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Role of each immunoglobulin-like loop of nectin for its cell–cell adhesion activity☆

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Abstract

Nectins are Ca^{2+}-independent immunoglobulin (Ig)-like cell–cell adhesion molecules that form cell–cell junctions, cooperatively with or independently of cadherins, in a variety of cells. Nectins comprise a family of four members, nectin-1, -2, -3, and -4. All nectins have one extracellular region with three Ig-like loops, one transmembrane segment, and one cytoplasmic tail. It has been shown mainly by use of cadherin-deficient L fibroblasts stably expressing each nectin that nectins first form homo-*cis*-dimers and then homo- or hetero-*trans*-dimers, causing cell–cell adhesion, and that the formation of the *cis*-dimers is necessary for the formation of the *trans*-dimers. However, kinetics of the formation of these dimers have not been examined biochemically by use of pure nectin proteins. We prepared here pure recombinant proteins of extracellular fragments of nectin-3 containing various combinations of Ig-like loops, all of which were fused to the Fc portion of IgG and formed homo-*cis*-dimers through the Fc portion, and of an extracellular fragment of nectin-1 containing three Ig-like loops which was fused to secreted alkaline phosphatase and formed homo-*cis*-dimers. We showed here by use of these proteins that the first Ig-like loop of nectin-3 was essential and sufficient for the formation of *trans*-dimers with nectin-1, but that the second Ig-like loop of nectin-3 was furthermore necessary for its cell–cell adhesion activity.

Keywords: Nectin; Cell–cell adhesion molecule; Immunoglobulin-like loop; Adherens junctions

Nectins are Ca^{2+}-independent immunoglobulin (Ig)-like cell–cell adhesion molecules and expressed in a wide variety of cells [1]. Nectins form cell–cell adherens junctions (AJs) cooperatively with E-cadherin in epithelial cells and fibroblasts [2–8], form synapses cooperatively with N-cadherin in neurons [9], and form Sertoli cell–spermatid junctions in a cadherin-independent manner in the testis [10].

Nectins comprise a family of four members, nectin-1, -2, -3, and -4, each of which has two or three splicing variants [1]. All nectins have one extracellular region with three Ig-like loops, one transmembrane segment, and one cytoplasmic tail. It has been shown mainly by use of cadherin-deficient L fibroblasts expressing each nectin that nectins first form homo-*cis*-dimers and then homo-*trans*-dimers (*trans*-interaction), causing cell–cell adhesion [3,5,6,11,12]. Nectin-3 furthermore forms hetero-*trans*-dimers with either nectin-1 or -2 and the adhesion activity of each hetero-*trans*-dimer is stronger than that of each homo-*trans*-dimer [6,13]. Nectin-4 also forms hetero-*trans*-dimers with nectin-1 [8]. It has furthermore been shown, by use of L cells expressing a point-mutated form of nectin-2, which does not form homo-*trans*-dimers, or each fragment of nectin-2 containing various combinations of Ig-like loops, that the first Ig-like loop is necessary for the formation of

☆ *Abbreviations:* Ig, immunoglobulin; AJs, adherens junctions; MDCK cells, Madin–Darby canine kidney cells; Nef, the extracellular fragment of nectin fused to the Fc portion of IgG; Ab, antibody; mAb, monoclonal Ab; aa, amino acid(s); SEAP, secreted alkaline phosphatase; nectin-1-SEAP, the extracellular fragment of nectin-1 fused to SEAP; RU, resonance unit(s); K_D, the dissociation constant; BS3, bis-(sulfosuccinimidyl) suberate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol.

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homo-*trans*-dimers, that the second Ig-like loop is necessary for the formation of homo-*cis*-dimers, and that the formation of homo-*cis*-dimers is necessary for the formation of homo-*trans*-dimers [5,12]. It has been shown, by use of Madin–Darby canine kidney (MDCK) cells stably expressing each mutant of nectin-1 containing various combinations of Ig-like loops or COS cells transiently expressing nectin-1, -2, -3, or -4, and pure recombinant proteins of the extracellular fragments of nectin-1, -2, -3, and -4 fused to the Fc portion of human IgG (Nef-1, -2, -3, and -4, respectively) or various mutants of Nef-1, that the first Ig-like loop of nectin-1 is necessary for the formation of hetero-*trans*-dimers with nectin-3 and -4 [8,14]. However, the detailed kinetics for these interactions by use of pure nectin proteins have not been studied.

We first examined here by the surface plasmon resonance method which Ig-like loops of nectin-3 are necessary for the formation of hetero-*trans*-dimers with nectin-1. For this purpose, we prepared and used pure recombinant proteins of extracellular fragments of nectin-3 containing various combinations of Ig-like loops, all of which were fused to the Fc portion of IgG and formed homo-*cis*-dimers through the Fc portion, and of an extracellular fragment of nectin-1 containing three Ig-like loops which was fused to secreted alkaline phosphatase and formed homo-*cis*-dimers.We then examined which fragments of nectin-3 are necessary for its cell–cell adhesion activity. For this purpose, we assessed antagonistic and agonistic effects of each nectin-3 fragment using the assays that we recently developed [13]. The antagonistic effect was assayed by measuring the ability of each nectin-3 fragment to reduce the formation of the *trans*-dimers mediated by nectin-1 of L cells stably expressing nectin-1 (nectin-1-L cells) and nectin-3 of L cells stably expressing nectin-3 (nectin-3-L cells). The agonistic effect was assayed by measuring the ability of each nectin-3 fragment coated on micro-beads to form *trans*-dimers with cellular nectin-1 and to recruit E-cadherin at the bead-cell contact sites in nectin-1-L cells stably expressing E-cadherin (nectin-1-EL cells) as described [13].

Materials and methods

Antibodies. A rabbit anti-nectin-1α polyclonal antibody (Ab), which was raised against the cytoplasmic region of nectin-1α, was prepared as described [4]. A rat anti-E-cadherin monoclonal Ab (mAb) (ECCD-2) was supplied by Dr. M. Takeichi (RIKEN, Kobe, Japan). Anti-secreted alkaline phosphatase (SEAP) and anti-His6 mAbs were purchased from Seradyn (Indianapolis, IN) and Qiagen, respectively. FITC-conjugated anti-rabbit IgG and TRICT-conjugated anti-rat IgG Abs (Chemicon) were used for the secondary Abs.

Plasmid construction. pFastBac1-Msp-Fc, a baculovirus transfer vector, was constructed to express a chimera protein fused to the N-terminal honeybee melittin signal peptide and the C-terminal Fc portion of human IgG [7,11]. pFastBac1-Msp-Fc-Nef-3-Full (amino acids (aa) 55–400) was prepared as described [6]. pFastBac1-Msp-Fc-Nef-3-Δ3 (aa 55–290; deletion of the third Ig-like loop), pFastBac1-Msp-Fc-Nef-3-Δ1 (aa 149–400; deletion of the first Ig-like loop), pFastBac1-Msp-Fc-Nef-3-Δ2Δ3 (aa 55–192; deletion of the second and third Ig-like loops), and pFastBac1-Msp-Fc-Nef-3-Δ1Δ3 (aa 149–290; deletion of the first and third Ig-like loops) were similarly prepared. Baculoviruses bearing the cDNAs encoding Nef-3-Full, -Δ3, -Δ1, -Δ2Δ3, and -Δ1Δ3 were prepared with pFastBac1-Msp-Fc-Nef-3-Full, -Δ3, -Δ1, -Δ2Δ3, and -Δ1Δ3, respectively, according to the manufacturer's protocol (Invitrogen). pDREF-SEAP(His)6-Hyg-nectin-1-EX was constructed by inserting a cDNA fragment encoding the extracellular fragment of nectin-1 (aa 1–347) into pDREF-SEAP(His)6-Hyg [15].

Purification of proteins. Nef-3 fragments were expressed in High Five insect cells (Invitrogen) and purified as described [13]. The extracellular fragment of nectin-1 fused to SEAP (nectin-1-SEAP) was expressed in 293/EBNA-1 cells transfected with pDREF-SEAP(His)6-Hyg-nectin-1-EX and purified as described [15].

Cell lines. L and EL cells were kindly supplied from Dr. Sh. Tsukita (Kyoto University, Kyoto, Japan). EL cells were cloned by introduction of the exogenous E-cadherin cDNA to cadherin-deficient L cells [16]. L cell lines stably expressing exogenous full-length nectin-1α or -3α (nectin-1- or -3-L cells, respectively) were prepared as described [4,6]. EL cells stably expressing full-length nectin-1α (nectin-1-EL cells) were prepared as described [7].

Surface plasmon resonance analysis. A BIAcore X surface plasmon resonance-based biosensor (BIAcore, Piscataway, NJ) was used to measure kinetic parameters for the interaction between each Nef-3 fragment and immobilized nectin-1-SEAP. The anti-SEAP mAb was immobilized to the sensor chip surface by the amine-coupling method according to the manufacturer's protocol (BIAcore). Nectin-1-SEAP or SEAP as a control was immobilized at a concentration of approximately 2800 resonance units (RU) (2.8 ng/mm^2) to the sensor chip via the anti-SEAP mAb. The solution of each Nef-3 fragment (90 nM) was injected and each assay cycle was performed at 20 μl/min at 25 °C for 450 s. The dissociation constant (K_D) was obtained using the BIA-evaluation software version 3.2 (BIAcore).

Chemical cross-linking assay. Nectin-1-SEAP (1.0 μg) was incubated in 100 μl of phosphate-buffered saline with 5 mM bis-(sulfosuccinimidyl) suberate (BS3) (Pierce) at room temperature for 30 min. After the incubation, the reaction was stopped with the addition of 50 μl of 1 M Tris–HCl, pH 7.5. The sample was subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), followed by Western blotting with the anti-His6 mAb.

Cell aggregation assay. The cell aggregation assay was done as described [6]. Briefly, to obtain a single-cell suspension, nectin-1- or -3-L cells were washed with PBS, incubated with 0.2% trypsin and 1 mM EDTA at 37 °C for 5 min, and dispersed gently by pipetting. Dispersed nectin-1-L cells were mixed with an equal number of dispersed nectin-3-L cells in the presence of each Nef-3 fragment or human IgG as a control. After the 10-min incubation, the reaction was stopped and observed by phase-contrast illumination.

Bead-cell adhesion assay. The bead-cell adhesion assay was done as described [13]. Briefly, micro-beads coated with each Nef-3 fragment or human IgG as a control were added to nectin-1-EL cells (1×10^4) on a 24-well dish. The cells were incubated at 37 °C for 1 h and then fixed, followed by immunostaining for nectin-1α and E-cadherin.

Other procedures. Immunofluorescence microscopy of cultured cells was done as described [13]. Protein concentrations were determined with bovine serum albumin as a reference protein [17]. SDS–PAGE was done as described [18].

Results

We made pure recombinant proteins of various mutants of Nef-3 (the extracellular fragment of nectin-3

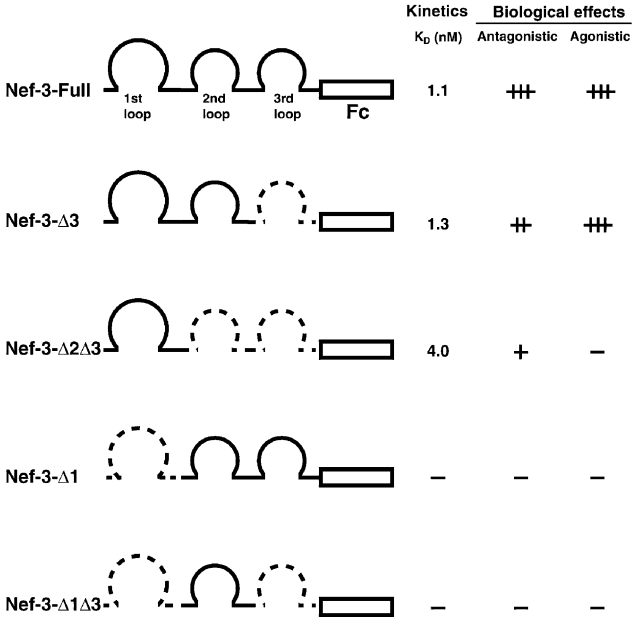

Fig. 1. Summaries of schematic representation, kinetics, and biological effects of various Nef-3 fragments. Dashed lines, deleted regions; Fc, the Fc portion of human IgG; K_D, the K_D value of each Nef-3 fragment binding to nectin-1. The antagonistic and agonistic effects of each Nef-3 fragment are summaries of Figs. 4 and 5, respectively. (+++) Very strong; (++) strong; (+) weak; and (–) not detected.

fused to the Fc portion of IgG) as schematically shown in Fig. 1: these include fragments which contained all the three Ig-like loops (Nef-3-Full), the first and second Ig-like loops (Nef-3-Δ3), the first Ig-like loop (Nef-3-Δ2Δ3), the second and third Ig-like loops (Nef-3-Δ1), and the second Ig-like loop (Nef-3-Δ1Δ3). All of these samples formed homo-*cis*-dimers through the Fc

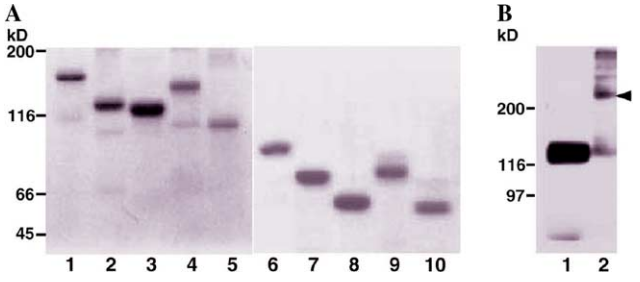

Fig. 2. Formation of homo-*cis*-dimers of Nef-3 fragments and nectin-1-SEAP. (A) Nef-3 fragments. A pure recombinant sample of each Nef-3 fragment (2 μg protein per lane) was subjected to SDS–PAGE (a 10% SDS–polyacrylamide gel) in the presence or absence of 260 mM 2-mercaptoethanol (2-ME) followed by Coomassie brilliant blue staining. (1–5) In the absence of 2-ME; (6–10) in the presence of 2-ME; (1 and 6) Nef3-Full; (2 and 7) Nef3-Δ3; (3 and 8) Nef3-Δ2Δ3; (4 and 9) Nef3-Δ1; (5 and 10) Nef3-Δ1Δ3. (B) Nectin-1-SEAP. A pure recombinant nectin-1-SEAP was incubated in the presence or absence of BS3 for 30 min. After the incubation, the reaction was stopped and the samples were subjected to SDS–PAGE, followed by Western blotting with the anti-His6 mAb. (1) In the absence of BS3; (2) in the presence of BS3. The arrowhead indicates homo-*cis*-dimers of nectin-1-SEAP. The results shown are representative of three independent experiments.

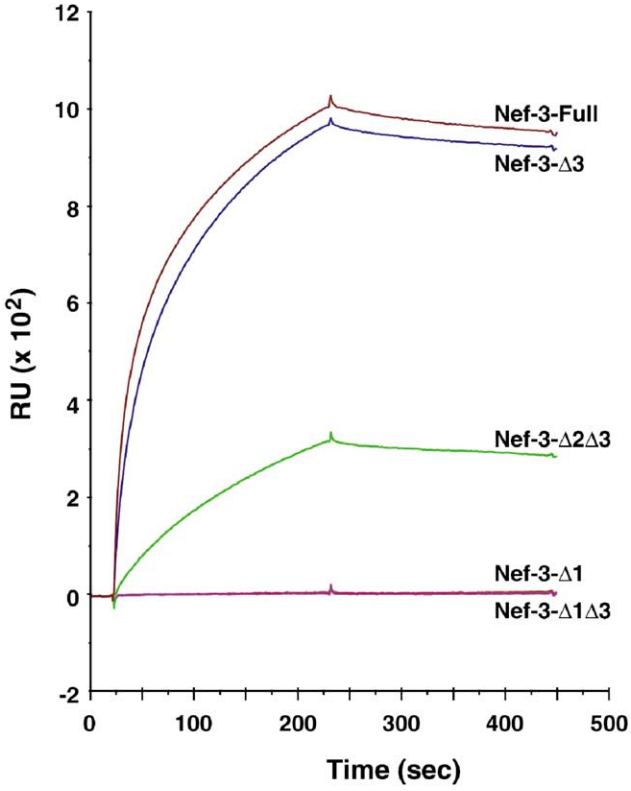

Fig. 3. Surface plasmon resonance analysis of the binding of nectin-1-SEAP to Nef-3 fragments. Sensorgrams are shown for different Nef-3 fragments injected on immobilized nectin-1-SEAP. Analysis was performed at a flow rate of 20 μl/min. The results shown are representative of three independent experiments.

portion of IgG (Fig. 2A). We furthermore made a pure recombinant nectin-1-SEAP (the extracellular fragment of nectin-1 fused to SEAP). Nectin-1-SEAP also formed homo-*cis*-dimers (Fig. 2B).

We examined by the surface plasmon resonance method using these samples which Nef-3 fragments form *trans*-dimers with nectin-1-SEAP. Each Nef-3 fragment was injected on nectin-1-SEAP immobilized to the sensor chip and the association and dissociation of the two proteins were measured. Nef-3-Full bound to nectin-1-SEAP with a high affinity (K_D value of 1.1 nM) (Fig. 3), consistent with the previous report that the K_D value of Nef-3 binding to nectin-1, which was overexpressed in MDCK cells, was 1 nM [14]. Nef-3-Δ3 and Nef-3-Δ2Δ3 bound to nectin-1-SEAP with the K_D values of 1.3 and 4.0 nM, respectively. In contrast, the binding activity of Nef-3-Δ1 or Nef-3-Δ1Δ3 to nectin-1-SEAP was not detected in this assay system.

We then examined which Nef-3 fragments are necessary for the cell–cell adhesion activity of nectin-3. For this purpose, we examined the antagonistic effect of each Nef-3 fragment on the formation of the *trans*-dimers mediated by nectin-1 of nectin-1-L cells and nectin-3 of nectin-3-L cells. Nectin-1- and -3-L cells were mixed in the presence of each Nef-3 fragment (1×10^{-6} M) or

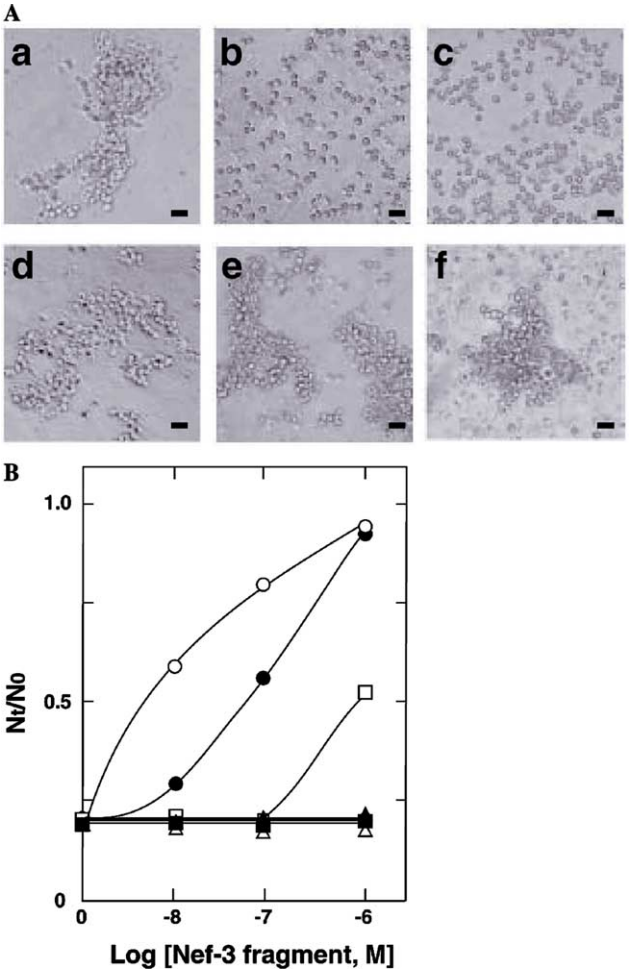

Fig. 4. Effects of various Nef-3 fragments on the formation of cell aggregates by the mixture of nectin-1- and -3-L cells. (A) Inhibition by Nef-3-Full, -Δ3, or -Δ2Δ3 of the formation of the cell aggregates. The mixture of single-cell suspensions of nectin-1- and -3-L cells was incubated in the presence of each Nef-3 fragment (1×10^{-6} M) or human IgG (1×10^{-6} M) as a control for 10 min. (a) Human IgG; (b) Nef-3-Full; (c) Nef-3-Δ3; (d) Nef-3-Δ2Δ3; (e) Nef-3-Δ1; (f) Nef-3-Δ1Δ3. Bars, 50 μm. (B) Dose-dependent inhibitory activity. The mixture of single-cell suspensions of nectin-1- and -3-L cells was incubated in the presence of the indicated concentrations of each Nef-3 fragment for 10 min. (○) Nef-3-Full; (●) Nef-3-Δ3; (□) Nef-3-Δ2Δ3; (■) Nef-3-Δ1; (▲) Nef-3-Δ1Δ3; (△) human IgG. The extent of aggregation of cells is represented by the ratio of the total particle number at time t of incubation (N_t) to the initial particle number (N_0). The results shown are representative of three independent experiments.

human IgG (1×10^{-6} M) and subjected to the cell aggregation assay. Nef-3-Full markedly inhibited the cell aggregation of nectin-1- and -3-L cells (Fig. 4A) as described [13]. Nef-3-Δ3 also markedly inhibited the cell aggregation. Nef-3-Δ2Δ3 showed a smaller inhibitory effect, but neither Nef-3-Δ1 nor Nef-3-Δ1Δ3 showed any effect. The dose–response curve of each mutant is shown in Fig. 4B. Nef-3-Full showed the strongest inhibitory effect; Nef-3-Δ3 showed a less effect; and Nef-3-Δ2Δ3 showed a much lesser effect. Nef-3-Δ1 and Nef-3-Δ1Δ3 showed no effect at any concentrations tested.

We finally examined the agonistic effect of each Nef-3 fragment on the formation of its *trans*-dimers with cellular nectin-1 of nectin-1-EL cells. Each Nef-3 fragment or human IgG was coated on micro-beads, the beads were put on nectin-1-EL cells, and it was examined whether each Nef-3 fragment recruits cellular nectin-1 and E-cadherin to the bead-cell contact sites. Nef-3-Full recruited both cellular nectin-1 and E-cadherin as described [13], whereas human IgG did not (Fig. 5). Nef-3-Δ3 recruited both cellular nectin-1 and E-cadherin, but other Nef-3 fragments, Nef-3-Δ2Δ3, Nef-3-Δ1, and Nef-3-Δ1Δ3, did not show this agonistic activity (data not shown).

Discussion

We have shown here by use of pure recombinant nectin proteins that the first Ig-like loop of Nef-3 directly interacts with nectin-1 with a high affinity (the K_D value of 4.0 nM). The affinity of this interaction is somewhat enhanced by the second Ig-like loop (the K_D value of 1.3 nM). This result is consistent with our earlier observation that L cells stably expressing nectin-2 containing a point-mutated aa in the first Ig-like loop lose cell aggregation activity [5], and also consistent with the recent report that Nef-1 lacking the second and third Ig-like loops binds to COS cells transiently expressing nectin-3 [8]. Taken together, these results indicate that the first Ig-like loop of nectins is necessary and sufficient for the formation of *trans*-dimers.

We have moreover shown here that the second Ig-like loop of nectin-3 in addition to the first one is necessary for its cell–cell adhesion activity as estimated by measuring the antagonistic and agonistic effects of the Nef-3 fragments containing various combinations of Ig-like loops, although Nef-3-Full containing three Ig-like loops is the most effective in the antagonistic capacity. Nef-3-Δ2Δ3, that contains the first Ig-like loop but lacks the second Ig-like loop, shows little antagonistic activity and does not show the agonistic activity. We have previously shown that the second Ig-like loop of nectin-2 is necessary for the formation of homo-*cis*-dimers and that the formation of homo-*cis*-dimers is essential for the formation of *trans*-dimers [12]. We have used here Nef-3 fragments all of which form homo-*cis*-dimers through the Fc portion of IgG. Therefore, the present results suggest that the second Ig-like loop plays roles not only in the formation of homo-*cis*-dimers and *trans*-dimers but also in the conformational change of nectin-3 for its cell–cell adhesion activity.

The role of the third Ig-like loop of nectin-3 still remains to be elucidated, but the present results, that full-length Nef-3 (Nef-3-Full) shows a stronger antagonistic effect than that of Nef-3 lacking the third Ig-like loop (Nef-3-Δ3), suggest that the third Ig-like loop

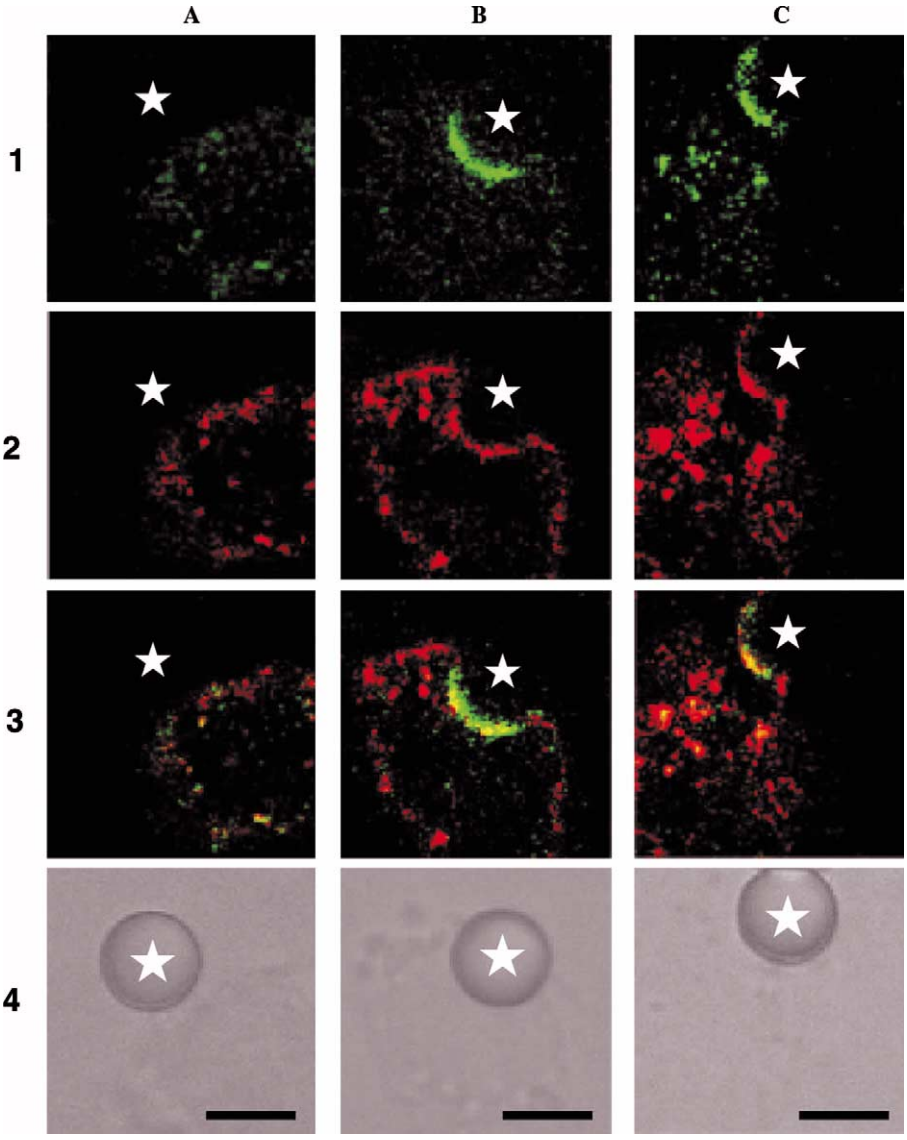

Fig. 5. Effects of various Nef-3 fragments on the recruitment of cellular nectin-1α and E-cadherin to the Nef-3-coated bead-cell contact sites. Nectin-1-EL cells were incubated with the micro-beads coated with Nef-3-Full, Nef-3-Δ3 or human IgG as a control for 1 h. After the incubation, the cells were fixed, followed by immunostaining for nectin-1 and E-cadherin using the anti-nectin-1α and anti-E-cadherin Abs, respectively. (A) Human IgG ($n = 100$); (B) Nef-3-Full ($n = 100$); (C) Nef-3-Δ3 ($n = 100$); (1) the signal for nectin-1α; (2) the signal for E-cadherin; (3) merge; (4) differential interference contrast images. The positions of the beads are marked with asterisks. Bars, 5 μm. The results shown are representative of three independent experiments.

also plays an unknown role in the cell–cell adhesion activity of nectins.

Acknowledgments

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