Lateral Dimerization of the E-cadherin Extracellular Domain Is Necessary but Not Sufficient for Adhesive Activity*

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Cadherins are transmembrane glycoproteins involved in Ca\(^{2+}\)-dependent cell-cell adhesion. Using L cells coexpressing E-cadherin constructs with different epitope tags, we examined the lateral dimerization of E-cadherin and its adhesive activity by co-immunoprecipitation and aggregation assays, respectively. Although the transmembrane domain is required for dimerization, tail-less constructs possessing the transmembrane domain of either N-cadherin or CD45 show dimerization and are active in aggregation assays. Two mutant constructs having either of two amino acid substitutions, W2A or substitutions that disrupt the recognition sequence for endoproteolytic enzymes involved in removal of the precursor segment, cannot form dimers and are inactive in aggregation. These monomeric proteins, like their wild-type dimerizing counterparts, retain their Ca\(^{2+}\)-dependent resistance to trypsin digestion, suggesting that dimerization per se does not induce a large conformational change. Two other constructs, having either an amino acid substitution, D134A, or a C-terminal deletion of 70 amino acid residues, retain the ability to associate laterally but are inactive in aggregation assays. Staurosporine treatment of cells expressing the latter construct increases aggregation but does not increase the extent of lateral dimerization. Thus, lateral dimerization is necessary, but not sufficient for adhesive activity.

The classic cadherins (e.g. E-, N-, and P-cadherins) are single transmembrane domain proteins involved in Ca\(^{2+}\)-dependent cell-cell adhesion (1–3). Ca\(^{2+}\) protects the extracellular domains of cadherins from proteolytic degradation and is necessary for their function. They share a common primary structure, with five tandemly repeated extracellular domains of ~110 amino acids each, a transmembrane segment, and a cytoplasmic domain. Their cytoplasmic domain interacts with intracellular proteins termed catenins, which mediate connections between the cadherins and the actin cytoskeleton (4–7). These interactions seem to be involved in regulation of cadherin adhesive activity (8–13).

The five extracellular domains of classic cadherins together coordinate several Cu\(^{2+}\) ions to maintain the rodlike conformation of the entire extracellular region (14, 15) and allow interaction with other cadherins, resulting in their adhesive activity (16). Experiments with cadherin mutants and chimeric molecules have shown that the first extracellular domain (EC1) governs the homophilic binding specificity of the cadherins (17).

Recent studies determined the three-dimensional structure of the EC1 (18, 19) and EC1-EC2 domains of E- and N-cadherins (15, 20). Analysis of cadherin crystals suggested that these proteins may form lateral homodimers (15, 19). According to one model (19), two cadherin molecules extending from the same cell surface laterally interact through hydrophobic interactions, i.e. the mutual incorporation of a conserved Trp residue, localized at the second position of mature classic cadherins, Trp-2, into the hydrophobic core of the paired molecule. In an alternative model (15, 21), lateral cadherin dimers are stabilized by mutually coordinating Ca\(^{2+}\). The second model is consistent with the observation that, at high concentrations, recombinantly expressed extracellular regions of E-cadherin dimerize in a Ca\(^{2+}\)-dependent manner (22, 23). Electron microscopy of a recombinant E-cadherin ectodomain pentamered by the assembly domain of cartilage oligomeric matrix protein showed that the artificially assembled recombinant protein self-associates in a Ca\(^{2+}\)-dependent manner, whereas the ectodomain alone does not (21, 24). The protein also showed Ca\(^{2+}\)-dependent adhesive interactions. Mutation of the Trp-2 to alanine (W2A) abolishes the adhesive interactions, but not the lateral interactions in the recombinant E-cadherin construct (21). This work is consistent with other studies (15, 25, 26), which all support the presence of Ca\(^{2+}\)-dependent lateral interactions. Takeda et al. (25) used chemical cross-linking studies at the cell surface to show that Ca\(^{2+}\) ions are required for lateral E-cadherin dimer formation.

Regardless of the mechanism that governs cadherin lateral dimerization, these dimeric structures are thought to represent the functional units required for adhesion. Adhesive bonds between cadherins of interacting cell membranes are predicted to result from binding between EC1 domains of oppositely oriented cis-dimers to form trans-interacting anti-parallel tetramers, which are termed adhesion dimers. The necessity for lateral dimers is supported by experiments with recombinant cadherin extracellular segments (24, 27) that show that only

* This work was supported by a grant-in-aid for science research on priority area (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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the dimeric form can mediate adhesion. Furthermore, arti-
cially induced multimerization of a chimeric tail-less C-cad-
herin strengthened adhesion in cells expressing this molecule (28).
Thus, it appears likely that lateral dimers are a funda-
mental unit for cadherin function, at least for the classical
cadherins. Chitaev and Troyanovsky (29) showed, however,
that a relatively small pool of E-cadherin exists as lateral
homodimers. This result raises the possibility that, by control-
ling the number of lateral dimers versus monomers on the cell
surface, cells can regulate cadherin activity. In the present
study, we show several lines of evidence that dimerization of
the cadherin extracellular domain is not the sole critical step in
regulating cadherin adhesion activity, and that a post-dimer-
ization event is also important in this regulation.

Experimental Procedures
cDNA Construction—A cDNA encoding full-length mouse E-cadherin
was described previously (4). All the following constructs were cloned
into an expression vector, pCAGGSneo (30) (a gift from Dr. K.
Yamamura, Institute of Molecular Embryology and Genetics, Kum-
amoto University, Kumamoto, Japan). The cDNA encoding a mutant
E-cadherin protein with an aspartate-to-alanine substitution at posi-
tion 134 in the extracellular Ca\(^{2+}\)-binding motif (D134A) was described previously (16). The cDNA encoding a mutant
E-cadherin protein with amino acid substitutions (Arg-Arg-Gln-Lys-Arg to Arg-Thr-Gln-Thr-Arg) in the endoproteolytic cleavage site for removal of the precursor
segment, was described previously (31).

To detect co-immunoprecipitation by immunoblotting, the E-cad-
erin construct was fused to either a GFP or HA epitope at its C
terminus (Fig. 1). An EcoRV site was created by PCR at the C-terminal
site of the E-cadherin construct, using the following primers for full-
length E-cadherin (5'-TATACCGCTGGAGAGCGG and 5'-ATCTAGA-
GAGAGCCGG) and the tail-less E-cadherin construct (5'-TATACCG-
TCGAGAGCCGG and 5'-ATCTACAGAGAGCCGG), the partially
truncated EC81 construct (5'-ATCTAGAAGCAGC), the tail-less EC0 construct (5'-TATACCGCTCAGAGAGCCGG and 5'-ATC-
GATGAAGTTCCATTTTACAT), and the soluble and secreted Ex con-
struct (5'-TATACCGCTGGAGAGCCGG and 5'-ATCTAGTGCCGAC-
AATTGTCAAT). The EcoRV site was used for ligation of the cDNA for
the E-cadherin constructs with the unique EcoRV site upstream of the
GFP or HA epitope sequence in the pCAGGSneo vector.

cDNAs encoding two E-cadherin constructs whose transmembrane
domains were replaced by that of either N-cadherin or human CD45
(CD45) were cloned into plasmids. The cDNA encoding a mutant
E-cadherin protein with an arginine-to-alanine substitution in the end-
oproteolytic cleavage site for removal of the precursor segment, was
described previously (31).

Site-directed mutagenesis of the EC1 domain of E-cadherin was
performed as described above, using the following primer pair (5'-GAT-
CCGACTTGCATCTCGGGC and 5'-GTCACAAATGTCCTGCAGCCG and
EC0-HA) with antibodies against GFP, HA, and E-cadherin
(Sigma), the cells were washed, resuspended, and incubated for 30 min
at 37 °C with constant rotation at 70 rpm.

Antibodies—DECA-1, a rat mAb to E-cadherin, was used for im-
munoblotting and immunofluorescence staining, and rabbit anti-E-cad-
erin antibodies were used for immunoprecipitation. A mouse mAb
(12CA5) directed against HA was kindly provided by Dr. A. Yoshimura
(Medical Institute of Bioregulation, Kyushu University, Fukuoka, Ja-
pan). A rat mAb against HA (3F10) was purchased from Roche Molec-
ular Biochemicals. Rabbit anti-GFP antisera was purchased from Mo-
lecular Probes and were used for immunoprecipitation. A mouse mAb
against GFP was purchased from CLonTECH and used for immu-

Immunoblotting and Immunoprecipitation—Immunoprecipita-
tion and immunoblot analyses were carried out as described previously (11).
In brief, cells (2 x 10^6) were cultured in 60-mm tissue culture dishes at
37 °C for 24 h and lysed in PI lysis buffer (25 mM Tris-HCl buffer, pH
7.4, containing 0.5% Nonidet P-40, 2 mM EDTA, 10 mM sodium pyro-
phosphate, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin,
and 25 μg/ml aprotinin). In coculture experiments, 2 x 10^6 cells
expressing EC0-GFP and EC0-HA mixed in a 1:1 ratio were cultured for 24 h.
To analyze E-cadherin constructs secreted into the medium (Ex-
GFP and Ex-HA), cells expressing these constructs were cultured for 3
days. The medium was collected, clarified by centrifugation, and then
subjected to analysis. The E-cadherin proteins were collected with
rabbit anti-GFP or anti-E-cadherin antibodies, which had been pre-
absorbed to protein G-agarose (Sigma). The immune complex was washed
with the same buffer three times and then boiled for 5 min in SDS-
PAGE sample buffer. In some immunoprecipitation experiments, cell
lysis and washing of the immunoprecipitates were done with the fol-
lowing buffers or a combination of buffers: 1) 10 mM Tris-HCl buffer, pH
8.0, containing 1% Triton X-100, 60 mM octyl glucoside, 0.15 mM NaCl, 5
mM EDTA, and 1 mM Na_3VO_4; 2) cells were lysed with phosphate-
buffered saline containing 1% Triton X-100, 1% Nonidet P-40, 1 mM
CaCl_2, and 1 mM PMSF, and the immunoprecipitates were washed with
50 mM Tris-HCl buffer, pH 8.5, containing 0.05% Nonidet P-40, 0.5 mM
NaCl, 1 mM CaCl_2, and 1 mM PMSF; 3) RIPA (10 mM Tris-HCl buffer,
pH 7.4, containing 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS,
1 mM Na_3VO_4, 1 mM EDTA, and 1 mM PMSF).

Results
Lateral Association of the Tail-less E-cadherin—Using an
immunoprecipitation approach, lateral dimers of E-cadherin
that are soluble in nonionic detergents were detected in epi-
theial cells (29). This approach was also used successfully to
identify lateral heterodimerization between N-cadherin and
R-cadherin (32). We expressed tail-less E-cadherin (EC0) constructs
tagged with either a GFP or HA epitope (EC0-GFP and EC0-HA, respectively) (Fig. 1) in L cells and
and established stable single and double cadherin expressors. Im-
munoblot analysis of total cell lysates of EC0-GFPL (L cells
expressing EC0-GFP), EC0-HAL (L cells expressing EC0-HA),
and EC0-GFP/EC0-HAL (L cells expressing both EC0-GFP and
EC0-HA) with antibodies against GFP, HA, and E-cadherin
showed that each transfectant produces its respective protein
(Fig. 2, upper panel, Total), but that the amount of EC0-HA
detected is much higher than that of EC0-GFP. The latter
finding suggests that EC0-HA is more stable than EC0-GFP in L
cells.

When total cell lysates from EC0-GFPL, EC0-HAL, and EC0-
GFP/EC0-HAL were immunoprecipitated with anti-GFP anti-
bodies and detected by immunoblotting with anti-HA antibodies,
EC0-HA protein was detected in immunoprecipitates isolated
from EC0-GFPL/EC0-HAL cell lysates but not in those from
EC0-GFPL or EC0-HAL cell lysates (Fig. 2, lower panel, IP: GFP),
suggesting a lateral interaction between EC0-GFP and EC0-
HA expressed on the same cells. These results were con-
sistent with a previous report (29) and suggest that lateral
dimerization does not depend on association with catenins or
p120. Because cell lysis and immunoprecipitation were per-
formed in the presence of 2 mM EDTA, divalent cations, includ-
ing Ca^{2+}, are not required for dimer formation. Immunoblot-
ting of the immunoprecipitates with E-cadherin antibodies that
recognize both EC0-GFP and EC0-HA revealed that the amount of EC0-HA coprecipitated was similar to that of EC0-GFP, indicating that most, if not all, of the EC0-GFP is in a dimer with EC0-HA. The results are in contrast with a previous report (29) showing that a relatively small pool of E-cadherin forms lateral dimers.

The Transmembrane Domain Is Required for Lateral Association

Although a soluble, secreted extracellular segment of C-cadherin has been shown to form lateral dimers (27), the ability of E-cadherin is not definitely known (24). To address this issue, constructs encoding the extracellular domain of E-cadherin tagged with GFP or the HA epitope (Ex-GFP or Ex-HA, respectively; see Fig. 1) were expressed in L cells. Immunoblot analysis of culture media collected from these transfectants revealed that these proteins were expressed and secreted into the medium as soluble proteins (Fig. 3, upper panel, Total). When immunoprecipitates collected from media of Ex-GFP and Ex-HA coexpressors (Ex-GFP/Ex-HA cells) with anti-GFP antibodies were blotted with anti-HA or E-cadherin antibodies, no Ex-HA band was detected (Fig. 3, lower panel, IP: GFP). Lack of the coprecipitation of Ex-HA with Ex-GFP indicates that the transmembrane domain is required for the lateral association.

The Transmembrane Domain of N-cadherin or CD45 Is Sufficient for Lateral Association—To determine whether a specific amino acid sequence in the transmembrane domain is required, the transmembrane domains of EC0-GFP and EC0-HA were replaced by that of N-cadherin (EC0TMN-GFP and EC0TMN-HA) or CD45 (EC0TMC-GFP and EC0TMC-HA) (Fig. 4A). These constructs were introduced into L cells in various combinations to determine whether the transmembrane domain of N-cadherin or CD45 can substitute for the role of the E-cadherin transmembrane domain and whether heterodimerization between EC0 molecules with different transmembrane domains can occur. HA-tagged chimeric constructs consisting of the E-cadherin extracellular domain and the transmembrane domain of either N-cadherin or CD45, respectively, pEC0, constructs with amino acid substitutions in the endoproteolytic cleavage site for removal of the precursor segment, thus resulting in a immature, uncleaved protein expressed on the cell surface. TMD, transmembrane domain.
extracellular domains show the same trypsin resistance in the presence of Ca\(^{2+}\). These constructs are deficient in adhesion, because cells expressing them on their surface (EC0WA-HAL and EC0WA-GFP/EC0WA-HAL) showed no activity in aggregation assays (Fig. 5). Cells coexpressing EC0-GFP and EC0WA-HAL do not aggregate either, indicating that the mere presence of EC0WA-HA seems to disrupt the potential homodimeric association of EC0-GFP molecules between cells in a dominant-negative fashion. At present we do not know the reason for this.

We previously reported that an amino acid substitution at residue 134, from aspartic acid to alanine, abolishes E-cadherin-mediated cell-cell adhesion (16). To determine whether this effect is the result of a failure of the mutant E-cadherin to form lateral dimers, we expressed HA-tagged EC0 with the D134A mutation (EC0DA-HA) together with GFP-tagged constructs with or without the same mutation (EC0DA-GFP or EC0-GFP, respectively) (Fig. 6A, upper panel). Immunoblot analysis of the GFP immunoprecipitates with anti-HA or anti-E-cadherin antibodies revealed that EC0DA-HA coprecipitated with both EC0DA-GFP and EC0-GFP, indicating that this substitution has no effect on lateral dimerization (Fig. 6A, lower panel). Cells expressing any D134A mutant EC0 (EC0DA-HAL, EC0DA-GFP/EC0DA-HAL, and EC0-GFP/EC0DA-HAL), however, do not exhibit any adhesion (Fig. 5). Thus, Aep-134 must play a critical role in post-lateral dimerization events. Like the EC0WA-HA construct, EC0DA-HA seems to have dominant-negative effect on the EC0-GFP construct because cells coexpressing these constructs show no aggregation.

**The Presence of the Precursor Segment Prevents Dimerization**—Cadherins are synthesized as precursor polypeptides, which are processed by a series of posttranslational modifications, including proteolytic cleavage. The mature proteins are formed intracellularly and transported to the cell surface. The precursor E-cadherin includes a 129-residue segment that is cleaved off to generate the mature protein. We previously showed that amino acid substitutions in the recognition site for processing proteases inhibit intracellular processing of E-cadherin (31). The unprocessed polypeptides are expressed efficiently on the cell surface and had other features in common with mature E-cadherin, such as complex formation with catenins and Ca\(^{2+}\)-dependent resistance to proteolytic degradation. However, cells expressing the unprocessed proteins showed no E-cadherin-mediated adhesion. Removal of the precursor region results in activation of E-cadherin function (31). To determine whether or not the presence of the precursor segment prevents dimerization, we expressed HA-tagged EC0 with a mutation in the proteolytic recognition site (pEC0-HA) together with GFP-tagged constructs with or without the same mutation (pEC0-GFP or EC0-GFP, respectively), or EC0-HA together with pEC0-GFP (Fig. 8A, upper panel). Immunoblot analysis of the GFP immunoprecipitates with anti-HA or anti-E-cadherin revealed that pEC0-HA did not coprecipitate with pEC0-GFP or EC0-GFP, and that EC0-HA did not coprecipitate with pEC0-GFP (Fig. 8A, lower panel), indicating that the presence of the precursor segment prevents lateral dimerization. Lack of coprecipitation does not seem to be because of different localization of the constructs, because immunofluorescence microscopy revealed that they are both expressed on the cell surface (data not shown). Trypsin digestion (0.01%) in the presence of 1 mM EGTA, which would remove all Ca\(^{2+}\) from the system, revealed that significant portions of the constructs were expressed on cell surfaces (Fig. 7A). In the presence of 2 mM Ca\(^{2+}\), these constructs remained on the surface after digestion. Thus, the typical Ca\(^{2+}\)-dependent trypsin resistance does not appear to rely upon lateral dimerization. Incubation of cells expressing E-cadherin in a high concentration of trypsin (0.1%) in the presence of Ca\(^{2+}\) results in release of an 84-kDa E-cadherin fragment from the cells (Fig. 7B). Incubation of cells expressing both EC0WA-GFP and EC0WA-HA protein under the same conditions also yielded the same 84-kDa fragment (Fig. 7B). Therefore, the monomeric as well as the dimeric E-cadherin

**Fig. 3.** The transmembrane domain is required for the dimerization. Total proteins from cell culture medium concentrated by ethanol precipitation (upper panel, Total) or the materials collected by immunoprecipitation with anti-GFP antibodies (lower panel, IP: GFP) from single transfectants (Ex-GFP and Ex-HA) and double transfectants (Ex-GFP/Ex-HA) were detected by GFP, HA, and E-cadherin (E-cad) antibodies.
in the presence of Ca^{2+} resulted in release of an 84-kDa fragment from the cells (Fig. 7B). Removal of the precursor segment by trypsin digestion results in dimer formation between the processed pEC0-GFP and pEC0-HA but not between pEC0-GFP and EC0-HA (Fig. 8C). The latter finding suggests that the exchange of components between the dimers does not take place, or that it occurs very slowly.

Activation of the Partially Truncated E-cadherin Polypeptides by Staurosporine Does Not Change the Degree of the Lateral Dimerization—We previously showed that, although the tail-less E-cadherin is active in aggregation assays, partially truncated E-cadherin, such as EC071, with a 71-residue C-terminal deletion, is not (8). When L cells expressing this construct are treated with staurosporine, a kinase inhibitor, the nonfunctional E-cadherin becomes activated (11). We postulated that the inability of the partially truncated E-cadherin to mediate cell adhesion can be ascribed to the association of Ser/Thr-phosphorylated p120 to the membrane-proximal region of the protein, which may prevent its lateral dimerization. To determine whether or not the partially truncated E-cad-
herin can form lateral dimers, GFP- or HA-tagged E-cadherin constructs with a 70-residue C-terminal deletion (EC81-GFP or EC81-HA) were introduced into L cells, and clones expressing both proteins were isolated. As expected, L cells expressing these constructs (EC81-GFP/EC81-HA) showed no adhesion in aggregation assays (Fig. 5). Immunoblot analysis of the GFP immunoprecipitates obtained from these cells with anti-HA and E-cadherin antibodies revealed that EC81-HA coprecipitated with EC81-GFP (Fig. 5). Thus, the C-terminally deleted EC81 protein still retains the ability to dimerize but has lost the ability to form adhesive interactions. Staurosporine treatment, which activates this particular nonfunctional E-cadherin (Fig. 5), did not change the degree of lateral dimerization (Fig. 5).

Stability of the Lateral Dimers of EC0 Constructs—To determine the extent to which several different lysis and washing conditions affect lateral dimerization, L cells coexpressing EC0-GFP and EC0-HA were lysed using different buffer conditions and immunoprecipitated with anti-GFP antibodies. After washing with different buffers, immunoprecipitates were subjected to immunoblot analysis with antibodies against GFP, HA, and E-cadherin. Inclusion of octyl glucoside (60 mM), an agent that disrupts membrane rafts (33), did not affect lateral dimerization, nor did lysis and washing with RIPA, another highly stringent buffer containing 0.1% SDS in addition to 1% Triton X-100 and 0.5% deoxycholate (data not shown). However, when immunoprecipitates were washed with a buffer containing 0.5 M NaCl and 50 mM Tris-HCl, pH 8.5, EC0-HA no longer coprecipitated with EC0-GFP (data not shown). These results support the idea that the dimerization process is driven not only by hydrophobic interactions but also by ionic interactions between the extracellular domains of E-cadherin.

**DISCUSSION**

Cadherin-mediated adhesion has been shown to be a regulated process during tissue morphogenesis and in some pathophysiological states. Little is known, however, about the mechanisms underlying cadherin regulation. The facts that the extracellular segment alone, without the cytoplasmic domain, possesses intrinsic homophilic binding activity and that its activity depends on lateral dimerization indicate possible mechanisms through which regulation could occur. Adhesive activity could range from a weak state mediated by the intrinsic activity of the extracellular segment when the cytoplasmic tail is inactive to a strong state when the contribution of the cytoplasmic tail is maximal. Regulatory signals most likely influence the contribution of the cytoplasmic tail to the strength of adhesion. It may also be possible to regulate cadherin activity by controlling the number of lateral dimers versus monomers on the cell surface. Finally, a dimeric structure could allow for conformational changes in the extracellular segment, resulting in changes in the homophilic binding affinity. Therefore, lateral dimerization of cadherins is believed to be a critical factor regulating the adhesive affinity of cadherins (21, 25, 27). The present data provide a strong biochemical argument in favor of the model that lateral dimerization of the cadherin extracellular domain is necessary for adhesion. The data also demonstrate that dimerization itself is not sufficient for this activity.

In the present study, we analyzed lateral dimerization of E-cadherin constructs, especially those with deletions of the cytoplasmic domain. These tail-less (EC0) constructs have experimental advantages over a full-length construct. Recently, it has been demonstrated that expression of exogenous cadherins
Cadherin Lateral Dimerization

**Fig. 7. Dimerization and Ca\(^{2+}\)-dependent resistance to trypsin digestion.**

A, dimerization is not necessary for Ca\(^{2+}\)-dependent resistance to trypsin digestion. The double transfectants (EC0-GFP/EC0-HA, EC0DA-GFP/EC0DA-HA, and EC0WA-GFP/EC0WA-HA) were incubated with 0.1% trypsin for 60 min at 37 °C in the presence of 2 mM Ca\(^{2+}\) (TC) or 1 mM EGTA (TE). The protein bands marked by asterisks correspond to the intracellular, incompletely processed proteins having the precursor segment. B, extensive trypsin digestion yields a soluble 84-kDa fragment from the cells. The double transfectants were incubated with 0.1% trypsin for 60 min at 37 °C in the presence of 2 mM Ca\(^{2+}\). The soluble 84-kDa fragment (gp84) was collected by immunoprecipitation with rabbit anti-E-cadherin (E-cad) antibodies and detected by immunoblotting with a rat anti-E-cadherin monoclonal antibody. Consistent with our previous observation (18), EC0DA constructs show sensitivity to the high concentration of trypsin and were cleaved into smaller fragments.

**Mechanisms for Lateral Dimerization**—Evidence for the role of Trp-2 and Ca\(^{2+}\) in lateral dimerization is complex and partly conflicting. Our observation, that mutating Trp-2 in E-cadherin destroys lateral dimer formation, is consistent with other results (29) showing that a double mutation (W2A/V3G) completely abolishes dimerization. However, it conflicts with other findings of Pertz et al. (21) that demonstrated a loss of adhesive but not lateral interactions upon Trp-2 mutation. We do not know the reason for the discrepancy in results, but it is possible that it is because of differences in the methodology used to detect dimers. We and Chitaev and Troyanovsky (29) used immunoprecipitation, whereas Pertz et al. (21) used electron microscopy to detect dimers. Weak interaction of Trp-2 mutant may dissociate during immunoprecipitation experiments. We believe that dimerization detected by immunoprecipitation may be driven by Trp-2 and hydrophobic core interaction. Mutual insertion of Trp-2 into the hydrophobic cores seems to be critical, because we did not detect any dimers between EC0-GFP and EC0WA-HA. In addition to this hydrophobic interaction, ionic interactions seem to be critical for dimerization, because washing of the complexes with an alkaline buffer containing 0.5 M NaCl disrupts the association.

Ca\(^{2+}\)-dependent dimerization of the first two domains of E-cadherin has been reported (21–23). Pertz et al. (21) have proposed a model for homophilic adhesion in which lateral dimerization occurs entirely in a Ca\(^{2+}\)-dependent and Trp-2-independent manner. In contrast to this, Ca\(^{2+}\)-independent lateral dimerization has been detected with C-cadherin (27) and E-cadherin (29). Recently, an alternative model, based on results using the E-cadherin extracellular domain fused to the Fc portion of IgG, has been proposed (36). In this case, the lateral association was assessed by analyzing the reactivity of
the fusion proteins to antibodies that recognize sequences that are buried after dimer formation. The analysis revealed that the Trp-2/Val-3 mutant dimerizes in the presence of Ca\(^{2+}\)/H\(_{11001}\), but not in its absence. We could not detect, however, any dimerization of the Trp-2 mutant irrespective of the presence or absence of Ca\(^{2+}\)/H\(_{11001}\) under our experimental settings. Further studies are necessary to clarify the role of Ca\(^{2+}\)/H\(_{11001}\) and Trp-2 in lateral dimerization.

Specific interactions between the transmembrane domains seem to be important for folding and/or oligomerization of many integral proteins (37). The transmembrane segment of E-cadherin has shown to self-assemble and thereby support the lateral interactions between cadherin molecules in the plasma membrane of adhesive cells (38). We showed that the E-cadherin extracellular domain must be linked to the transmembrane segment to dimerize. A specific conserved amino acid sequence of the transmembrane segment is not important for this activity. Persistence of dimers after detergent solubilization of the membrane suggests that the transmembrane domain is involved only in the formation, but not in maintenance of the dimer. In that case, assembly of lateral dimers must take place only on the membrane. Another possibility is the nonspecific interaction of the transmembrane domains even in the presence of detergents allows the dimeric structure to be driven by the interactions of the extracellular domains. A monomer-dimer equilibrium with significantly lower affinity was demonstrated for the extracellular segment of C-cadherin by sedimentation analysis (39).

Post-dimerization Event(s): The Site of Regulation—Observations that the nonfunctional EC81 forms lateral dimers and that its activation by staurosporine treatment did not change the extent of dimerization suggests that its nonfunctionality is not because of a failure to dimerize. Furthermore, EC0DA, a mutant in which Asp-134, which is involved in Ca\(^{2+}\)-binding, was replaced with alanine, retained the ability to form lateral dimers but lacked adhesive properties. Thus, although the exact nature of cadherin adhesion is still unknown, post-dimerization events seem to be a critical for subsequent adhesive interactions.
interactions, and potentially for the formation of zipper-like adhesive structures.

What are these post-dimerization events? Multimerization or clustering of E-cadherin is the most probable mechanism. So far, however, others, including our research group, have not been able to acquire definitive biochemical evidence for the presence of multimers or clusters of E-cadherin. The reason for the difficulty seems to be the sensitivity of the structure to detergents.

A model, called the “zipper model,” proposes that the formation of trans adhesive contacts between the NH2-terminal domains of cis lateral dimers generates an alternating ribbon of anti-parallel dimers, which forms the intercellular junction (19, 40). Contrary to this, recent studies suggested that homophilic C-cadherin binding involves multiple, distinct binding interactions, which involve more than just the N-terminal EC1 domain (39, 41). As shown in the present study, Asp-134, which is main (39, 41). As shown in the present study, Asp-134, which is

**Acknowledgments**—We thank Drs. Rolf Kemler, Akihiko Yoshimura, Shintaro T. Suzuki, Haruo Saito, Tadashi Kaname, and Ken-ichi Yamamura for providing reagents, and Kumiko Sato for secretarial assistance.

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J. Biol. Chem. 2002, 277:19600-19608.
doi: 10.1074/jbc.M202029200 originally published online March 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202029200

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