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Regulation by nectin of the velocity of the formation of adherens junctions and tight junctions☆

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Abstract

Cadherins are key Ca^{2+}-dependent cell–cell adhesion molecules at adherens junctions (AJs) in fibroblasts and epithelial cells, whereas claudins are key Ca^{2+}-independent cell–cell adhesion molecules at tight junctions (TJs) in epithelial cells. The formation and maintenance of TJs are dependent on the formation and maintenance of AJs. Nectins are Ca^{2+}-independent immunoglobulin-like cell–cell adhesion molecules which comprise a family of four members, nectin-1, -2, -3, and -4, and are involved in the formation of AJs in cooperation with cadherins, and the subsequent formation of TJs. We show here that the velocity of the formation of the E-cadherin-based AJs is increased by overexpression of nectin-1 and is reduced by addition of the nectin-1 inhibitors to the medium in L cells stably expressing E-cadherin and Madin–Darby canine kidney cells. Moreover, the velocity of the formation of the claudin-based TJs is increased by overexpression of nectin-1 and is reduced by addition of the nectin-1 inhibitors to the medium in Madin–Darby canine kidney cells. These results indicate that nectins regulate the velocity of the formation of the E-cadherin-based AJs and the subsequent formation of the claudin-based TJs.

Keywords: Nectin; Cadherin; Claudin; Adherens junctions; Tight junctions

Cell–cell adhesion is critical for tissue patterning and morphogenesis as well as maintenance of normal tissues [1,2]. In epithelial cells, a junctional complex comprised of tight junctions (TJs) and cell–cell adherens junctions (AJs) plays key roles in cell–cell adhesion, whereas there are AJs, but not TJs, in fibroblasts [3]. The formation and maintenance of TJs are regulated by AJs in epithelial cells [4]. E-Cadherin is a Ca^{2+}-dependent cell–cell adhesion molecule and regulates the formation and maintenance of AJs [5,6]. E-Cadherin is a member of the cadherin superfamily that comprises over 80 members [5,6]. E-Cadherin forms *cis*-dimers and then *trans*-dimers (*trans*-interactions) 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 [7]. At TJs, claudins are Ca^{2+}-independent cell–cell adhesion molecules that form TJ strands [3]. Claudins comprise a family of 15 members [3]. The cytoplasmic tail of claudins is linked to the actin cytoskeleton through ZO-1, -2, and -3 [3]. Two other transmembrane proteins, occludin and junctional adhesion molecule (JAM), also localize at TJs [3,8]. The function of occludin has not been established, but JAM is reported to be involved in the formation of cell polarity by direct binding of polarity protein Par-3, which forms a ternary complex with other polarity proteins, atypical PKC and Par-6 [8].

Nectins and afadin constitute another cell–cell adhesion unit at AJs in epithelial cells and fibroblasts [9]. Nectins are Ca^{2+}-independent immunoglobulin-like cell–cell adhesion molecules and comprise a family of four members, nectin-1, -2, -3, and -4. All nectins form homo-

☆ *Abbreviations:* TJs, tight junctions; AJs, adherens junctions; JAM, junctional adhesion molecule; Nef-3, a recombinant extracellular fragment of nectin-3 fused to the human IgG Fc; gD, a recombinant extracellular fragment of glycoprotein D fused to the human IgG Fc; pAb, polyclonal antibody; mAb, monoclonal Ab; MDCK cells, Madin–Darby canine kidney cells.

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cis-dimers and then homo-*trans*-dimers (*trans*-interactions), causing cell–cell adhesion. Nectin-3 furthermore forms hetero-*trans*-dimers with nectin-1 and -2. Nectin-4 also forms hetero-*trans*-dimers with nectin-1. Nectins are associated with the actin cytoskeleton through afadin, a nectin- and F-actin-binding protein. We have recently made the recombinant extracellular fragment of nectin-3 or glycoprotein D, an envelope glycoprotein of herpes simplex virus type I, fused to the human IgG Fc, Nef-3, and gD, respectively [10,11]. Using Nef-3 and gD, we have found that Nef-3 *trans*-interacts with cellular nectin-1 and thereby diminishes the formation of the nectin-1-based cell–cell adhesion, resulting in reduction of the formation of the E-cadherin-based AJs in L fibroblasts stably expressing both exogenous nectin-1 and E-cadherin (nectin-1-EL cells), and resulting in reduction of the formation of the E-cadherin-based AJs and the claudin-based TJs in Madin–Darby canine kidney (MDCK) cells stably expressing exogenous nectin-1 (nectin-1-MDCK cells) [12–14]. Conversely, Nef-3 coated on micro-beads recruits first the nectin-1-afadin complex and then the E-cadherin-catenin complex to the bead-cell contact sites in nectin-1-EL and nectin-1-MDCK cells [12].

Thus, the nectin–afadin unit plays roles in the organization of AJs in cooperation with cadherins in fibroblasts, and AJs and TJs in cooperation with E-cadherin and claudins in epithelial cells, but it remains unknown how the nectin–afadin unit plays such roles. We show here that the nectin–afadin unit kinetically increases the velocity of the formation of AJs and TJs.

Materials and methods

Antibodies and proteins. A rabbit anti-human nectin-1 polyclonal antibody (pAb) was generated against human nectin-1α, as described [11,15]. A mouse anti-afadin monoclonal Ab (mAb) was prepared, as described [16]. A rat anti-E-cadherin mAb (ECCD-2) was supplied from Dr. M. Takeichi (RIKEN Center for Developmental Biology, Kobe, Japan). A rabbit anti-JAM pAb was supplied from Dr. T. Kita (Kyoto University, Kyoto, Japan). Rabbit anti-claudin-1 and anti-occludin pAbs (Zymed) and secondary Abs (Chemicon) were purchased from commercial sources. Nef-3 and gD were expressed in High Five insect cells (Invitrogen) and purified, as described [10,11].

Cell lines. EL cells were 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 [17]. Nectin-1-EL cells were prepared, as described [18]. MDCK cells were supplied from Dr. W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). Nectin-1-MDCK cells were prepared, as described [15].

A Ca^{2+} switch assay. Cell–cell adhesion of nectin-1-EL, EL, nectin-1-MDCK, and MDCK cells were assayed, as described [12]. Briefly, these cells were washed with PBS and cultured at 2 mM Ca^{2+} in DMEM for 1 h. The medium was replaced with DMEM containing 5 mM EGTA (2 μM Ca^{2+}) in the presence or absence of a mixture of 1 μM Nef-3 and 1 μM gD, and then nectin-1-EL, EL, nectin-1-MDCK, and MDCK cells were cultured for 4, 4, 2, and 2 h, respectively. After the culture, the cells were washed with DMEM and further cultured in DMEM (2 mM Ca^{2+}) in the presence or absence of the mixture of 1 μM Nef-3 and 1 μM gD for indicated periods of time. The cells were then fixed, followed by immunostaining for nectin-1, afadin, E-cadherin, claudin-1, JAM, and occludin with the anti-nectin-1 pAb, anti-afadin mAb, anti-E-cadherin mAb, anti-claudin-1 pAb, anti-JAM pAb, and anti-occludin pAb, respectively. The indices of E-cadherin and claudin-1 recruitment represent the percentages of the E-cadherin and claudin-1 signal-positive cell–cell adhesion sites in 50 cell–cell contact sites counted, respectively.

Other procedures. Immunofluorescence microscopy of cultured cells was done, as described [15]. Protein concentrations were determined with bovine serum albumin as a reference protein [19].

Results

Regulation by nectin-1 of the velocity of the formation of AJs in EL cells

We first examined the kinetics of the formation of the E-cadherin-based AJs in EL and nectin-1-EL cells. The signal for E-cadherin was concentrated at the cell–cell adhesion sites in both EL and nectin-1-EL cells at 2 mM Ca^{2+} (Fig. 1A, control). EL and nectin-1-EL cells were then cultured at 2 μM Ca^{2+} for 4 h and re-cultured at 2 mM Ca^{2+} for 30 and 60 min. The signal for E-cadherin was diffusely observed along the plasma membrane in both EL and nectin-1-EL cells after the 4-h culture at 2 μM Ca^{2+} (Fig. 1A, low Ca^{2+}). The signal for E-cadherin was re-concentrated at the cell–cell adhesion sites in a time-dependent manner after both the cells were re-cultured at 2 mM Ca^{2+} (Fig. 1A, normal Ca^{2+}), but the time course of the accumulation of E-cadherin at the cell–cell adhesion sites in nectin-1-EL cells was much faster than that in EL cells (Fig. 1C). In EL cells, endogenous nectin-1 and afadin are expressed as estimated by Western blotting using the anti-nectin-1 pAb and the anti-afadin mAb, respectively [12]. Afadin is clearly stained at the E-cadherin-based AJs, but the nectin-1 staining is hardly detected [12]. This might be just due to the low sensitivity of the anti-nectin-1 pAb. Therefore, we stained here afadin instead of nectin-1. The signal for afadin was concentrated at the cell–cell adhesion sites in both EL and nectin-1-EL cells at 2 mM Ca^{2+} (Fig. 1B, control). The signal for afadin was slightly reduced at the cell–cell adhesion sites and, furthermore observed along the free surface of the plasma membrane in both EL and nectin-1-EL cells at 2 μM Ca^{2+} (Fig. 1B, low Ca^{2+}). The signal for afadin was observed only at the cell–cell adhesion sites after both the cells were re-cultured at 2 mM Ca^{2+} (Fig. 1B, normal Ca^{2+}). These results indicate that overexpression of nectin-1 increases the velocity of the formation of the E-cadherin-based AJs in fibroblasts.

The result, that nectin-1 increases the velocity of the formation of the E-cadherin-based AJs, suggests that inhibition of nectin-1 conversely decreases the velocity of the formation of the E-cadherin-based AJs. We used a mixture of Nef-3 and gD as the nectin-1 inhibitors,

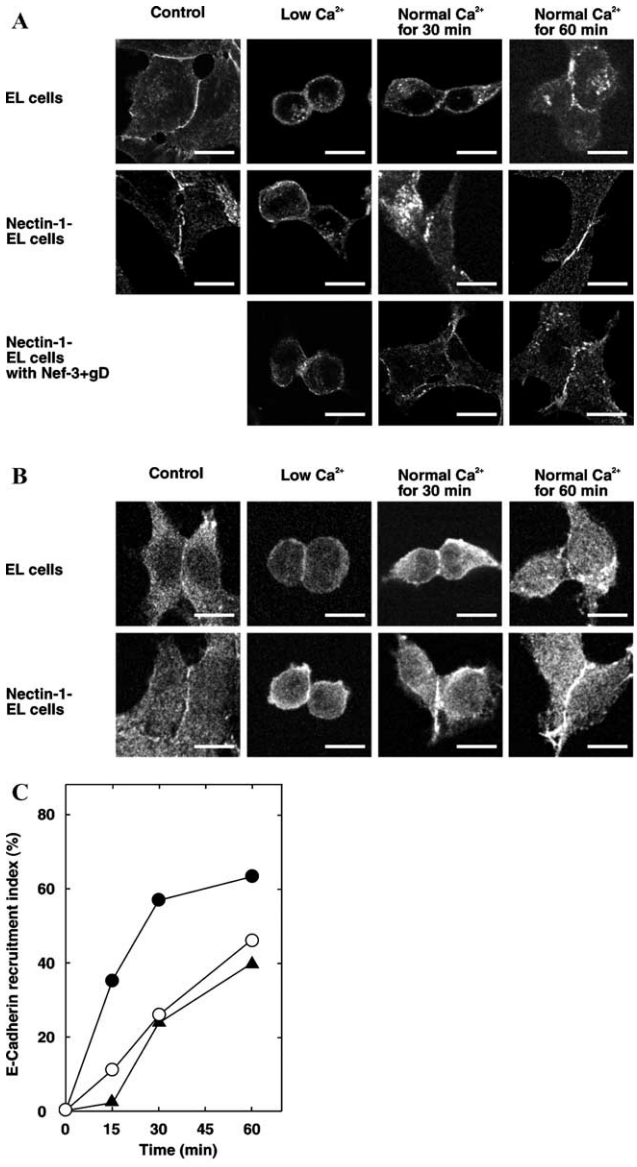

Fig. 1. Regulation by nectin-1 of the velocity of the formation of the E-cadherin-based AJs in EL cells. (A) Time courses of formation of the E-cadherin-based AJs. EL and nectin-1-EL cells were cultured at 2 mM Ca^{2+} (control). The cells were then cultured with or without the mixture of 1 μM Nef-3 and 1 μM gD at 2 μM Ca^{2+} for 4 h (low Ca^{2+}). After the culture, the cells were re-cultured with or without the mixture of 1 μM Nef-3 and 1 μM gD at 2 mM Ca^{2+} for 30 and 60 min (normal Ca^{2+} for 30 and 60 min, respectively). The cells were fixed, followed by immunostaining for E-cadherin using the anti-E-cadherin mAb. Bars, 10 μm. (B) Time courses of accumulation of afadin at the cell–cell adhesion sites. EL and nectin-1-EL cells were cultured without the nectin-1 inhibitors, as described in (A). The cells were then fixed, followed by immunostaining for afadin using the anti-afadin mAb. Bars, 10 μm. (C) Quantitative analysis of (A). (○) EL cells without the nectin-1 inhibitors; (●) nectin-1-EL cells without the nectin-1 inhibitors; and (▲) nectin-1-EL cells with the nectin-1 inhibitors. E-Cadherin recruitment index was calculated, as described in Materials and methods. The results shown are representative of three independent experiments.

because the mixture of Nef-3 and gD showed more inhibitory activity than Nef-3 or gD alone [13]. Nectin-1-EL cells were cultured at 2 μM Ca^{2+} for 4 h and then re-cultured at 2 mM Ca^{2+} in the presence or absence of the nectin-1 inhibitors for 30 and 60 min. The time course of the accumulation of E-cadherin at the cell–cell adhesion sites in the absence of the nectin-1 inhibitors was much faster than that in the presence of the nectin-1 inhibitors after nectin-1-EL cells were re-cultured at 2 mM Ca^{2+} (Figs. 1A and C), indicating that inhibition of the formation of the nectin-1-based cell–cell adhesion decreases the velocity of the formation of the E-cadherin-based AJs in fibroblasts. The signal for nectin-1 in the absence of the nectin-1 inhibitors remained at the cell–cell adhesion sites, whereas the signal for nectin-1 in the presence of the nectin-1 inhibitors was diffusely observed along the plasma membrane at 2 μM Ca^{2+} and after the cells were re-cultured at 2 mM Ca^{2+}, as described [12] (data not shown). Taken together, these results indicate that nectin-1 regulates the velocity of the formation of AJs in fibroblasts.

Regulation by nectin-1 of the velocity of the formation of AJs and TJs in MDCK cells

We next examined whether nectin-1 regulates the velocity of the formation of the E-cadherin-based AJs and the claudin-based TJs in epithelial cells. We assayed the time courses of the formation of these cell–cell junctions in MDCK and nectin-1-MDCK cells. The signals for E-cadherin and claudin-1 were concentrated at the cell–cell adhesion sites in both MDCK and nectin-1-MDCK cells at 2 mM Ca^{2+} (Figs. 2A and 3A, control). MDCK and nectin-1-MDCK cells were cultured at 2 μM Ca^{2+} for 2 h and then re-cultured at 2 mM Ca^{2+} for indicated periods of time. The signals for E-cadherin and claudin-1 were not observed at any site along the plasma membrane in both MDCK and nectin-1-MDCK cells after the 2-h culture at 2 μM Ca^{2+} (Figs. 2A and 3A, low Ca^{2+}). The signals for E-cadherin and claudin-1 were re-concentrated at the cell–cell adhesion sites in a time-dependent manner after both the cells were re-cultured at 2 mM Ca^{2+} (Figs. 2A and 3A, normal Ca^{2+}), but the time courses of the accumulations of E-cadherin and claudin-1 at the cell–cell adhesion sites in nectin-1-MDCK cells were much faster than those in MDCK cells (Figs. 2B and 3B). When occludin and JAM were immunostained, the essentially same results as those obtained for claudin-1 were obtained (data not shown). We could not stain endogenous nectin-1 in MDCK cells, as described [12]. Therefore, we stained here afadin instead of nectin-1. The signal for afadin remained at the cell–cell adhesion sites in nectin-1-MDCK cells at low and normal Ca^{2+}, as described [12,13] (data not shown). In contrast, the signal for afadin in MDCK cells was diffusely observed along the entire surface of the plasma membrane in MDCK cells at 2 μM Ca^{2+}, but was concentrated at the cell–cell adhesion sites after MDCK cells were re-cultured at 2 mM Ca^{2+} (data not shown).

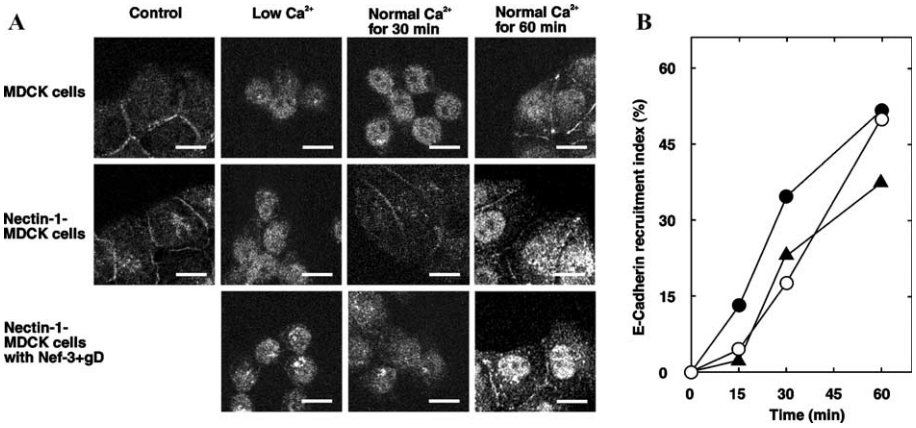

Fig. 2. Regulation by nectin-1 of the velocity of the formation of the E-cadherin-based AJs in MDCK cells. (A) Time courses of formation of the E-cadherin-based AJs. MDCK and nectin-1-MDCK cells were cultured at 2 mM Ca^{2+} (control). The cells were then cultured with or without the mixture of 1 μM Nef-3 and 1 μM gD at 2 μM Ca^{2+} for 2 h (low Ca^{2+}). After the culture, the cells were re-cultured with or without the mixture of 1 μM Nef-3 and 1 μM gD at 2 mM Ca^{2+} for 30 and 60 min (normal Ca^{2+} for 30 and 60 min, respectively). The cells were fixed, followed by immunostaining for E-cadherin using the anti-E-cadherin mAb. Bars, 10 μm. (B) Quantitative analysis of (A). (○) MDCK cells without the nectin-1 inhibitors; (●) nectin-1-MDCK cells without the nectin-1 inhibitors; and (▲) nectin-1-MDCK cells with the nectin-1 inhibitors. E-Cadherin recruitment index was calculated, as described in Materials and methods. The results shown are representative of three independent experiments.

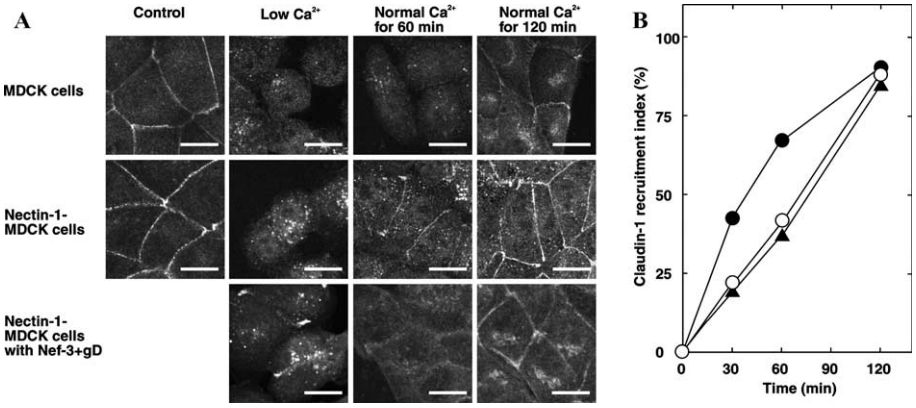

Fig. 3. Regulation by nectin-1 of the velocity of the formation of the claudin-based TJs in MDCK cells. (A) Time courses of formation of the claudin-based TJs. MDCK and nectin-1-MDCK cells were cultured at 2 mM Ca^{2+} (control). The cells were then cultured with or without the mixture of 1 μM Nef-3 and 1 μM gD at 2 μM Ca^{2+} for 2 h (low Ca^{2+}). After the culture, the cells were re-cultured with or without the mixture of 1 μM Nef-3 and 1 μM gD at 2 mM Ca^{2+} for 60 and 120 min (normal Ca^{2+} for 60 and 120 min, respectively). The cells were fixed, followed by immunostaining for claudin-1 using the anti-claudin-1 pAb. Bars, 10 μm. (B) Quantitative analysis of (A). (○) MDCK cells without the nectin-1 inhibitors; (●) nectin-1-MDCK cells without the nectin-1 inhibitors; (▲) nectin-1-MDCK cells with the nectin-1 inhibitors. Claudin-1 recruitment index was calculated, as described in Materials and methods. The results shown are representative of three independent experiments.

These results indicate that overexpression of nectin-1 increases the velocity of the formation of the E-cadherin-based AJs and the subsequent formation of the claudin-based TJs in epithelial cells.

To confirm that inhibition of nectin-1 decreases the velocity of the formation of the E-cadherin-based AJs and the claudin-based TJs in epithelial cells, we assayed the time courses of the formation of these cell–cell junctions in nectin-1-MDCK cells using the nectin-1 inhibitors. Nectin-1-MDCK cells were cultured at 2 μM Ca^{2+} for 2 h and then re-cultured at 2 mM Ca^{2+} in the presence or absence of the nectin-1 inhibitors for indicated periods of time. The time courses of the accumulations of E-cadherin and claudin-1 at the cell–cell adhesion sites in the absence of the nectin-1 inhibitors were much faster than those in the presence of the nectin-1 inhibitors after nectin-1-MDCK cells were re-cultured at 2 mM Ca^{2+} (Figs. 2A and B; 3A, and B), indicating that inhibition of the formation of the nectin-1-based cell–cell adhesion decreases the velocity of the formation of the E-cadherin-based AJs and the subsequent formation of the claudin-based TJs in epithelial cells. When occludin and JAM were immunostained, the essentially same results as those obtained for claudin-1 were obtained (data not shown). The signal for nectin-1 in the absence of the nectin-1 inhibitors remained at the cell–cell adhesion sites, whereas the signal for nectin-1 in the presence of the nectin-1 inhibitors was diffusely

observed along the plasma membrane at low and normal Ca^{2+}, as described [12] (data not shown). Taken together, these results indicate that nectin-1 regulates the velocity of the formation of AJs and TJs in epithelial cells.

Discussion

We have previously shown that inhibition of the formation of the nectin-1-based cell–cell adhesion by the nectin-1 inhibitors results in reduction of the formation of the E-cadherin-based AJs and that the nectin-1-based cell–cell adhesion recruits E-cadherin there [12,18]. We have furthermore shown that nectin-1 is involved in the recruitment of claudin-1, occludin, and JAM to the apical side of AJs, resulting in the formation of TJs [13,14]. In this study, we have examined the kinetics of the formation of the E-cadherin-based AJs in fibroblasts, and the kinetics of the formation of the E-cadherin-based AJs and the claudin-based TJs in epithelial cells. We have found that overexpression of nectin-1 kinetically increases the velocity of the formation of the E-cadherin-based AJs in EL cells and the velocity of the formation of the E-cadherin-based AJs and the claudin-based TJs in MDCK cells, whereas inhibition of the formation of the nectin-1-based cell–cell adhesion by the nectin-1 inhibitors decreases the velocity of the formation of the E-cadherin-based AJs in EL cells and the velocity of the formation of the E-cadherin-based AJs and the claudin-based TJs in MDCK cells. Because the formation and maintenance of TJs are totally dependent on the formation and maintenance of AJs, the nectin-1-enhanced velocity of the formation of TJs may be just due to the nectin-1-enhanced velocity of the formation of AJs. The nectin-1 inhibitors do not completely abolish the nectin-1-based cell–cell adhesion in nectin-1-EL and nectin-1-MDCK cells [12,13]. Therefore, it is unknown whether the nectin-based cell–cell adhesion is essential for the formation of the E-cadherin-based AJs or regulates the velocity of the formation of the E-cadherin-based AJs. It is also unknown whether the nectin-based cell–cell adhesion is necessary for the maintenance of the E-cadherin-based AJs. Further studies are necessary for our understanding of the molecular mechanism of how nectins regulate the velocity of the formation of AJs and TJs.

Acknowledgments

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