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The extracellular domains of E- and N-cadherin determine the scattered punctate localization in epithelial cells and the cytoplasmic domains modulate the localization

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The accumulation of classical cadherins is essential for their function, but the mechanism is poorly understood. Hence, we investigated the accumulation of E- and N-cadherin and the formation of cell junctions in epithelial cells. Immunostaining revealed a scattered dot-like accumulation of E- and N-cadherin throughout the lateral membrane in MDCK II and other epithelial cells. Mutant E-cadherin lacking the β-catenin binding site accumulated granularly at cell–cell contact sites and showed weak cell aggregation activity in cadherin-deficient epithelial cells, MIA PaCa2 cells. Mutant E-cadherin lacking the p120-catenin binding site exhibited scattered punctate accumulation and strong cell adhesion activity in MIA PaCa2 cells. Electron microscopy demonstrated that MIA PaCa2 transfectants of E-cadherin containing β-catenin binding site formed adherens junction, whereas E-cadherin lacking the binding site did not. Mutant N-cadherins showed accumulation properties similar to those of corresponding mutant E-cadherins. Moreover, wild type and mutant N-cadherin lacking the p120-catenin binding site showed subapical accumulation in polarized DLD-1 cells, whereas mutant N-cadherin lacking β-catenin binding site did not. These results indicate that the extracellular domains of E- and N-cadherin determines the basic localization pattern, whereas the cytoplasmic domains modulate it thereby affects the cell adhesion activity, subapical accumulation, and the formation of adherens junction.

Keywords: adherens junction/catenin/E-cadherin/epithelial cells/localization.

Abbreviations: cad-5, cadherin-5; DLD-1/Δα, DLD-1 mutant cell lacking α-catenin expression; E-cad, E-cadherin; E-ΔCP, E-cadherin lacking the cytoplasmic domain; E-CPΔβ, E-cadherin lacking the β-catenin binding site; E-CPΔp120, E-cadherin lacking the p120-catenin binding site; FBS, fetal bovine serum; HBS, HEPES-buffered saline; MIA2, MIA PaCa2; N-cad, N-cadherin; N-ΔCP, N-cadherin lacking the cytoplasmic domain; N-CPΔβ, N-cadherin lacking the β-catenin binding site; N-CPΔp120, N-cadherin lacking the p120-catenin binding site; TBS, Tris-buffered saline.

Many investigators have morphologically studied the cell junctions that connect apposing cells, since intercellular adhesion is one of the most fundamental features of multicellular organisms. The results have revealed that a characteristic junctional complex is constructed just beneath the apical surface in epithelial cells of vertebrates (*1*). This complex is composed of three different types of cell junctions, *i.e.* the zonula occludens or tight junction, the zonula adherens and the macula adherens or desmosome (*1–3*). The zonula adherens is a belt-like cell junction (*1*) connecting adjacent cells with an ~15–20 nm gap between them. The cytoplasmic region is connected with actin filaments, which effectively ensures the strength of the intercellular adhesion. A similar but discontinuous junction is described as the fascia adherens in some tissues such as myocardium. Many investigators have collectively termed these cell junctions as adherens junctions. In this report, we use the term adherens junction to refer to the zonula adherens, fascia adherens or their related cell adhesion structures.

Various cell adhesion proteins, on the other hand, have been isolated and characterized to elucidate the mechanism of cell–cell adhesion (*4*). Among them, a Ca^{2+}-dependent cell–cell adhesion protein named cadherin was identified in 1982 (*5*). Since then, a large number of different cadherins have been identified in a variety of multicellular organisms, and their properties have been extensively characterized (*6*). E-cadherin was the first cadherin identified. It belongs to a subfamily of cadherins termed classical cadherins and is mainly expressed in epithelial cells. It is thought that the primary role of E-cadherin is the formation and maintenance of epithelial tissue structure, which it achieves by establishing an intercellular junction termed the zonula adherens, an adherens junction that supports the strength and the specificity of

intercellular adhesions (*7*). Moreover, E-cadherin is required for the formation of tight junction, desmosome, gap junction and apico-basal polarity (*6*, *7*).

N-cadherin is another classical cadherin that has been studied extensively. N-cadherin is expressed in various tissues, especially in nervous tissue. The structure is highly homologous to that of E-cadherin as well as other classical cadherins and is thought to have similar function.

It is generally believed that the biological function of cadherins is based on their homophilic intercellular adhesion activity. To elucidate the mechanism of cell adhesion Shapiro *et al.* (*8*) proposed a model named the 'zipper' model based on the structure of the extracellular domain of E-cadherin as determined by X-ray crystallography. This model indicates that E-cadherin molecules make cis dimers within a plasma membrane that then form trans dimers between the extracellular domains on adjacent cells. Accordingly, the combination of the two activities constructs a zipper-like macro-structure that makes a strong intercellular connection. On the other hand, the cytoplasmic domains of classical cadherins form complexes with catenins, which are linked to actin cytoskeletons (*9*). Thus, the linkage supports the firm intercellular adhesion by classical cadherins. Yamada *et al.* (*10*) and Drees *et al.* (*11*); however, reported that the interaction of E-cadherin with actin fibres is indirect.

As described above, E-cadherin is an adhesion protein that plays a central role in the strong intercellular adhesion in epithelial cells (*7*) and is localized at zonula adherens to which actin filaments are connected (*12*). Moreover, adherens junctions are not formed without E-cadherin (*13*). Hence, many investigators assume that the accumulation of E-cadherin at a particular site indicates the formation of an adherens junction. In epithelial cells, however, the localization of E-cadherin is rather complex. It has been known that E-cadherin superficially shows a thick linear localization at the subapical site where the zonula adherens is located in epithelial cells. However, various studies have suggested that E-cadherin is localized not only at the subapical site of the lateral membrane, but also at other parts of the lateral membrane (*14–18*). Recently, Kametani and Takeichi (*19*) reported that cadherin-5 formed dot-like clusters in the lateral membrane of one type of cancer cell. The authors suggested that these clusters were transient and would eventually construct mature adherens junctions, but it was unclear how these clusters would actually construct the cell junction or it was a general feature of classical cadherins. On the other hand, Meng *et al.* (*20*) suggested that the zonula adherens is formed and/or maintained by microtubule structures via p120-catenin. However, no details were given.

Thus, it is still an open question as to how classical cadherins accumulate in the lateral membrane and thereby the cell junctions are constructed. Hence, we carefully examined the localization of E- and N-cadherin in various epithelial cells. Then, we constructed various deletion mutants of E- and N-cadherin, and clarify the region in the molecule necessary for accumulation of cadherins and formation of cell junctions. Here, we describe the results and discuss the role of the extracellular domains and the cytoplasmic domains of classical cadherins in the formation and localization of adherens junctions.

Materials and methods

Chemicals

Antibodies against E-cadherin, β-catenin and p120-catenin were the product of Transduction Laboratories (Lexington, KY, USA); and those against claudin-1, occludin, N-cadherin and ZO-1 were from Zymed (South San Francisco, CA, USA). Antibodies against cadherin-5 and α-catenin were obtained from ICOS Corporation (Bothell, WA, USA) and Sigma-Aldrich Corporation (St. Louis, MO, USA), respectively. Antibody against desmoplakin was the product of Progen (Heidelberg, Germany) and antibody against HA was purchased from Medical and Biological Laboratories (Nagoya, Japan). Anti-mouse and anti-rabbit antibodies conjugated with Alexa Fluor-488 or Alexa Fluor-568 and phalloidin conjugated with Alexa Fluor-568 were obtained from Molecular Probe (Eugene, OR, USA). Alkaline phosphatase-conjugated anti-mouse and anti-rabbit antibodies were the products of Promega (Madison, WI, USA). Collagen type I was obtained from Nitta Gelatin (Osaka, Japan). Sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-Dithiopropionate was the product of Pierce (Rockford, IL, USA).

Cell culture

DLD-1, HeLa, MDCK II, MIA PaCa-2 (MIA2) and NMuMG cells were cultured in Dulbecco's modified Eagle's medium-F12 (1:1) containing 10% fetal bovine serum (FBS) in 5% CO_2 at 37°C. Three-dimensional cultures of MDCK II cells in collagen gel were prepared according to the method described by O'Brien *et al.* (*21*). Rat cardiomyocytes were cultured by the method described by Oyamada *et al.* (*22*). For immunostaining, the cells were cultured on cover glasses or in collagen gel.

Constructs of cDNAs for mutant cadherins and expression in cultured cells

The cDNAs for mutant E-cadherins were constructed by PCR using human E-cadherin cDNA as a template and appropriate primers shown in Table 1. Briefly, the cDNA for mutant E-cadherin lacking the entire cytoplasmic domain (E-ΔCP) was constructed as follows: The cDNA corresponding to a C-terminal region of the mutant E-cadherin with a stop codon at the start site of the cytoplasmic domain was synthesized by PCR using E-ECs and E-ΔCPas (Table 1) as primers. Then, the *Bbr* PI-*Not* I fragment was prepared from the cDNA, and the corresponding region of the expression construct of full-length E-cadherin was substituted with this fragment. The cDNA for a deletion mutant of the p120-catenin binding region (E-CPΔp120) was prepared by linking the two fragments corresponding to the 5′ and 3′ regions next to the sequence for p120-catenin binding site by PCR. The resultant cDNA was cut with *Bbr* PI and *Not* I, and the corresponding region of the expression construct of full-length E-cadherin was substituted with the fragment as described above. A cDNA for mutant E-cadherin lacking the β-catenin binding site (E-CPΔβ) was constructed as follows: First, the sequence corresponding to the β-catenin binding site was deleted as described for the synthesis of E-ΔCP cDNA. Then, the sequence corresponding to the possible clathrin binding site was deleted from the resultant cDNA by the method used for E-CPΔp120 cDNA synthesis.

The cDNAs for mutant N-cadherins lacking the entire cytoplasmic domain (N-ΔCP), β-catenin binding site (N-CPΔβ) and p120-catenin binding site (N-CPΔp120) were similarly constructed as described for the constructs of E-CPΔβ and E-CPΔp120 cDNAs, respectively.

The resultant cDNAs were subcloned into the pEF1-MycHis vector (Invitrogen, Carlsbad, CA, USA) or the pEGFP vector (Clontech Laboratories, Mountain View, CA, USA). The resultant constructs were used to transfect cultured cells by the calcium phosphate precipitation method. Stable transfectants were obtained by G418 selection.

Table 1. Primers used for PCR.

The following primers were used for the synthesis of cDNAs for mutant E-cadherins	
Name	Sequence
E-ECs	CTACACTGCCCAGGAGC
E-ΔCPas	GCGGCCGCTCACCGAAGAAACAGCAAGAG
E-CPΔβ-as	GCGGCCGCTCAAGCCGCTTTCAGATTTTC
E-CPΔβΔCTR-1s	CTACACTGCCCAGGAGC
E-CPΔβΔCTR-1as	TCTGGGGGCGCTCTCCTCCGAAGAAA
E-CPΔβΔCTR-2s	GGAGAGCGCCCCCAGAGGATGACA
E-CPΔβΔCTR-2as	GCGGCCGCTCAAGCCGCTTTCAGATTTTC
E-CPΔp120-1s	CTACACTGCCCAGGAGC
E-CPΔp120-1as	CCCCTGTGGTAATAAACGTTGTCCCGG
E-CPΔp120-2s	ACGTTTATTACCACAGGGGCCTGGACGC
E-CPΔp120-2as	GCGGCCGCCCTAGTCGTCCTCGCC
N-ECs	GCAACCGTGTCTGTTAC
N-CPΔβ-as	ACTAGTAGCCGCTTTAAGGCCCTC
N-CPΔp120as	ACTAGTTTTAAAATATTATCTCTTACATC
N-CPΔp120s	TCTAGACAGCAGCCTGACACTGTG
N-CPas	CGCCGGCGGTCATCACCTCCACCATA

Immunofluorescence staining

Cells grown on cover slips for 2 days were fixed with 1% paraformaldehyde in HEPES-buffered saline (HBS) for 10 min at room temperature. Mouse colon was dissected and fixed with 4% paraformaldehyde for 1 h. The resultant tissue samples were washed three times with HBS and equilibrated overnight with HBS containing 30% sucrose. Then the samples were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen. Frozen sections (14 μm) were cut from the samples by using a Leica CM1850 cryostat (Leica Microsystems, Marburg, Germany). In some experiments, 10% TCA in HBS, cold methanol or cold methanol/acetone (1:1) was used as the fixative.

The fixed cells and tissue sections were washed three times with Tris-buffered saline (TBS) and then treated with 0.2% Triton X-100 in TBS for 10 min. After the samples had been washed with TBS, they were incubated with 1% BSA in TBS for 30 min and then reacted with primary antibody for 3 h at room temperature. Next, the samples were washed with TBS three times and reacted further with Alexa Fluor-conjugated secondary antibody for 90 min at room temperature. The resultant samples were washed again three times with TBS and mounted in GelTol mounting medium (Thermo Electron Corporation, Waltham, MA, USA). Staining of MDCK II cells cultured in collagen gel was carried out as described by Yu *et al*. (*23*) with some modification. The stained samples were observed and photographed by using a Zeiss LSCM 1552 laser confocal microscope (Carl Zeiss, Jena, Germany) or a Nikon Eclipse TE2000-U fluorescence microscope equipped with a cell culture apparatus (Nikon, Tokyo, Japan).

Electron microscopy

Cells were prepared for conventional electron microscopy as described earlier (*24*). Briefly, cells were fixed with paraformaldehyde and glutaraldehyde, then with osmium tetroxide, dehydrated with ethanol series, and embedded in Poly/Bed 812 (Polysciences, Inc., Warrington, USA). Thin sections were cut, double-stained with uranyl acetate and lead citrate, and examined by using an H7500 electron microscope (Hitachi, Japan).

Other procedures

Cultured cells were solubilized with 0.1% or 1.0% NP-40 in HBS for 10 min and centrifuged at 100,000 $\times g$ for 30 min, and the supernatant and the pellet were designated as the soluble fraction and the insoluble fraction, respectively (*25*). The resultant samples were subjected to immunoblot analysis as described earlier (*26*).

Surface labelling was carried out according to the method described by Murata *et al*. (*27*) and cell aggregation assay was performed as described earlier (*26*).

Results

Scattered punctate accumulation of E-cadherin in MDCK II cells

To define the precise localization of E-cadherin in epithelial cells, we carefully examined the localization of E-cadherin in MDCK II cells by confocal microscopy, since MDCK II cells have been frequently used as model epithelial cells. When MDCK II cells were cultured on cover glasses, E-cadherin was apparently localized diffusely throughout the lateral membranes (Fig. 1A, arrowheads) and the localization appeared to be granular in some areas. In contrast, zonula occludens proteins, *i.e*. ZO-1, claudin-1, and occludin were always localized linearly just beneath the apical membrane (Figs 1A and S1, arrows). Actin fibres were similarly localized near the luminal surface.

For further clarification of the localization of E-cadherin, the regions where the lateral membrane was oblique to the light axis of the microscope were examined at higher magnification by using a conventional fluorescence microscope; because the latter easily provides a wider view than obtained by confocal microscopy, although the resolution is compromised (Fig. 1B). The results indicated that E-cadherin exhibited scattered dots and thread-like accumulation throughout the lateral membrane except for some parts of the most apical regions where E-cadherin showed relatively thin but dense linear accumulation, forming a sharp boundary and no accumulation of E-cadherin above the boundary (Fig. 1B, asterisks). Accumulation of E-cadherin at the boundary was mostly continuous, but broken in some parts (Fig. 1B, arrows). At some sites, E-cadherin showed thick linear localization (Fig. 1B, circles), corresponding to the region of the lateral membrane parallel to the light axis. When several different fixatives were singly used for the sample preparation, essentially the same localization pattern was obtained (data not shown).

Then, GFP-tagged E-cadherin was expressed in MDCK II cells; and the localization was observed without fixation in order to address more directly the

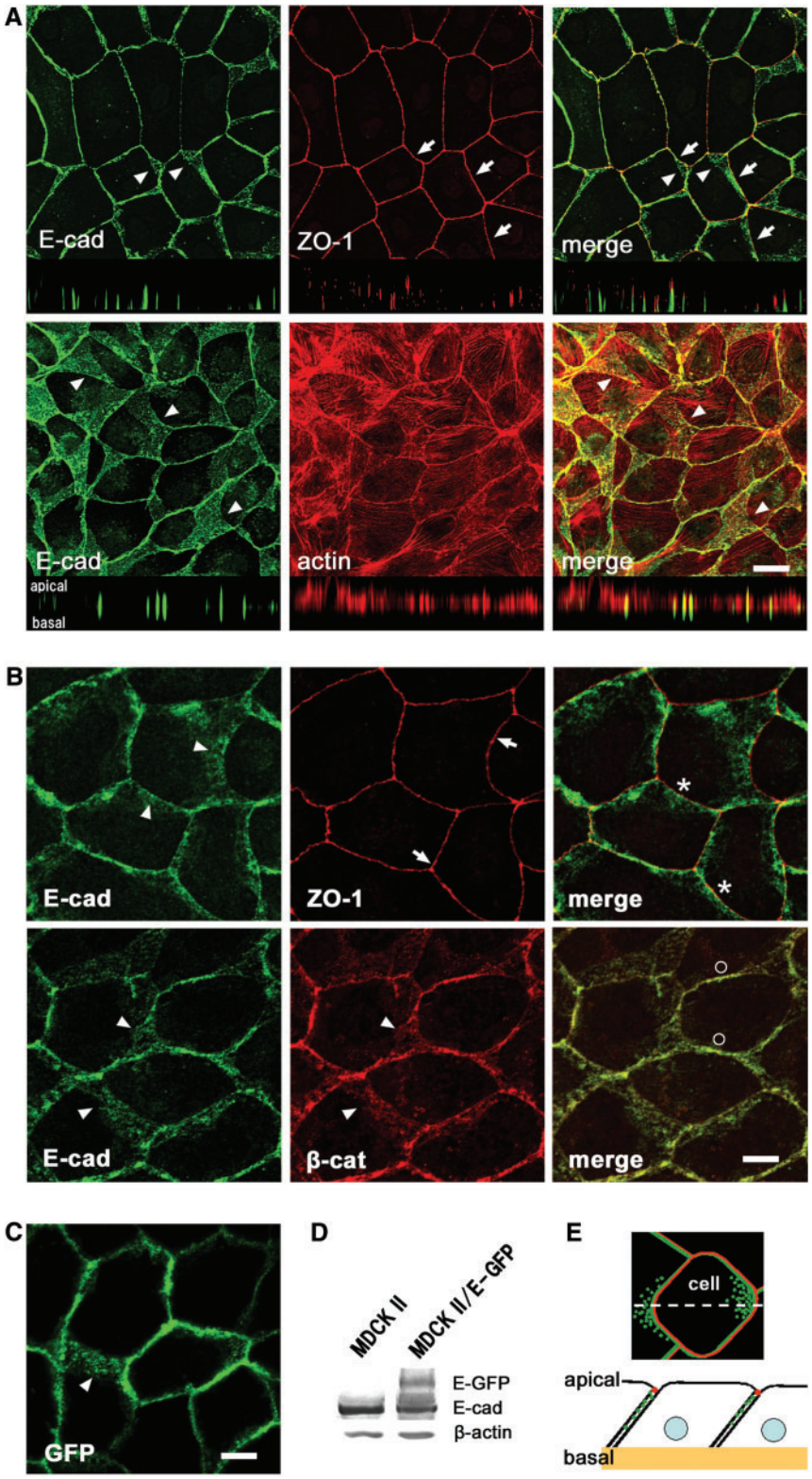

Fig. 1 Scattered dot-like accumulation of E-cadherin in MDCK II cells. MDCK II cells were cultured on cover glasses and were stained with phalloidin (actin, red) or antibodies against E-cadherin (E-cad, green), β-catenin (β-cat, red) or ZO-1 (ZO-1, red). (A) Photographs were taken by using a confocal microscope, and localization of these proteins on the *Z*-axis plane was constructed from the confocal micrographs. E-cadherin and β-catenins are detected throughout the lateral membrane (arrowheads), whereas ZO-1 is localized just beneath the luminal surface (arrows). Scale bar, 20 μm. (B) At higher magnification by a conventional fluorescence microscope, E-cadherin and β-catenin show fine dots and thread-like accumulation (arrowheads), whereas ZO-1 is localized linearly at the subapical sites of the lateral membrane (asterisks). Arrows indicate the lack of E-cadherin accumulation at the boundary. At some sites, E-cadherin shows thick linear localization (circles), corresponding to the region of the lateral membrane parallel to the light axis. Scale bar, 8 μm. (C) GFP in live MDCK II transfectants of GFP-tagged E-cadherin shows dot-like accumulation (arrowhead) similar to that of E-cadherin seen in 'B.'Scale bar, 8 μm. (D) Expression of GFP-tagged E-cadherin was examined by immunoblotting. (E) Schematic drawing of the relationship between stained cells and observed image.

question as to whether the scattered dot-like accumulation of E-cadherin was an artefact produced by sample preparation or not. The result indicated again that E-cadherin showed scattered dots and thread-like accumulation in the lateral membrane (Fig. 1C, arrowhead), demonstrating that the localization pattern was not an artefact but a physiological one.

It is known that E-cadherin and actin filaments are localized closely together in epithelial cells (*25*). The present results showed that the actin ring was localized circumferentially near the luminal surface and roughly co-localized with E-cadherin in MDCK II cells cultured on cover glasses, but not well co-localized with proteins of zonula occludens (Fig. 1A).

Localization of classical cadherins in different cells and formation of adherens junctions

The localization of several classical cadherins expressed endogenously or ectopicly in different cells was examined by the same method as used for the staining of E-cadherin in MDCK II cells to answer the question as to whether the scattered punctate accumulation was specific to E-cadherin expressed in a special cell type. Endogenous E-cadherin expressed in DLD-1 cells was localized mainly at subapical sites with a small number of scattered clusters throughout the lateral membrane, forming a clear boundary between the locations of ZO-1 and E-cadherin (Fig. 2B, DLD-1, arrows). Endogenous E-cadherin in NMuMG cells showed similar localization (Fig. S2).

The localization pattern of N-cadherin expressed ectopicly in MDCK II cells was very similar to that of E-cadherin, with a sharp boundary at the subapical region (Fig. 2B, MDCK II). Endogenous N-cadherin in cardiomyocytes was mainly detected at the cell termini (Fig. 2B, cardiomyocyte), as already reported (*28*), which was in sharp contrast to the localization pattern of E-cadherin in epithelial cells. Moreover, close examination revealed that the accumulation was partly granular. Endogenous N-cadherin in HeLa cells accumulated at cell–cell contact sites, and some parts of the accumulation were rather diffuse and streak-like (Fig. S2, HeLa).

Cadherin-5 expressed ectopicly in MIA2 cells and MDCK II cells showed scattered dot-like accumulation very similar to that of endogenous E-cadherin in MDCK II cells (Fig. 2B MDCK II/cad-5 and Fig. S2, MIA2/cad-5). A clear boundary between Cadherin-5 and ZO-1 locations was also obtained in the MDCK II cells but not in the MIA2 cells. Interestingly, the size of the clusters was quite different from that of E-cadherin. The clusters appeared to be fused to each other and formed large clusters.

Next, the localization pattern of E-cadherin in mouse colon epithelial cells and in the cysts of MDCK II cells was examined to clarify the pattern of epithelial cells *in vivo* or cultured cells under more physiological conditions. The results of colon cells and MDCK II cells were essentially the same as those of cultured cells, but formation of dot-like structure was unclear (Fig. 2B).

Then, formation of cell junctions in MDCK II cells and colon epithelial cells was examined by electron

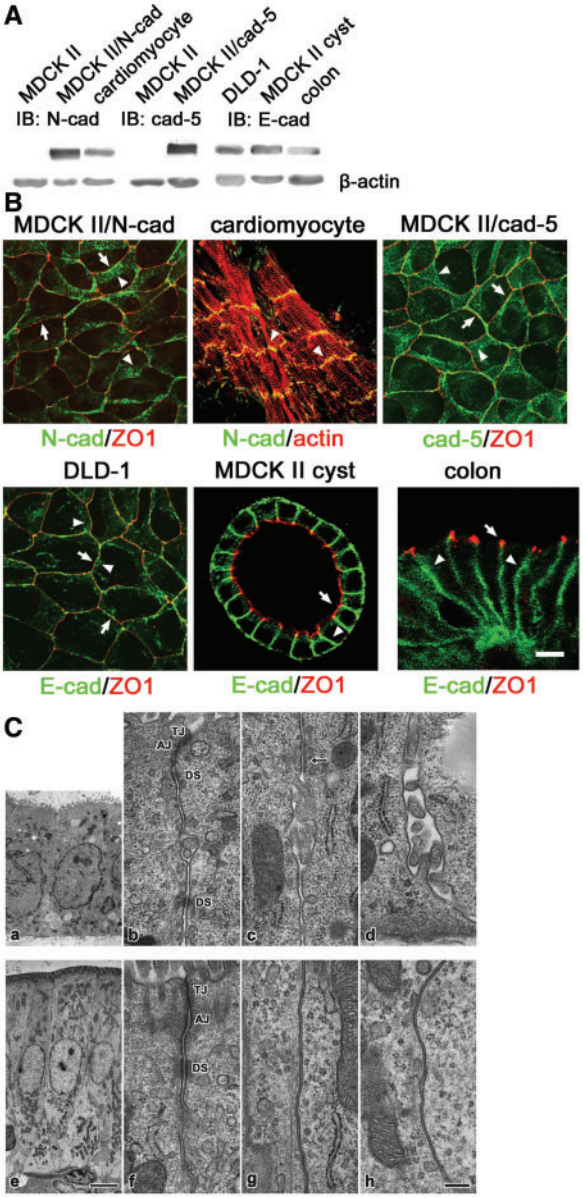

Fig. 2 Localization pattern of classical cadherins in different cells and formation of adherens junctions. (A) Expression of cadherins was examined by immunoblotting. (B) Mouse colon, MDCK II cysts, and cultured cells grown on cover slips were stained doubly with antibodies against ZO-1 (red) and E-cadherin (E-cad, green), N-cadherin (N-cad, green) or cadherin-5 (cad-5, green) or with phalloidin (actin, red) and antibody against N-cadherin (green). N-cadherin and cadherin-5 in MDCK II cells as well as E-cadherin in DLD-1 cells, MDCK II cysts, and colon cells show diffuse granular localization in the lateral membrane, whereas N-cadherin in cardiomyocytes exhibits localization mainly at cell ends. N-cadherin in MDCK II cells, cadherin-5 in MDCK II cells and E-cadherin in DLD-1 cells reveal a sharp boundary at subapical sites (arrows). Scale bar, 20 μm. (C) Cell junctions of MDCK II cysts (a–d) and mouse colon epithelial cells (e–h) were examined by electron microscopy. Typical junctional complexes are observed just beneath the luminal surface of MDCK II cells (b) and mouse colon epithelial cells (f). A cell junction-like structure is occasionally detected in the middle region of the lateral membrane of MDCK II cells (c, arrow). However, this structure is not observed in the basal region of MDCK II cells, or the middle or lower region of mouse colon epithelial cells (g). AJ, adherens junction; DS, desmosome; TJ, tight junction. Scale bars, 5 μm (a, e) and 0.2 μm (b–d, f–h).

microscopy to compare the localization of E-cadherin (Fig. 2C) and the sites of adherens junctions (Fig 2C). In the cystic MDCK II cells, typical tight junctions and adherens junctions or adherens junction-like structures were easily discernible in the subapical region of the lateral membrane (Fig. 2C, b); whereas no specific cell junction structure was evident in other regions (Fig. 2C, c and d). However, careful examination of the cysts revealed that a cell junction-like structure or adherens junction-like structure was occasionally present in the middle region but not in the basal region (Fig. 2C, c and d).

Mouse colon epithelium is composed of columnar cells that show a typical junctional complex in their subapical region (Fig. 2C, f), as has been known for a long time (*1*). Thorough examination of the colon epithelial cells did not reveal any specific cell junction structure in other regions of the lateral membrane as far as we examined. However, cell–cell contacts with a constant inter-membrane distance of ~15–20 nm were observed in the middle and the lower parts of the lateral membrane, suggesting the formation of intercellular adhesion at these sites (Fig. 2C, g and h).

Expression of mutant E-cadherins in cultured epithelial cells

To clarify which part of the cytoplasmic domain of E-cadherin was necessary for the patch-like accumulation and/or adherens junction formation, several deletion mutants of the cytoplasmic domain were constructed (Fig. 3A) and were expressed in epithelial MIA2 cells: In this experiment, MIA2 cells were used instead of widely used L cells to characterize the properties of mutant E-cadherins in E-cadherin-deficient epithelial cells. We chose multiple clones for each mutant that expressed a similar amount of cadherin and used them in the subsequent experiments. Surface labelling experiments indicated that more than half of the E-cadherins expressed were located on the plasma membrane (Fig. 3B).

MIA2 cells expressed essentially no classical cadherins and exhibited a slightly spherical shape (data not shown). In contrast, MIA2 transfectants of wild-type E-cadherin exhibited epithelial morphology and strong cell aggregation activity, although the cells did not form the typical epithelial apico-basal polarity. E-cadherin accumulated almost linearly at cell–cell contact sites, but close examination revealed that the accumulation was rather granular at least in some areas (Fig. 3C, MIA2/E-cad, arrowheads). In contrast, MIA2 transfectants of mutant E-cadherin lacking the entire cytoplasmic domain (E-ΔCP) or the β-catenin binding site (E-CPΔβ) did not show the epithelial morphology; but the truncated E-cadherins accumulated densely and sometimes linearly at intercellular contact sites. The granular accumulation was also observed in some areas (Fig. 3C, MIA2/E-ΔCP and MIA2/E-CPΔβ, arrowheads). The transfectant cells of these mutant E-cadherins showed clear cell aggregation activity, forming a couple of large aggregates within 20 min of incubation (*29*, *30*); but the aggregates were fragile and easily disintegrated into smaller aggregates by gentle pipetting. In the above E-CPΔβ construct, a possible clathrin binding site was also deleted from the juxtamembrane region in addition to the β-catenin binding site, since the mutant E-cadherin lacking only the β-catenin binding site could not localize well to the plasma membrane (*30*).

Then, an 18-amino acid sequence containing the reported p120-catenin binding site (*31*) was deleted from the juxtamembrane region of the cytoplasmic domain (E-CPΔp120) to examine the role of p120-catenin in the localization process of E-cadherin. The resultant MIA2 transfectant cells of E-CPΔp120 showed spread morphology and strong cell aggregation activity. Interestingly, E-CPΔp120 showed scattered punctate accumulation in the transfectants (Fig. 3C). When the p120-catenin binding activity of E-cadherin was inactivated by amino acid substitution (*32*) and the properties were examined, similar results were obtained (data not shown). The results indicate that p120-catenin is not involved in the subapical localization of classical cadherins.

Examination of cell junction formation in the transfectants of mutant E-cadherins by electron microscopy

Next, the MIA2 transfectants of the mutant E-cadherins as well as wild-type E-cadherin were subjected to electron microscopy to examine whether the transfectants formed specific cell junctions. The transfectant cells of wild-type E-cadherin formed adherens junctions or adherens junction-like structures, but the location was not restricted to the subapical region; and the cell junctions were observed in various regions of lateral membrane including near the basal region (Fig. 4, b). In contrast, the transfectant cells of E-ΔCP and E-CPΔβ did not form adherens junction or adherens junction-like structure, and only revealed cell–cell contacts with a constant gap of about 20 nm (Fig. 4, c, d, h and i). Interestingly, these transfectant cells and even parental MIA2 cells occasionally formed a structure that resembled the adherens junction in ~3 ~ 5 % of cell–cell contact sites (Fig. 4, inlet in h). The linkage to actin filaments was unclear. E-CPΔp120 transfectant cells, on the other hand, formed adherens junctions or adherens junction-like structures as in the case of the transfectant cells of wild type E-cadherin (Fig. 4A, e and j).

Solubilization of E- and N-cadherin

It is widely believed that E-cadherin is firmly integrated into the cell junctions at subapical sites and resistant to detergent solubilization (*25*). The present study, however, revealed new features of localization of E- and N-cadherin and formation of adherens junctions. Hence, the solubility of E- and N-cadherin was carefully re-examined by using various cells. When E-cadherin of MDCK II cell, DLD-1 cells and L cell transfectants were briefly treated with 0.1% or 1.0% NP-40, most of the E-cadherin was solubilized; and only a small amount of it remained in the insoluble fraction. In contrast, when cultured rat cardiomyocytes, in which N-cadherin is thought to be firmly integrated into the intercalated disks (*28*), were solubilized

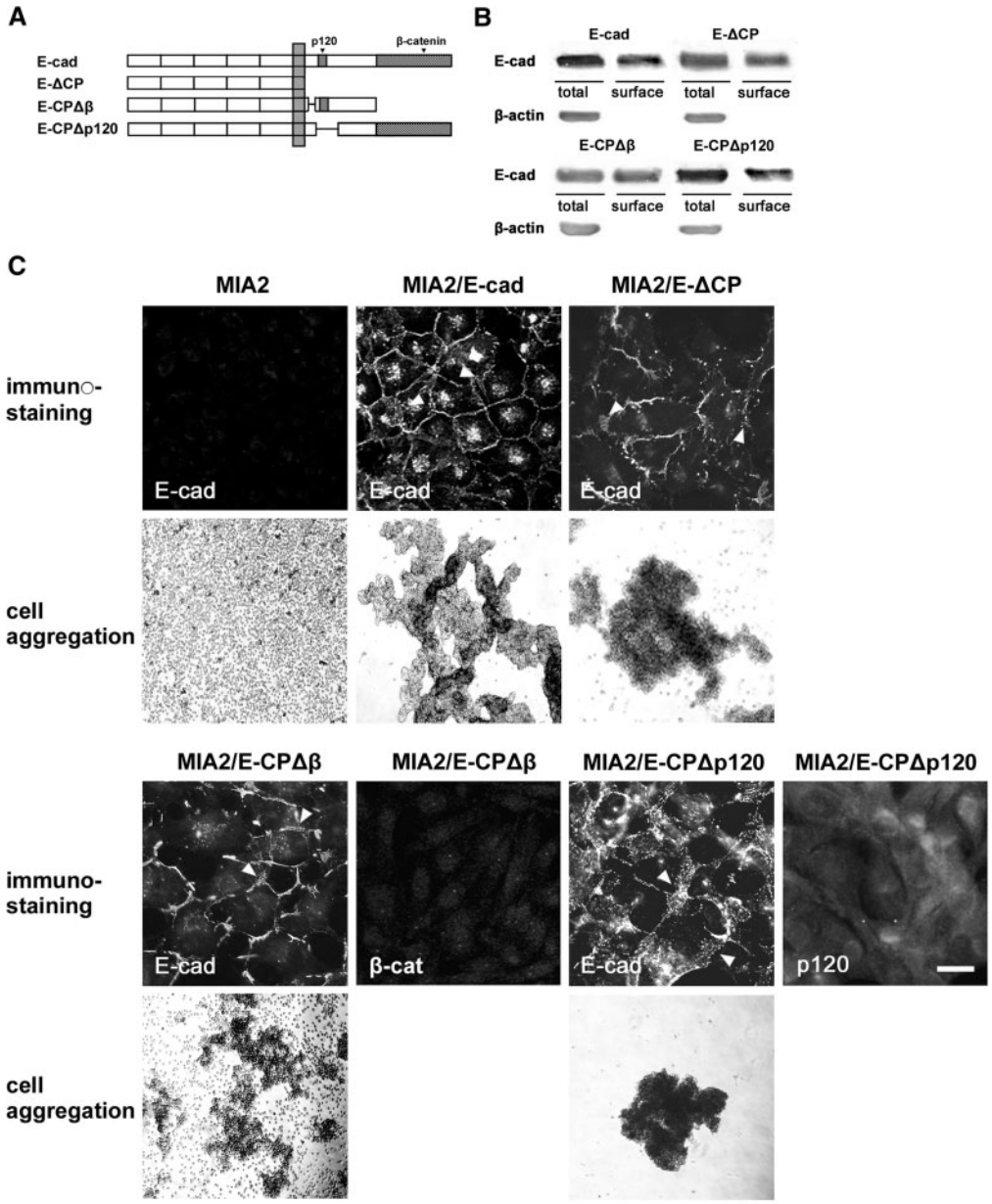

Fig. 3 Expression of mutant E-cadherins in MIA2 cells. (A) Mutant E-cadherins constructed are schematically shown. p120, p120-catenin binding region; β-catenin, β-catenin binding region. (B) Surface-labelling experiments indicate that more than half of the expressed E-cadherins are localized on the cell surface. (C) Immunofluorescence staining of the transfectants with anti-E-cadherin antibody (E-cad) indicates that wild-type E-cadherin, E-ΔCP, E-CPΔβ and E-CPΔp120, are localized at cell–cell contact sites and show patch-like accumulation at some sites (arrowheads). No significant localization of p120-catenin (p120) and β-catenin (β-cat) is observed in the transfectants of E-CPΔp120 and E-CPΔβ. Aggregation activities of MIA2 transfectants of E-cadherin, E-ΔCP, E-CPΔβ and E-CPΔp120 are detected. Scale bars, 20 μm.

by the same method, a substantial amount of N-cadherin remained in the insoluble fraction (Fig. 5). The solubility of α-catenin was similar to that of cadherin in these cells.

The staining for E-cadherin in MDCK II cells was greatly reduced after NP-40 treatment, whereas that for actin did not change much (Fig. S3). In contrast, N-cadherin in cardiomyocytes remained well after NP-40 treatment. These results suggest that most of E-cadherin as well as N-cadherin did not form tight cell junction in MDCK II cells and DLD-1 cells, which is very different from the case of N-cadherin in cardiomyocytes.

Expression of mutant N-cadherins in DLD-1 cells

Next, we examined the localization properties of mutant E-cadherins expressed in typical epithelial cells that exhibit well-developed apico-basal polarity. Before the experiments, we first expressed E-ΔCP in DLD-1 cells lacking α-catenin expression (DLD-1/Δα) and examined the interaction between the mutant E-cadherin and the endogenous E-cadherin, because it seemed likely that ectopicly expressed E-cadherins interacted with endogenous E-cadherin and localization patterns of the cadherins might change. The results showed that the endogenous E-cadherin and the ectopicly expressed E-ΔCP co-precipitated with

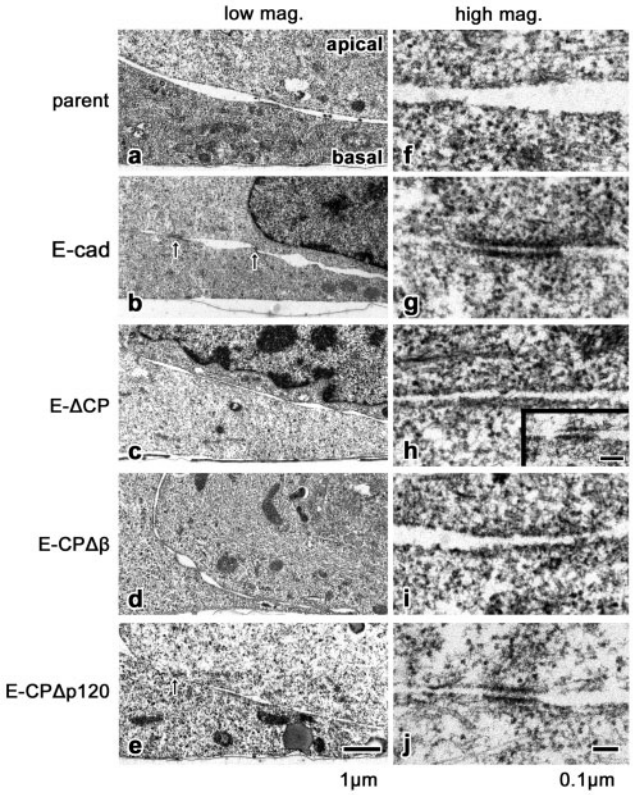

Fig. 4 Examination of cell junction formation in the transfectants of mutant cadherins by electron microscopy. (A) Cell junctions of parental MIA2 cells (a, f), and MIA2 transfectants of wild-type E-cadherin (b, g), E-ΔCP (c, h), E-CPΔβ (d, i), and E-CPΔp120 (e, j) were examined by electron microscopy. MIA2 transfectants of wild-type E-cadherin (arrows in b, g) and E-CPΔp120 (arrow in e, j) form adherens junction, whereas MIA2 transfectants of E-ΔCP (c, h) and E-CPΔβ (d, i), and parental MIA2 cells (a, f) do not. However, these cells occasionally form adherens junction-like structures (inlet in h). Scale bars, 1 μm (a–e) and 0.1 μm (f–j).

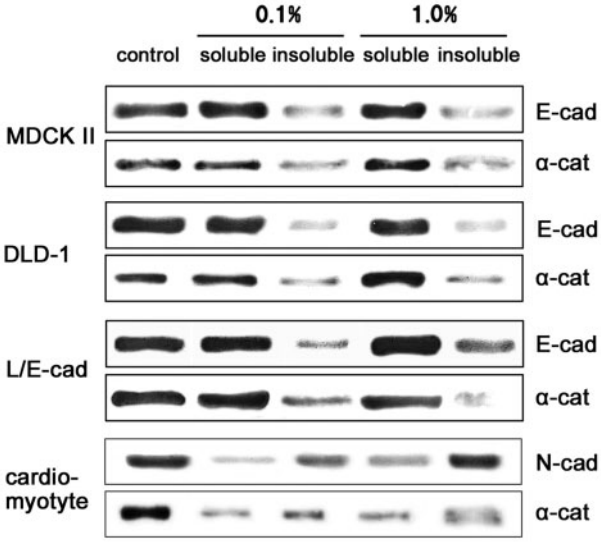

Fig. 5 Detergent solubilization of E- and N-cadherin. MDCK II cells, DLD-1 cells, L cell transfectants of E-cadherin (L/E-cad), and rat cardiomyocytes were solubilized with 0.1% or 1.0% NP-40, and the amount of cadherin in the soluble and insoluble fractions was determined by immunoblotting. Most of the E-cadherin in MDCK II cells, in DLD-1 cells, and in L cell transfectants is easily solubilized; and only a small amount of cadherins remains in the insoluble fraction. In contrast, the N-cadherin in cardiomyocytes is mostly insoluble.

an antibody against the cytoplasmic domain of E-cadherin in immunoprecipitation (Fig. S4A). Moreover, the endogenous E-cadherin did not show significant accumulation in DLD-1/Δα cells, but it became accumulated at cell–cell contact sites after E-ΔCP was expressed in the cells (Fig. S4B). These results clearly indicate that ectopicly expressed E-cadherin associates with endogenous E-cadherin.

Hence, in the subsequent experiments, mutant N-cadherins were used instead of mutant E-cadherins to examine the mechanism of the subapical localization of classical cadherins in polarized epithelial cells (Fig. 6A). The mutant N-cadherins constructed were the same types as those of mutant E-cadherins. In MIA2 cells, these mutant N-cadherins showed accumulation properties similar to those of corresponding mutant E-cadherins (data not shown). When N-ΔCP and N-CPΔβ were expressed in DLD-1 cells (Fig. 6B), the mutant cadherins showed a diffuse and punctate localization and no subapical localization (Fig. 6C, arrowheads). Moreover, the mutant N-cadherins showed a clear boundary between the locations of N-cadherin and ZO-1 and dense accumulation at some subapical sites of DLD-1 cells (Fig. 6C, arrows) as in the cases of E-cadherin and N-cadherin in MDCK II cells (Figs 1B and 2B).

On the other hand, N-CPΔp120 and wild type N-cadherin in DLD-1 cells showed a localization pattern that was similar to those of E-cadherin in DLD-1 cells and N-cadherin in MDCK II cells. However, N-CPΔp120 showed more diffuse localization than that of wild-type N-cadherin (Fig. 6C).

Discussion

E- and N-cadherin show a scattered punctate localization in epithelial cells

Many investigators have thought that E-cadherin localizes linearly at subapical region of epithelial cells, since apparent thick accumulation of E-cadherin at the subapical region of epithelial cells has been reported frequently. However, the present study clearly demonstrated that E-cadherin actually exhibits a scattered dot-like localization in the lateral membrane of various epithelial cells (Figs 1 and 2). We conclude from the present results that most of the reported linear thick localization of E-cadherin in the lateral membrane is attributable to an apparent result derived from the observation of the scattered accumulation throughout the lateral membrane from the upper position, since broad lateral membrane was not observed at these sites (Fig. 1B, circles).

The scattered dot-like clustering was not specific to E-cadherin, and a similar localization pattern was observed for N-cadherin and cadherin-5 in different cells (Fig. 2A). Various other reports also described the diffuse and/or granular localization pattern of classical cadherins, although most of them were not discussed in detail (*14–19*). On the other hand, the sizes

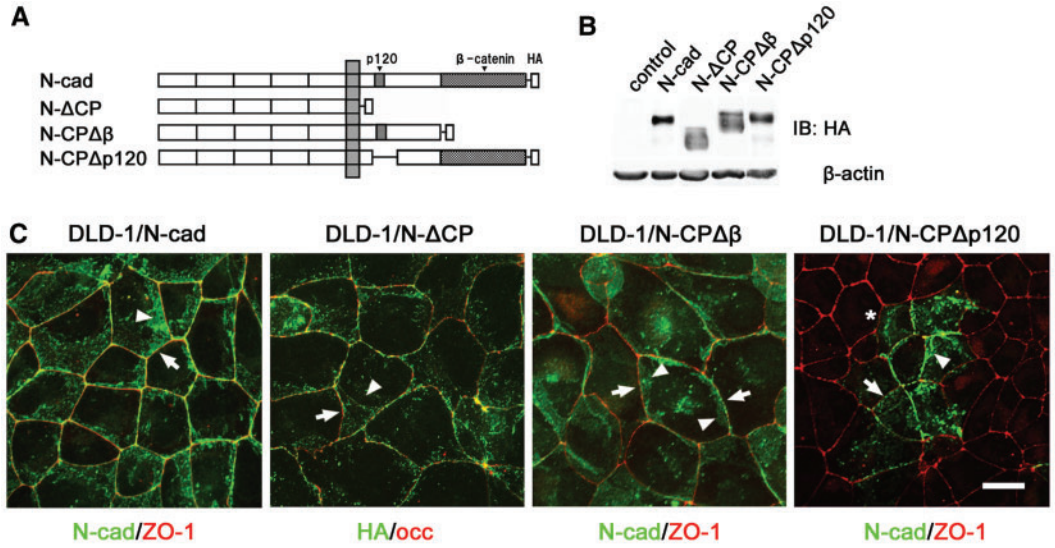

Fig. 6 Expression of mutant N-cadherins in DLD-1 cells. (A) Mutant N-cadherins constructed are schematically shown. Hemagglutinin-tag was linked at the C-termini of these constructs. p120, p120-catenin binding region; β-catenin, β-catenin binding region; HA, hemagglutinin-tag. (B) Expression of mutant N-cadherins was examined by immunoblotting. (C) Immunofluorescence staining of the DLD-1 cell transfectants indicates that wild-type and the mutant N-cadherins show diffuse punctate localization (arrowheads). N-cadherin and N-CPΔp120 exhibit a clear boundary (arrows) between the locations of N-cadherins (N-cad, green) and ZO-1 (red), but the other mutant N-cadherins did not. N-CPΔp120 is not localized at the cell–cell contact sites between DLD-1 transfectants of N-CPΔp120 and wild type DLD-1 cells (asterisk). Scale bar, 20 μm.

and the density of the clusters were variable. For example, the cluster size of cadherin-5 was significantly larger than that of E-cadherin or N-cadherin, and the density of patch-like clusters of E-cadherin in DLD-1 cells was much lower than that in MDCK II cells (Fig. 2A).

Although E-cadherin is localized diffusely in the lateral membrane of various epithelial cells, it should be noted that a large portion of E-cadherin accumulate at the subapical region in some cells such as DLD-1 cells, and N-cadherin is exclusively localized at cell ends of cardiomyocytes and at subapical sites of neuroepithelial cells. Thus, the present results indicate that the scattered punctate accumulation is an intrinsic feature of classical cadherins; but the localization pattern is dependent on the cells expressing the cadherins and cadherin species. Indeed, misexpressed E-cadherin was localized at the cell ends of cardiomyocytes (*33*).

The function of the scattered punctate accumulation of E-cadherin is an intriguing question. Kametani and Takeichi (*19*) recently reported a similar dot-like clustering of cadherin-5 in the lateral membrane of cancer cells. They suggested that the clusters of cadherin-5 represented a transient state and was eventually transported to the subapical region to form adherens junctions. Their model neatly explains the subapical localization of E-cadherin in epithelial cells, but the clusters do not seem to correspond to mere cargos of E-cadherin transported. The present clusters, on the contrary, appear to form intercellular adhesion sites: The clusters were stably present in mature epithelial cells and easily labelled by surface labelling method. Moreover, this notion is consistent with the various reports that most of the apposing lateral membranes of different epithelial cells are intimately connected with a constant narrow gap as shown in Fig. 2C. However, it cannot be completely excluded that the clusters serve as the precursor of adherens junctions at subapical regions in some cases.

The extracellular domains of E- and N-cadherin determine the scattered punctate accumulation and the cytoplasmic domains modulate it

An interesting result of the present study is that the extracellular domain of classical cadherins has intrinsic activity of punctate accumulation in the lateral membrane (Figs 3C and 6C). Although this conclusion may sound slightly odd, it seems reasonable: The extracellular domain has the homophilic interaction site (*8*); and different members of cadherin superfamily accumulate in the lateral membrane, although they have highly divergent cytoplasmic domains.

One may argue that the transmembrane domains may be involved in the localization of classical cadherins. We cannot completely exclude this possibility at the present, but we think that the extracellular domains play a major role in the localization, since EΔCP and NΔCP did not show the localization at the cell–cell contact sites between the transfectant cells of these constructs and their parental cells.

It is generally thought that the cytoplasmic domains of classical cadherins are essential for their functions and β-catenin that binds to the domain plays a key role in it. Indeed, the present results are consistent with the notion: The β-catenin binding site of the cytoplasmic domain was required for the subapical localization of N-cadherin (Fig. 6C) or the formation of adherens junction (Fig. 4). In contrast, the role of p120-catenin has been enigmatic. Meng *et al.* (*20*) reported recently the possible involvement of p120-catenin in the polarized localization of E-cadherin. The present results,

however, did not support the function. Instead, we noticed one possible function that p120-catenin organizes the clusters of classical cadherins: E-CPΔp120 exhibited scattered punctate accumulation in MIA2 cells (Fig. 3C), whereas wild-type E-cadherin showed dense linear accumulation (Fig. 3C). Moreover, E-CPΔβ containing the p120-catenin binding site exhibited continuous accumulation, whereas E-ΔCP lacking the site showed mostly dot-like accumulation (Fig. 3C). This issue should be addressed next.

Accumulation of E-cadherin is necessary but not sufficient for formation of adherens junctions

Another intriguing result of this study is the apparent discrepancy in the locations of E-cadherin accumulation and morphologically identifiable adherens junctions or adherens junction-like structures; *i.e.* E-cadherin accumulated throughout the lateral membrane, but adherens junctions were constructed basically at the subapical region of mature epithelial cells (Figs 1 and 2C). This result raises a basic question as to why concentration of E-cadherin at a site does not necessarily result in the construction of adherens junction, despite the general belief that E-cadherin concentrated at a site indicates the formation of adherens junctions (*12*, *13*).

It is hard to answer clearly this question at the present. One possible explanation may be that adherens junctions are formed only at the sites where E-cadherin becomes concentrated relatively densely as shown in DLD-1 cells. However, dense accumulation of E-cadherin at the subapical site was not always observed in various cells (Fig. 1B, arrows). Alternatively, the clusters of E-cadherin at the subapical site and at the middle or lower sites of the lateral membrane may differ in some properties whereby they form different cell junctions: The formation of adherens junctions in epithelial cells may require the accumulation of not only E-cadherin but also other proteins as essential components. In this connection, it is noteworthy that several proteins such as Scribble, ZO proteins and vinculin were reported to interact with cell junctional complex of E-cadherin and be involved in adherens junction formation (*34*–*37*). On the other hand, complex cell junctions containing classical cadherins have been postulated in endothelial cells and cardiomyocytes (*38*, *39*). Taken together, it may be possible that adherens junctions are heterogeneous and complex junctional structures are formed depending on the tissues. This question should also be addressed further.

Obviously, further experiments are necessary to elucidate the mechanism of the formation and localization of adherens junction and the role of E-cadherin in these processes. However, the present results reveal some new aspects of these processes and provide a basis for future studies.

Supplementary data

Supplementary Data are available at *JB* Online.

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Conflict of interest

None declared.

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