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Role of the second immunoglobulin-like loop of nectin in cell–cell adhesion☆

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Abstract

Nectin is a Ca^{2+}-independent immunoglobulin (Ig)-like cell–cell adhesion molecule that forms cell–cell adherens junctions cooperatively with E-cadherin in a variety of cells. Nectin has one transmembrane segment and three Ig-like loops in the extracellular region. The first Ig-like loop is essential for the *trans*-dimer formation of nectin of two neighboring cells, causing cell–cell adhesion. We show here that the second Ig-like loop is essential for the *cis*-dimer formation of nectin on the same cell, and that the *cis*-dimer formation is essential for the *trans*-dimer formation.

Keywords: Nectin; Ig-like cell adhesion molecule; *cis*-Dimers; *trans*-Dimers; Cell–cell adhesion; Adherens junctions

Nectin is a Ca^{2+}-independent immunoglobulin (Ig)-like cell–cell adhesion molecule that localizes to E-cadherin-based cell–cell adherens junctions (AJs) and forms AJs cooperatively with E-cadherin in epithelial cells and fibroblasts [1–7]. In neurons, nectin colocalizes with N-cadherin to synapses and forms synaptic junctions cooperatively with N-cadherin [8]. In testis, nectin localizes to spermatid–Sertoli cell junctions and forms the heterotypic junctions[1]. At this type of junctions, nectin, but not cadherin, is a major adhesion molecule.

Nectin comprises a family consisting of four members, nectin-1, -2, -3, and -4, each of which except nectin-4 has two or three splicing variants [3,5,7,9–13]. Nectin-1 and -2 were originally identified as the poliovirus receptor-related protein [9–12] and have recently been shown to serve as the α-herpes virus entry and cell–cell spread mediators [13–17]. Each nectin family member forms homo-*cis*-dimers and homo-*trans*-dimers, causing cell–cell adhesion [2,4,5,7,17]. 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*-dimers [5]. Nectin-4 also forms hetero-*trans*-dimers with nectin-1 [7].

All the nectin family members have one transmembrane segment and three Ig-like loops in the extracellular region [5,7,9–13]. We have previously shown that the first Ig-like loop of nectin-2 is essential for the *trans*-dimer formation [4], but it remains unknown which loop is involved in the *cis*-dimer formation. We show here that the second Ig-like loop of nectin-2 is essential for the *cis*-dimer formation, and that the *cis*-dimer formation is essential for the *trans*-dimer formation.

Materials and methods

Antibodies. A rabbit polyclonal anti-nectin-2 antibody (Ab), which was raised against the cytoplasmic region of nectin-2α, was prepared as described [3]. A rat monoclonal anti-nectin-2 Ab, which was raised against the extracellular region, was prepared as described [3]. This monoclonal Ab recognized the first Ig-like loop of nectin-2.

☆ *Abbreviations:* Ig, immunoglobulin; Ab, antibody; nectin-2-full, full-length nectin-2; nectin-2-ΔIg1, nectin-2 lacking the first Ig-like loop; nectin-2-ΔIg2, nectin-2 lacking the second Ig-like loop; nectin-2-ΔIg3, nectin-2 lacking the third Ig-like loop; aa, amino acid(s); BS3, bis-(sulfosuccinimidyl) suberate; PAGE, polyacrylamide gel electrophoresis.

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Construction of expression vectors. Mammalian expression vectors of mouse nectin-2α were constructed in pCAGIPuro [4] and FLAG-pCAGIPuro by use of standard molecular biology methods [18]. FLAG-pCAGIPuro was constructed to express the preprotrypsin-signal peptide and the FLAG epitope as described [3]. The expression vectors of nectin-2 shown in Fig. 1 contained the following amino acids (aa): pCAGIPuro-nectin-2-full, aa 1–467 (full length); FLAG-pCAGIPuro-nectin-2-ΔIg1, aa 156–467 (deletion of the first Ig loop); pCAGIPuro-nectin-2-ΔIg2, aa 1–467 carrying an internal deletion of aa 166–250 (deletion of the second Ig-like loop); pCAGIPuro-nectin-2-ΔIg3, aa 1–467 carrying an internal deletion of aa 265–329 (deletion of the third Ig-like loop).

Cells and transfection. COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. To prepare COS7 cells transiently expressing nectin-2-full, -ΔIg1, -ΔIg2, or -ΔIg3, the cells were transfected with pCAGIPuro-nectin-2-full, FLAG-pCAGIPuro-nectin-2-ΔIg1, pCAGIPuro-nectin-2-ΔIg2, or pCAGIPuro-nectin-2-ΔIg3, respectively, by use of Lipofectamine reagent (GIBCO BRL) according to the manufacturer's protocols. The cells were cultured for 48 h, followed by chemical cross-linking as described below. L cells stably expressing nectin-2-full (nectin-2-full-L cells) or -ΔIg2 (nectin-2-ΔIg2-L cells) were prepared as described [4]. Briefly, L cells were transfected with pCAGIPuro-nectin-2-full or pCAGIPuro-nectin-2-ΔIg2 and cultured for 24 h. The cells were then replated and selected by culturing in the presence of 5 μg/ml of puromycin (Sigma–Aldrich Chemical).

Chemical cross-linking and cell aggregation assay. Chemical cross-linking was done as described [4]. Briefly, a single-cell suspension (1×10^6 cells/ml) was incubated at 14 °C for 15 min in phosphate-buffered saline with 1 mM bis-(sulfosuccinimidyl) suberate (BS3) (Pierce). After the incubation, the reaction was stopped with the addition of 10 mM Tris/Cl at pH 7.5. The cells were washed with phosphate-buffered saline and counted to confirm that there was no aggregation in the cell suspension.

Cell aggregation assay was performed as described [3–5]. Briefly, a single-cell suspension (1×10^6 cells/ml) was placed in twelve-well plates pre-coated with bovine serum albumin, rotated on a gyratory shaker at 37 °C for indicated periods of time. Aggregation was stopped with the addition of glutaraldehyde. The extent of aggregation of cells was represented by the ratio of the total particle number at time *t* of incubation (*Nt*) to the initial particle number (*No*).

Other procedures. Immunofluorescence microscopy of cultured cells was done as described [3–6]. Protein concentrations were determined with bovine serum albumin as a reference protein [19]. SDS–polyacrylamide electrophoresis (PAGE) was done as described [20].

Results and discussion

We have previously shown that nectin-2 forms homo-*cis*-dimers [4]. To examine which Ig-like loop of nectin-2 is required for the *cis*-dimer formation, we generated various deletion mutants, including nectin-2 lacking the first, second, or third Ig-like loop (nectin-2-ΔIg1, -ΔIg2, or -ΔIg3, respectively) (Fig. 1). Full-length nectin-2 (nectin-2-full) or each mutant was transiently expressed in COS7 cells. These cells were dissociated to single-cell suspensions and incubated with a cell surface cross-linker, BS3, followed by Western blotting with the polyclonal anti-nectin-2 Ab that recognized the cytoplasmic region of nectin-2. In the absence of the cross-linker, nectin-2-full, -ΔIg1, -ΔIg2, and -ΔIg3 showed molecular mass values of about 70, 55, 65, and 60 kD, respectively (Fig. 2). Consistent with previous reports [2,4], the cross-linking of COS7 cells expressing nectin-2-full formed an additional band with a molecular mass of about 140 kDa that corresponded to the *cis*-dimers. A band with a higher molecular mass, which corresponded to the oligomers, was also detected. The cross-linking of COS7 cells expressing nectin-2-ΔIg1 or -ΔIg3 also

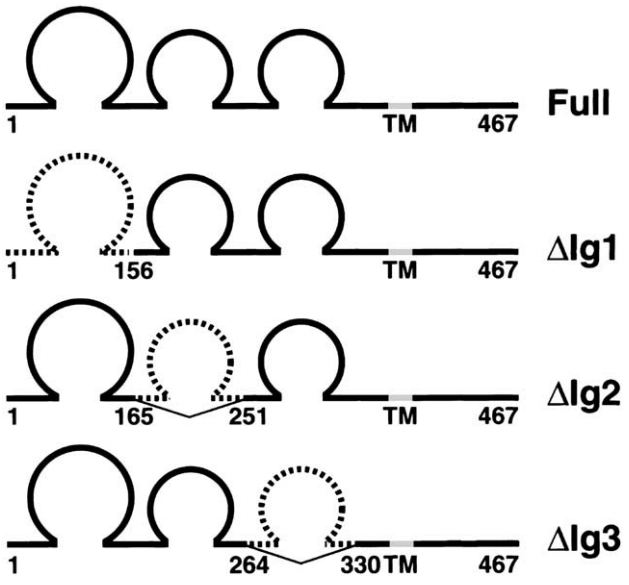

Fig. 1. Structures of various constructs of nectin-2. Dashed lines, deletion regions; TM, transmembrane segment.

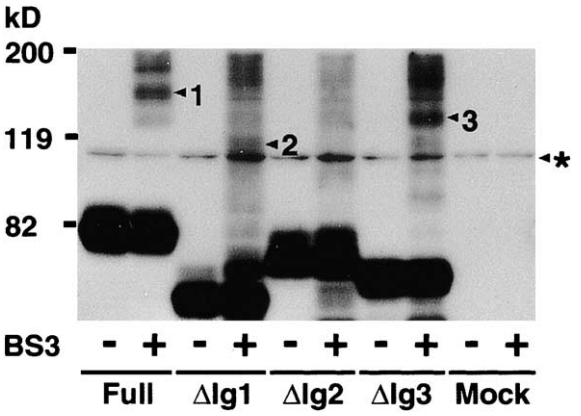

Fig. 2. Requirement of the second Ig-like loop of nectin-2 for the *cis*-dimer formation as estimated by transient transfectants. The full length or deletion mutants of nectin-2 were transiently expressed in COS7 cells. Single-cell suspensions of these cells were incubated in the presence or absence of BS3. Each cell lysate (20 μg of protein each) was subjected to SDS–PAGE (10% polyacrylamide gel), followed by Western blotting with the polyclonal anti-nectin-2 Ab: (1) *cis*-Dimers of nectin-2-full; (2) *cis*-Dimers of nectin-2-ΔIg1; (3) *cis*-Dimers of nectin-2-ΔIg3; and (asterisk) cross-reacted protein band of endogenous proteins in COS7 cells. The relationship of the band (asterisk) with nectin-2 was not clear. These results shown are representative of three independent experiments.

formed the *cis*-dimers with a molecular mass of about 110 or 130 kD, respectively. In these cells, the cross-linking furthermore formed the oligomers with higher molecular masses. In contrast, the cross-linking of COS7 cells expressing nectin-2-ΔIg2 formed neither *cis*-dimers nor oligomers. The same results were obtained with the monoclonal anti-nectin-2 Ab except that nectin-2-ΔIg1 was not detected because this monoclonal Ab recognized the first Ig-like loop of nectin-2 (data not shown). These results indicate that the second Ig-like loop of nectin-2 is responsible for the *cis*-dimer formation.

To further examine the role of the second Ig-like loop, we established an L cell line stably expressing nectin-2-ΔIg2 (nectin-2-ΔIg2-L cells) as well as an L cell line stably expressing nectin-2-full (nectin-2-full-L cells). Western blot analysis with the polyclonal anti-nectin-2 Ab revealed that the expression level of the nectin-2-ΔIg2 protein in nectin-2-ΔIg2-L cells was similar to that of the nectin-2-full protein in nectin-2-full-L cells (Fig. 3). We confirmed that the cross-linking of nectin-2-ΔIg2-L cells formed no *cis*-dimers whereas that of nectin-2-full-L cells formed them. By use of these L cell lines, we then performed the cell aggregation assay to examine whether the second Ig-like loop is required for the homo-*trans*-dimer formation. Consistent with previous reports [1,2,4], nectin-2-full-L cells formed cell aggregates in a time-dependent manner (Fig. 4A and B). In contrast, nectin-2-ΔIg2-L cells as well as parental L cells formed no cell aggregates. To confirm this result, we performed immunofluorescence microscopic analysis. Nectin-2-ΔIg2 did not concentrate cell–cell contact sites in nectin-2-ΔIg2-L cells under the conditions where nectin-2-full localized to cell–cell contact sites in nectin-2-full-L cells (Fig. 5A and B). These results indicate that the second loop is required for the *trans*-dimer formation.

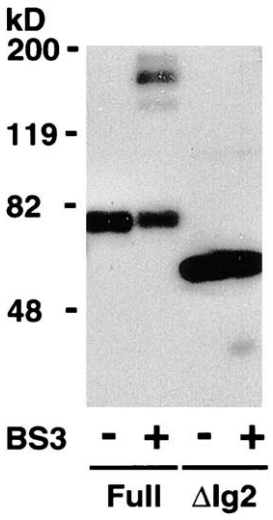

Fig. 3. Requirement of the second Ig-like loop of nectin-2 for the *cis*-dimer formation as estimated by stable transfectants. Single-cell suspensions of nectin-2-full-L and -ΔIg2-L cells were incubated in the presence or absence of BS3. Each cell lysate (20 μg of protein each) was subjected to SDS–PAGE (10% polyacrylamide gel), followed by Western blotting with the polyclonal anti-nectin-2 Ab. These results shown are representative of three independent experiments.

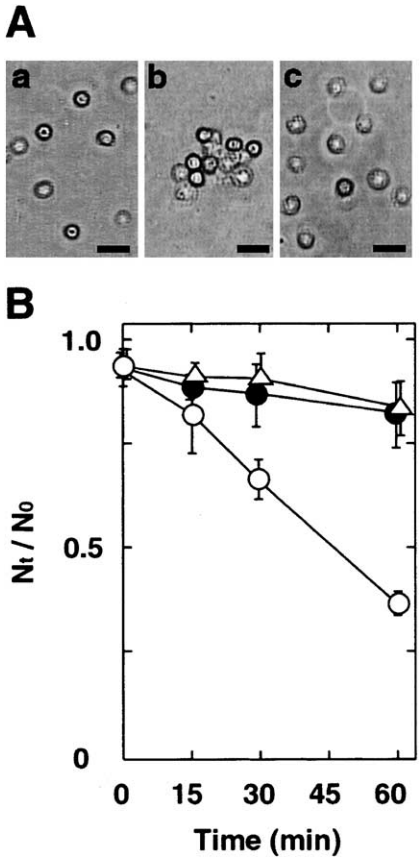

Fig. 4. Requirement of the second Ig-like loop of nectin-2 for the *trans*-dimer formation as estimated by cell aggregation assay. (A) Cell aggregation. Single-cell suspensions of parental L, nectin-2-full-L, and -ΔIg2-L cells were rotated for 30 min. (a) Parental L cells; (b) Nectin-2-full-L cells; (c) Nectin-2-ΔIg2-L cells. Bars, 50 μm. (B) Cell aggregation activity. The single-cell suspensions were rotated for indicated periods of time. The extent of aggregation of cells was represented by the ratio of the total particle number at time *t* of incubation (*Nt*) to the initial particle number (*No*). (△) Parental L cells; (○) Nectin-2-full-L cells; (●) Nectin-2-ΔIg2-L cells. These results shown are representative of three independent experiments.

Several lines of evidence indicate that the first Ig-like loop of nectin is responsible for the *trans*-dimer formation, but not for the *cis*-dimer formation: (1) an Ab against the first Ig-like loop of either nectin-1 or -2 inhibits the *trans*-dimer formation [1,7]; (2) a point mutation in the first Ig-like loop of nectin-2 abolishes the *trans*-dimer formation, but not the *cis*-dimer formation [4]; (3) glycoprotein D, an envelop glycoprotein of herpes simplex virus that binds to the first Ig-like loop of nectin-1, inhibits the *trans*-dimer formation of nectin-1, but not the *cis*-dimer formation [13,17,21]; (4) a chimeric

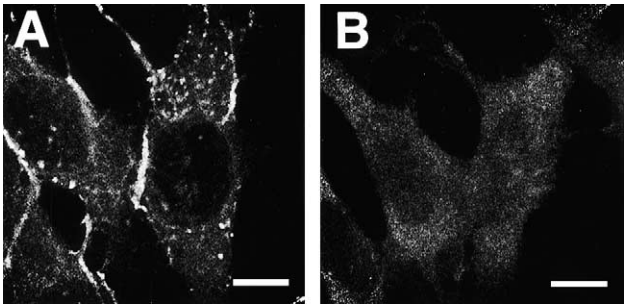

Fig. 5. Requirement of the second Ig-like loop of nectin-2 for the *trans*-dimer formation as estimated by immunofluorescence microscopy. Nectin-2-full-L and -ΔIg2-L cells were stained with the monoclonal anti-nectin-2 Ab, followed by immunofluorescence microscopy. (A) Nectin-2-full-L cells; (B) Nectin-2-ΔIg2-L cells. Bars, 10 μm. These results shown are representative of three independent experiments.

protein of the first Ig-like loop of nectin-1 with IgG Fc forms the *trans*-dimers [7]. Thus, nectin forms the *trans*-dimers through the first Ig-like loop. The *trans*-dimer formation is not required for the *cis*-dimer formation. Consistently, we have shown here that the deletion of the first Ig-like loop of nectin-2 does not abolish the *cis*-dimer formation. On the other hand, we have shown here that the deletion of the second Ig-like loop of nectin-2 abolishes both the *cis*- and *trans*-dimer formation. Nectin-2 forms the *cis*-dimers through the second Ig-like loop. The *cis*-dimer formation is required for the *trans*-dimer formation. It is likely that nectin forms the *cis*-dimers through the second Ig-like loop, followed by the *trans*-dimer formation through the first Ig-like loop, causing cell–cell adhesion. In contrast to the first and second loops of nectin, little is known about the role of the third loop. The third loop may interact laterally with an unidentified membrane protein(s) on the same cell. Further studies are necessary for our understanding of the adhesion mechanism of nectin.

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