Functional Domains of α-Catenin Required for the Strong State of Cadherin-based Cell Adhesion

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Abstract. The interaction of cadherin-catenin complex with the actin-based cytoskeleton through α-catenin is indispensable for cadherin-based cell adhesion activity. We reported previously that E-cadherin-α-catenin fusion molecules showed cell adhesion and cytoskeleton binding activities when expressed in nonepithelial L cells. Here, we constructed deletion mutants of E-cadherin–α-catenin fusion molecules lacking various domains of α-catenin and introduced them into L cells. Detailed analysis identified three distinct functional domains of α-catenin: a vinculin/α-actinin-binding domain, a ZO-1-binding domain, and an adhesion-modulation domain. Furthermore, cell dissociation assay revealed that the fusion molecules containing the ZO-1-binding domain in addition to the adhesion-modulation domain conferred the strong state of cell adhesion activity on transfectants, although those lacking the ZO-1-binding domain conferred only the weak state. The disorganization of actin-based cytoskeleton by cytochalasin D treatment shifted the cadherin-based cell adhesion from the strong to the weak state. In the epithelial cells, where α-catenin was not precisely colocalized with ZO-1, the ZO-1-binding domain did not completely support the strong state of cell adhesion activity. Our studies showed that the interaction of α-catenin with the actin-based cytoskeleton through the ZO-1-binding domain is required for the strong state of E-cadherin-based cell adhesion activity.

Key words: α-catenin • ZO-1 • vinculin • E-cadherin • adhesion

The cadherins are a family of transmembrane proteins responsible for Ca²⁺-dependent cell–cell adhesion (Takeichi, 1991). Intracellularly, they interact with a group of proteins collectively termed catenins (Ozawa et al., 1989; Nagafuchi et al., 1993). Association with catenins is necessary for cadherins to express their full function as cell adhesion molecules, and this complex is now regarded as a functional unit for cell adhesion. A unique property of this cadherin-catenin complex is its intimate interaction with the actin-based cytoskeleton. At cell-cell contact sites, this complex is colocalized with actin filaments and resists nonionic detergent extraction (Hirano et al., 1987). This complex is a major constituent of intercellular adherens junctions (A J), where actin filaments are densely associated with the plasma membrane through its well-developed plaque structure (Tsukita et al., 1993). Although the cytoskeletal interaction of cadherin-catenin complex is thought to be essential for adhesion, the detailed molecular mechanism of this interaction remains elusive.

α-Catenin associates with the COOH-terminal end of the cadherin cytoplasmic domain (catenin-binding site) via β-catenin. Sequence analysis showed that α-catenin has similarity to vinculin, another constituent of A J (Herrenknecht et al., 1991; Nagafuchi et al., 1991), which interacts with various actin-based cytoskeletal components including actin itself (Jockusch et al., 1995). This suggested that α-catenin may interact with the actin-based cytoskeleton. α-A ctinin, ZO-1, vinculin, and actin itself were reported to interact directly with α-catenin (Rimm et al., 1995; Itoh et al., 1997; Nieset et al., 1997; Watabe-Uchida et al., 1998; Weiss et al., 1998). ZO-1 was identified originally as a tight junction (T J)-associated peripheral membrane protein (Stevenson et al., 1986; A nderson et al., 1988), but it is also concentrated at cadherin-based cell-cell contact sites together with vinculin in nonepithelial cells lacking T J such as fibroblasts and cardiac muscle cells (Itoh et al., 1993).

The functions of α-catenin have been analyzed mainly using two cell lines, the human lung carcinoma cell line PC9, and L cells, a mouse fibroblast cell line. PC9 cells express E-cadherin and β-catenin but not α-catenin. They do...
Materials and Methods

Cells and Antibodies

Mouse L cells were grown in DME supplemented with 10% FCS. Transfectants expressing E-cadherin (EL1) (Nose et al., 1988), nE\textsubscript{150} (1-508), and nE\textsubscript{alpha}(327-906), expression vectors for nE\textsubscript{alpha}(327-906), nE\textsubscript{alpha}(631-906), nE\textsubscript{alpha}(509-643), nE\textsubscript{alpha}(1-325/509-906), and nE\textsubscript{alpha}(1-402/509-906), respectively (see Fig. 1). For construction of these vectors, we used three plasmids: (a) pBATE\textsubscript{alpha}(1-184/509-643)-HA, mouse E-cadherin expression vector (Nose et al., 1988), the Clal-XbaI fragment of which corresponds to the cadherin-binding site and was replaced with the alpha-catenin cDNA fragments in constructs; (b) pSK102B, which contains the alpha-catenin cDNA with a PstI-BglII adaptor inserted into the PstI site just before the initiation methionine codon; and (c) pBATE\textsubscript{alpha}, the Clal-Xbal fragment of pBATE\textsubscript{alpha} was replaced with the Clal-Xbal fragment of pSK102B including the whole ORF of alpha-catenin cDNA (Nagafuchi et al., 1994). In the open reading frame of the alpha-catenin cDNA sequence, we used four restriction sites, PmaCI, Scal, Clal, and Smal, corresponding to amino acid residues 326, 403, 508, and 670, respectively. The Clal site at the 3' terminal of alpha-catenin cDNA in pSK102B was also used. For the production of pBATE\textsubscript{alpha}(327-906), the Clal-Xbal fragment of pBATE\textsubscript{alpha} was replaced with the PmaCI-Xbal fragment of pSK102B. For construction of pBATE\textsubscript{alpha}(631-906), a Clal site (630Clal) was introduced at the position corresponding to amino acid residue 630 of alpha-catenin cDNA in pSK102B by PCR, then the Clal-Xbal fragment of pBATE\textsubscript{alpha} was replaced with the Clal-Xbal fragment of pBATE\textsubscript{alpha}.

We constructed pGEX\textsubscript{alpha}(1-184/509-643)-HA, an EcoRI site (185 EcoRI) was introduced at the position corresponding to amino acid residue 185 of alpha-catenin cDNA in pSK102B by PCR. The BglII-XhoI fragment, ClaI-44Xbal fragment, and a HA and alpha3 fragment, which contains an HA epitope tag sequence, a stop codon, and a 3' noncoding region of alpha-catenin, were tandemly ligated and inserted into the pEfMC1-neo expression vector (Vissader et al., 1992).

For construction of pEGX\textsubscript{alpha}(1-325/509-906), pEGX\textsubscript{alpha}(327-906), and pEGX\textsubscript{alpha}(671-906), expression vectors for GST-alpha-catenin fusion molecules, pEGX102B and pEGX vectors (Pharmacia LKB Biotechnology). For production of pEGX\textsubscript{alpha}(1-325/509-906), pEGX\textsubscript{alpha}(327-906), pEGX\textsubscript{alpha}(671-906), the BglII-ClaI, Clal-Xbal, and Smal-ClaI fragments of pSK102B were inserted into the BamHI-L-Smal sites of pEGX-27, the Smal site of pEGX-3X, and the Smal-KpnI sites of pEGX-4T-3 (Pharmacia LKB Biotechnology), respectively.

Transfection

L cells (5 \times 10^4 per 3-cm plate) were cotransfected with 1 \mu g of each expression vector and 0.05 \mu g of pSPTNeo (Kato et al., 1987) by the lipofectamine method (Life Technologies, Inc.). After 48 h of incubation, the cells were replated on pairs of 9-cm dishes and cultured in the presence of 400 \mu g/ml of G418 to select stable transfectants. Colonies of G418-resistant cells were isolated, recloned, and subsequently maintained in complete medium with 150 \mu g/ml of G418. We isolated several stable clones for each transfection experiment. Since nE\textsubscript{alpha}(327-906)L-1, nE\textsubscript{alpha}(631-906)L-7, nE\textsubscript{alpha}(644-663)-9, nE\textsubscript{alpha}(509-643)L-32, nE\textsubscript{alpha}(1-325/509-906)L-2, and nE\textsubscript{alpha}(1-402/509-906)L-23 clones expressed relatively large amounts of fusion molecules, we mainly used these in this study.

Trypsinized PC9 cells (10^6) were suspended in 500 \mu l of Hepes-buffered (pH 7.4) Ca\textsuperscript{2+} and Mg\textsuperscript{2+}-free saline and mixed with 10 \mu g of expression vector and 1 \mu g of pSPTneoB. Electroporation was performed at 960 \mu F, 250 V. The cells were selected in G418 (0.2 mg/ml)-containing medium.

Immunohistochemistry

All procedures were performed at room temperature. Cells cultured on coverslips were fixed with 3.5% (for hVIN-1 and BM-75.2) or 10% (for T-754) formaldehyde solution in HMF (Hepes-buffered magnesium-saline) for 15 min. After three washes with PBS, cells were soaked in blocking solution (1% BSA in PBS) for 30 min and subsequently incubated with ECD-D2 diluted with PBS containing 1% BSA for 30–60 min at room temperature. The cells were then washed three times with PBS.
and soaked in 0.2% Triton X-100 in PBS for 15 min. After rinsing with PBS, the cells were treated with 1% BSA in PBS for 30 min and subsequently incubated with hVIN-1, T-8754, or BM-75.2 for 30–60 min. After extensive washing with PBS, the specimens were incubated with fluorescein-conjugated secondary antibodies (Cy2- or DTAF-labeled goat anti-rat IgG for ECD-2 and Cy3-labeled donkey anti-mouse IgG [H&L] for hVIN-1, T-8754, and BM-75.2) diluted with PBS containing 1% BSA for 30 min at room temperature. After washing thoroughly with PBS, the preparation was mounted with 90% glycerol PBS containing 0.1% para-phenylendiamine and 1% n-propyglycate. Samples were observed with a Zeiss Axiophot photomicroscope (Carl Zeiss). Images were recorded with a cooled CCD camera (SenSys 0400, 768 × 512 pixels; Photometrics) controlled by a Power Macintosh 7600/132 and the software package IPLab Spectrum V3.1 (Signal A nalytic Corp.).

**SDS-PAGE and Immunoblotting**

SDS-PAGE (10 or 7.5%) and immunoblotting were performed as described previously (Nagafuchi et al., 1994). Samples were solubilized in SDS sample buffer, separated by SDS-PAGE, and gels were stained with Coomassie brilliant blue R-250. For immunoblotting, proteins were electrothermally transferred onto nitrocellulose sheets. Nitrocellulose membranes were then incubated with ECD-2, T-8754, or 12CA5. A nitrotyrosine detection was performed using an A mersham biotin-streptavidin kit with biotinylated anti-rat or anti-mouse Ig and NBT-BCIP.

**In Vitro Binding Assay Using GST Fusion Proteins**

In vitro binding assays were performed as previously described (Itoh et al., 1997). GST-α-catenin fusion proteins were expressed in Echerichia coli and purified using glutathione-Sepharose 4B beads (Pharmacia LKB Biotechnology) as previously described (Itoh et al., 1997). Then, 2 ml of the cell lysate of 5 × 10^6 cells expressing N-ZO-1 was added, followed by incubation for 3 h at 4°C. The beads were again washed with PBS containing 0.1% Triton X-100, 2 mM PMSF, and 4 μg/ml of leupeptin, and then bound proteins were eluted with 1 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM glutathione. The amounts of GST fusion proteins in each eluate were determined by SDSPAGE.

**Detergent Extraction of Cells**

Confluent cultured cells (4 × 10^6 cells per 6-cm dish) were extracted with 2.5% NP-40 in HMF, then centrifuged at 100,000 rpm for 30 min as described previously (Nagafuchi and Takeichi, 1988). To the supernatant, 2× SDS sample buffer was added to make the total volume 0.4 ml and used as the detergent-soluble fraction. On the other hand, the pellet fraction was dissolved in 0.4 ml of 1× SDS sample buffer used and as the detergent-insoluble fraction.

**Trypsin Treatment, Aggregation, and Dissociation of Cells**

Cells were trypsinized by two different methods for the differential removal of E-cadherin or its fusion molecules, as described by Takeichi (1977). In brief, cells were treated with 0.01% trypsin in the presence of 1 mM CaCl₂ (TC treatment) or 1 mM EGTA (TE treatment) at 37°C for 30 min. Generally, cadherins are left intact after TC treatment, but are digested by TE treatment.

For the cell aggregation assay, cells were dispersed after TC treatment as described by Takeichi (1977). L and R/727 transfectants were pretreated with 1 μM cytochalasin D in culture medium for 2 h. A 1 μl of a 10 mg/ml suspension in PBS containing 0.1% Triton X-100 containing 1% BSA was added to each well of a Falcon 12-well plate with 0.5 ml HMF and allowed to aggregate for 3 h at 4°C. The beads were again washed with PBS containing 0.1% Triton X-100, 2 mM PMSF, and 4 μg/ml of leupeptin, and then bound proteins were eluted with 1 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM glutathione. The amounts of GST fusion proteins in each eluate were determined by SDSPAGE.

**Results**

**Involvement of α-Catenin in the Recruitment of Vinculin and ZO-1 to Cadherin-based Cell–Cell Adhesion Sites**

In L cell transfectants expressing E-cadherin, two cytoskeletal proteins, vinculin and ZO-1, are precisely colocalized with E-cadherin at cell–cell contact sites (Itoh et al., 1993). In parental L cells, vinculin is concentrated exclusively at cell-substrate A1, and ZO-1 does not show specialized localization but some condensation at tips of cellular processes (data not shown).

To determine whether α-catenin is involved in the recruitment of vinculin and ZO-1 to E-cadherin–based cell adhesion sites, we used L cell transfectants expressing nEα(1-906), which is a fusion molecule consisting of nonfunctional E-cadherin lacking its catenin-binding domain and full-length α-catenin (Fig. 1B; Nagafuchi et al., 1994). A s previously reported, this molecule showed similar cell adhesion and cytoskeleton interaction activities to the normal E-cadherin–α-catenin complex. Immunocytochemical analysis clearly revealed that both vinculin and ZO-1 were precisely colocalized with nEα(1-906) at cell–cell contact sites in L cell transfectants (Fig. 2). A s reported previously, nonfunctional E-cadherin does not interact with the cytoskeleton and nEα(1-906) is not associated with endogenous β-catenin (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989; Nagafuchi et al., 1994). These observations indicated that α-catenin was crucial for the recruitment of vinculin and ZO-1 to cell–cell contact sites in transfected L cells.

**Involvement of Residues 327–402 of α-Catenin in the Recruitment of Vinculin**

To determine the domain of α-catenin necessary for the recruitment of vinculin or ZO-1, we constructed several expression vectors encoding various E-cadherin–α-catenin fusion molecules, in which distinct domains of α-catenin were deleted (Fig. 1B). These expression vectors were introduced into mouse L cells, and stable transfectant clones were isolated for each construct. Each mutant molecule expressed in transfectants had the expected apparent molecular mass (Fig. 1C).

The subcellular localization of vinculin was compared with those of expressed fusion molecules in various transfectants (Fig. 3). nEα(327–906) in which the NH₂-terminal 326 residues had been truncated was precisely colocalized with vinculin (Fig. 3, a and a'). However, nEα(C509-906) with a longer NH₂-terminal deletion showed no colocalization with vinculin (Fig. 3, b and b'). In cells expressing nEα(C1-508) with truncation of the COOH-terminal 398 residues, expressed fusion molecule was also colocalized with vinculin at sites where it was heavily condensed (data not shown). These observations suggested that residues 327–508 are important for the recruitment of vinculin. Consistently, vinculin was not colocalized with nEα(C1-325/509-906) lacking residues 326–508 (Fig. 3, c and c'). Residues 326–508 of α-catenin include the direct α-actinin-binding site (325–394 residues) reported previously (Nieset al., 1997). When this α-actinin-binding site was added to nEα(1-325/509-906), the resultant fusion molecule nEα(1-402/509-906) retained the ability to colocalize with vinculin.
Double-immunostaining for E-cadherin and α-actinin revealed that the constructs which recruited vinculin, such as nEα(327-906) and nEα(1-402/509-906), could recruit α-actinin (Fig. 4, a, a’, c, and c’). In contrast, nEα(1-325/509-906) was not colocalized with either vinculin or α-actinin (Fig. 4, b and b’). These results demonstrated that residues 327-402 of α-catenin are crucial for the recruitment of vinculin as well as α-actinin to cadherin-based cell adhesion sites of L cell transfectants (see Fig. 11 A).

Involvement of the COOH-terminal Domain of α-Catenin (Residues 631-906) in the Recruitment of ZO-1

To determine the domain(s) of α-catenin involved in the recruitment of ZO-1, we compared the subcellular localization of ZO-1 with that of expressed fusion molecules in transfectants. In contrast with vinculin, ZO-1 was not precisely colocalized with nEαN(1-508) but with nEαC(509-906) (Fig. 5, a, a’, b, and b’). nEα(631-906), which had the longest NH2-terminal deletion and showed no cell adhesion activity (see below), was also colocalized with ZO-1 at cell–cell boundaries (Fig. 5, c and c’), although their condensation at cell–cell boundaries was not as exclusive compared with those in other transfectants. These observations strongly suggested that the COOH-terminal domain of α-catenin (631-906 residues) is crucial for the recruitment of ZO-1 (see Fig. 11 A).

We have reported previously that the NH2-terminal half of ZO-1 (N-ZO-1) directly interacts with α-catenin (Itoh et al., 1997). Therefore, it was expected that N-ZO-1...
would directly interact with the COOH-terminal domain of β-catenin. To test this possibility, we produced four GST fusion proteins, GST-α(1-906), GST-αN(1-508), GST-αC(509-906), and GST-α(671-906), which contained the full-length α-catenin, its NH2-terminal half, its COOH-terminal half, or the COOH-terminal 236 residues, respectively (Fig. 6 A). Then, we analyzed in vitro binding abilities of these fusion molecules with recombinant N-ZO-1 produced in Sf9 cells by baculovirus infection. As shown in Fig. 6 B, N-ZO-1 bound to not only GST-α(1-906) but also GST-αC(509-906) and GST-α(671-906), although the binding affinities to the latter two were lower than that to the former. N-ZO-1 did not bind to GST-αN(1-508). These results strongly suggested that the COOH-terminal 276 amino acids (residues 631-906) of α-catenin recruited ZO-1 to cadherin-based cell–cell contact sites through its direct binding to ZO-1 in transfected L cells.

Involvement of Residues 509-643 of α-Catenin in Cell Adhesion Activity of Fusion Molecules

We have reported previously that nEαC(509-906) shows similar cell adhesion activity to the normal E-cadherin-α-catenin complex (Nagafuchi et al., 1994). To determine the domain which is required for this function, we compared the reaggregative properties of L cell transfectants ex-

Figure 3. Subcellular localization of vinculin and E-cadherin-α-catenin fusion proteins in transfectants expressing nEα(327-906) (a and a'), nEαC(509-906) (b and b'), nEα(1-325/509-906) (c and c'), and nEα(1-402/509-906) (d and d'). Cells were doubly stained with anti-vinculin mAb (a–d) and anti-E-cadherin mAb (a'–d') mixture. Arrows indicate the colocalization of fusion molecules with vinculin. Arrowheads indicate the absence of vinculin at cell–cell boundaries where fusion molecules were condensed. Bar, 25 μm.

Figure 4. Subcellular localization of α-actinin and E-cadherin-α-catenin fusion proteins in transfectants expressing nEα(327-906) (a and a'), nEα(1-325/509-906) (b and b'), and nEα(1-402/509-906) (c and c'). Cells were doubly stained with anti-α-actinin mAb (a-c) and anti-E-cadherin mAb (a'-c'). Arrows indicate the colocalization of fusion molecules with α-actinin. Arrowheads indicate the absence of α-actinin at cell–cell boundaries where fusion molecules were condensed. Bar, 25 μm.

Figure 5. Subcellular localization of ZO-1 and E-cadherin-α-catenin fusion proteins in transfectants expressing nEαN(1-508) (a and a'), nEαC(509-906) (b and b'), and nEα(631-906) (c and c'). Cells were doubly stained with anti-ZO-1 mAb (a-c) and anti-E-cadherin mAb (a'-c'). Arrows indicate the colocalization of fusion molecules with ZO-1. Arrowheads indicate the absence of ZO-1 at cell–cell boundaries where fusion molecules were condensed. Bar, 25 μm.
pressing nE\(\alpha\)C(509-643) and nE\(\alpha\)(631-906); the former was an nE\(\alpha\)C(509-906) derivative lacking the ZO-1-binding domain, and the latter contained only the ZO-1-binding domain (see Fig. 1 B). Cells expressing nE\(\alpha\)C(509-906) aggregated as rapidly as those expressing nE\(\alpha\)C(509-906). In contrast, the reaggregation of cells expressing nE\(\alpha\)(631-906) was indistinguishable from that of parent L cells (Fig. 7 A). These observations suggested that residues 509-643 of \(\alpha\)-catenin are required for cell adhesion activity of fusion molecules. Indeed, when residues 509-643 were added to nE\(\alpha\)N(1-508), cells expressing the resultant fusion molecule nE\(\alpha\)(1-643) aggregated rapidly, although nE\(\alpha\)N(1-508) did not (Fig. 7 A). The levels of expression of nE\(\alpha\)(631-906) or nE\(\alpha\)N(1-508) in the transfectants examined were relatively low among the fusion molecules examined (Fig. 1 C, lanes 4 and 5). However, the reduced level of fusion molecule expression did not seem to be the cause of loss of their cell adhesion activity since L cells expressing similar or lesser amounts of nE\(\alpha\)(1-643) still aggregated rapidly (data not shown). These results demonstrated that residues 509-643 of \(\alpha\)-catenin are required for cell adhesion activity of E-cadherin-\(\alpha\)-catenin fusion molecules in transfected L cells (see Fig. 11 A). We tentatively called this an adhesion-modulation domain (see Fig. 11 B).

Figure 6. Association of \(\alpha\)-catenin with N-ZO-1 in vitro. (A) Schematic representation of fusion molecules between GST and various domains of \(\alpha\)-catenin. The amino acid residues of \(\alpha\)-catenin remaining in fusion molecules are shown in parentheses. (B) Detection of proteins bound to GST-\(\alpha\)(1-906), GST-\(\alpha\)N(1-508), GST-\(\alpha\)C(509-906), and GST-\(\alpha\)(671-906). GST fusion proteins were bound to glutathione-Sepharose beads, and incubated with the lysate of Sf9 cells expressing N-ZO-1. After washing, GST fusion proteins were eluted together with their binding proteins from the beads with a buffer containing glutathione. Proteins in the eluates were separated by SDS-PAGE followed by Coomassie brilliant blue staining to estimate the amounts of GST fusion protein in each eluate (GST fusion), or followed by immunoblotting with anti-ZO-1 mAb, T8-754, to detect N-ZO-1 bound to GST fusion proteins (Bound N-ZO-1).

Figure 7. Aggregation and dissociation assay of L cell transfectants. (A) Aggregation of L cells (open circles) and their transfectants expressing nE\(\alpha\)C(509-906) (closed circles), nE\(\alpha\)(509-643) (open squares), nE\(\alpha\)(631-906) (closed squares), nE\(\alpha\)(1-643) (open triangles), and nE\(\alpha\)N(1-508) (closed triangles). Cells dissociated by TC treatment in the presence of cytochalasin D were allowed to aggregate in the presence of 1 mM Ca\(^{2+}\). The lower value on the ordinate represents the higher degree of aggregation. (B and C) Dissociation of L cell transfectants expressing nE\(\alpha\)C(509-906) (black bar), nE\(\alpha\)(509-643) (hatched bar), and nE\(\alpha\)(1-643) (white bar) in the absence (B) or in the presence (C) of cytochalasin D. The lower value on the ordinate represents the lower degree of dissociation.

The cell adhesion activities of E-cadherin and its \(\alpha\)-catenin fusion molecules were reported to be associated with their interactions with the cytoskeleton (Nagafuchi et al., 1994; Sako et al., 1998). For example, about half of the nE\(\alpha\)C(509-906) was resistant to extraction with NP-40 (Fig. 8) Interestingly, most of the nE\(\alpha\)(509-643) carrying only the adhesion-modulation domain was extracted with NP-40, suggesting that this fusion molecule did not interact with the cytoskeleton (Fig. 8). In contrast, nE\(\alpha\)N(1-508) (data not shown; see Nagafuchi et al., 1994) and nE\(\alpha\)(631-906) (Fig. 8) did not show cell adhesion activity but interacted with the cytoskeleton as judged from their resistance to NP-40 extraction. In the cell aggregation assay, the disorganization of actin-based cytoskeleton by cy-
the cell dissociation assay (Fig. 7B), indicating that these
easily dissociated into single cells under the conditions of
of 200, 116, and 97 kD are indicated on the left.

Imamura et al. demonstrated previously that cells assumed two states
of cadherin-based cell adhesion, strong and weak (Takeda
et al., 1995). The cell-cell adhesion in the strong state could hardly be dissociated by pipetting, although that in
the weak state was easily dissociated. Using the cell disso-
ciation assay, we examined the states of cell adhesion of
various L cell transfectants. As reported previously, L cell
transfectants expressing nE (1-643) were hardly dissociated in the cell dissocia-
tion assay (Fig. 7B; Nagafuchi et al., 1995). The cell–cell adhesion in the strong state
of cadherin-based cell adhesion, strong and weak (Takeda
et al., 1995). R2/7 transfectants expressing α- catenin–deficient colon carci-
noma line. R2/7 transfectants expressing α-catenin dele-
tion mutants were previously reported (Watabe-Uchida
et al., 1998). We used four of these transfectants in this
study. α-Catenin deletion mutants expressed in these
transfectants are shown in Fig. 9A. Using the cell aggrega-
tion assay, we compared the regenerative properties of
R2/7 and its transfectants (Fig. 9B). R2/7 itself showed
aggregation activity which was blocked in the presence
of E-cadherin blocking antibodies (data not shown, see
Watabe-Uchida et al., 1998). R2/7 transfectants expressing
αE (1-890), which is indistinguishable from cells expressing
intact α-catenin, aggregated more rapidly than parental
R2/7 cells. Not only cells expressing αE (1-325/510-890) but
also those expressing αE (1-509), which lacks the adhesion-
modulation domain, also aggregated more rapidly than parental
R2/7 cells. R2/7 cells expressing αE (1-325) showed
similar aggregation activity to the parental cell line R2/7.
These results demonstrated that the residues 325-509 includ-
ing the vinculin/α-actinin-binding domain are also involved
in cadherin-dependent cell aggregation activity in R2/7. In
the cell dissociation assay, R2/7 was readily dissociated but
R2/7 transfectants expressing αE (1-890) were hardly disso-
ciated (Fig. 9C). Although cells expressing αE (1-325/
510-890) showed some degree of resistance to pipetting, the
strong state of cell adhesion activity was not fully re-
stored (Fig. 9C). This partial restoration of the strong
state of cell adhesion activity was also observed in cells ex-
pressing not only αE (1-509) but also αE (1-325) (Fig. 9C).
These results suggested that multiple domains of α-catenin
were required for the strong state of cell adhesion activity in
R2/7.

To confirm the importance of adhesion-modulation do-
main in epithelial cells, we used PC9 cells, a human lung
carcinoma cell line lacking α-catenin expression. It was re-

Figure 8. Detergent extraction of E-cadherin–α-catenin fusion proteins. Soluble (S) and insoluble (I) fractions derived from 2 ×
10⁶ cells expressing nEαC (509-906), nEαC (509-643), nEα (631-906), and nEα (1-643) were separated by SDSPAGE (10%) and
immunoblotted with anti-α-catenin mAb. Several minor bands with lower molecular mass, which may have been degradation
products of fusion molecules, were detected. Cell adhesion activity for each transfectants is represented by + or –. Size markers
of 200, 116, and 97 kD are indicated on the left.

Functions of α-Catenin in Epithelial Cells

We examined the role of α-catenin in epithelial cell adhe-
sion using α-catenin-deficient epithelial cell lines and their
transfectants expressing α-catenin deletion mutants. Since
cadherin-catenin complex is not colocalized with ZO-1 in
epithelial cells, the roles of α-catenin in epithelial cells are
expected to be different, at least in some aspects, from
those in nonepithelial cells such as L cells. DLD-1/R2/7,
abbreviated to R2/7, is an α-catenin-deficient colon carci-
noma line. R2/7 transfectants expressing α-catenin dele-
tion mutants were previously reported (Watabe-Uchida
et al., 1998). We used four of these transfectants in this
study. α-Catenin deletion mutants expressed in these
transfectants are shown in Fig. 9A. Using the cell aggrega-
tion assay, we compared the reaggregate properties of
R2/7 and its transfectants (Fig. 9B). R2/7 itself showed
aggregation activity which was blocked in the presence
of E-cadherin blocking antibodies (data not shown, see
Watabe-Uchida et al., 1998). R2/7 transfectants expressing
αE (1-890), which is indistinguishable from cells expressing
intact α-catenin, aggregated more rapidly than parental
R2/7 cells. Not only cells expressing αE (1-325/510-890) but
also those expressing αE (1-509), which lacks the adhesion-
modulation domain, also aggregated more rapidly than parental
R2/7 cells. R2/7 cells expressing αE (1-325) showed
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ciated (Fig. 9C). Although cells expressing αE (1-325/
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strong state of cell adhesion activity was not fully re-
stored (Fig. 9C). This partial restoration of the strong
state of cell adhesion activity was also observed in cells ex-
pressing not only αE (1-509) but also αE (1-325) (Fig. 9C).
These results suggested that multiple domains of α-catenin
were required for the strong state of cell adhesion activity in
R2/7.

To confirm the importance of adhesion-modulation do-
main in epithelial cells, we used PC9 cells, a human lung
carcinoma cell line lacking α-catenin expression. It was re-
ported that PC9 showed aggregation activity to some extent and that this activity was dependent on E-cadherin-β-catenin complex without α-catenin (Shimoyama et al., 1992). We constructed an expression vector encoding α(1-184/509-643), in which only an adhesion-modulation domain was covalently connected to the NH₂-terminal β-catenin-binding domain of α-catenin (Fig. 10 A). This vector was introduced into PC9 cells, and several transfec-
tant clones were isolated. α(1-184/509-643) with the ex-
pected size was expressed in the transfectants (Fig. 10 B) and colocalized with E-cadherin–β-catenin complex (data not shown). Cell aggregation assay revealed that cells expressing α(1-184/509-643) aggregated more rapidly and more extensively than parental PC9 cells (Fig. 10 C). These aggregates were readily dissociated into single cells under the dissociation assay conditions (data not shown). These observations indicated that an adhesion-modulation domain is involved in the weak state of cell adhesion activity even in epithelial cell lines.

Discussion

It is generally accepted that the cadherin-catenin cell ad-
hesion complex plays fundamental roles not only in the formation of cell–cell junctions but also in the morphogenesis of tissue and organs, dependent on its strong state of cell–cell adhesion activity and its interaction with the actin-based cytoskeleton (Takeichi, 1991). We identified three distinct functional domains of α-catenin required for the interaction with vinculin, for direct binding to ZO-1, and for the adhesion activity of E-cadherin–α-catenin fusion molecules (Fig. 11 B). Here, we will discuss possible functions of each domain of α-catenin and the relationship between the cell adhesion activity and the interaction of α(1-184/509-643), in which only an adhesion-modulation domain was covalently connected to the NH₂-terminal β-catenin-binding domain of α-catenin (Fig. 10 A). This vector was introduced into PC9 cells, and several transfec-tant clones were isolated. α(1-184/509-643) with the ex-
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cadherin–catenin complex with the cytoskeleton. We will also discuss the difference of α-catenin function in nonepithelial and in epithelial cells.

Functional Domains of α-Catenin

ZO-1-Binding Domain. α-Catenin was reported to be directly associated with β-catenin, α-actinin, and actin filaments, and the domains responsible for their binding have been narrowed down on the α-catenin molecule (Rimm et al., 1995; Nieset et al., 1997; Obama and Ozawa, 1997). It was reported recently that ZO-1 bound to α-catenin directly (Itoh et al., 1997), but the domain responsible remained elusive. In this study, using deletion constructs, we showed that the COOH-terminal domain (residues 631-906) of α-catenin recruited ZO-1 to the cell adhesion sites and directly bound to NH2-terminal half of ZO-1 in vitro. We also demonstrated that this ZO-1-binding domain interacted with cytoskeletons judging from the resistance of fusion molecules to NP-40 extraction. It was reported previously that the COOH-terminal halves of ZO-1 are directly associated with actin filaments (Itoh et al., 1997). Based on these properties, we can imagine that the ZO-1-binding domain interact with the actin-based cytoskeleton through ZO-1. We demonstrated that this domain is essential for the strong state of cadherin-based cell adhesion in L cell transfectants, which was dependent on the intact actin-based cytoskeleton. The functional importance of the ZO-1-binding domain was also reported using mouse embryos expressing mutant α-catenin (Torres et al., 1997). Taken together, we concluded that the ZO-1-binding domain (COOH-terminal 276 residues) of α-catenin plays a fundamental role in the cadherin–catenin cell adhesion system probably through its interaction with ZO-1 and/or the actin-based cytoskeleton.

We cannot exclude the possibility that other cytoskeletal proteins interact with the ZO-1-binding domain. This domain is known to interact with actin in vitro (Rimm et al., 1995). The physiological role of this interaction remains to be elucidated. The ZO-1-binding domain was also shown to directly interact with vinculin in vitro (Weiss et al., 1998). However, we found that the ZO-1-binding domain in fusion molecules did not recruit vinculin to the cell–cell boundaries in L cell transfectants. Since the reported binding constant of the ZO-1-binding domain to vinculin was lower than that to ZO-1 (Itoh et al., 1997; Weiss et al., 1998), the binding of vinculin to the ZO-1-binding domain may be prevented by ZO-1 in L cell transfectants. ZO-2
and ZO-3, homologues of ZO-1, are other candidates as binding proteins to the ZO-1-binding domain. In fact, it was reported recently that ZO-2 showed very similar properties with ZO-1 and directly bound to \(\alpha\)-catenin (Itoh et al., 1999). However, these proteins are not involved in the cadherin-based cell adhesion in L cell transfectants, since their expression was not detected in L cell transfectants (our unpublished observation).

**Domain Required for the Recruitment of Vinculin.** We found that residues 327-402 of \(\alpha\)-catenin were required for fusion molecules to recruit not only \(\alpha\)-actin but also vinculin in transfected L cells. This is consistent with previous data that this domain directly binds to both vinculin (Watabe-Uchida et al., 1998) and \(\alpha\)-actin (Nieset et al., 1997), and these two molecules interact with each other (Wachsstock et al., 1987). It is not clear whether vinculin and \(\alpha\)-actin interact with this short domain with 76 residues simultaneously or competitively in vivo. E-cadherin-\(\alpha\)-catenin fusion molecules conferred full adhesion activity in L cell transfectants even if they lacked the vinculin/\(\alpha\)-actinin-binding domain. This raised the question of what is the function of the vinculin/\(\alpha\)-actinin-binding domain. We reported previously that intact E-cadherin conferred a flexible adhesive phenotype upon L cells, but E-cadherin-\(\alpha\)-catenin fusion molecules conferred inflexible phenotypes (Nagafuchi et al., 1994). If the vinculin/\(\alpha\)-actinin-binding domain is involved in this flexible adhesion activity, its function would not be observed using E-cadherin-\(\alpha\)-catenin molecules expressed in L cells. On the other hand, it was reported recently that this domain is involved in the organization of apical junctional complex and the activation of cadherin-based cell adhesion in epithelial cells (Watabe-Uchida et al., 1998). It has been reported also that vinculin is colocalized with the cadherin-catenin complex in epithelial cells but not in some fibroblastic cell lines (Knudsen et al., 1995), and that vinculin is one of the major components of cell–cell AJs in epithelial cells (Geiger et al., 1980). These observations suggest that the vinculin/\(\alpha\)-actinin-binding domain is required for the function of cadherin–catenin complex, especially for junctional complex formation, only in epithelial cells but not in fibroblastic cells.

**Functional Domain Involved in Cell Adhesion Activity.** Deletion constructs showed that residues 509-643 of \(\alpha\)-catenin are required for fusion molecules to function as cell adhesion molecules. We tentatively called this domain an adhesion-modulation domain. When mutant \(\alpha\)-catenin containing the \(\beta\)-catenin-binding and the adhesion-modulation domains was expressed in \(\alpha\)-catenin-deficient PC9 cells, such cells aggregated more rapidly than parental PC9 cells. These results suggested that the adhesion-modulation domain is involved in cell adhesion in the “natural” cadherin/catenin complex.

Although several sites of \(\alpha\)-catenin were reported to be required for the interaction with the cytoskeleton, the adhesion-modulation domain does not correspond to these cytoskeletal interaction sites. Moreover, the fusion molecule carrying only this domain was easily extracted with NP-40, suggesting that this molecule did not interact with the cytoskeleton. These findings indicated that the adhesion-modulation domain might function without the interaction with the cytoskeleton. It has been accepted that the insoluble fraction of E-cadherin was active in cell adhesion and the soluble one was not, since E-cadherin molecules which could not be extracted with NP-40 were strictly localized at cell–cell contact sites (Nagafuchi and Takeichi, 1988). However, our present results suggested that some fraction of soluble E-cadherin–catenin complex was also active in cell adhesion.

The molecular mechanism of the activation of E-cadherin extracellular domain remains unclear. One simple interpretation is that the adhesion-modulation domain supports the lateral aggregation of E-cadherin molecules, which may mediate the weak state of cell adhesion. Alternatively, this domain may trigger off the other adhesion-modulation system. Fusion molecules used contained the membrane proximal, p120-binding domain of E-cadherin (Yap et al., 1998) and are colocalized with endogenous p120 protein in transfected L cells (our unpublished observation). It was reported that this membrane proximal domain might positively or negatively regulate cadherin-based cell adhesion (Ozawa and Kemler, 1998; Yap et al., 1998). It is possible that the adhesion-modulation domain of \(\alpha\)-catenin affects the potential activity of the membrane proximal domain of E-cadherin.

Some fusion molecules lacking the adhesion-modulation domain were detected at cell–cell boundaries in transfectants, although nonfunctional E-cadherin itself was not (Nagafuchi and Takeichi, 1988). These observations raised the question of how these fusion molecules were condensed at cell–cell boundaries, although they did not function as cell adhesion molecules. The main difference between these fusion molecules and nonfunctional E-cadherin is that the former interacted with cytoskeletal proteins such as ZO-1 or vinculin/\(\alpha\)-actinin but the latter did not. As discussed below, ZO-1 is likely to facilitate the lateral aggregation of its membrane binding partners. Vinculin is also expected to form clusters through its interaction with various cytoskeletal components (Otto, 1990). So, the interaction with cytoskeletal components may cause the clustering of fusion molecules in the plasma membrane, which then induces the association of cadherin complexes on apposed cell membranes (Shapiro et al., 1995).

**Relationship between Interaction with the Cytoskeleton and Adhesion Activity of Cadherin–Catenin Complex**

As previously reported, cadherin-based cell adhesion can be classified into the strong state and the weak state, using cell dissociation and aggregation assays (Takeda et al., 1995). In the cell aggregation assay, cells form aggregates in both adhesive states. In the cell dissociation assay, however, cells in the strong state were hardly dissociated into single cells but those in the weak state were dissociated readily. It is known that the adhesive state is regulated by the phosphorylation level of the cytoplasmic components (Matsuyoshi et al., 1992; Behrens et al., 1993). We also demonstrated that disorganization of the actin-based cytoskeleton shifted the cadherin-based cell adhesion from the strong to the weak state. Since the level of cadherin expression on the cell surface was not affected in either case, the strong state and the weak state may reflect qualitative differences in cell adhesion activity but not quantitative differences in cell adhesion molecules. The present results...
are consistent with this idea, since cells in both adhesive states aggregated in a similar manner.

We found that all of the fusion molecules that showed the strong state of cell adhesion activity interacted with the cytoskeleton, suggesting that the interaction with the cytoskeleton is required for the strong state of cell adhesion activity. This was supported by the present observation that cytochalasin D treatment shifted cadherin-based cell adhesion to the weak state. Interestingly, the fusion molecule lacking only the ZO-1-binding domain showed the weak state of cell adhesion activity, although it contained a vinculin/α-actinin-binding domain and may interact with the cytoskeletons, judging from the refractoriness to NP-40 extraction. Thus, we concluded that the cytoskeleton interaction through the ZO-1-binding domain, but not through the vinculin/α-actinin binding domain or other domain(s), is required for the strong state of cell adhesion activity in L cell transficients. As discussed above, ZO-1 and actin are possible binding proteins to this domain in L cell transficients. ZO-1 is a member of the MAGUK family. A member of this family, PSD 95, is known to facilitate the lateral aggregation of its membrane binding partners such as NMDA receptors and K⁺ channels (Kim et al., 1995; Niethammer et al., 1996). Thus, it is possible that ZO-1 strengthens the cell–cell adhesion activity not only by cross-linking α-catenin to actin filaments (Itoh et al., 1997) but also by facilitating the lateral aggregation of E-cadherin or its fusion molecules in L cell transficients. The role of actin binding to this domain remains unclear. Furthermore, we cannot exclude the possibility that unknown factor(s) interacted with the ZO-1-binding domain and supported the strong state of cell adhesion activity.

Function of α-Catenin in Epithelial Cells

At the immunoelectron microscopic level, cadherin–catenin complex is known to be colocalized with ZO-1 in nonepithelial cells such as L cells but not in epithelial cells. In epithelial cells, ZO-1 is highly condensed at the TJ and is functionally used in nonepithelial and epithelial cells. We found that all of the fusion molecules that showed the strong state of cell adhesion activity interacted with the cytoskeleton, suggesting that the interaction with the cytoskeleton is required for the strong state of cell adhesion activity. This was supported by the present observation that cytochalasin D treatment shifted cadherin-based cell adhesion to the weak state. Interestingly, the fusion molecule lacking only the ZO-1-binding domain showed the weak state of cell adhesion activity, although it contained a vinculin/α-actinin-binding domain and may interact with the cytoskeletons, judging from the refractoriness to NP-40 extraction. Thus, we concluded that the cytoskeleton interaction through the ZO-1-binding domain, but not through the vinculin/α-actinin binding domain or other domain(s), is required for the strong state of cell adhesion activity in L cell transficients. As discussed above, ZO-1 and actin are possible binding proteins to this domain in L cell transficients. ZO-1 is a member of the MAGUK family. A member of this family, PSD 95, is known to facilitate the lateral aggregation of its membrane binding partners such as NMDA receptors and K⁺ channels (Kim et al., 1995; Niethammer et al., 1996). Thus, it is possible that ZO-1 strengthens the cell–cell adhesion activity not only by cross-linking α-catenin to actin filaments (Itoh et al., 1997) but also by facilitating the lateral aggregation of E-cadherin or its fusion molecules in L cell transficients. The role of actin binding to this domain remains unclear. Furthermore, we cannot exclude the possibility that unknown factor(s) interacted with the ZO-1-binding domain and supported the strong state of cell adhesion activity.

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At the immunoelectron microscopic level, cadherin–catenin complex is known to be colocalized with ZO-1 in nonepithelial cells such as L cells but not in epithelial cells. In epithelial cells, ZO-1 is highly condensed at the TJ and is thought to directly interact with TJ membrane proteins including occludin (Furuse et al., 1994). These results suggested that the ZO-1-binding domain of α-catenin may have different functions in nonepithelial and in epithelial cells. In fact, it was demonstrated that this domain could not cause redistribution of ZO-1 in R2/7, an epithelial colon carcinoma cell line (Watabe-Uchida et al., 1998). It remains unclear why the ZO-1-binding domain does not associate with ZO-1 and how this domain functions in epithelial cells. Further studies to address this question will provide important information regarding the mechanisms of junctional complex formation in epithelial cells.

It was reported recently that the vinculin/α-actinin-binding domain directly binds to vinculin and this interaction functions to organize the apical junctional complex, including occludin (Furuse et al., 1994). These results suggested that the ZO-1-binding domain of α-catenin may have different functions in nonepithelial and in epithelial cells. In fact, it was demonstrated that this domain could not cause redistribution of ZO-1 in R2/7, an epithelial colon carcinoma cell line (Watabe-Uchida et al., 1998). It remains unclear why the ZO-1-binding domain does not associate with ZO-1 and how this domain functions in epithelial cells. Further studies to address this question will provide important information regarding the mechanisms of junctional complex formation in epithelial cells.

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