Involvement of the Annexin II-S100A10 Complex in the Formation of E-cadherin-based Adherens Junctions in Madin-Darby Canine Kidney Cells*

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E-cadherin and nectins are major cell-cell adhesion molecules at adherens junctions (AJs) in epithelial cells. When Madin-Darby canine kidney (MDCK) cells stably expressing nectin-1 (nectin-1-MDCK cells) are cultured at normal Ca\(^{2+}\), E-cadherin and nectin-1 are concentrated at the cell-cell contact sites. When these cells are cultured at low Ca\(^{2+}\), E-cadherin disappears from the cell-cell contact sites, but nectin-1 persists there. When these cells are re-cultured at normal Ca\(^{2+}\), E-cadherin is recruited to the nectin-based cell-cell contact sites. We found here that this recruitment was dependent on protein synthesis, because a protein synthesis inhibitor, cycloheximide, prevented the accumulation of E-cadherin. When nectin-1-MDCK cells, precultured at low Ca\(^{2+}\) in the presence of a proteasome inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal), were re-cultured at normal Ca\(^{2+}\), E-cadherin was recruited to the nectin-based cell-cell contact sites but the level of E-cadherin was reduced. Similar results were obtained when wild-type MDCK cells were used instead of nectin-1-MDCK cells. These results suggest that degradation of one or more protein factors and de novo synthesis of the same or different protein factor(s) are needed for the formation of the E-cadherin-based AJs. We biochemically identified the annexin II-S100A10 complex as such a candidate. Depletion of plasma membrane cholesterol, which abolished the localization of the annexin II-S100A10 complex at the plasma membrane, inhibited the re-concentration of E-cadherin at the nectin-based cell-cell contact sites in the Ca\(^{2+}\) switch experiment. Knockdown of annexin II by RNA interference also inhibited the re-concentration of E-cadherin. These results indicate that the annexin II-S100A10 complex is involved in the formation of the E-cadherin-based AJs in MDCK cells.

Cell-cell junctions have essential roles in various cellular functions, including morphogenesis, differentiation, proliferation, and migration (1–5). In polarized epithelial cells, cell-cell adhesion is mediated through a junctional complex composed of tight junctions (TJs), adherens junctions (AJs), and desmosomes (6). These junctional structures are typically aligned from the apical to basal sides, although desmosomes are independently distributed in other areas. These junctions are generally made between homotypic cells and mediated by homophilic interactions of cell adhesion molecules. The formation and maintenance of TJs and desmosomes are generally dependent on the formation of AJs (7). At AJs, the transmembrane protein E-cadherin functions as a Ca\(^{2+}\)-dependent cell adhesion molecule (1, 2, 8). E-cadherin is a member of the cadherin superfamily, comprising over 80 members (5, 9). E-cadherin forms homo-cis-dimers and then homo-trans-dimers (trans-interactions) through the extracellular region, causing cell-cell adhesion. The cytoplasmic tail is linked to the actin cytoskeleton through many peripheral membrane proteins (PMPs), including α-catenin, β-catenin, vinculin, and α-actinin (8, 10, 11). Cadherins directly bind β-catenin, which in turn binds α-catenin. α-Catenin then binds vinculin and α-actinin. Of these PMPs, α-catenin, vinculin, and α-actinin are actin filament (F-actin)-binding proteins (11). The association of E-cadherin with the actin cytoskeleton through these PMPs strengthens the cell-cell adhesion activity of E-cadherin (10, 11).

Nectins, which constitute a family of four members, have recently emerged as Ca\(^{2+}\)-independent Ig-like cell adhesion molecules at AJs (12, 13). Nectins form homo-cis-dimers and then homo- and hetero-trans-dimers through the extracellular region, causing cell-cell adhesion. The cytoplasmic tail of nectins interacts with afadin, an F-actin-binding protein, which links nectins to the actin cytoskeleton. Nectins first form cell-cell adhesion where cadherins are recruited, eventually forming AJs in epithelial cells and fibroblasts. Nectins are thought to recruit cadherins through the association mediated by their associating PMPs. The detailed molecular mechanisms for this association are not fully understood, but afadin and α-catenin are essential for this association and several proteins, which connect afadin and α-catenin, have been identified (12–16). The first putative connector unit for nectins and E-cadherin is a ponsin-vinculin-α-actinin. Ponsin is an afadin and vinculin-binding protein, and vinculin is an F-actin- and α-catenin-binding protein. The second connector is an afadin dilute domain.
main-interacting protein (ADIP)-α-actinin unit (15). ADIP is an afadin- and α-actinin-binding protein, and α-actinin is an α-catenin-binding protein. The third connector is a LIM domain involved in the formation of the E-cadherin-based AJs in MDCK cells.

EXPERIMENTAL PROCEDURES

Antibodies, Chemicals, and Expression Vectors—A rabbit anti-nectin-1 polyclonal antibody (pAb) was prepared as described (30). A mouse anti-afadin monoclonal antibody (mAb) and a rabbit anti-afadin pAb were prepared as described (31). A rabbit anti-ADIP pAb was prepared as described (15). A rabbit anti-LMO7 pAb was prepared as described (33).

Cell Culture and Transfection—MDCK cells were kindly supplied from Dr. W. Birchmeier (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany). MDCK cells stably expressing FLAG-tagged nectin-1 (nectin-1-MDCK cells) were prepared as described (30). MDCK cells stably expressing GFP-E-cadherin (GFP-E-cadherin-MDCK cells) were prepared as described (32). Nectin-1-MDCK or wild-type MDCK cells were transfected with pBS-H1-annexin II using Lipofectamine 2000 (Invitrogen).

Ca2+ Switch Assay—Ca2+ switch experiments using nectin-1-MDCK or wild-type MDCK cells were done as described (33). Briefly, nectin-1-MDCK or wild-type MDCK cells were seeded on 18-mm glass coverslips in 12-well culture dishes. Forty-eight hours later, the cells were washed with phosphate-buffered saline (PBS) and cultured at 2 mM Ca2+. The cells were then cultured at 2 mM Ca2+ for 12 h, or 2 mM Ca2+ for 12 h followed by 50 μM ALLN for 3 h. The cells were washed with DMEM and cultured at 2 mM Ca2+ in DMEM without serum in the presence or absence of 10 μM cycloheximide, 50 μM ALLN, or 50 μM ALLM for 3 h. After the culture, the cells were washed with DMEM and cultured at 2 mM Ca2+ in DMEM without serum in the presence or absence of 10 μM cycloheximide, 50 μM ALLN, or 50 μM ALLM for 3 h. The cells were then washed three times with PBS containing 1 mM CaCl2 for 5 min and incubated for 30 min in PBS containing 1% bovine serum albumin and 1 mM CaCl2 with the secondary pAbs. The samples were then washed three times with PBS containing 1 mM CaCl2 for 5 min and mounted in GEL/MOUNT (Biomeda). The samples were analyzed by a Radiance 2100 confocal laser scanning microscope (Bio-Rad Laboratories).

Cell Dissociation Assay—The cell dissociation assay was done as described (36). In brief, nectin-1-MDCK or wild-type MDCK cells (1 × 106) were seeded in a 35-mm dish. Seventy-two hours later, the cells were washed with PBS and cultured at 2 mM Ca2+ in DMEM without serum for 1 h. The cells were then cultured at 2 mM Ca2+ (DMEM with 5 mM EGTA) in the presence or absence of 10 μM cycloheximide, 50 μM ALLN, or 50 μM ALLM for 3 h. The cells were then washed three times with PBS containing 1 mM CaCl2 for 5 min and incubated for 30 min in PBS containing 1% bovine serum albumin and 1 mM CaCl2 with the secondary pAbs. The cells were then washed three times with PBS containing 1 mM CaCl2 for 5 min and mounted in GEL/MOUNT (Biomeda). The samples were analyzed by a Radiance 2100 confocal laser scanning microscope (Bio-Rad Laboratories).
the index $N_{TC}/N_{TE}$, where $N_{TC}$ and $N_{TE}$ were the total particle number after the TC and TE treatments, respectively.

Cell Surface Biotinylation—Nectin-1-MDCK cells grown on filters were incubated with 0.5 mg/ml sulfosuccinimidyl 2-(biotinamido)ethyl-dithioproprionate (sulfo-NHS-SS-biotin) (Pierce Chemical Co.), which was applied to both apical and basal sides of the filter, followed by washing with PBS containing 50 mM NH$_4$Cl to quench free sulfo-NHS-SS-biotin, followed by several further washes in PBS. The cells were then scraped off the filters and suspended in radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 50 μg/ml ALLN). The cell lysates were centrifuged, and the supernatant was incubated with streptavidin beads (Sigma) to collect bound, biotinylated protein. The samples were then subjected to SDS-PAGE, followed by Western blotting with the anti-nectin-1, anti-E-cadherin, and anti-ERK1/2 Abs.

Isolation of the Plasma Membrane Fraction—After the Ca$^{2+}$ switch, nectin-1-MDCK cells were washed with PBS, and then sonicated in Buffer A (10 mM HEPES-NaOH at pH 7.5, 100 mM KCl, 1 mM MgCl$_2$, and 25 mM NaHCO$_3$) on ice for 15 s six times at 3-min intervals. The homogenate was centrifuged at 1,000 g at 4 °C for 5 min. The supernatant was diluted with Buffer A into 8.0 mg/ml protein, and 0.45 ml each was applied on a 4.2-ml continuous sucrose density gradient (10–40% sucrose in Buffer A) with 0.3 ml of 50% sucrose cushion, followed by centrifugation at 200,000 g at 4 °C for 1 h with a swinging bucket rotor (P55ST2, Hitachi Ltd.). After the centrifugation, 0.3 ml of fraction each was collected. Each fraction was subjected to SDS-PAGE (8% polyacrylamide gel), followed by Western blotting with the anti-
FLAG and anti-ZO-1 Abs. The fractions in which nectin-1 and ZO-1 were concentrated were collected and used as the plasma membrane fraction.

Two-dimensional PAGE—The aliquot of the plasma membrane fraction (0.33 mg of protein) was precipitated with trichloroacetic acid. The precipitated protein was dissolved in 150 μl of Solution A (7M urea, 2M thiourea, 2% CHAPS, 0.8% Pharmalyte, and 10 mM dithiothreitol), to which 200 μl of Solution B (8M urea, 2% CHAPS, and 0.0025% orange G) was added. After centrifugation at 16,000 g for 5 min, 1.75 μl of IPG Buffer (an ampholyte-containing buffer concentrate for Immobiline DryStrip; Amersham Bioscience) was added to the supernatant. A sheet of Immobiline Drystrip (18-cm long, pH 3–10, Amersham Bioscience) was rehydrated with this solution for 12 h and subjected to isoelectric focusing at 500 V for 1 h, 1000 V for 1 h, and finally 8000 V for 6 h using the IPGphor isoelectric focusing system (Amersham Bioscience). The electrophoresed strip was placed in an equilibration solution (6 M urea, 50 mM Tris-HCl at pH 6.8, 0.25% dithiothreitol, 30% glycerol, and 1% SDS) for 15 min. The strip was then incubated in a re-equilibration solution (6 M urea, 50 mM Tris-HCl at pH 6.8, 0.25% dithiothreitol, 30% glycerol, 1% SDS, 4.5% iodoacetamide, and 0.01% bromphenol blue) for 15 min. The strip was placed on a 10% SDS-polyacrylamide gel without a stacking gel and subjected to electrophoresis at a constant current per gel of 10 mA for 30 min, 20 mA for 30 min, and finally 40 mA for 3 h. Protein spots were identified by silver staining as described (37).

Amino Acid Sequencing—Proteins separated by two-dimensional PAGE were stained with Coomassie Brilliant Blue R-250, the spots were excised, and amino acid sequence analysis was performed by the in-gel digestion method (38) using Procise 494 cLC Protein Sequencing Systems (Applied Biosystems). The determined peptide sequences (spot #1: Asp-Ala-Leu-Asn-Ile-Glu-Thr-Ala-Ile-Lys; spot #2: Pro-Ser-Gln...
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**RESULTS**

Requirement of Protein Synthesis for the Concentration of E-cadherin at the Nectin-based Cell-Cell Contact Sites after the Ca\(^{2+}\) Switch—We have previously shown that nectin-1 and E-cadherin are concentrated at the cell-cell contact sites of MDCK cells stably expressing nectin-1 (nectin-1-MDCK cells) cultured at 2 mM Ca\(^{2+}\) (41, 42). The sites of the signals for nectin-1 and E-cadherin correspond to AJs. When nectin-1-MDCK cells are cultured at 2 mM Ca\(^{2+}\) for 3 h, nectin-1 remains at the cell-cell contact sites, whereas E-cadherin disappears from these sites (41, 42). When these cells are re-cultured at 2 mM Ca\(^{2+}\), E-cadherin is re-concentrated at the cell-cell contact sites where nectin-1 is concentrated, resulting in the formation of AJs (41, 42). We first confirmed these earlier observations by staining of nectin-1 and E-cadherin (Fig. 1, Aa and Ac). We performed the Ca\(^{2+}\) switch experiment in the presence of cycloheximide, an inhibitor of protein synthesis. When nectin-1-MDCK cells were cultured at 2 mM Ca\(^{2+}\) for 3 h in the presence of cycloheximide, nectin-1 remained at the cell-cell contact sites, whereas E-cadherin disappeared from these sites (Fig. 1, Ab and Ac). However, when these cells were re-cultured at 2 mM Ca\(^{2+}\) in the presence of cycloheximide, E-cadherin was not re-concentrated at the cell-cell contact sites (Fig. 1, Ab and Ac). The staining pattern of nectin-1 was not affected. Similar results were obtained when wild-type MDCK cells were used instead of nectin-1-MDCK cells (Fig. 2, Aa and Ab). In this experiment, endogenous nectin-3 and afadin were stained instead of nectin-1, because endogenous nectin-1 was only faintly stained in wild-type MDCK cells as described (41, 42).

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**FIG. 3. Requirement of protein degradation for the concentration of E-cadherin at the nectin-based cell-cell contact sites after the Ca\(^{2+}\) switch in nectin-1-MDCK cells.** Aa, nectin-1-MDCK cells were cultured at 2 mM Ca\(^{2+}\) for 3 h and then incubated with 2 mM Ca\(^{2+}\) for 2 h in the presence of 50 μM ALLN or ALLM. After the incubation, the cells were fixed, followed by immunostaining for E-cadherin and nectin-1 using the anti-E-cadherin and anti-FLAG Abs, respectively. ALLN, in the presence of ALLN; ALLM, in the presence of ALLM; and bars, 10 μm. Ab, quantitative analysis of Aa. Bars represent percentage of cell-cell contact sites with the signal for E-cadherin of the total cell-cell contact sites counted (n = 50) and are expressed as means ± S.E. of the three independent experiments. Ba, stage of the degradation of one or more protein factors for the formation of the E-cadherin-based AJs. Left panels, nectin-1-MDCK cells were cultured at 2 mM Ca\(^{2+}\) for 3 h in the absence of 50 μM ALLN. Then, the cells were incubated with 2 mM Ca\(^{2+}\) for 2 h in the presence of 50 μM ALLN. After the incubation, the cells were fixed, followed by immunostaining for E-cadherin and nectin-1 using the anti-E-cadherin and anti-FLAG Abs, respectively. Right panels, nectin-1-MDCK cells were cultured at 2 mM Ca\(^{2+}\) for 3 h in the presence of 50 μM ALLN. Then, the cells were washed with DMEM and incubated with 2 mM Ca\(^{2+}\) for 2 h in the absence of 50 μM ALLN. After the incubation, the cells were fixed, followed by immunostaining for E-cadherin and nectin-1 using the anti-E-cadherin and anti-FLAG Abs, respectively. Bars represent percentage of cell-cell contact sites with the signal for E-cadherin of the total cell-cell contact sites counted (n = 50) and are expressed as means ± S.E. of the three independent experiments.
We then examined the cell-cell adhesion activity of E-cadherin in the presence of ALLN or ALLM by the cell dissociation assay. When nectin-1-MDCK or wild-type MDCK cells precultured at 2 μM Ca\(^{2+}\) were re-cultured at 2 mM Ca\(^{2+}\) in the presence of ALLN, these cells formed aggregates (N\(_{TC}/N_{TE} = 0.31\) in nectin-1-MDCK cells; N\(_{TC}/N_{TE} = 0.27\) in wild-type MDCK cells). When nectin-1-MDCK or wild-type MDCK cells precultured at 2 μM Ca\(^{2+}\) were re-cultured at 2 mM Ca\(^{2+}\) in the presence of ALLN, these cells formed fewer aggregates (N\(_{TC}/N_{TE} = 0.87\) in nectin-1-MDCK cells; N\(_{TC}/N_{TE} = 0.68\) in wild-type MDCK cells). These results suggest that degradation of one or more protein factors is necessary for the formation of the E-cadherin-based AJs.

We next examined at which stage, during the culture at 2 μM Ca\(^{2+}\) or the re-culture at 2 mM Ca\(^{2+}\), the degradation of one or more protein factors is necessary for the formation of the E-cadherin-based AJs. When nectin-1-MDCK cells, precultured at 2 μM Ca\(^{2+}\) for 3 h in the presence of ALLN, were then re-cultured at 2 mM Ca\(^{2+}\) in the absence of ALLN, then re-cultured at 2 mM Ca\(^{2+}\) in the presence of ALLN, the re-concentrated E-cadherin at the nectin-based cell-cell contact sites was reduced (Fig. 3, Aa, right panels, and Ab). In contrast, when nectin-1-MDCK cells, precultured at 2 μM Ca\(^{2+}\) for 3 h in the absence of ALLN, were then re-cultured at 2 mM Ca\(^{2+}\) in the presence of ALLN, the re-concentrated E-cadherin at the nectin-based cell-cell contact sites was not reduced (Fig. 3, Ba, left panels, and Bb). These results suggest that degradation of one or more proteins during the culture at 2 μM Ca\(^{2+}\) is necessary for the formation of the E-cadherin-based AJs.

Amount of E-cadherin on the Plasma Membrane after the Ca\(^{2+}\) Switch in the Presence of Cycloheximide or ALLN—We then examined whether E-cadherin diffusely remained on the plasma membrane but was not detected by immunofluorescence microscopy in nectin-1-MDCK cells, which were treated with cycloheximide or ALLN in the Ca\(^{2+}\) switch experiment. The Ca\(^{2+}\) switch experiment was performed in the presence of cycloheximide or ALLN, and then the extracellular regions of E-cadherin and nectin-1 of nectin-1-MDCK cells were labeled with sulfo-NHS-SS-biotin. Biotinylation of E-cadherin and nectin-1 was also performed using nectin-1-MDCK cells before the Ca\(^{2+}\) switch experiment. After free sulfo-NHS-SS-biotin was removed by extensive washing, the detergent-soluble, surface-biotinylated proteins on the plasma membrane were recovered on streptavidin beads and analyzed by SDS-PAGE, followed by Western blotting with the anti-nectin-1 and anti-E-cadherin Abs. The amount of nectin-1 or E-cadherin was not affected by the Ca\(^{2+}\) switch experiment in the presence of ALLN (Fig. 4A, lane 4). However, the amounts of nectin-1 and E-cadherin were reduced to 50% of the control in the presence of cycloheximide (Fig. 4A, lane 3). To exclude the possibility that the failure of
the re-concentration of E-cadherin at the nectin-based cell-cell contact sites in the presence of cycloheximide was simply due to a decrease of the amount of E-cadherin on the plasma membrane, we performed the Ca\(^{2+}\) switch experiment using MDCK cells overexpressing GFP-E-cadherin (GFP-E-cadherin-MDCK cells). The level of exogenous GFP-E-cadherin was 3-fold higher than that of endogenous E-cadherin (Fig. 5A). When GFP-E-cadherin-MDCK cells, precultured at 2 \(\mu M\) Ca\(^{2+}\) for 3 h in the presence of cycloheximide, were re-cultured at 2 \(\mu M\) Ca\(^{2+}\) in the presence of cycloheximide, GFP-E-cadherin was re-concentrated at the nectin-based cell-cell contact sites, but the level of the accumulated GFP-E-cadherin was reduced compared with that in the absence of cycloheximide (Fig. 5B). These results suggest that the failure of the re-concentration of E-cadherin at the nectin-based cell-cell contact sites in the presence of cycloheximide was not simply due to the reduced level of E-cadherin on the plasma membrane. The amounts of the residual GFP-E-cadherin remained on the plasma membrane after the cycloheximide treatment was comparable to that of endogenous E-cadherin before the cycloheximide treatment, but the level of the accumulated GFP-E-cadherin at the cell-cell contact sites in the presence of cycloheximide was apparently reduced com-
pared with endogenous E-cadherin in the absence of cycloheximide (Fig. 5, A and B). When GFP-E-cadherin-MDCK cells, precultured at 2 μM Ca^{2+} for 3 h in the presence of ALLN, were re-cultured at 2 mM Ca^{2+} in the presence of ALLN, GFP-E-cadherin was re-concentrated at the nectin-based cell-cell contact sites, but the level of the accumulated GFP-E-cadherin was reduced compared with that in the presence of ALLM, an inactive analogue of ALLN (Fig. 5C). Taken together, these results suggest that one or more additional protein factors, different from E-cadherin, is necessary for the formation of the E-cadherin-based AJs.

We then examined the amounts of the nectin- and E-cadherin-associating PMPs, afadin, α-catenin, β-catenin, p120⁹⁰⁰⁰, α-actinin, vinculin, ADIP, and LMO7 in the presence of cycloheximide or ALLN. These nectin- and E-cadherin-associating PMPs are involved in the formation of E-cadherin-based AJs (15, 16, 41, 42). The amount of afadin, α-actinin, vinculin, ADIP, or LMO7 was not affected by the Ca^{2+} switch experiment in the presence of cycloheximide or ALLN (Fig. 4B). The amounts of α-catenin, β-catenin, and p120⁹⁰⁰⁰ were slightly reduced in the presence of cycloheximide and somewhat increased in the presence of ALLN (Fig. 4B). These subtle effects of cycloheximide and ALLN on the amount of these PMPs are not likely responsible for the failure of the re-concentration of E-cadherin at the nectin-based cell-cell contact sites in the presence of cycloheximide or ALLN. These results suggest that...
degradation and de novo synthesis of one or more protein factors different from these PMPs are necessary for the formation of the E-cadherin-based AJs.

Identification of a Protein Factor(s) to be the Annexin II-S100A10 Complex—We next attempted to identify the protein factor(s) involved in the formation of the E-cadherin-based AJs. For this purpose, we first prepared the plasma membrane fractions of the nectin-1-MDCK cells treated with or without cycloheximide or ALLN by sucrose density gradient ultracentrifugation. The proteins were extracted from each plasma membrane fraction and subjected to two-dimensional gel electrophoresis, followed by protein staining with silver. We found two protein spots whose amounts were decreased when nectin-1-MDCK cells were cultured at 2 mM Ca\(^{2+}\) for 3 h (Fig. 6, A and B). The amounts of these two protein spots were increased when nectin-1-MDCK cells were re-cultured at 2 mM Ca\(^{2+}\) (Fig. 6C). The amounts of the two protein spots were decreased by the treatment with cycloheximide, but increased by the treatment with ALLN as compared with that with ALLM (Fig. 6, D and E). Although the amounts of some additional proteins varied under these conditions, none of them behaved like these two protein spots, which were decreased at 2 mM Ca\(^{2+}\) and increased at 2 mM Ca\(^{2+}\). The two protein spots (#1 and #2) were separately excised out from the gel, digested by lysylendopeptidase, and their partial amino acid sequences were determined. The sequences (spot #1: Asp-Ala-Leu-Asn-Ile-Glu-Thr-Ala-Ile-Lys; spot #2: Pro-Ser-Gln-Met-Glu-His-Ala-Met-Glu-Thr-Met-Met-Phe-Thr-Phe) were 100% identical to those of human annexin II and S100A10, respectively. Annexin II was previously called p11 or calpactin I light chain (25). These results suggest that at least one of the protein factors responsible for the formation of the E-cadherin-based AJs in the Ca\(^{2+}\) switch experiment is the annexin II-S100A10 complex.

Inhibition of the E-cadherin-based Formation of AJs by a Cholesterol-sequestering Agent and Annexin II RNAi—In the last set of experiments, we examined whether the annexin II-S100A10 complex is indeed involved in the formation of the E-cadherin-based AJs in the Ca\(^{2+}\) switch experiment. It has been reported that annexin II associates with the plasma membrane lipid raft microdomains in a cholesterol-dependent manner (27). We examined the effect of a cholesterol-sequestering agent, methyl-\(\beta\)-cyclodextrin, on the re-concentration of E-cadherin at the nectin-based cell-cell contact sites in the Ca\(^{2+}\) switch experiment. When nectin-1-MDCK or wild-type MDCK cells, precultured at 2 mM Ca\(^{2+}\) for 3 h in the absence of methyl-\(\beta\)-cyclodextrin, were re-cultured at 2 mM Ca\(^{2+}\) in the presence of methyl-\(\beta\)-cyclodextrin, neither annexin II nor E-cadherin was re-concentrated at the nectin-based cell-cell contact sites (Fig. 7, A–C). In contrast, the staining pattern of nectin-1 or afadin was not affected (Fig. 7, A and B).

We finally examined whether annexin II was required for the formation of the E-cadherin-based AJs by knockdown of annexin II with RNAi. The expression level of annexin II was reduced in MDCK cells transfected with the vector expressing small interfering RNA oligonucleotides against annexin II (Fig. 8A). When the Ca\(^{2+}\) switch experiment was performed using nectin-1-MDCK or wild-type MDCK cells in which annexin II was knocked-down, the re-concentration of E-cadherin at the nectin-based cell-cell contact sites was inhibited as compared with the control cells (Fig. 8, B–D). In contrast, the staining pattern of nectin-1 or afadin was not affected (Fig. 8, B and C). These results suggest that annexin II is indeed involved in the formation of the E-cadherin-based AJs.

**DISCUSSION**

We have found conditions where E-cadherin is not concentrated at the cell-cell contact sites where nectin-1 is concentrated in nectin-1-MDCK cells. When nectin-1-MDCK cells pre-cultured at low Ca\(^{2+}\) are re-cultured at normal Ca\(^{2+}\) in the presence of cycloheximide, E-cadherin is not re-concentrated at the nectin-based cell-cell contact sites. When nectin-1-MDCK cells, precultured at low Ca\(^{2+}\) in the presence of ALLN, a proteasome inhibitor, are re-cultured at normal Ca\(^{2+}\), the re-
concentrated E-cadherin at the nectin-based cell-cell contact sites is also reduced. Similar results are obtained when wild-type MDCK cells are used instead of nectin-1-MDCK cells. Although the amount of E-cadherin is reduced in the presence of cycloheximide, this reduction of the amount of E-cadherin does not account for the failure of the re-concentration of E-

FIG. 8. Inhibition of the re-concentration of E-cadherin at the nectin-based cell-cell contact sites by annexin II RNAi in wild-type MDCK and nectin-1-MDCK cells. A, decreased level of annexin II by RNAi. Wild-type MDCK cells were transfected with pBS-H1-annexin II or the control vector and cultured for 72 h. The cells were subjected to SDS-PAGE, followed by Western blotting with the anti-annexin II and anti-actin Abs. B, inhibition of the re-concentration of E-cadherin at the cell-cell contact sites by RNAi of annexin II in wild-type MDCK cells. Wild-type MDCK cells were transfected with pBS-H1-annexin II and pEGFP vector and cultured for 72 h. The cells were then incubated at 2 μM Ca\(^{2+}\) for 3 h and further incubated at 2 mM Ca\(^{2+}\) for 2 h. The cells were fixed and stained for E-cadherin, annexin II, and afadin with the anti-E-cadherin, anti-annexin II, and anti-afadin Abs, respectively. C, inhibition of the re-concentration of E-cadherin at the cell-cell contact sites by RNAi of annexin II in nectin-1-MDCK cells. Nectin-1-MDCK cells were transfected with pBS-H1-annexin II and pEGFP vector and cultured for 72 h. The cells were then incubated at 2 μM Ca\(^{2+}\) for 3 h and further incubated at 2 mM Ca\(^{2+}\) for 2 h. The cells were fixed and stained for E-cadherin, annexin II, and nectin-1 with the anti-E-cadherin, anti-annexin II, and anti-nectin-1 Abs, respectively. Bars, 10 μm. D, quantitative analyses of B and C. Bars represent percentage of cell-cell contact sites with the signal for E-cadherin of the total cell-cell contact sites counted (n = 50) and are expressed as means ± S.E. of the three independent experiments.
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The E-cadherin-based AJs (49). Annexin II has been identified with the 11 S complex to the 16 S complex during the formation of E-cadherin at the cell-cell contact sites. Additionally, the 11 S complex to the 16 S complex during the formation of E-cadherin on the plasma membrane. The amounts of the PMPs associating with nectin and E-cadherin are also not changed dramatically in the presence of cycloheximide or ALLN. Thus, degradation and de novo synthesis of one or more protein factors, which is different from the known nectin- and E-cadherin-associating PMPs, are necessary for the formation of the E-cadherin-based AJs.

We have biochemically identified a candidate of such a protein factor(s) to be the annexin II-S100A10 complex. The amounts of both annexin II and S100A10 on the plasma membrane are decreased by the treatment with cycloheximide but increased by the treatment with ALLN. Depletion of plasma membrane cholesterol in nectin-1-MDCK and wild-type MDCK cells abolishes the localization of annexin II in the plasma membrane, causing the inhibition of the accumulation of E-cadherin at the cell-cell contact sites. This observation is consistent with the previous finding that depletion of plasma membrane cholesterol impaired the formation of AJs in endothelial cells (45). In addition, knockdown of annexin II by RNAi inhibits the formation of the E-cadherin-based AJs in nectin-1-MDCK and wild-type MDCK cells. These results indicate that degradation and de novo synthesis of the annexin II-S100A10 complex is involved in the formation of the E-cadherin-based AJs in MDCK cells. However, it remains unclear if the annexin II-S100A10 complex is the only cycloheximide- or ALLN-sensitive factor.

There are several possible mechanisms of the annexin II-S100A10 complex in the formation of the E-cadherin-based AJs. Because it has been shown that the annexin II-S100A10 complex is involved in the recycling endosomes (29), the annexin II-S100A10 complex might be involved in recycling of E-cadherin during the formation of the E-cadherin-based AJs. Because annexin II is implicated in the anchoring of lipid rafts with the actin cytoskeleton (27, 28), the annexin II-S100A10 complex is involved in the recycling endosomes (29), the annexin II-S100A10 complex might be involved in the actin cytoskeleton formed by the actin cytoskeleton (27, 28). The annexin II-S100A10 complex is then recruited to the actin cytoskeleton formed by the actin cytoskeleton (27, 28), the annexin II-S100A10 complex is involved in the recycling endosomes (29), the annexin II-S100A10 complex might be involved in recycling of E-cadherin during the formation of the E-cadherin-based AJs. 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Involvement of the Annexin II-S100A10 Complex in Formation of AJs

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Involvement of the Annexin II-S100A10 Complex in the Formation of E-cadherin-based Adherens Junctions in Madin-Darby Canine Kidney Cells
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