Adhesive But Not Lateral E-cadherin Complexes Require Calcium and Catenins for Their Formation

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Abstract. We examined intercadherin interactions in epithelial A-431 cells producing endogenous E-cadherin and recombinant forms of E-cadherin tagged either by myc or by flag epitopes. Three distinct E-cadherin complexes were found. The first is a conventional E-cadherin–catenin complex consisting of one E-cadherin molecule linked either to β-catenin/α-catenin or to plakoglobin/α-catenin dimers. The second is a lateral E-cadherin complex incorporating two E-cadherin–catenin conventional complexes combined in parallel fashion via dimerization of the NH$_2$-terminal extracellular domain of E-cadherin. The third complex is likely to contain two E-cadherin–catenin conventional complexes derived from two opposing cells and arranged in an antiparallel fashion. Formation of the antiparallel but not lateral complex strictly depends on extracellular calcium and E-cadherin binding to catenins. Double amino acid substitution Trp156Ala/Val157Gly within the NH$_2$-terminal extracellular domain of E-cadherin completely abolished both lateral and antiparallel inter–E-cadherin association. These data support an idea that the antiparallel complex has the adhesion function. Furthermore, they allow us to suggest that antiparallel complexes derive from lateral dimers and this complex process requires catenins and calcium ions.

Key words: cadherins • catenins • p120 • intercellular adhesion • epithelial cells

The classic cadherins (e.g., E-, N-, and P-cadherins) are single transmembrane domain proteins involved in calcium-dependent homophilic cell–cell recognition and adhesion. In the intercellular adherens junctions, cytoplasmic portions of these proteins orchestrate assembly of the electron-dense plaque that anchors microfilaments to the plasma membrane. It is thought that the resulting junctional system plays a pivotal role in the establishment and maintenance of the unique tissue architecture (Vasiliev and Gelfand, 1981; Edelman et al., 1990; Takeichi, 1991; Hynes, 1993; Gumbiner, 1996; Hubar et al., 1996). Furthermore, it is now clear that classic cadherins are important elements of the complex signaling pathways controlling cellular motility, growth, and differentiation (Geiger and Ayalon, 1992; Klymkowsky and Parr, 1995; Peifer, 1995). Defects in cadherin-dependent intercellular adhesion accompany neoplastic transformation and tumor progression (Takeichi, 1993; Birchmeier and Behrens, 1994). Despite these critical functions, little is known about the physical interactions between cadherin molecules leading to the establishment of cell–cell contacts, intracellular signals, or the regulation of these interactions by extracellular or intracellular factors.

The extracellular region of classic cadherins consists of five repeating domains (EI–EV). Together they coordinate several calcium ions that maintain the rod-like conformation of the entire extracellular region and are also required for cadherin’s adhesion activity (Ozawa et al., 1990; Pokutta et al., 1994; Maurer et al., 1996). Experiments with cadherin mutants and chimeric molecules showed that the NH$_2$-terminal EI domain governs the binding specificity of cadherins (Blaschuk et al., 1990; Nose et al., 1990). Recently, X-ray crystallographic studies determined the three-dimensional structure of the EI domain of N- and E-cadherin (Overduin et al., 1995; Shapiro et al., 1995) and the EI–EII domains of E-cadherin (Nagar et al., 1996). All reports confirmed that calcium ions stabilize the interdomain organization of the extracellular cadherin region. Furthermore, analysis of cadherin crystals suggested that these proteins may form lateral homodimers. The models of organization of such dimers, however, differed significantly in two reports. According to one model (Shapiro et al., 1995) two cadherin molecules extending from the same cell surface laterally interact through hydrophobic interactions. A major feature of this interaction is the mutual incorporation of the conserved

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Trp residue, localized at the second position in mature
classic cadherins, into the hydrophobic core of the paired
molecule. This model was supported by experiments with
the recombinant extracellular portion of Xenopus C-cad-
herin showing that homodimerization is calcium indepen-
dent (Briever et al., 1996). In the alternative model (Nagar et al., 1996), lateral cadherin dimers are stabilized by mu-
tually coordinating calcium ions. The second model is con-
sistent with the observation that at high concentrations the
recombinantly expressed extracellular region of mouse
E-cadherin dimerized in a calcium-dependent manner
(Koch et al., 1997). The existence of any of such dimers on
the cell surface was not confirmed by direct experiments.
Dimerization of the extracellular E-cadherin segment,
however, was demonstrated in pentameric structures as-
sembled by chimeric proteins consisting of the extracelu-
lar portion of E-cadherin and the transmembrane region
of cartilage oligomeric matrix protein (Tomschy et al.,
1996).

It has been proposed that lateral cadherin dimers associ-
ate into antiparallel complexes that result in cell–cell ad-
hesion (Shapiro et al., 1995). The structural basis of this
interaction is not clear. Available data suggest that lateral
cadherin dimers are more efficient in adhesive interactions
than monomers and their formation may regulate intercel-
lular adhesion (Briever et al., 1996; Tomschy et al., 1996).
Extracellular calcium ions were shown to be required for
cadherin-dependent cell–cell adhesion (Kartenbeck et
al., 1982; Volberg et al., 1986; Ozawa et al., 1990a). Some
evidence, however, suggested that disruption of cadherin
adhesion by calcium removal may have a complex
mechanism and may be triggered via remodeling of the
microfilament cytoskeleton (Citi, 1992; Citi et al., 1994;
Shapiro et al., 1995).

Another well-established feature of classic cadherins is
their interaction with cytoplasmic proteins, catenins. The
intracellular cadherin region directly interacts either with
β-catenin or with plakoglobin (γ-catenin). Both these pro-
teins associate with α-catenin, which is thought to link the
cadherin–catenin complex to the actin cytoskeleton. Dele-
tion of the catenin-binding domain completely abolished
the adhesion activity of classic cadherins (Nagafuchi and
Takeichi, 1988; Ozawa et al., 1989, 1990b). Some evidence,
however, suggested that disruption of cadherin
adhesion by calcium removal may have a complex
mechanism and may be triggered via remodeling of the
microfilament cytoskeleton (Citi, 1992; Citi et al., 1994;
Shapiro et al., 1995).

Cloning of Human E-cadherin and Its Mutants

Fragments of the human E-cadherin gene between nucleotides 89 and 982
(fragment 1); 756 and 1,652 (fragment 2); 1,372 and 2,363 (fragment 3);
2,105 and 2,802 (fragment 4; nucleotide and amino acid sequences of
E-cadherin are numbered accordingly to the sequence with GenBank/
EMBL/DDBJ accession number Z13009, Bussemakers et al., 1993) were
amplified by PCR from a human salivary gland cDNA (Clontech, Palo
Alto, CA) using the following pairs of primers: 5'-CCACCCATGGGG-
CCTTGGAGCCCG-3' and 5'-TATAGAGGATCCATGGCCCATACA-3' and 5'-TCTGTTC-
CATAAATGTTGCTGTGCTG-3'; 5'-CAACAAATCCAGTGAAACAACG-
ATGG-3' and 5'-CATATAGTAAAAACGTTGTC-3'; 5'-GAT-
AACAGAATAAAGGCAAGTG-3' and 5'-GAAGCGTGATTTCC-
TGACTTCCAA-3'. The fragment 4 was blunt ended, cleaved by EcoRI,
and then ligated with the EcoRI-SmaI-digested Bluescript II KS+ vector
(Stratagene, La Jolla, CA). The resulting plasmid was cleaved by KpnI
EcoRI and ligated with the KpnI-EcoRI-digested fragment 3 that gener-
ates a plasmid BlEc3-4. In parallel experiments, the fragment 1 was blunt
ended, treated with BamHI, and then ligated with the BamHI-EcoRV-
digested Bluescript vector. The resulting plasmid was combined with the
fragment 2 using overlapping EcoRI site that generates a plasmid BlEc1-2.
Finally, to construct the BlEctw plasmid encoding a full-length E-cadherin
cDNA, the KpnI fragment of the plasmid BlEc2-1 was inserted into the
KpnI-cleaved BlEc3-4 plasmid. E-cadherin cDNA of this plasmid was
completely identical to the 89–2,802 nucleotide sequence of E-cadherin
published by Bussemakers et al. (1993).

To facilitate detection and immunoprecipitation, E-cadherin or its mu-
tants were tagged COOH terminal by 6x myc or by a single flag
epitope. For this procedure, the BamHI site was substituted for the E-cad-
herin stop codon in the BIEctw using PCR-mediated mutagenesis. This
site was used for ligation of the E-cadherin gene with the unique BamHI
site of 6x myc sequence in the plasmid CS26Mt (Chitaga and Troy-
ansky, 1997) or with the BgIII site of the Flag vector the plasmid
pFLAG-CTS (Sigma Chemical Co., St. Louis, MO). PCR-mediated muta-
genesis was used to construct the plasmid BlEc1M encoding E-cadherin
containing the internal deletion in the cytosolic domain between His73
and Leu96 (Ec1M). This deletion as was shown in preliminary experi-
ments specifically eliminates the recognition site of anti-E-cadherin mAb
clon C20820, Transduction Laboratories, Lexington, KY). To introduce
this deletion, two E-cadherin fragments (1,699–2,406 and 2,468–2,740
nucleotides) were amplified by PCR using primers 5'-TAATTCCCGG-
CAATGGTT and 5'-AAAAATCGGTCGACAAGTCC and 5'-AAA-
CAATTGGTAGTGCTCCCGGGTAT and 5'-GGATCGGTCGATCTC-
GGCGCTCCC. They were then ligated through the unique MunI site
(underlined) and the resulting fragment was inserted between the BspEI
and BamHI sites of the BlEc1M plasmid.

To construct the deletion mutant Ec1A(159–536), the unique NcoI/BspEI
fragment (941–702 nucleotides) in the BlEc1M was replaced with the
fragment (94–573) resulting from PCR performed with primers 5'-
CGGCCAGCCATGGGCCCCCT and 5'-AAAGTCCCGGAAATAC-
CCAGTCT. Plasmids BlEc1A(172–882) and BlEc1A(748–882) encod-
ing two different COOH terminally truncated E-cadherin mutants were
also constructed using PCR. The BspEI/BamHI fragment (1,703–2,745) of
the BlEc1M plasmid was replaced with the fragments amplified between
1,699 and 2,410 or between 1,699 and 2,339 nucleotides of E-cadherin
cDNA, respectively. Identical sense primer 5'-TAATTCCCGCACGTGT
and different antisense primers 5'-AAAGGATCCACGCTGCTAAGTCC
and 5'-AAAGGATCCGTCGATCTCCGTGGGG were used.

PCR-mediated mutagenesis was also used to introduce point mutations. In
the mutant Ec1 WVM, a nucleotide sequence GACTGCGTT encoding the
Asp94→Trp amino acid sequence was replaced with GAGGGCGG,
resulting in a new Nael site. In the mutant Ec1QMN, the nucleotide se-
quence GATCAGAAT encoding Asp34GlnAsn tripeptide was replaced with
GATGCTGCA. For transfection of A-431 cells, the corresponding
Bluescript clones were further subcloned into the eukaryotic expression
vector pRcCMV (Invitrogen, Carlsbad, CA) containing a neomycin resis-
tance gene and cytomegalovirus (CMV) promoter.
The integrity of the constructs was confirmed by restriction endonuclease mapping. All fragments obtained by PCR were completely sequenced using the sequencing core facility of Washington University Medical School (St. Louis, MO).

Cell Culture, DNA Transfection, and Immunofluorescence Microscopy
HaCat human keratinocytes (Boukamp et al., 1988) were provided by W.W. Franke (German Cancer Research Center, Heidelberg, Germany). Transfection of human epidermoid carcinoma A-431 cells (CRL1555; American Type Culture Collection, Rockville, MD) and selection, growth, and immunofluorescence microscopy were done as described (Chitaev et al., 1996). The following mouse monoclonal antibodies were used: anti-plakoglobin (clone 11 E4; Zymed Laboratories, San Francisco, CA); anti-desmoglein (clone 310; provided by W.W. Franke); anti-myc (clone 9E10; provided by R. Kopan, Washington University, St. Louis, MO); rabbit anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-flag (Sigma Chemical Co.), and anti-α-catenin, anti-E-cadherin (mAbs C20820 and C37020), anti-β-catenin, and anti-pli20 (Transduction Laboratories). In all cases, anti-E-cadherin antibody C20820 was used except where indicated.

Immunoprecipitation and Sedimentation Analysis
For most immunoprecipitation experiments, 2 × 10⁶ cells were cultured in 10-cm tissue culture dishes at 37°C for 72 h. In coculture experiments, 6 × 10⁶ cells producing myc- and flag-tagged forms of E-cadherin mixed in a ratio 1:1 were cultured in a 10-cm dish for 24 h. The confluent monolayer (~10⁶ cells) was then washed with PBS and extracted in 1.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 20 μM p-APMSF, 2 mM EDTA, and 1% NP-40) for 10 min. After clarification in a microfuge (model 5415C; Eppendorf Scientific, Inc., Hamburg, Germany) for 10 min at 14,000 rpm, the lysates were immunoprecipitated for 1.5 h at 4°C in the presence of specific antibody (~1 μg per sample). The antigen–IgG complexes were precipitated with sequential incubation with protein A-Sepharose as described previously (Chitaev et al., 1996). For the E-cadherin immunodepletion experiments, cell lysates were incubated with 5 μg of anti–E-cadherin (clones C37020 or C20820) or anti-Dsg 3.10 monoclonal antibodies. Then the antibodies were collected by two rounds of anti-mouse IgG–agarose (Sigma Chemical Co.) chromatography. After repeating this step once (second round of immunodepletion), the lysates were immunoprecipitated by anti-plakoglobin antibody as described above. For sucrose gradient centrifugation, confluent monolayer cells from three 10-cm dishes, obtained as described above, were washed with PBS and lysed with 2 ml of lysis buffer. For metabolic labeling experiments, cells were cultured in the presence of [³⁵S]methionine, 50 μCi/ml for 16 h. Lysates (1 ml) were centrifuged (model TL-100; Beckman Instruments, Inc., Palo Alto, CA) at 100,000 g for 1 h, were loaded on top of a 12-mL linear 5–20% (wt/wt) sucrose gradient prepared in lysis buffer. In some experiments, either 10 mM EGTA was added into gradient fractions before immunoprecipitation were mixed with BSA, 4.5 S; IgG, 7.5 S; catalase, 11.35 S; apoferritin, 17 S. Biotinylation of Cell Surface Proteins

The cells of three 10-cm dishes reaching near confluent growth were washed with ice-cold PBS containing 0.5 mM CaCl₂ (PBS-C). Each plate was incubated at 4°C with 7 ml of 0.5 mg/ml of sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) in PBS-C for 1 h. The reaction was quenched by washing the cells with 1 M Tris/100 mM glycine buffer, pH 7.5. In some experiments, cells were dissociated into a single cell suspension by 5 min of treatment with 10 mM EGTA in PBS at 37°C after biotinylation. Then cells were lysed in lysis buffer and analyzed by sucrose gradient centrifugation and coimmunoprecipitation assays as described above. Biotinylated proteins were visualized with streptavidin-HRP conjugate (Pierce Chemical Co.) in conjunction with enhanced chemiluminescence (Boehringer Mannheim, Indianapolis, IN).

Results

Lateral Dimers of E-cadherin Are Exposed on the Surface of Epithelial Cells
The cytoplasmic domain of E-cadherin binds to either β-catenin or plakoglobin (Butz and Kemler, 1994; Hinck et al., 1994) that in turn, interacts directly with α-catenin (Jou et al., 1995). This gives rise to two distinct E-cadherin–catenin complexes, E-cadherin–β-catenin–α-catenin and E-cadherin–plakoglobin–α-catenin. The presence of a plakoglobin–β-catenin complex may indicate the assembly of these conventional cadherin–catenin complexes into higher order structures. To test this hypothesis, we subjected Triton X-100–soluble proteins from A-431 or HaCat cells to sucrose gradient fractionation followed by immunoprecipitation with anti-plakoglobin (Fig. 1 a) or anti-β-catenin (data not shown) mAbs. Both types of experiments showed that the conventional E-cadherin–catenin complexes sedimented at ~9 S, in agreement with published results (Nelson et al., 1990; Ozawa and Kemler, 1992; Hinck et al., 1995). However, the plakoglobin–β-catenin complex separated into a distinct peak, sedimenting at 13 S. Both complexes were stable upon recentrifugation (Fig. 1, b and c), showing that the 9 S complex does not associate into a 13 S complex during in vitro manipulation. To determine whether E-cadherin is part of the β-catenin–plakoglobin complex, the 13 S fraction from metabolically labeled proteins of A-431 cells was immunoprecipitated with either anti–E-cadherin or anti-plakoglobin mAbs. Immunoprecipitates were found to contain an identical set of proteins, E-cadherin, plakoglobin and α- and β-catenins (Fig. 2 a). Furthermore, depletion of the 13 S fraction with any of two anti–E-cadherin mAbs used in this work before anti-plakoglobin immunoprecipitation strongly reduced β-catenin communoprecipitation. Depletion with mAb against desmoglein, that is desmosome-specific cadherin of the A-431 cells, had no effect (Fig. 2 b). This suggests that the E-cadherin found in the 13 S fraction is complexed with plakoglobin and β-catenin. The plakoglobin–β-catenin complex detectable after E-cadherin immunodepletion is likely to contain P-cadherin, another classic cadherin of A-431 cells.

To show that the formation of this complex requires dimerization of E-cadherin, we expressed in A-431 cells human E-cadherin containing a 19-amino acid (aa)¹ His792-Leu801) long internal deletion in the cytoplasmic domain and COOH-terminal 6× myc epitope (Ec1M, see Fig. 3 a for details). As demonstrated by Western blotting (Fig. 3 b), this deletion completely abolished binding of E-cadherin to mAb C20820. Thus, cells stably transfected with the Ec1M DNA construct produce two forms of E-cadherin that could be specifically recognized by mAb C20820 (endogenous form) and by mAb against the myc epitope (Ec1M form). Only cell subclones producing Ec1M at the level similar to the level of endogenous

1. Abbreviation used in this paper: aa, amino acid(s).
E-cadherin, as estimated by immunoblotting of the total lysates of the selected clones with C37020 antibody, was detected in both 9 S and 13 S fractions (Fig. 3 c and not shown). Thus, the His° catenin and E-cadherin in the anti-plakoglobin immunoprecipitate. (b) Western blot analysis of the total proteins from fraction 6 released with anti-E-cadherin (Ec), β-catenin (βc), and plakoglobin (Pg) antibodies before (lane 1), and after depletion with anti-E-cadherin C20820 (first round, lane 2; second round, lane 3) or anti-desmoglein (first round, lane 4; second round, lane 5) mAbs. The samples depleted with anti-E-cadherin antibody (lane 6), or not desmoglein antibody (lane 7), reduces recovery of the plakoglobin-β-catenin complex.

E-cadherin is a transmembrane protein that mediates cell-cell adhesion and plays a crucial role in the maintenance of cell polarity and cellular integrity. It is composed of a cytoplasmic domain, an extracellular domain, and a transmembrane domain. The cytoplasmic domain contains binding sites for β-catenin and α-catenin, which are integral components of the cadherin-catenin complex. This complex is involved in various cellular processes, including cell migration, morphogenesis, and tissue development.

In the context of the provided information, E-cadherin is studied in relation to its interaction with plakoglobin and β-catenin. The study also investigates the effects of deletions and the addition of tags to the E-cadherin molecule on its properties and localization within the cell. The results suggest that E-cadherin, when tagged with a myc epitope, retains its ability to dimerize with other proteins and participate in cell-cell adhesion processes.

The findings are supported by experiments that demonstrate the preservation of E-cadherin's interaction properties in various conditions. This understanding is further supported by the observed sedimentation properties of the E-cadherin–plakoglobin–β-catenin complex, which remains stable even after depletion with anti-E-cadherin antibodies.

These findings contribute to our understanding of the molecular mechanisms underlying cell-cell adhesion and the role of E-cadherin in maintaining cellular integrity, providing insights that are valuable for the study of developmental biology, tissue engineering, and potentially for therapeutic applications in various disease states involving cell adhesion and migration.
Taken together, these data demonstrate a presence on the cell surface of the E-cadherin complex in which both E-cadherin molecules are aligned in parallel orientation.

The Extracellular EI Domain, But Not Binding to Catenins or p120, Is Required for Lateral E-cadherin Dimerization

Next, we examined whether binding to cytosolic proteins is essential for lateral association of EclM with endogenous E-cadherin. The truncated version of EclM, the EclD(772–882)M mutant (refer to Fig. 3a and Fig. 4 for details), lacking the catenin-binding domain was expressed in A-431 cells. In agreement with the previous data (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990b), immunofluorescent microscopy and degradation of this mutant by trypsin treatment of the intact cells indicated that despite deletion of the catenin-binding domain, the EclD(772–882)M protein was from the same cell. Taken together, these data demonstrate a presence on the cell surface of the E-cadherin complex in which both E-cadherin molecules are aligned in parallel orientation.
transported to the cell surface. Results presented in Fig. 4 show that this mutant associates efficiently with endogenous E-cadherin. Besides catenins, in agreement with previous observations (Reynolds et al., 1994; Shibamoto et al., 1995), minor fractions of the 9 S and 13 S E-cadherin–catenin complexes were found to be associated with the tyrosine kinase substrate p120 (refer to Fig. 1a and Fig. 4a). Deletion of the catenin-binding domain in the Ecl1Δ(772–882)M mutant did not affect association with p120 (Fig. 4a), whereas it was completely abolished by a deletion of an additional 24-aa residues from the COOH terminus of the Ecl1Δ(772–882)M protein (Fig. 4a). The resulting deletion mutant Ecl1Δ(748–882)M was unable to bind p120 but formed hybrid complexes with endogenous E-cadherin (Fig. 4a and b). As expected, the mobility of the hybrid complex in a sucrose gradient shifted from fraction 6 to 7 due to the absence of the catenin-binding site in the mutant. This complex was not affected by EGTA-induced dissociation of the cells. These experiments show that lateral dimerization of E-cadherin is mediated by its extracellular domain and does not depend on an interaction with the cytoplasmic proteins p120, plakoglobin, and catenins.

X-ray crystallographic analysis (Shapiro et al., 1995; Nagar et al., 1996) showed that the extracellular domain of cadherin forms homodimers. Shapiro et al. (1995) suggested that the side chain of Trp156 of each cadherin molecule protrudes into the hydrophobic core of the neighbor molecule. In the second model, the lateral dimers are stabilized by mutually coordinating calcium ions (Nagar et al., 1996). Both models are consistent with our observation that deletion of the extracellular cadherin-like repeats 1–3 completely abolishes dimerization, whereas the lateral dimerization of the resulting Ec1WVM mutant and the other extracellular mutant Ec1WVM(772–882)M protein (Fig. 4c) is an effect of the removal of extracellular calcium, which Trp156 and a neighbor residue, Val157, were substituted for Ala and Gly, respectively (refer to Fig. 3a). In A-431 cells, this mutant was translocated to the cell surface (data not shown) and formed a conventional 9 S trimer, but not the 13 S complex (Fig. 4a). Additionally, the Ecl1WVM mutant and the other extracellular mutant Ecl1Δ(159–536)M, induced a strong dominant-negative effect on cell–cell adhesion (Fig. 4a and b). A similar effect has been described previously for different cadherins in lateral dimerization. To determine whether the Trp156 residue is required for E-cadherin dimerization, we constructed an Ec1WVM combinatorial mutant in which Trp156 and a neighbor residue, Val157, were substituted for Ala and Gly, respectively (refer to Fig. 3a). In A-431 cells, this mutant was translocated to the cell surface (data not shown) and formed a conventional 9 S trimer, but not the 13 S complex (Fig. 4a). Additionally, the Ecl1WVM mutant and the other extracellular mutant Ec1Δ(159–536)M, induced a strong dominant-negative effect on cell–cell adhesion (Fig. 4a and b). A similar effect has been described previously for different cadherins lacking the extracellular domain (Kintner, 1992; Fujiwara and Takeichi, 1993). Because this phenotypic effect may abolish lateral E-cadherin association indirectly, we determined whether these cells contain the 13 S plakoglobin/β-catenin complex incorporating endogenous E-cadherin. This was done by analyzing the association between plakoglobin and β-catenin using anti-plakoglobin (or anti-β-catenin) coimmunoprecipitation assays as described above. The data showed that the amount and sedimentation properties of the 13 S complex were unchanged in control and in transfected cells (data not shown), suggesting that a failure of the Ec1WVM mutant to interact with endogenous E-cadherin was not caused by a dominant-negative effect. Thus, our data imply that lateral interaction between two E-cadherin molecules in the 13 S complex is mediated by the mechanism proposed by Shapiro et al. (1995).

Adhesive, Head to Head, and Not Lateral E-cadherin Interaction Requires Catenins and Calcium

The data presented provide clear evidence that the lateral dimerization of the E-cadherin is independent of calcium and catenins. In contrast, homophilic cadherin-mediated cell–cell adhesion strictly depends on both these factors, suggesting that cadenin and calcium may directly control adhesive, but not lateral association between cadherins. Our experiments with Ec1M protein, however, failed to demonstrate any multicadherin complexes other than lateral E-cadherin–catenin hexamers. The adhesive intercadherin association, however, could be hidden in the experiments described above if adhesive and lateral E-cadherin complexes have the same compositions and, hence, similar sedimentation properties. To test for the formation of adhesive complexes in which E-cadherin–catenin complexes are arranged in antiparallel orientations, we applied an approach successfully used in our recent experiments with desmosomal cadherins (Chitaev and Troyanovsky, 1997). A-431 cells expressing Ec1M were cocultured overnight with A-431 cells stably producing the Ec1F form of E-cadherin. The latter protein contained the same internal deletion as Ec1M and was selectively tagged by flag, but not the myc epitope. In this coculture system, specific coimmunoprecipitation of Ec1F with anti-myc antibody would indicate the adhesive association between two forms of E-cadherin, whereas the presence of endogenous E-cadherin in the same immunoprecipitates, assessed by mAb C20, would show both lateral and adhesive types of E-cadherin complexes.

Sedimentation–immunoprecipitation experiments with the total lysates obtained from such cocultures demonstrated that in the 13 S fractions anti-myc antibody did not coimmunoprecipitate both flag-tagged and endogenous E-cadherin (Fig. 5). To show that the association between flag- and myc-tagged forms of E-cadherin depends on the integrity of cell–cell contacts, the cocultured cells were incubated for 10 min in 10 mM EGTA at 37°C before lysis in the regular lysis buffer. Complete disappearance of Ec1F, but not endogenous E-cadherin, in the anti-myc immunoprecipitates after EGTA treatment (Fig. 5b, lanes 1 and 2) showed that the 13 S fraction contained two distinct lateral and adhesive E-cadherin complexes. To study whether reestablishment of the cell–cell contacts is accompanied by restoration of adhesive E-cadherin complexes, the cocultured cells, after incubation in 10 mM EGTA at 37°C, were further cultivated for the next 30 min either in low calcium or in normal medium (Fig. 5b, lanes 3 and 4). The data show that the normal medium completely restored the amount of adhesive dimers (Ec1M–Ec1F complexes), whereas only Ec1M–E-cadherin complexes were detected in low calcium medium. Surprisingly, depletion of the calcium ions by EGTA did not reduce the amount of the E-cadherin–Ec1M complexes, which is a sum of the lateral and adhesive complexes (Fig. 5 and refer to Fig. 3). It can be suggested that the removal of extracellular calcium, while causing dissociation of the adhesive complexes, might increase the solubility of the lateral complexes which would account for the unchanged amount of the total complexes detected.

To further evaluate the function of calcium ions in adhesive E-cadherin interactions, two additional experiments
were performed. First, the cocultured cells were incubated before lysis for 10 min with EGTA at 4°C. At this temperature, in agreement with published observations (Citi, 1992), EGTA did not affect cell–cell junctions. In the second experiment, 10 mM EGTA was added to the lysis buffer. In this case, the cells were not incubated with EGTA before lysis. Surprisingly, we found that in both these conditions EGTA did not decrease the adhesive association between flag- and myc-tagged forms of cadherins (Fig. 5b, lane 5 and data not shown). Taken together, these data strongly suggest that calcium ions do not directly participate in the formation of the structure of the adhesive interface between two E-cadherin molecules. This is consistent with the crystallography model reported by Shapiro at al. (1995). Our experiments, however, did not exclude the possibility that calcium might be important for one of the steps leading to the formation of adhesive dimers. To test this assumption, a new Ec1M mutant, Ec1QNM, in which residues Gln\(^{255}\) and Asn\(^{256}\) were replaced with two alanines, was constructed. According to X-ray analysis, the replaced amino acids participate in coordination of two of the three Ca\(^{2+}\) ions filling the cleft between the ECI and ECII domains of E-cadherin (Nagar et al., 1996). Furthermore, it was shown that ablation of one of these sites blocked E-cadherin cell–cell adhesion activity (Ozawa et al., 1990a). We found that the Ec1QNM mutant upon expression in A-431 cells, similar to the Ec1WVM mutant described above, was delivered to the cell surface and induced a severe dominant-negative effect on cell–cell adhesion (Fig. 6). This mutant normally associated with endogenous E-cadherin, but not with Ec1F (Fig. 5b, lane 7), suggesting that defects in the Ca\(^{2+}\)-binding sites, while unable to change lateral dimerization, affect the adhesion interaction between two E-cadherin molecules. However, we could not discard the possibility that the absence of such an interaction is caused indirectly by the dominant-negative effect of the Ec1QNM mutant on the cellular phenotype. Although additional experiments are needed to completely understand the role of calcium in cadherin-mediated adhesion, our data imply that binding of calcium ions to cadherins is important for the formation of adhesive dimers, that become independent of calcium after their formation.

Finally, we determined whether mutations constructed for the characterization of lateral E-cadherin dimerization were performed. First, the cocultured cells were incubated before lysis for 10 min with EGTA at 4°C. At this temperature, in agreement with published observations (Citi, 1992), EGTA did not affect cell–cell junctions. In the second experiment, 10 mM EGTA was added to the lysis buffer. In this case, the cells were not incubated with EGTA before lysis. Surprisingly, we found that in both these conditions EGTA did not decrease the adhesive association between flag- and myc-tagged forms of cadherins (Fig. 5b, lane 5 and data not shown). Taken together, these data strongly suggest that calcium ions do not directly participate in the formation of the structure of the adhesive interface between two E-cadherin molecules. This is consistent with the crystallography model reported by Shapiro et al. (1995). Our experiments, however, did not exclude the possibility that calcium might be important for one of the steps leading to the formation of adhesive dimers. To test this assumption, a new Ec1M mutant, Ec1QNM, in which residues Gln\(^{255}\) and Asn\(^{256}\) were replaced with two alanines, was constructed. According to X-ray analysis, the replaced amino acids participate in coordination of two of the three Ca\(^{2+}\) ions filling the cleft between the ECI and ECII domains of E-cadherin (Nagar et al., 1996). Furthermore, it was shown that ablation of one of these sites blocked E-cadherin cell–cell adhesion activity (Ozawa et al., 1990a). We found that the Ec1QNM mutant upon expression in A-431 cells, similar to the Ec1WVM mutant described above, was delivered to the cell surface and induced a severe dominant-negative effect on cell–cell adhesion (Fig. 6). This mutant normally associated with endogenous E-cadherin, but not with Ec1F (Fig. 5b, lane 7), suggesting that defects in the Ca\(^{2+}\)-binding sites, while unable to change lateral dimerization, affect the adhesion interaction between two E-cadherin molecules. However, we could not discard the possibility that the absence of such an interaction is caused indirectly by the dominant-negative effect of the Ec1QNM mutant on the cellular phenotype. Although additional experiments are needed to completely understand the role of calcium in cadherin-mediated adhesion, our data imply that binding of calcium ions to cadherins is important for the formation of adhesive dimers, that become independent of calcium after their formation.

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abolish the adhesive cadherin interaction. For this, we co-cultivated the cells expressing these mutants with Ec1F-producing cells as described above. Anti-myc immunoprecipitation experiments presented in Fig. 5 b clearly show that the adhesive Ec1M–Ec1F association was completely abolished either by deletion of the catenin-binding region or by Trp156Ala/Val157Gly replacement (Fig. 5 b, lanes 6 and 8, respectively). The same factors, catenin and calcium, are therefore required for E-cadherin to form adhesive dimers and to establish cell–cell adhesion. This strongly suggests that the revealed dimers are structural elements of cadherin-mediated cell–cell adhesion.

Discussion

It is well established that the transmembrane protein E-cadherin forms complexes with intracellular β-catenin (or plakoglobin) and with α-catenin (in this paper they are designated conventional E-cadherin–catenin complexes). This association is absolutely required for E-cadherin to mediate intercellular adhesion (Nagafuchi and Takeichi, 1988; Ozawa et al., 1990b). The exact role of catenins in this process is still unknown. It was also suggested (Shapiro et al., 1995; Briehler et al., 1996; Nagar et al., 1996; Tomschy et al., 1996), although never shown directly, that E-cadherin may form lateral dimers on the cell surface. These lateral complexes, in which two E-cadherin molecules are aligned in parallel orientation, were proposed to participate, in turn, in the antiparallel interactions resulting in cell–cell adhesion. The structural basis of both adhesive and lateral interactions is not clear. In the present investigation, we analyzed intercadherin interactions using an immunoprecipitation approach. To trace such interactions, we expressed in A-431 cells producing endogenous E-cadherin recombinant forms of the same protein tagged either by myc or by flag epitopes. Our findings not only present compelling evidence for two distinct lateral and adhesive E-cadherin complexes, but also allows us to clarify their structures.

First, we obtained a strong evidence that the conventional E-cadherin–catenin complexes may self-associate on the cell surface through E-cadherin dimerization. Our data strongly support the idea that this process is driven by hydrophobic interactions between the extracellular E1 domains of E-cadherin. This mechanism was previously proposed by Shapiro et al. (1995). As suggested by sedimentation analysis (refer to Figs. 1 and 3), only a relatively small pool of E-cadherin is incorporated into the lateral E-cadherin complexes. The formation of these E-cadherin complexes is independent of interactions of E-cadherin with cytoplasmic proteins, such as catenins or p120. The fact that even the longest deletion within the intracellular E-cadherin region did not abolish lateral dimerization suggests that cytoplasmic factors are unlikely to regulate assembly of these dimers. The amount of the lateral complexes was also independent of cell–cell interactions, since it was stable (a) upon EGTA treatment leading to separation of cells, (b) in cells cultivated up to 1 wk in low calcium medium preventing establishment of cell–cell contacts (data not shown), and (c) in the cells with strong dominant-negative abnormalities in cell–cell adhesion caused by Ec1QNFM expression. These data imply that lateral E-cadherin dimerization is not triggered in response to head to head interaction with a protein(s) extended from the opposing cell surface. Thus, if the E-cadherin lateral complex is not formed spontaneously, its assembly is regulated either by soluble (humoral) factor(s) or by transmembrane protein(s) extended from the same cellular surface.

Interestingly, independence of E-cadherin dimerization from catenins indicates that the cell surface may contain dimers in which E-cadherin molecules are linked either to the same or to different catenins (plakoglobin and β-catenin). This possibility was supported by our experiments (Chitaev et al., 1998) with A-431 cells expressing myc-tagged versions of β-catenin or plakoglobin. In both cell types, anti-myc coimmunoprecipitation shows that the 13 S complex may contain two β-catenin or two plakoglobin molecules. Diversity of the lateral E-cadherin complexes might even be larger since some may also incorporate different isoforms of p120. It is not clear whether such variety has any biological significance. In theory, E-cadherin dimers associated with different sets of catenins may have distinct signaling or adhesive properties.

The second type of inter–E-cadherin interaction detected in our work is an adhesive interaction leading to the formation of antiparallel E-cadherin complexes. Our experiments did not allow us to evaluate the exact structure of such complexes, since these and lateral complexes were present in the same fractions of the sucrose gradient. On the other hand, several observations strongly suggest that, similar to lateral complexes, they consist of two conventional E-cadherin–catenin complexes, but arranged in an antiparallel fashion. First, both lateral and antiparallel complexes have the same sedimentation value. Second, no differences were detected in protein composition of antiparallel E-cadherin (or anti-β-catenin) radioimmunoprecipitates obtained from the 13 S fraction from cells exhibiting (control), and not exhibiting (EGTA-treated) antiparallel complex (data not shown). Finally, immunoblotting experiments failed to reveal association with 13 S E-cadherin complex other possible E-cadherin/catenin–associated proteins, such as EGF-receptor, α-actinin, vinculin, or actin. These observations indicate similar protein composition of the lateral and antiparallel E-cadherin complexes, although we cannot exclude that some of the peripheral proteins dissociate from these complexes during their solubilization in Triton X-100. The most important and specific feature of adhesive complexes is the requirement for their assembly of the same factors, extracellular calcium and catenins, necessary for cell–cell adhesion. Such similarities strongly suggest that the revealed antiparallel complexes are structural units of cadherin-mediated cell–cell adhesion and their clustering, via interaction with the actin cytoskeleton (Knudsen et al., 1995; Rimm et al., 1995), may be a step in adherens junction assembly.

The critical question is whether adhesive and lateral complexes are the consecutive steps of one process, or are unrelated, and participate in distinct processes, such as signaling and adhesion. Although additional experiments are needed to understand this question, there is evidence in favor of the first hypothesis. We show that the Trp156Ala/Val157Gly substitution which, according to Shapiro et al. (1995), leaves an intact adhesive interface,
abolishing both lateral and adhesive association. The latter association, however, could be specifically blocked by removal of the catenin- or calcium-binding sites. This suggests that the adhesive complex is derived from lateral complexes. A similar scenario was suggested by Briehner et al. (1996), who found that lateral dimers of the recombinant extracellular portion of C-cadherin, participate in adhesion much more efficiently than monomers. Examination of intra-E-cadherin interactions by chimeric ECAD-COMP molecules also supports this view (Tomschy et al., 1996). The obvious problem is that, the head to head association of two lateral complexes must result in a structure containing at least four conventional E-cadherin–catenin complexes. Our attempts to reveal E-cadherin complexes greater than 13 S using different centrifugation protocols were unsuccessful. Thus, the mechanism of the assembly of the adhesive complexes remains open. One interpretation of our results is that a hypothetical antiparallel complex consisting of the four conventional E-cadherin–catenin units is very unstable. In normal cells this complex dissociates at a high rate into two equal adhesive complexes characterized in our work. Alternatively, a lateral E-cadherin-catenin complex may dissociate, giving rise to two activated E-cadherin–catenin complexes. They are subsequently used in adhesive associations. Importantly, both models would predict a special step, lateral-adhesive transition, in which two lateral E-cadherin dimers form two antiparallel dimers. Catenins and calcium ions might be functionally important for E-cadherin to proceed to this transition. In the simplest model, catenins in response to the lateral dimerization of E-cadherin would trigger a conformational change of each cadherin resulting in the activation of the E-cadherin adhesive site and concomitant dissociation of the lateral dimers. This model is consistent with our data suggesting that calcium ions, which are known to stabilize overall cadherin conformation (Pokutta et al., 1996), could be involved only in formation, but not in maintenance of the adhesive association.

In summary, our data present direct experimental evidence that E-cadherin may form lateral and adhesive complexes. They also show that interactions of E-cadherin with catenins and Ca\(^{2+}\) are essential for adhesive, but not for lateral intercadherin interactions. The major points for further investigation are the precise mechanisms leading to the formation of adhesive complexes and the role of catenins and other intracellular factors in this process.

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