with the anti-BrdU mAb and DAPI. The pEGFP-tub–positive cells were measured. (a) Control; (b) Necl-5 siRNA; (c) quantification of BrdU-positive cells. Bars, 20 μm. *, *P < 0.001. The results shown in A are representative of three independent experiments, and the results shown in B and C are the mean ± SEM of the three independent experiments.
In contrast, this spotlike signal for Necl-5 was not observed in the cells cultured at the higher cell density in the absence of Nef-3, although the cell surface signal for Necl-5 markedly decreased. The exact reason for this apparent discrepancy is not known, but one possible explanation is that Necl-5 was endocytosed and transported to the late endosome in the cells cultured at the higher density in the absence of Nef-3 but its amount in the late endosome was too low to be detected. The other possible explanation is that when the cells were treated with Nef-3, Necl-5 was coendocytosed with Nef-3 and the Necl-5–Nef-3 complex was delivered to the late endosome and was not further transported to the lysosome, where the proteins might be degraded, whereas Necl-5 free of nectin-3 was transported to the lysosome, where it was degraded in the cells cultured at the higher density in the absence of Nef-3.

We previously showed that Necl-5 has a potency to enhance cell movement and proliferation induced by growth factors such as PDGF and FGF (Ikeda et al., 2004; Kakunaga et al., 2004). Consistent with this finding, our current results show that the cell–cell contact-induced down-regulation of Necl-5 causes reduction of cell movement and proliferation. The inhibition of this down-regulation by the anti–Necl-5 mAb-i or the knockdown of nectin-3 blocks the reduction of cell movement and proliferation. The artificial knockdown of Necl-5 by the siRNA method similarly reduces cell movement and proliferation. Therefore, it is likely that the interaction of Necl-5 with nectin-3 causes the down-regulation of Necl-5, which then reduces cell movement and proliferation. As cultured cells become confluent, they form cell–cell adhesion, gradually reducing and then finally stopping movement and proliferation (Abercrombie and Heaysman, 1953; Fisher and Yeh, 1967). This phenomenon has for a long time been known as contact inhibition of cell movement and proliferation, and several mechanisms have been reported. For instance, the vascular endothelial cadherin–β-catenin complex inhibits the VEGF signaling for endothelial cell proliferation (Grazia Lampugnani et al., 2003); the E-cadherin–mediated cell–cell adhesion down-regulates extracellular regulated protein kinase signaling through the phosphoinositide 3-kinase–Akt pathway for cell proliferation (Laprise et al., 2004); the neurofibromatosis-2 gene product, Merlin, and a transmembrane hyaluronic acid receptor, CD44, form a molecular switch that specifies cell growth arrest or proliferation (Morrison et al., 2001); and the effector of Rac/Cdc42, PAK1, and the PAK-interacting Rac-guanine nucleotide exchange factor, PIX, regulate contact inhibition during epithelial wound healing (Zegers et al., 2003). The present results that the cell–cell contact-induced interaction of Necl-5 with nectin-3 and the subsequent endocytosis-mediated down-regulation of Necl-5 and reduction of cell movement and proliferation suggest that this mechanism is an additional mechanism for the contact inhibition of cell movement and proliferation. All the experiments shown here were performed by use of NIH3T3 cells, but essentially the same results were obtained for MEFs (see the online supplemental material and Figs. S1–S5, available at http://www.jcb.org/cgi/content/full/jcb.200501090/DC1). Therefore, the down-regulation of Necl-5 induced by its interaction with nectin-3 upon cell–cell contacts is observed not only in NIH3T3 cells but also in MEFs.

Materials and methods

Cell culture

NIH3T3 cells were obtained from American Type Culture Collection. NIH3T3 cells were maintained in DMEM supplemented with 10% calf serum. For cultures at the lower or higher density, NIH3T3 cells were plated at a density of 5 × 10^4 or 4 × 10^5 cells per cm², respectively, and cultured for 24 h. For DNA transfection, Lipofectamine 2000 reagent (Invitrogen) was used.

Antibodies (Abs) and reagents

A rat IgG2a mAb against the extracellular region of Nef-5 (1A8-8, mAb-b) was prepared as described previously (Ikeda et al., 2003). A rabbit biotinylational Ab against the extracellular region of Nef-5 was used for Western blotting. Rabbit anti–nectin-1 pAb, rat anti–nectin-3 mAb, and rabbit anti–nectin-3 xA1 pAb were prepared as described previously (Sato–Horikawa et al., 2000; Sakisaka et al., 2001). Hybridoma cells (9E10) expressing a mouse anti-myc mAb were purchased from American Type Culture Collection. A rabbit anti–N-cadherin pAb was purchased from Takara. A mouse anti-tubulin mAb was purchased from Chemicon. A mouse anti-HA mAb was purchased from Babco. A goat anti–Rab7 pAb was purchased from Santa Cruz Biotechnology, Inc. HRP-conjugated and fluorophore-conjugated secondary Abs were purchased from GE Healthcare, Chemicon, Jackson ImmunoResearch Laboratories, and ICN Biomedicals. DAPI was purchased from Nacalai Tesque. Nef-1 or -3 was prepared as described previously (Honda et al., 2003) and cross-linked by a rabbit anti–human IgG Fc pAb (Jackson ImmunoResearch Laboratories) before use.

pEFBoSmyc-epsin epsin NHI-terminal homology was supplied by A. Kikuchi (Hirosima University, Hiroshima, Japan); pEGFPN15 DII by A. Benmerah (Cochin Institute, Paris, France); pCNeo-HA-dynamin K44A by S.L. Schmid (The Scripps Research Institute, La Jolla, California); pEGFP-DGY-caveolin by T. Fujimoto (Nagoya University, Nagoya, Japan); and pBSH1 vector by H. Shibuya (Tokyo Medical and Dental University, Tokyo, Japan).

Immunofluorescence microscopy

The cells were fixed with 1% formaldehyde in PBS for 15 min and permeabilized with 100% methanol. Alternatively, the cells were fixed with aceto-methanol [1:1] for 1 min at −20°C. The cells, which were preincubated with the anti–Nef-5 mAb, were washed with a stripping buffer (0.2 M acetic acid and 0.5 M NaCl) before fixation. For blocking, the samples were incubated with 1% BSA in PBS and then with 20% BlockAce (Dainippon Seiyaku) in PBS. The samples were stained with the various combinations of the primary Abs and then with appropriate fluorophore-conjugated secondary Abs. The samples were analyzed using a confocal laser scanning microscope (Radiance 2000 or 2100; Bio-Rad Laboratories).

siRNA experiments

For the knockdown of nectin-3, double-stranded 25-nt RNA duplex, stealth RNA interference, to nectin-3 (5′-UGUAACAAUGUGCUUGUCAA-3′) and control stealth RNA interference duplex were purchased from Invitrogen. Duplexes were transfected using Lipofectamine 2000 reagent according to the manufacturer’s protocol. The transfection efficiency was monitored using FITC-labeled RNA oligo (Invitrogen). For the knockdown of Nef-5, the pBS-H1 vector containing H1 promoter was used for the expression of siRNA. To generate a vector for knockdown of Nef-5 (pBS-H1–Necl-5), a specific insert for Nef-5 was subcloned into pBS-H1. The insert was used as follows: mouse Necl-5 gene-specific insert was a 19-nt sequence. A pBS-H1 control vector had an insert including a 19-nt sequence. A pBS-H1–Necl-5 DNA, which was separated by a 9-nt noncomplementary spacer (5′-TTCAAGAGA-3′) from the reverse complement of the same 19-nt sequence. A pBS-H1 control vector had an insert including a 19-nt sequence (luciferase; 5′-GGTAGTGTGGCTCCTACTA-3′) of mouse Necl-5, with some modifications. NIH3T3 cells were plated at a density of 10⁶ cells per well. The cells were incubated at 37°C for 9 h in the presence of 10% serum. The migrated cells, which were stained with crystal violet, were expressed EGF-Pub, were counted by phase contrast or fluorescence microscopic examination, respectively.

Boyden chamber assay

The Boyden chamber assay was performed as described previously (Ikeda et al., 2004), with some modifications. NIH3T3 cells were seeded at a density of 10⁴ cells per well. The cells were incubated at 37°C for 9 h in the presence of 10% serum. The migrated cells, which were stained with crystal violet, were expressed EGF-Pub, were counted by phase contrast or fluorescence microscopic examination, respectively.
Other procedures
Cell growth assay and FACS analysis were performed as described previously (Kakunaga et al., 2004). Quantification of cell surface proteins and endocytosed Necl-5 by the biotinylation method were performed as described previously (Ike et al., 1999). The endocytosis assay was performed at 18°C to inhibit recycling. Real-time RT-PCR was performed as described previously (Hirota et al., 2003). The levels of Necl-5 mRNA were determined as the absolute value to total RNA. Bead-cell adhesion assay was performed by using a BrdU labeling and detection kit I (Roche) according to the manufacturer’s protocol.

Online supplemental material
Online supplemental text describes that the down-regulation of Necl-5 induced by its interaction with nectin-3 upon cell–cell contacts is observed in MEFs. It also describes additional materials and methods for the experiments using MEFs. Figs. S1 and S2 show that the cell density–dependent down-regulation of Necl-5 is induced by its interaction with nectin-3 in MEFs. Fig. S4 shows that Necl-5 is down-regulated by the clathrin-dependent endocytosis in MEFs. Fig. S5 shows that the down-regulation of Necl-5 reduces cell movement and proliferation in MEFs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200501090/DC1.

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