Involvement of Nectin-activated Cdc42 Small G Protein in Organization of Adherens and Tight Junctions in Madin-Darby Canine Kidney Cells*

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Nectins, Ca\(^{2+}\)-independent immunoglobulin-like cell-cell adhesion molecules, trans-interact and form cell-cell adhesion, which increases the velocities of the formation of the E-cadherin-based adherens junctions (AJs) and the claudin-based tight junctions (TJs) in Madin-Darby canine kidney (MDCK) cells. The trans-interactions of nectins furthermore induce activation of Cdc42 and Rac small G proteins, but the roles of these small G proteins activated in this way remain unknown. We examined here the role and the mode of action of Cdc42 in the organization of AJs and TJs in MDCK cells. We first made the NWASP-Cdc42 and Rac interactive binding (CRIB) domain, an inhibitor of activated Cdc42, fused to the Ki-Ras CAAX motif (NWASP-CRIB-CAAX; where A is aliphatic amino acid), which was targeted to the cell-cell adhesion sites. We then found that overexpression of NWASP-CRIB-CAAX reduced the velocities of the formation of AJs and TJs. Conversely, overexpression of a constitutively active mutant of Cdc42 (V12Cdc42) increased their velocities, and the inhibitory effect of NWASP-CRIB-CAAX was suppressed by co-expression with V12Cdc42. The inhibitory effect of NWASP-CRIB-CAAX on the formation of AJs and TJs was suppressed by co-expression of nectin-1 of which trans-interaction activated endogenous Cdc42. Moreover, the formation of the claudin-based TJs required a greater amount of activated Cdc42 than that of the E-cadherin-based AJs. These results indicate that the Cdc42 activated by the trans-interactions of nectins is involved in the organization of AJs and TJs in different mechanisms in MDCK cells.

Cell-cell adhesion is an essential feature of epithelia that ensures their polarized status and therefore their differentiation and physiological functions. In polarized epithelial cells, cell-cell adhesion is mediated through a junctional complex composed of tight junctions (TJs);\(^1\) cell-cell adherens junctions (AJs), and desmosomes (DSs) (1). These junctional structures are typically aligned from the apical to the basal side, although DSs are independently distributed in other areas (1). The formation and maintenance of TJs and DSs are totally dependent on the formation and maintenance of AJs (2). At AJs, E-cadherin is a key Ca\(^{2+}\)-dependent cell-cell adhesion molecule (3, 4). 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 \(\alpha\)-catenin, \(\beta\)-catenin, vinculin, and \(\alpha\)-actinin, which strengthen the cell-cell adhesion activity of E-cadherin (5). At TJs, claudins are key Ca\(^{2+}\)-independent cell-cell adhesion molecules that form TJ strands (2). The cytoplasmic tail of claudins is linked to the actin cytoskeleton through ZO-1, -2, and -3.

Nectins are Ca\(^{2+}\)-independent Ig-like cell-cell adhesion molecules at AJs in epithelial cells and fibroblasts (6). Nectins compose a family of four members, nectin-1, -2, -3, and -4. All nectins form homo-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. Nectins play roles in the organization of AJs and TJs in cooperation with E-cadherin and claudins in epithelial cells. Kinetically, nectins increase the velocities of the formation of AJs and TJs (7, 8).

The Rho family small G proteins, Rho, Rac, and Cdc42, have all been reported to affect the formation and/or maintenance of AJs (9–11). The formation of AJs is suppressed by inhibition of Rho (12, 13), although expression of a constitutively active mutant of Rho (V14Rho) does not affect the formation of the E-cadherin-mediated cell-cell AJs (14). Rac activity is necessary for the establishment of AJs in epithelial cells (12–15). The activation of Rac leads to a dramatic reorganization of intercellular contacts in Madin-Darby canine kidney (MDCK) cells. Tight contact is restricted to the apical area of the lateral plasma membrane in wild-type MDCK cells, whereas tight contact extends over the entire lateral plasma membrane in MDCK cells expressing a constitutively active mutant of Rac1 (V12Rac1) (14, 16). In addition, the lateral plasma membrane displays extensive interdigititation in MDCK cells expressing V12Rac1. In MDCK cells expressing a constitutively active

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†The abbreviations used are: TJs, tight junctions; AJs, adherens junctions; CRIB domain, Cdc42 and Rac interactive binding domain; DSs, desmosomes; GFP, green fluorescent protein; EGFP, enhanced GFP; F-actin, actin filaments; mAb, monoclonal antibody; MDCK cells, Madin-Darby canine kidney cells; pAb, polyclonal antibody; PI, phosphatidylinositol; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; FCS, fetal calf serum.

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DSs, desmosomes; GFP, green fluorescent protein; EGFP, enhanced (GFP); F-actin, actin filaments; mAb, monoclonal antibody; MDCK cells, Madin-Darby canine kidney cells; pAb, polyclonal antibody; PI, phosphatidylinositol; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; FCS, fetal calf serum.

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Role of Nectin-activated Cdc42 in Cell-Cell Junction Formation

AAX

Expression of EGFP-NWASP-CRIB-CAX in wild-type MDCK cells. A, confocal laser scanning microscopy analysis. Wild-type MDCK cells were microinjected with pEGFP-NWASP-CRIB-CAX, pEGFP-NWASP-CRIB, pEGFP-CAX, pEGFP, or pEGFP-NWASP-CRIB[H208D]-CAX. After a 3-h incubation, the cells were fixed and stained for F-actin with rhodamine-phalloidin. Bars, 10 μm. B, Western blotting. MDCK cells transiently expressing either EGFP-NWASP-CRIB-CAX, EGFP-NWASP-CRIB, EGFP-CAX, EGFP, or EGFP-NWASP-CRIB[H208D]-CAX were homogenized, and the homogenate was centrifuged. After the centrifugation, the supernatant was collected as the post-nuclear fraction. The pellet was suspended with the same volume as that of the supernatant and collected as the nuclear fraction. Twenty μl of each fraction was subjected to SDS-PAGE, followed by Western blotting using the anti-GFP pAb. Lane H, the homogenate; lane N, the nuclear fraction; and lane P, the post-nuclear fraction. The results shown are representative of three independent experiments.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Expression vectors for enhanced green fluorescent protein (EGFP)-tagged NWASP-Cdc42 and Rac interactive binding (CRIB) domain (pEGFP-NWASP-CRIB) and Myc-tagged V12Cdc42 (pEFBOS-myc-V12Cdc42) were prepared as described (17, 26). pEGFP-NWASP-CRIB-CAX for EGFP-NWASP-CRIB-CAX was constructed by inserting the cDNA fragment encoding the C-terminal region of human Ki-Ras (GenBank™ accession number NM_004985) (amino acid sequence GKKKK(S)KTCIVM) into the 3' end of the cDNA fragment encoding NWASP-CRIB in pEGFP-NWASP-CRIB, pEGFP-NWASP-CRIB[H208D]-CAX for EGFP-NWASP-CRIB[H208]-CAX, in which a histidine residue at amino acid 208 of NWASP was replaced by an aspartate, was prepared by site-directed mutagenesis with pEGFP-NWASP-CRIB-CAX as described (28). pEGFP-CAX for EGFP-CAX was constructed by inserting the cDNA fragment encoding the C-terminal region of human Ki-Ras into the 3' end of the cDNA fragment encoding EGFP in pEGFP. The cDNA fragment of NWASP-CRIB was kindly supplied from Dr. T. Takenawa (Tokyo University, Tokyo, Japan). pEGFP-V12Cdc42 for EGFP-V12Cdc42 and pEGFP-Cdc42 for EGFP-Cdc42 (wild type) were kindly supplied from Dr. S. Narumiya (Kyoto University, Kyoto, Japan). All constructs and a mutation were confirmed by sequencing.

Cell Lines and Transfection—Wild-type MDCK cells were kindly supplied from Dr. W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). MDCK cells stably expressing FLAG-tagged nectin-1α (nectin-1-MDCK cells) were prepared as described (29). MDCK cells stably expressing V12Cdc42 (V12Cdc42-MDCK cells) were prepared as described (17). Transfection was done as described (29) with pEGFP, pEGFP-CAX, pEGFP-NWASP-CRIB, pEGFP-NWASP-CRIB-CAX, and pEGFP-NWASP-CRIB[H208D]-CAX.

Antibodies—A mouse anti-afadin monoclonal antibody (mAb) was prepared as described (30). A rat anti-E-cadherin mAb (ECCD-2) was kindly supplied from Dr. M. Takei (Center for Developmental Biology, RIKEN, Kobe, Japan). Hybridoma cells expressing a mouse anti-Myc mAb (9E10) were obtained from American Type Culture Collection as described (17). A rabbit anti-claudin-1α polyclonal Ab (pAb) (Zymed Laboratories Inc., South San Francisco, CA), a mouse anti-EA1 mAb (BD Transduction Laboratories), a rabbit anti-Rab7 pAb (Santa Cruz Biotechnology, Santa Cruz, CA), a mouse anti-α-tubulin mAb (Sigma), a rabbit anti-GFP pAb (BD Biosciences, Clontech, Palo Alto, CA), and secondary Abs (Chemicon, Temecula, CA) were purchased from commercial sources.

Subcellular Fractionation—Subcellular fractionation was performed as described previously (31) with some modifications. Briefly, MDCK cells (6 × 10⁶ cells/10-cm dish) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) on four 10-cm dishes at 37 °C for 24 h. After the culture, the cells (2.4 × 10⁷ cells/10-cm dish) were homogenized, and the homogenate was centrifuged. After the centrifugation, the supernatant was collected as the post-nuclear fraction. The pellet was suspended with the same volume as that of the supernatant and collected as the nuclear fraction. Twenty μl of each fraction was subjected to SDS-PAGE, followed by Western blotting using the anti-GFP pAb.
RESULTS

Reduction of the Velocities of the Formation of Ads and TJs by NWASP-CRIB-CAAX—To inhibit the action of the Cdc42 activated by the trans-interactions of nectins at the cell-cell junctions, we first expressed EGFP-NWASP-CRIB, a specific inhibitor of activated Cdc42 (35), in MDCK cells. The CRIB domain of NWASP specifically binds to activated Cdc42 and inhibits the action of activated Cdc42 (35). Most of the signal for EGFP-NWASP-CRIB appeared in nuclei, and the signal was hardly observed at the cell-cell adhesion sites (Fig. 1A). The signal for EGFP alone was diffusely observed in the cytosol (Fig. 1A). If NWASP-CRIB is preferentially targeted to the cell-cell adhesion sites, the NWASP-CRIB would effectively inhibit the action of activated Cdc42 there. Thus, we prepared a plasmid for EGFP-NWASP-CRIB fused to the Ki-Ras CAAX motif with the polybasic sequence (pEGFP-NWASP-CRIB-CAAX). The CAAX motif is required for targeting of Ki-Ras to the plasma membrane (36). When EGFP-NWASP-CRIB-CAAX was expressed in MDCK cells, the signal for EGFP-NWASP-CRIB-CAAX was markedly observed at the cell-cell adhesion sites (Fig. 1A). The signal for EGFP fused to the Ki-Ras CAAX motif with the polybasic sequence (EGFP-CAAX) was also observed at the cell-cell adhesion sites (Fig. 1A).

To confirm that EGFP-NWASP-CRIB-CAAX is targeted to the cell-cell adhesion sites where E-cadherin localizes, we performed the subcellular fractionation of MDCK cells expressing EGFP-NWASP-CRIB-CAAX. First, we examined the expression levels of the EGFP-NWASP-CRIB, EGFP-NWASP-CRIB-CAAX, EGFP, and EGFP-CAAX proteins by Western blotting using the anti-GFP pAb. MDCK cells transiently expressing either EGFP-NWASP-CRIB-CAAX, EGFP-NWASP-CRIB, EGFP-CAAX, or EGFP were homogenized and kept as the homogenate. The expression levels of these proteins were approximately equal in the homogenate (Fig. 1B, lane H). The homogenate was fractionated into the nuclear fraction and the post-nuclear fraction by centrifugation. Most of EGFP-NWASP-CRIB-CAAX, EGFP-CAAX, and EGFP were detected in the post-nuclear fraction, whereas most of EGFP-NWASP-CRIB was detected in the nuclear fraction (Fig. 1B, lanes N and P). To examine the more detailed subcellular distribution of EGFP-NWASP-CRIB-CAAX, the post-nuclear fraction was subjected to sucrose density gradient ultracentrifugation. After the centrifugation, each fraction was collected from the bottom (fraction 1) to the top (fraction 15) in the tube, followed by Western blotting of each fraction. EGFP-NWASP-CRIB-CAAX was indeed detected in the plasma membrane fraction where E-cadherin was detected (Fig. 2A, fractions 1–4). EGFP-NWASP-CRIB-CAAX was also detected in the intracellular vesicular organelle fraction (Fig. 2A, fractions 5–14) where Rab7 and EEA1, the markers for late and early endosomes (37), respectively, were detected, and in the cytosolic fraction (Fig. 2A, fractions 14 and 15) where α-tubulin, the marker for cytoplasm, was detected. In contrast, EGFP-NWASP-CRIB was hardly detected in the plasma membrane fraction (Fig. 2B, fractions 1–4). EGFP-CAAX was detected in the plasma membrane fraction, whereas EGFP was hardly detected in the plasma membrane fraction (data not shown). It is not known why there are two differently migrating bands of EGFP-NWASP-CRIB-CAAX and EGFP-NWASP-CRIB, but it may be due to the degradation or unprocessed forms. These results indicate that the addition of the Ki-Ras CAAX motif with the polybasic sequence to NWASP-CRIB enhances targeting of NWASP-CRIB to the plasma membrane.
By use of NWASP-CRIB-C\textsubscript{AAX}, we then examined a role of activated Cdc42 in the formation of cell-cell adhesion. If activated Cdc42 is involved in proper formation of AJs and/or TJs, overexpression of NWASP-CRIB-C\textsubscript{AAX} in wild-type MDCK cells would affect the accumulation of nectins, afadin, E-cadherin, or claudin-1 during the formation of cell-cell adhesion. Wild-type MDCK cells were transfected with a plasmid for EGFP-NWASP-CRIB-C\textsubscript{AAX} or EGFP-C\textsubscript{AAX} and cultured at 2 mM Ca\textsuperscript{2+} for 24 h. After the culture, the cells were incubated at 2 mM Ca\textsuperscript{2+} for 2 h and then incubated at 2 mM Ca\textsuperscript{2+} for 2 h. The cells were fixed and stained for afadin, E-cadherin, and claudin-1 with the anti-afadin mAb, the anti-E-cadherin mAb, and the anti-claudin-1 pAb, respectively.

The signals for afadin, E-cadherin, and claudin-1 were concentrated at the cell-cell adhesion sites in wild-type MDCK cells after the 24-h culture as described (8) (Fig. 3, A). On the other hand, the signals for afadin, E-cadherin, and claudin-1 were reduced at the cell-cell adhesion sites between the wild-type MDCK cells expressing EGFP-NWASP-CRIB-C\textsubscript{AAX} (Fig. 3, A).

These results indicate that NWASP-CRIB-C\textsubscript{AAX} inhibits the accumulation of afadin, E-cadherin, and claudin-1 at the newly formed cell-cell adhesion sites, resulting in a reduction of the velocities of the formation of AJs and TJs in wild-type MDCK cells.

We confirmed that the inhibitory effect of NWASP-CRIB-C\textsubscript{AAX} is due to its specific interaction with activated Cdc42. For this purpose, we prepared a mutant [H208D] in which a histidine residue conserved in all CRIB motifs is replaced by an aspartate residue. This mutant does not bind activated Cdc42 (28). When EGFP-NWASP-CRIB[H208D]-C\textsubscript{AAX} was expressed in wild-type MDCK cells, its distribution and expression patterns were the same as those of EGFP-NWASP-CRIB-C\textsubscript{AAX} (Fig. 1, A and B, and data not shown). Then transfected wild-type MDCK cells with a plasmid for EGFP-NWASP-CRIB[H208D]-C\textsubscript{AAX} and cultured them at 2 mM Ca\textsuperscript{2+}.
for 24 h. After the culture, the cells were incubated at 2 μM Ca²⁺ for 2 h. After the incubation, the cells were incubated at 2 mM Ca²⁺ for the indicated times. The cells were fixed, followed by immunostaining for afadin, E-cadherin, and claudin-1 using the anti-afadin mAb, the anti-E-cadherin mAb, and the anti-claudin-1 pAb, respectively. A, afadin; B, E-cadherin; and C, claudin-1. Bars, 10 μm. The results shown are representative of three independent experiments. D, quantitative analysis of A–C. Closed and open circles, afadin; closed and open squares, E-cadherin; closed and open triangles, claudin-1; closed circles, squares, and triangles, V12Cdc42-MDCK cells; and open circles, squares, and triangles, wild-type MDCK cells. The indices of afadin, E-cadherin, and claudin-1 recruitment represent the percentages of the afadin, E-cadherin, and claudin-1 signal-positive cell-cell adhesion sites in 50 cell-cell adhesion sites counted, respectively. The values are means ± S.E. of three independent experiments.

FIG. 4. Increase of the velocities of the formation of AJs and TJs by V12Cdc42. A–C, confocal laser scanning microscopy analysis. Wild-type MDCK and V12Cdc42-MDCK cells were incubated at 2 μM Ca²⁺ for 2 h and then incubated at 2 mM Ca²⁺ for 2 h. The signals for afadin, E-cadherin, and claudin-1 were re-concentrated at the cell-cell adhesion sites between the wild-type MDCK cells expressing EGFP-NWASP-CRIB[H208D]-CAAX after the 2-h incubation at 2 mM Ca²⁺ (Fig. 3, A–D). These results indicate that the inhibitory effect of NWASP-CRIB-CAAX is due to its specific interaction with activated Cdc42.

Increase of the Velocities of the Formation of AJs and TJs by V12Cdc42—The result that inhibition of the action of activated Cdc42 reduces the velocities of the formation of AJs and TJs suggests that overexpression of V12Cdc42 conversely increases their velocities. To test this possibility, V12Cdc42-MDCK cells (MDCK cells stably overexpressing V12Cdc42) and wild-type MDCK cells were incubated at 2 μM Ca²⁺ for 2 h and then incubated at 2 mM Ca²⁺ for 2 h. The signals for afadin, E-cadherin, and claudin-1 were re-concentrated at the cell-cell adhesion sites in a time-dependent manner after both the cells were incubated at 2 mM Ca²⁺ (Fig. 4, A–D), but the time courses of the accumulation of afadin, E-cadherin, and claudin-1 at the cell-cell adhesion sites in V12Cdc42-MDCK cells were much faster than those in wild-type MDCK cells. These results indicate that overexpression of V12Cdc42 increases the velocities of the formation of the E-cadherin-based AJs and the claudin-based TJs in epithelial cells.

Suppression of the Inhibitory Effect of NWASP-CRIB-CAAX by V12Cdc42—We next examined whether the inhibitory effect of NWASP-CRIB-CAAX is suppressed by co-expression with V12Cdc42. A plasmid for Myc-tagged V12Cdc42 was co-microinjected with a plasmid for EGFP-NWASP-CRIB-Cₐₓₐₓ into wild-type MDCK cells. After a 30-min incubation at 2 mM Ca²⁺, the cells were incubated at 2 μM Ca²⁺ for 30 min and then incubated at 2 mM Ca²⁺ for 2 h. The signals for afadin, E-cadherin, and claudin-1 were reduced at the cell-cell adhesion sites between the wild-type MDCK cells expressing only EGFP-NWASP-CRIB-CAAX (Fig. 5, A–D), consistent with the results in Fig. 3. These results indicate that the inhibitory effect of NWASP-CRIB-CAAX is mediated through the inhibition of the action of activated Cdc42.

Suppression of the Inhibitory Effect of NWASP-CRIB-CAAX by Nectin-1—We then examined whether the inhibitory effect of NWASP-CRIB-CAAX is suppressed by the trans-interactions of nectins which induce activation of Cdc42. To test this possibility, we used nectin-1-MDCK cells (MDCK cells stably overexpressing exogenous nectin-1). Nectin-1-MDCK cells were transfected with a plasmid for EGFP-NWASP-CRIB-CAAX or EGFP-CAAX and
cultured for 24 h. After the culture, the cells were incubated at 2 μM Ca²⁺ for 2 h and then incubated at 2 mM Ca²⁺ for 2 h. The signals for afadin and E-cadherin, but not that for claudin-1, were re-concentrated at the cell-cell adhesion sites between the nectin-1-MDCK cells expressing EGFP-NWASP-CRIB-CAX (Fig. 6, A–D). These results indicate that the trans-interaction of nectin-1 suppresses the inhibitory effect of NWASP-CRIB-CAX on the accumulation of afadin and E-cadherin, but not that of claudin-1, at the newly formed cell-cell adhesion sites.

Different Sensitivities of the Formation of AJs and TJs to Activated Cdc42—The result that the trans-interaction of nectin-1 suppresses the inhibitory effect of NWASP-CRIB-CAX on the accumulation of afadin and E-cadherin, but not that of claudin-1, led us to questions of whether the formation of TJs requires a greater amount of activated Cdc42 than that of AJs and of whether the amount of the Cdc42 activated by the trans-interaction of nectin-1 is not enough to suppress the inhibitory effect of NWASP-CRIB-CAX. We microinjected various doses of a plasmid for EGFP-NWASP-CRIB-CAX from 0.01 to 0.1 mg/ml into nectin-1-MDCK and wild-type MDCK cells. After a 30-min incubation at 2 mM Ca²⁺, the cells were incubated at 2 μM Ca²⁺ for 30 min and then incubated at 2 mM Ca²⁺ for 2 h. The microinjection with a lower concentration (0.01 mg/ml) of the plasmid resulted in accumulation of the signal for claudin-1 at the cell-cell adhesion sites between the nectin-1-MDCK cells expressing EGFP-NWASP-CRIB-CAX after the 2-h incubation at 2 mM Ca²⁺, but resulted in a reduction of that between the wild-type MDCK cells expressing EGFP-NWASP-CRIB-CAX (Fig. 7). In contrast, the microinjection with a higher concentration (0.1 mg/ml) of the plasmid resulted in a reduction of the signal for claudin-1 at the cell-cell adhesion sites between the nectin-1-MDCK cells expressing EGFP-NWASP-CRIB-CAX and between the wild-type MDCK cells expressing EGFP-NWASP-CRIB-CAX. These results indicate that the trans-interaction of nectin-1 indeed suppresses the inhibitory effect of NWASP-CRIB-CAX on the formation of the claudin-1-based TJs and that the formation of TJs and AJs has different sensitivities to activated Cdc42.

Formation of Filopodia by V12Cdc42—Activated Cdc42 is well known to induce formation of filopodia in fibroblasts (39). The trans-interactions of nectins increase the velocities of the formation of AJs and TJs (8). Therefore, it is possible that the Cdc42 activated by the trans-interactions of nectins induces formation of filopodia to increase the number of cell-cell contact sites in wild-type MDCK cells, resulting in an increase of the velocities of the formation of AJs and TJs. In the last experiment, we tested whether a constitutively active mutant of Cdc42 induces formation of filopodia in wild-type MDCK cells.
Wild-type MDCK cells were microinjected with a plasmid for EGFP-V12Cdc42, EGFP-Cdc42 (wild type), or EGFP and incubated at 2 mM Ca\(^{2+}\)/H\(_{11001}\) for 3 h. The cells expressing EGFP-V12Cdc42 induced formation of filopodia, whereas the cells expressing EGFP-Cdc42 (wild type) or EGFP did not (Fig. 8 and data not shown). These results suggest that the Cdc42 activated by the trans-interactions of nectins during the formation of cell-cell adhesion induces formation of filopodia in MDCK cells, resulting in an increase of the velocities of the formation of AJs and TJs.

DISCUSSION

We have shown here that inhibition of the action of activated Cdc42 by NWASP-CRIB-CAAX results in a decrease in the
velocities of the formation of AJs and TJs in MDCK cells. Conversely, a constitutively active mutant of Cdc42 increases their velocities. The inhibitory effect of NWASP-CRIB-CAAX on the formation of AJs and TJs is suppressed by the trans-interactions of nectins. We have demonstrated previously (7, 8, 26) that the trans-interactions of nectins increase the velocities of the formation of AJs and TJs and induce activation of Cdc42 and Rac in MDCK cells. Taken together, it is likely that the Cdc42 activated by the trans-interactions of nectins increases the velocities of the formation of AJs and TJs in MDCK cells.

We have proposed that at the initial stage of the formation of AJs, cell-cell contact sites are formed mainly by the trans-interactions of nectins, because nectins kinetically form microclusters more rapidly than E-cadherin (6, 7). We have shown here that V12Cdc42, a constitutively active mutant of Cdc42, induces formation of filopodia in MDCK cells. Thus, the Cdc42 activated by the trans-interactions of nectins is likely to increase the number of filopodia at the cell-cell contact sites at the initial stage of the formation of AJs, resulting in an increase of the number of cell-cell contact sites like “fork initiation” described for DNA replication (40). It has been reported that the activation of Rac induces formation of lamellipodia and increases the velocity of the formation of the E-cadherin-based cell-cell adhesion, resulting in maturation of the E-cadherin-based cell-cell AJs in a phosphatidylinositol (PI) 3-kinase-independent manner (15). The trans-interaction of E-cadherin induces activation of Rac (20–24), whereas the trans-interactions of nectins induce not only activation of Cdc42 but also that of Rac (26, 27). Two studies (22, 24) have shown that the activation of Rac by the trans-interaction of E-cadherin is independent of PI 3-kinase, whereas two other studies (20, 23) have shown that PI 3-kinase is required for the full activation of Rac by the trans-interaction of E-cadherin. On the other hand, the trans-interactions of nectins induce activation of Rac and Cdc42 in a PI 3-kinase-independent manner (27). Therefore, it is likely that the Rac activated by the trans-interactions of nectins induces formation of lamellipodia that efficiently expands the cell-cell adhesion between the filopodia, acting like a so-called “zipper.” It remains unknown whether the E-cadherin-induced activation of Rac is involved in the formation of cell-cell adhesion.

Our data support a model in which the formation of the claudin-based TJs requires a larger amount of activated Cdc42 than that of the E-cadherin-based AJs, indicating that there are different sensitivities of the formation of AJs and TJs to the activation of Cdc42. Activated Cdc42 increases the velocity of the formation of AJs and TJs in wild-type MDCK cells (46). The discrepancy between this study and our previous study might be due to the different assay conditions. In this study, wild-type MDCK cells were microinjected after the cells were serum-starved for 24 h, and the phenotypes of the cells were observed 3 h after the microinjection. On the other hand, in the previous study, wild-type MDCK cells were microinjected without serum starvation, and the phenotypes of the cells were observed 12 h after the microinjection.

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