The Membrane-proximal Region of the E-Cadherin Cytoplasmic Domain Prevents Dimerization and Negatively Regulates Adhesion Activity

Masayuki Ozawa* and Rolf Kemler‡

*Department of Biochemistry, Faculty of Medicine, Kagoshima University, Kagoshima 890-8520, Japan; and ‡Max-Planck-Institut für Immunbiologie, Abteilung Moleculare Embryologie, D-79108 Freiburg, Germany

Abstract. Cadherins are transmembrane glycoproteins involved in Ca\textsuperscript{2+}-dependent cell–cell adhesion. Deletion of the COOH-terminal residues of the E-cadherin cytoplasmic domain has been shown to abolish its cell adhesive activity, which has been ascribed to the failure of the deletion mutants to associate with catenins. Based on our present results, this concept needs revision. As was reported previously, leukemia cells (K562) expressing E-cadherin with COOH-terminal deletion of 37 or 71 amino acid residues showed almost no aggregation. Cells expressing E-cadherin with further deletion of 144 or 151 amino acid residues, which eliminates the membrane-proximal region of the cytoplasmic domain, showed E-cadherin–dependent aggregation. Thus, deletion of the membrane-proximal region results in activation of the nonfunctional E-cadherin polypeptides. However, these cells did not show compaction. Chemical cross-linking revealed that the activated E-cadherin polypeptides can be cross-linked to a dimer on the surface of cells, whereas the inactive polypeptides, as well as the wild-type E-cadherin polypeptide containing the membrane-proximal region, can not. Therefore, the membrane-proximal region participates in regulation of the adhesive activity by preventing lateral dimerization of the extracellular domain.

Key words: cadherin • catenin • compaction • adhesion • aggregation

The cadherins are a family of Ca\textsuperscript{2+}-dependent transmembrane proteins that play essential roles in the initiation and stabilization of cell–cell contacts (Takeichi, 1991; Kemler, 1993; Gumbiner, 1996; Marrs and Nelson, 1996). The extracellular domain of cadherins is responsible for specific homophilic binding (Nose et al., 1990), while the conserved cytoplasmic domain interacts with intracellular proteins, termed catenins (Ozawa et al., 1989, 1990; Ozawa and Kemler, 1992; Stappert and Kemler, 1994). Each cadherin molecule can bind to either β-catenin or plakoglobin (γ-catenin), which in turn binds to α-catenin (Aberle et al., 1994; Hinck et al., 1994; Hülsken et al., 1994; Jou et al., 1995). α-Catenin is an actin-binding protein (Rimm et al., 1995) and interacts with other actin-binding proteins, i.e., α-actinin (Nieset et al., 1997) and ZO-1 (Itoh et al., 1997). These interactions link cadherins to the actin cytoskeleton. Binding of the cadherin–catenin complexes to the actin cytoskeleton has been proposed to be essential for the binding activity. Deletion or truncation of the cytoplasmic domain of cadherin results in a loss of function, in spite of its continued expression on the cell surface (Nagafuchi and Takeichi, 1988, 1989; Ozawa et al., 1990). Additionally, cells expressing normal E-cadherin but lacking α-catenin do not aggregate (Shimoyama et al., 1992), and cell–cell adhesion can be restored by transfection of these cells with the α-catenin cDNA (Hirano et al., 1992; Watabe et al., 1994). These observations led to the conclusion that the extracellular segment alone is insufficient to mediate detectable homophilic adhesion.

Contrary to this model, however, evidence exists that in special situations the extracellular part of cadherins might retain (acquire) some biological activity in the absence of the catenin linkage. A chimeric molecule consisting of the extracellular part of E-cadherin, and the transmembrane and intracellular parts of N-CAM is able to function in adhesion (Jaffe et al., 1990). Likewise, the transmembrane and intracellular domains of desmoglein 3 together confer the adhesive function on the extracellular domain of E-cadherin (Roh and Stanley, 1995). Neither of these chimeric molecules uses catenins to mediate its adhesive
E-cadherin, which is anchored to the membrane by a glycosyl phosphatidylinositol group instead of a transmembrane domain, also does not interact with catenins, but can still mediate cell–cell adhesion to some extent (Vestal and Ranscht, 1992). Furthermore, LI-cadherin, which does not associate with catenins, and a glycosyl phosphatidylinositol–anchored form of LI-cadherin are able to induce cell–cell adhesion (Kreft et al., 1997).

A proteolytic fragment of the extracellular segment of E-cadherin has been shown to disrupt cell–cell contacts (Wheelock et al., 1987). Furthermore, N-cadherin–expressing cells were shown to preferentially attach to a purified, extracellular, proteolytic fragment of N-cadherin (Paradies and Grunwald, 1993). Neurite outgrowth (Bixby and Zhang, 1990) and astrocyte spreading (Payne and Lemmon, 1993) occur on purified N-cadherin. Finally, adhesive binding of C-cadherin–expressing cells to a recombinant C-cadherin extracellular domain has been observed (Brieher et al., 1996). These results indicate that isolated cadherin proteins, and even the extracellular segment alone, retain some degree of functional activity.

During the cloning of K562 cells transfected with an E-cadherin expression vector, we obtained a cell clone expressing a truncated E-cadherin polypeptide of 86 kD on the cell surface. Surprisingly, cells expressing the truncated protein showed E-cadherin-dependent cell aggregation. As was found using L cells (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989), mutant E-cadherin polypeptides with COOH-terminal deletion of 37 and 71 amino acid residues, respectively, expressed on K562 cells are nonfunctional, i.e., cells expressing these proteins can not form aggregates (Ozawa and Kemler, 1998). The 86-kD truncated protein failed to react with pan-cadherin antibodies, suggesting that the intracellular portion of the E-cadherin protein was missing, and the protein was much smaller than the mutant E-cadherin polypeptide with the COOH-terminal deletion of 71 amino acid residues. These findings suggested that further deletion of the cytoplasmic domain of E-cadherin activates the partially truncated nonfunctional E-cadherin. Therefore, we examined further the effects of COOH-terminal deletions of E-cadherin on the adhesive activity, and found that both an E-cadherin mutant polypeptide with a cytoplasmic domain of 7 amino acid residues and a completely tail-less E-cadherin protein was missing, and the protein was much smaller than the mutant E-cadherin polypeptide which does not associate with catenins, and a glycosyl phosphatidylinositol group instead of a transmembrane domain negatively regulates its activity.

Materials and Methods

cDNA Construction

The mammalian expression vector containing E-cadherin cDNA encoding either the wild-type or a mutant protein, E4C37 (Ozawa et al., 1989), was described previously (Ozawa and Kemler, 1998). The cDNA encoding another E-cadherin mutant protein, E4C71 (Ozawa et al., 1989), was cloned into the same expression vector, pcAGGSneo (Niwa et al., 1991) (a gift from Dr. K. Yamamura, Kumamoto University, Kumamoto, Japan).

We generated truncation mutants by placing a stop codon at residue Arg170, Glu280, Asp227, or Gly321 in the E-cadherin cDNA, producing mutant E-cadherin proteins, EC0, EC7, EC18, and EC43, respectively (see Fig. 1). For this, the polymerase chain reaction was carried out using Pwo DNA polymerase (Boehringer Mannheim GmbH, Mannheim, Germany), E-cadherin cDNA as a template, and the following four combinations of a sense primer (X1) and an antisense primer: X1 (TATACCGCTC- GAGGCCG) and C0 (ATCATAGAAACATAGGAGCAG), X1 and C7 (ATCATTTGACCCGGTTCCTC), X1 and C18 (ATCAC- CGGTTATCATCATCTG), and X1 and C43 (ATCACCTGTCG- TGGTCT). The sense primer contained a XhoI restriction sequence, whereas the antisense primers contained a sequence (TCA) near the 5′ end to introduce a termination codon. The respective cDNA fragment was cloned into the XhoI–EcoRV site of the pBluescript II KS(+) vector after digestion with XhoI, and the sequence was confirmed by sequencing. The expression vectors for the mutant E-cadherin proteins were constructed by replacing the XhoI–EcoRV fragment of the E-cadherin cDNA that encodes the COOH-terminal 373 amino acids including the transmembrane and cytoplasmic domains as well as a part of the extracellular domain of E-cadherin in the expression vector for the wild-type E-cadherin with the cDNA fragments generated by means of the polymerase chain reaction described above.

Cells and Transfection

Human leukemia K562 cells (provided by Dr. K. Sekiguchi, Research Institute, Osaka Medical Center for Maternal and Child Health, Osaka, Japan) were grown in DME supplemented with 10% FCS. K562 cells (5 × 10⁴) were transfected with the expression vectors (10 µg) by electroporation as described previously (Ozawa and Kemler, 1998) using a Bio-Rad Gene Pulser (Heracles, CA) set at 280 V and 960 µF. G418-resistant clones were isolated by limiting dilution and examined for E-cadherin expression by immunofluorescence staining as described previously (Ozawa et al., 1989). Positive cells were subcloned and used for further studies.

Cell Aggregation Assay

The cell aggregation assay was performed as described previously (Ozawa et al., 1990), except that the cells were passed through Pasteur pipettes several times to obtain single cells. After incubation, the cells were fixed by adding an equal volume of 6% PFA in PBS.

Antibodies

mAbs against α-, β-, and γ-catenin, and p120 (ppl20) were purchased from Transduction Laboratories (Lexington, KY). DECMA-1, a mAb to E-cadherin (Vestweber and Kemler, 1985), was used for immunoblotting and immunofluorescence staining, and rabbit anti-E-cadherin antibodies (Ozawa et al., 1989) were used for immunoprecipitation.

Immunoblotting and Immunoprecipitation

For immunoblot analysis, cells (10⁴) were boiled for 5 min in SDS gel sample buffer (Laemmli, 1970), run on 8% polyacrylamide gels, and then electroblotted onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS, and then incubated with mAbs and finally with peroxidase-conjugated antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). After washing with PBS containing 0.1% Tween-20, the protein bands were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham International, Little Chalfont, UK). Immunoprecipitation was carried out as described previously (Ozawa et al., 1989) with the following modifications. The cells (10⁴) were labeled with 50 µCi/ml of [35S]methionine (DuPont-NEN, Boston, MA) in DME without methionine, containing 10% dialyzed FCS (GIBCO BRL, Gaithersburg, MD), for 16 h. After washing with PBS, the cells were lysed in 10 mM Tris-HCl buffer, pH 7.6, containing 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1 mM CaCl₂, 0.1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, and 25 µg/ml aprotinin. The E-cadherin–catenin complex was collected with rabbit anti-E-cadherin antibodies, which had been preabsorbed to protein A-Sepharose CL4B (Pharmacia Biotech, Inc., Piscataway, NJ). The immunocomplex was washed with the same buffer four times and then boiled for 5 min in the SDS-PAGE sample buffer.

Chemical Cross-Linking and Two-Dimensional (Nonreducing/Reducing) SDS-PAGE

After dissociation by pipetting, cells were washed twice with PBS, and then incubated with the indicated concentrations of 3,3′-dithiothreitol/sulfo-
succinimidylpropionate) (DTSSP) or dithiobis(succinimidylpropionate) (DSP) (Pierce Chemical Co., Rockford, IL) for 30 min at room temperature. The cells were washed twice with PBS containing 50 mM NH4Cl, boiled in the SDS-PAGE sample buffer without reducing reagents, and then subjected to SDS-PAGE on 8% acrylamide gels (nonreducing conditions) and immunoblot analysis with DECMA-1. For two-dimensional SDS-PAGE analysis, cells labeled with [35S]methionine were incubated with 50 μg/ml of DTSSP as above, lysed with the lysis buffer, and then subjected to immunoprecipitation with E-cadherin antibodies. The immunoprecipitates were boiled in the SDS-PAGE sample buffer containing 5% 2-mercaptoethanol for 1 h at room temperature and then subjected to SDS-PAGE on 8% acrylamide gels (second dimension).

**Immunofluorescence Staining**

Cells were fixed with 3% PFA in PBS for 20 min at room temperature. After three washes with PBS containing 50 mM NH4Cl, the cells were soaked in a blocking solution (PBS containing 5% FCS) for 15 min, and then incubated with DECMA-1 diluted with the blocking solution for 30 min. The cells were then washed three times with PBS and incubated with FITC-conjugated rabbit anti–rat IgG (Jackson ImmunoResearch Laboratories).

**Results**

**Deletion of the Membrane-proximal Region Activates Partially Truncated Nonfunctional E-Cadherin Polypeptides**

The wild-type E-cadherin has a cytoplasmic domain of 151 amino acids at its COOH terminus. Deletion of the E-cadherin COOH-terminal 37, 50, or 71 amino acid residues has been reported by several different research groups to cause loss of the ability of E-cadherin to mediate aggregation (Nagafuchi and Takeichi, 1988; Jaffe et al., 1990; Ozawa et al., 1990; Roh and Stanley, 1995). To examine the possibility that the removal of the membrane-proximal region of the E-cadherin cytoplasmic domain activates these nonfunctional E-cadherin polypeptides, we generated a truncation mutant by placing a stop codon at residue Arg578 in E-cadherin cDNA, producing a mutant E-cadherin protein, EC0, which completely lacks the cytoplasmic tail; EK, K562 cells expressing mutant E-cadherin lacking the cytoplasmic tail; EC0K, K562 cells expressing the wild-type E-cadherin.

Figure 1. Schematic representation of the transmembrane and cytoplasmic domains of E-cadherin, and the mutant polypeptides with deletions of the 15-amino acid cytoplasmic domain. EC37 and EC71 are mutant E-cadherin polypeptides with COOH-terminal deletions of 37 and 71 amino acid residues, respectively. EC43, EC18, and EC7 are mutant E-cadherin polypeptides with COOH-terminal deletions of 108, 133, and 151 amino acid residues, respectively. Thus, EC43, EC18, and EC7 retain 43, 18, and 7 NH2-terminal residues of the cytoplasmic domain whereas EC0 completely lacks the cytoplasmic domain.

1. Abbreviations used in this paper: DSP, dithiobis(succinimidylpropionate); DTSSP, 3,3’-dithiobis(sulfosuccinimidylpropionate); EC0K, K562 cells expressing mutant E-cadherin lacking the cytoplasmic tail; EK, K562 cells expressing E-cadherin.
EC43 construct were stained weakly (data not shown). Furthermore, the expression of these mutant polypeptides seemed to be unstable, since two types of cells, one positive and the other negative for DECMA-1 staining, were present even after recloning of the cells. The reason for the instability of these polypeptides is unknown at present. The unstable expression of mutant E-cadherin polypeptides, one with the COOH-terminal deletion of 135 amino acids, 16 amino acids of the cytoplasmic domain thus remaining, and the other containing the extracellular domain of E-cadherin, and the transmembrane and intracytoplasmic domains of desmoglein 3, has been reported by others (Nagafuchi and Takeichi, 1988; Roh and Stanley, 1995).

Figure 3. Aggregation of K562 cells expressing the mutant E-cadherin polypeptides. K562 cells expressing the wild-type E-cadherin (EK cells) or the mutant polypeptides with deletions of the cytoplasmic domain as shown in Fig. 1 were lysed in SDS sample buffer, and then subjected to immunoblot analysis with DECMA-1, an E-cadherin mAb.

Cells expressing these deletion polypeptides were subjected to aggregation assays (Fig. 3). EC7K cells formed aggregates in an E-cadherin–dependent manner (Fig. 3). In contrast, K562 cells expressing EC18 and EC43, like those expressing EΔC35 and EΔC71, did not show aggregation activity (data not shown). As described above, the amounts of the mutant polypeptides (EC18 and EC43) expressed on K562 cells were much smaller compared with those of the other mutant polypeptides. Since the extent of aggregation depends on the amount of cadherin polypeptides expressed on cells (Nose et al., 1988), it is premature to conclude that these polypeptides are inactive.

**Adhesive Properties of the Tail-less E-Cadherin Transfectants**

To determine whether or not a lack of association with the actin cytoskeleton affects adhesive strength, EK cells and EC0K cells were subjected to aggregation assays with different shear forces. Generally, the number of collisions between cells in suspension is proportional to the rotational speed imposed upon the suspension. However, as the speed increases, the shear forces that disrupt new aggregates also become greater. In assays in which intercellular collisions were generated by means of rotational forces of 70-180 rpm, the EC0 polypeptide was as efficient in promoting the aggregation of transfectants as the wild-type molecule in any individual assay (Fig. 4a).

The kinetics of E-cadherin–mediated aggregation were measured over a time course of 60 min using a rotational force of 70 rpm. In both EK and EC0K cells, aggregation occurred very rapidly in a single exponential step (Fig. 4b). After 45 min incubation, the degree of aggregation approached the maximum. As was reported previously, the cadherin-mediated aggregation was sensitive to low temperature (Takeichi, 1977; Angres et al., 1996). The aggregation of both EK cells and EC0K cells was totally inhibited at 4°C (Fig. 4b).

**Lack of Compaction in Aggregates of Cells Expressing the Tail-less E-Cadherin Polypeptides**

Cadherin-mediated cell aggregation is accompanied by a morphological change known as “compaction” (Takeichi, 1977; Hyafil et al., 1980). The aggregates of EK cells showed extensive compaction, i.e., cells adhered tightly to each other, changing in shape to maximize the contact areas (Fig. 5, a and c). The aggregates of EC0K cells did not appreciably show such a morphological change, and that
each cell in the aggregates remained round and was easily distinguishable (Fig. 5, b and d).

The Functional Tail-less E-Cadherin Polypeptides Are Cross-Linked to a Dimer, but the Nonfunctional E-Cadherin and Wild-Type E-Cadherin Polypeptides Are Not

Recent studies suggested that lateral dimerization is required for the binding activity of cadherins (Shapiro et al., 1995; Brieher et al., 1996; Nagar et al., 1996). Therefore, we reasoned that the conserved membrane-proximal region might be involved in the regulation of cadherin activity by preventing dimerization of its extracellular domain. To examine this possibility, we analyzed the oligomeric state of the mutant E-cadherin polypeptides using the homobifunctional cross-linking reagents, DTSSP, and DSP. Both reagents react covalently with amino groups, and their internal disulfide bond can be cleaved by reducing reagents. The incubation of EC0K cells after dissociation by pipetting with DTSSP resulted in the formation of a band corresponding to 170 kD recognized by E-cadherin antibodies (Fig. 6). A cross-linked product of the same molecular weight was also obtained on incubation of EC0K cells with DSP at concentrations of 20–50 μg/ml (data not shown). The incubation of EK or EAC71K cells with DTSSP did not, however, result in the formation of a high molecular weight band (Fig. 6). The molecular weight (170 kD) of the cross-linked product is consistent with the molecular weight of an EC0 dimer. Similar analysis of cells expressing another functional mutant E-cadherin polypeptide, EC7, showed the presence of a high molecular weight cross-linked product of 175 kD (data not shown).

To confirm that the high molecular weight cross-linked product is indeed a dimer of the EC0 polypeptide, we performed two-dimensional (nonreducing/reducing) SDS-PAGE. Cells labeled with $[^{35}S]$methionine were lysed after chemical cross-linking, and then subjected to immunoprecipitation with E-cadherin antibodies. The immunocomplex was run on the first gel under nonreducing conditions, gel strips containing the material were incubated with 2-mercaptoethanol to cleave the intramolecular disulfide bond of the cross-linker, and then the complex was run on the second gel under reducing conditions. Under nonreducing conditions, a cross-linked product of 170 kD was detected together with the 86-kD EC0 monomer (Fig. 7 a). On reduction, the 170-kD product yielded a single 86-kD polypeptide species, which co-migrated with the EC0 monomer (Fig. 7 b), thus demonstrating that the 170-kD product is a dimer of the EC0 polypeptide.

p120 Is Associated with Partially Truncated Nonfunctional E-Cadherin Polypeptides

We next reasoned that the lack of adhesion activity and the failure to obtain cross-linked dimers of the partially truncated E-cadherin polypeptides were due to association of the mutant polypeptides with some cellular component(s). This possible complex formation was studied by means of communoprecipitation experiments involving EK cells, EAC71K cells, and EC0K cells. Cells were metabolically labeled with $[^{35}S]$methionine, and the E-cadherin polypeptides were immunoprecipitated from cell lysates with E-cadherin antibodies. As described previously (Ozawa...

![Image](https://example.com/image.png)
and Kemler, 1998), three proteins migrating to positions corresponding to 102, 88, and 82 kD were coprecipitated with E-cadherin (120 kD) in the case of EK cells (Fig. 8a). These coprecipitated proteins were identified as α-catenin, β-catenin, and plakoglobin by subjecting the immunoprecipitates to immunoblot analysis with the respective antibodies (Ozawa and Kemler, 1998). In the case of EΔC71K cells and ECOK cells, the respective mutant E-cadherin polypeptides were precipitated, however, there were apparently no other polypeptides (Fig. 8a).

p120 is a recently described component of the cadherin adhesion complex that can directly associate with cadherins (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995; Staddon et al., 1995). p120 is related to β-catenin and plakoglobin (Reynolds et al., 1992), but its function in the complex remains unknown. In rasttransformed human breast epithelial cells, an inverse correlation between the amount of p120 in the E-cadherin adhesion complex and that of β-catenin in the same complex has been reported (Kinch et al., 1995). Increased association of p120 with E-cadherin accompanied by a dysfunction of E-cadherin has also reported in human intestinal HT-29 cells (Skoudy et al., 1996). We therefore reasoned that the lack or failure of complex formation with β-catenin/plakoglobin and α-catenin might facilitate binding of p120 to the E-cadherin cytoplasmic domain. Since isoforms of p120 comigrate with α- and β-catenin (Reynolds et al., 1994) and the mutant E-cadherin polypeptides also migrate to the positions of these catenins, we performed immunoblot analysis of the E-cadherin immunoprecipitates with anti-p120 antibodies. In previous experiments (Ozawa and Kemler, 1998), however, we could not detect p120 in E-cadherin immunoprecipitates isolated from extracts of EK cells under conditions under which we could easily detect β-catenin, plakoglobin, and α-catenin. Therefore in the present experiments, a five times greater amount of material was applied to each well and a 2.5 times higher concentration of anti-p120 antibodies was used. Under these conditions, p120 was detected in the E-cadherin immunoprecipitates (Fig. 8b). In the cases of EΔC37 cells and EΔC71K cells, a significantly reduced amount of p120 (~10% of EK cells) was coprecipitated together with E-cadherin and, as expected, no p120 was detected in the precipitates of E7K cells and ECOK cells (Fig. 8b).

### Discussion

The most important findings presented here are: (a) the removal of the amino acid residues of the inner membrane-proximal region of E-cadherin activates the partially truncated nonfunctional E-cadherin polypeptides; (b) the activated state of E-cadherin is independent of anchorage to the actin cytoskeleton because increased aggregation activity is preserved in the absence of the distal portion of the E-cadherin cytoplasmic domain, a region required for binding to β-catenin or plakoglobin, whereby E-cadherin interacts with the actin cytoskeleton through
o-catenin; and (c) the tail-less E-cadherin mediates Ca^{2+}-dependent aggregation of transfected K562 cells to the same extent and strength as the wild-type E-cadherin.

Recent investigations of the extracellular domains of cadherins have provided descriptions of their structure and suggested models of the possible mechanisms underlying their functional activity. The extracellular segment of cadherins consists of five domains. X-ray crystallographic studies of the NH_{2}-terminal domain of N-cadherin showed that it forms a dimer, called the strand dimer, in which the monomers are oriented in parallel with their adhesive binding surfaces directed outward from the plasma membrane (Shapiro et al., 1995). Recombinant fragments of the C-cadherin extracellular domain as well as the E-cadherin extracellular domain in solution have been shown to form dimers, a configuration required for full expression of their homophilic binding activity (Brieher et al., 1996; Tomschy et al., 1996). Therefore, it seems likely that one of the active states of cadherin is dimeric. Consistent with this, the tail-less E-cadherin, an active form, can be cross-linked to a dimer on the surface of cells but the partially truncated E-cadherin, an inactive form, cannot. The membrane-proximal region of the E-cadherin cytoplasmic domain may serve, therefore, to constrain the adhesion receptor in a default low-affinity state by preventing lateral dimerization of the extracellular domain. Despite the presence of this constraint, the association of β-catenin/plakoglobin and α-catenin with the distal portion of the E-cadherin cytoplasmic domain may induce lateral oligomerization and clustering of the extracellular domain through linking to the actin cytoskeleton. As was shown previously, E-cadherin chimera proteins covalently linked with the COOH-terminal half or two-thirds of α-catenin show cell adhesive activity despite the presence of the membrane-proximal region of E-cadherin (Nagafuchi et al., 1994; Ozawa and Kemler, 1998). Thus, the connection with the actin cytoskeleton overcomes the negative regulation by the membrane-proximal region. At present, we do not know why the wild-type E-cadherin, an active form, cannot be cross-linked to a dimer, but the lack of chemical cross-linking of the wild-type cadherin to a dimer on the surface has also been reported in the case of C-cadherin (Brieher et al., 1996). The detergent insolubility of cadherin has been shown to be an indication of complex association with the actin cytoskeleton (Hirano et al., 1987; Ozawa et al., 1990). This association is a prerequisite for the cell adhesive activity of cadherins (Nagafuchi and Takei, 1988; Ozawa et al., 1990; Hirano et al., 1992). Additionally, 10% of the wild-type E-cadherin expressed in K562 cells was detected in the detergent-insoluble fraction (Ozawa, M., submitted for publication). The low degree of association with the actin cytoskeleton may explain the failure to detect dimers of the wild-type E-cadherin by chemical cross-linking.

The capacity of the membrane-proximal portion of the E-cadherin cytoplasmic domain to negatively regulate its aggregation activity may be due to its interaction with an intracellular partner. Obviously, p120 is a candidate molecule, because p120 can associate with partially truncated nonfunctional E-cadherin polypeptides. Although it has been reported that, in E-cadherin, p120 binds to a different, but juxtaposed, region from that for β-catenin and plakoglobin within the last 37 COOH-terminal residues (Shibamoto et al., 1995), we found p120 in the immunoprecipitates of both the EΔC37 and EΔC71 polypeptides. The amount of p120 coprecipitated with these two truncated E-cadherin polypeptides with the membrane-proximal region was, however, much less compared with that found in the immunoprecipitate of the wild-type polypeptide. A similar observation has been reported for VE-cadherin (Lampugnani et al., 1997). These results suggest that there may be multiple binding sites for p120 in the E-cadherin and VE-cadherin cytoplasmic tails. Importantly, the partially truncated VE-cadherin has been reported to be able to promote cell aggregation (Navarro et al., 1995; see below for a detailed discussion). It seems, therefore, less likely that p120 associated with the partially truncated E-cadherin polypeptides is responsible for the inability of the protein to act as an adhesion molecule. We must therefore identify a new binding partner(s) of this region.

The amino acid residues in the membrane-proximal region of the E-cadherin cytoplasmic domain are relatively conserved in other cadherins that have been shown to be cell–cell adhesion molecules (Suzuki et al., 1991). Thus, this region appears to be important in control of the activity states of multiple cadherin families. There have, however, been reports that differ from the results we present here. The VE-cadherin polypeptide has a cytoplasmic domain of 164 amino acids and a mutant VE-cadherin polypeptide lacking the COOH-terminal 82 amino acids expressed on CHO cells has been reported to be able to promote cell aggregation (Navarro et al., 1995). Similarly, a deletion mutant of C-cadherin retaining the juxtamembrane 94-amino acid region of the cytoplasmic tail expressed on CHO cells has been shown to display adhesive activity (Yap et al., 1998). Another truncated C-cadherin polypeptide containing the first, cytoplasmic, juxtamembrane lysine residue followed by two amino acids, histidine and methionine, has been, however, reported not to mediate as strong adhesion as the wild-type C-cadherin (Brieher et al., 1996; Yap et al., 1998). At present we do not know the reason for the discrepancies. It is not however, because of different methodologies or cell systems that gave different results. To assess adhesion activity of the partially truncated VE-cadherin and the wild-type VE-cadherin, an aggregation assay (see Fig. 7; Navarro et al., 1995), which is essentially identical to the assay used in the present study, was used. The truncated VE-cadherin was even more efficient in promoting Ca^{2+}-dependent aggregation of transfectedants than the wild-type molecule in the assay. Furthermore, an aggregation assay, which is also essentially identical to the assay used in the present study, was used to assess the adhesive activity of the C-cadherin mutants, besides a laminar flow detachment assay (see Fig. 7; Yap et al., 1998). Adhesive activity of the C-cadherin mutant with the membrane-proximal region was detectable in the aggregation assay, whereas that of the tail-less C-cadherin was not detectable. Therefore, it is not because of different methodologies that gave different results. Furthermore, the tail-less E-cadherin (EC0) is functional in L cells, a fibroblastic cell line. Although CHO cells were used to express and to assess the adhesion activity of the truncated VE-cadherin and C-cadherin polypeptides, it seems less likely that mutant E-cadherin polypeptides ex-
pressed on L cells and CHO cells show distinct properties. Therefore it seems to be obvious that the difference between the data in the present study and those from previous studies comes from the difference of cadherin subtypes analyzed in the different studies. It has been shown that two major cadherins in endothelial cells, VE-cadherin and N-cadherin, are differentially targeted to membranes; i.e., VE-cadherin is clustered at cell-cell junctions, whereas N-cadherin remains diffusely distributed on the cell membrane (Salomon et al., 1992; Navarro et al., 1998). The membrane-proximal region of VE-cadherin has been shown to have the ability to exclude N-cadherin from intercellular junctions (Navarro et al., 1998). Therefore it is possible that the membrane-proximal region of each cadherin exhibits specific functional features depending on the subtype, despite the certain degree of homology.

It has been suggested that the stabilization and strengthening of E-cadherin-mediated adhesion is dependent on linkage of E-cadherin to the actin cytoskeleton. However, no significant difference was found between the adhesiveness conferred by the wild-type E-cadherin and that of the mutant tail-less E-cadherin polypeptide. Therefore, at least in the cell system used in the present study, a certain degree of strengthening of E-cadherin-mediated cell adhesion is independent of the linkage to the actin cytoskeleton. Instead, as shown in the present study, compaction, an E-cadherin-mediated morphological change of cells in aggregates, is dependent on the linkage to the actin cytoskeleton. In mouse embryos, E-cadherin-mediated compaction has been shown to be sensitive to cytochalasins, which disrupt actin bundles (Surani et al., 1990). Our observation that aggregates formed by cells transfected with the wild-type E-cadherin, which is anchored to the actin cytoskeleton via catenins, showed compaction, whereas aggregates formed by cells expressing the tail-less E-cadherin, which no longer can be anchored to the actin filament, remained uncompacted is consistent with the idea that the anchorage to the actin cytoskeleton is a prerequisite for the morphological changes.

Based on observations with v-src–transformed MDCK cells and L cells expressing E-cadherin, cadherin-mediated adhesion was postulated to have two states (Takeda et al., 1995). The aggregates in the weak state are easily dissociated into single cells on passage several times through Pasteur pipettes, whereas the aggregates in the strong state are hardly affected by the same treatment. According to this definition, the state of EK cell aggregates is weak, because they were dissociated into single cells when passed several times through a Pasteur pipette, whereas the aggregates in the strong state are densely packed. According to this definition, the state of EK cell aggregates is weak, because they were dissociated into single cells when passed several times through a Pasteur pipette, whereas the aggregates in the strong state are densely packed. In the case of EK cells, however, cells in the aggregates, even those formed after overnight culture, in which each cell in the aggregates showed compaction, remain in the weak state. Therefore, compaction, the morphological change in cell shape in aggregates seems not to be accompanied by the transition from the weak state to the strong state. Absent expression in K562 cells of ZO-1, which binds to α-catenin and actin filaments (Itoh et al., 1997), may explain why EK cells remain in the weak state. Another possibility, for example, that cadherin-independent adhesiveness of MDCK cells and L cells is involved in the transition must be considered.

Cadherin-mediated aggregation has been shown to be sensitive to low temperature (Takeichi, 1977; Angres et al., 1996). The aggregation activity of the tail-less E-cadherin, as well as that of the wild-type E-cadherin, were affected by lowering of the temperature for the aggregation assay. The results suggest a reduction in membrane fluidity, which may prevent clustering of E-cadherin, or a temperature-dependent conformational change of the extracellular domain of E-cadherin as the underlying mechanism. The possibility that lowering of the temperature affected the aggregation-competent state of the intracellular apparatus required for aggregation must also be considered. We can, however, exclude the possibility that the anchorage to the actin cytoskeleton is the sole site affected by lowering of the temperature.

We thank Drs. K. Sekiguchi and K-I. Yamamura for providing us with the reagents, and K. Sato for her secretarial assistance.

This work was supported by grants from the Ministry of Education, Science and Culture of Japan; the Naito Foundation for the Promotion of Science; the Kodama Memorial Foundation; and the Max-Planck Gesellschaft.

Received for publication 2 June 1998 and in revised form 24 July 1998.

References


