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# Stable and Unstable Cadherin Dimers: Mechanisms of Formation and Roles in Cell Adhesion<sup>□</sup>

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Numerous attempts to elucidate the strength of cadherin dimerization that mediates intercellular adhesion have produced controversial and inconclusive results. To clarify this issue, we compared E-cadherin dimerization on the surface of living cells with how the same process unfolds on agarose beads. In both cases, dimerization was monitored by the same site-specific cross-linking assay, greatly simplifying data interpretation. We showed that on the agarose surface under physiological conditions, E-cadherin produced a weak dimer that immediately dissociated after the depletion of calcium ions. However, either at pH 5 or in the presence of cadmium ions, E-cadherin produced a strong dimer that was unable to dissociate upon calcium depletion. Both types of dimers were W156-dependent. Remarkably, only the strong dimer was found on the surface of living cells. We also showed that the intracellular cadherin region, the clustering of which through catenins had been proposed as stabilizer of weak intercadherin interactions, was not needed, in fact, for cadherin junction assembly. Taken together, our data present convincing evidence that cadherin adhesion is based on high-affinity cadherin–cadherin interactions.

## INTRODUCTION

Classic cadherins are a family of adhesion transmembrane receptors that are responsible for the structural integrity and the specific architecture of all solid tissues in vertebrates. Malfunctions in the cadherin adhesion system are often regarded as a factor in tumor cell invasion and metastasis (Takeichi, 1995; Provost and Rimm, 1999; Patel *et al.*, 2003; Gumbiner, 2005). It is widely accepted that cell–cell adhesion is produced by the homodimerization of cadherin molecules exposed on opposing cells. This interaction obviously determines many critical parameters of cell–cell adhesion including its strength, plasticity, and stability. Although extensive work has been done to characterize the molecular details of cadherin adhesion interactions, many basic aspects of this process remain unknown.

The principal question that is yet to be answered is the strength of the individual cadherin adhesion bonds. The uncertainty arises from two contradictory groups of observations (reviewed in Troyanovsky, 2005; Mege *et al.*, 2006). On one hand, numerous biophysical experiments with recombinant cadherin fragments have shown that the lifetime of such a bond is limited to the millisecond range. On the other hand, remarkably stable cadherin homodimers with an undetectable dissociation rate were demonstrated in cultured cells by coimmunoprecipitation experiments. Conse-

quently, there are two principally different models of cadherin adhesion. A low-affinity cadherin adhesion model suggests that the strength of a cell–cell adhesive contact is mediated by the clustering of cadherin receptors via cytoplasmic interactions (Yap *et al.*, 1998; Kusumi *et al.*, 1999). According to this model, the clustering of the short-lived adhesive bonds provides the essential stability for the entire junction. Little is known, however, about the molecular details of cadherin clustering. Moreover, some data clearly contradict the “clustering-stability” hypothesis. For example, in some experiments E-cadherin mutants entirely lacking the intracellular region (and thus disconnected from the hypothetical intracellular clustering machinery) provided an adhesive force sufficient to aggregate cells in the aggregation assay (Ozawa and Kemler, 1998). Such data circumstantially support an alternative, high-affinity model of cadherin adhesion. By this model, the cell–cell adhesion is based on the continuous formation of high-affinity cadherin adhesive dimers, which dissociate under a strict cellular control (Troyanovsky *et al.*, 2006). The most obscure aspect of the latter model is the mechanism of high-affinity cadherin dimerization: why was this process never detected *in vitro*? It is also not clear why, if adhesion is based on stable dimers, does cadherin recruitment into junctions depend on intracellular cadherin–catenin interactions?

The prime objective for this work was to clarify these two questions, very critical for high-affinity model of cadherin adhesion. We first asked whether stable cadherin dimers identical to those detected in cells could be assembled *in vitro*. To answer this question, we studied cadherin dimerization on the surface of agarose beads using a site-specific cross-linking assay. In complete agreement with the published *in vitro* experiments, we showed that under physiological conditions cadherin formed unstable homodimers that immediately dissociated after the depletion of calcium

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ions. However, under destabilizing conditions (such as at pH 5, in the presence of cadmium ions or at high temperature) E-cadherin produced stable dimers. By all parameters these dimers were indistinguishable from those detected in living cells. These experiments clearly showed that stable dimers are formed in living cells as a result of a specific reaction. This reaction can be one of the important regulatory steps of cadherin-based adhesion.

We then studied a tailless cadherin mutant Ec1Δ(748-882)M. This mutant, which is unable to interact with any known intracellular cadherin partners, neither is recruited into intercellular junctions nor forms adhesive dimers (Chitaev and Troyanovsky, 1998). According to the low-affinity model of cadherin adhesion, such a phenotype is based on the disconnection of this mutant from the intracellular clustering machinery. However, we now found that the inactivation of clathrin endocytosis by several different small interfering RNAs (siRNAs) completely restored the recruitment of this mutant into cell–cell junctions. Furthermore, even a complete depolymerization of actin filaments by latrunculin A did not prevent the clustering of this mutant. These observations showed that the recruitment of cadherin into junctions can be based solely on extracellular interactions. Taken together, our study presents new, critical evidence supporting the high-affinity model of cadherin adhesion.

## MATERIALS AND METHODS

### Cell Culture, DNA Transfection, and Plasmid Construction

All clones of human epidermoid carcinoma A-431 but Ec1M-C163A/V176C/D155A-expressing clones were previously reported (Chitaev and Troyanovsky, 1998; Troyanovsky *et al.*, 2003). New plasmids coding for this mutant were constructed using site-directed mutagenesis in the expression vector pRcCMV (Invitrogen, Carlsbad, CA). Cadherin sequences are numbered according to human E-cadherin (Bussemakers *et al.*, 1993). Transfection, growth, immunofluorescence microscopy, and immunoprecipitation of the cells were done as described (Troyanovsky *et al.*, 2003). In some experiments the actin filament inhibitors, cytochalasin D (Sigma, St. Louis, MO; final concentration 5  $\mu$ M) or latrunculin A (Molecular Probes, Eugene, OR; final concentration 0.2  $\mu$ M) were used.

### Antibodies

Mouse antibodies were as follows: anti-E-cadherin (C20820), anti-clathrin heavy chain, anti- $\alpha$ -adaptin, anti-AP50, anti-epidermal growth factor (EGF) receptor (all BD Bioscience, San Jose, CA); anti-E-cadherin HECD-1 and SHE78-7 (both from Zymed Laboratories, South San Francisco, CA); and anti-myc (clone 9E10) and anti-flag (both from Sigma). Rabbit anti-myc (Santa Cruz Biotechnology, Santa Cruz, CA) and fluorescein isothiocyanate (FITC)-phalloidin (Sigma) were used for double staining.

### siRNA, Transfection, and Transferrin Uptake Assay

The clathrin heavy-chain siRNAs (AAA UUC UUC UAA CUC UGC AAG GCG G, HC Oligo 1; CCG GAA AUU UGA CAA UAC UUC A, HC Oligo2); the AP-2 subunit  $\alpha$ -adaptin siRNA (CCU GGG CCG CAU GUA UCU CUU CUA U); the AP-2 mu2-subunit siRNA (GGU GGU CAU CAA GUC CAA CUU UAA A); and three negative control oligos (low, medium, and high GC) were obtained from Invitrogen. Three hours before transfection with siRNA the cells were trypsinized and plated on 5-cm dishes at a density of 105 cells per dish. Transfection was performed according to Invitrogen protocol using Lipofectamine 2000. On next day the cells were replated and assayed 48 or 64 h after transfection. The assay for uptake of FITC-conjugated transferrin (Molecular Probes) was done as described elsewhere (Hinrichsen *et al.*, 2003).

### Cross-Linking

For cell-surface cross-linking, cells (on 3-cm dishes) were first washed with ice-cold phosphate-buffered saline containing either 1 mM CaCl<sub>2</sub> (PBS-Ca) or EDTA (PBS-EDTA) and then cross-linked for 5 min by BM[PEO3] (1 mg/ml in PBS) at 4°C.

To cross-link proteins on the surface of protein A-Sepharose, confluent cultures from three 10-cm dishes were washed and extracted with 2 ml of immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40). The insoluble material was removed by centrifugation and the lysates (1 ml) were loaded on top of a 12-ml linear 5–20% sucrose

gradient prepared in lysis buffer. Gradients, centrifuged at 200,000  $\times$  g for 17 h in SW40Ti rotor (Beckman Instruments, Fullerton, CA) at 4°C, were fractionated bottom to top into 12 (1 ml each) fractions. The fractions 9 and 10 containing predominantly monomeric cadherin (in respect to cadherin molecules, cf. 5) were immunoprecipitated using subsequent incubations with an anti-myc antibody (~2  $\mu$ g per sample, 1 h) and protein A-Sepharose (100  $\mu$ l, 0.15 mg/ml, 1 h). The beads were then washed four times in PBS-EDTA supplemented with 0.1% Triton X-100. The precipitates obtained from one sample were divided on several aliquots (up to 10 equal portions) and used for cross-linking. The beads were first washed in PBS-0.1% Triton X-100 (or in experiments with Cd<sup>2+</sup> ions in HBS-0.1% Triton X-100 (10 mM HEPES, pH 7, 150 mM NaCl). In some experiments the beads after that were incubated for 10 min with PBS or HBS containing different concentrations of Ca<sup>2+</sup> or Cd<sup>2+</sup> ions at different temperatures or pH and then washed with buffers containing the indicated concentrations of divalent ions or EDTA. Finally beads were cross-linked by BM[PEO3] (1 mg/ml) for 5 min. The reaction was terminated by adding an equal volume (100  $\mu$ l in typical experiments) of SDS-gel sample buffer containing 200 mM dithiothreitol. Samples were separated by SDS-5% PAGE and then analyzed by immunoblotting as described previously (Troyanovsky *et al.*, 2003).

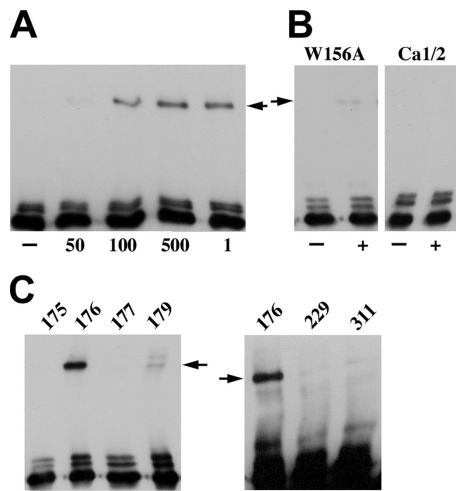
## RESULTS

### Unstable Cadherin Dimers

Our recent work described a simple and reliable technique for detecting cadherin dimerization *in vivo* (Troyanovsky *et al.*, 2003). We demonstrated that lateral and adhesive homodimers of the cadherin cysteine mutant Ec1M-C163A/V176C can be efficiently cross-linked by the cysteine-specific homobifunctional cross-linker BM[PEO3] on the surface of A-431 cells. By all tested parameters the dimers revealed by this approach correspond to the cadherin dimers detected in the same A-431 cells by a coimmunoprecipitation assay. To understand whether such dimers could be formed outside the cell context, we studied the homodimerization of the same Ec1M-C163A/V176C mutant on the surface of agarose beads. To this end, the monomeric fraction of the Ec1M-C163A/V176C mutant extracted from Ec1M-C163A/V176C-expressing A-431 cells was loaded on protein A-Sepharose through an anti-myc antibody. All these manipulations were performed in an EDTA-containing buffer. Then cadherin-coated beads were exposed to BM[PEO3] in the presence or absence of calcium ions. These experiments showed that calcium ions at concentrations above 100  $\mu$ M triggered cadherin cross-linking (Figure 1A).

We first checked whether the formation of this cross-linked dimeric product is abolished by point mutation W156A. It had been shown that this mutation, which does not significantly change the secondary cadherin structure, specifically inactivates cadherin dimerization (Chitaev and Troyanovsky, 1998; Shan *et al.*, 2000; Ozawa, 2002). Figure 1B (lanes W156A) shows that this mutation did abolish calcium-dependent cross-linking of the Ec1M-C163A/V176C mutant. Cross-linking of the Ec1M-C163A/V176C mutant was also completely blocked by point inactivation of the EC1-EC2 calcium-binding site (Figure 1B, lanes Ca1/2). Similar to W156A, this mutation was shown to inactivate cadherin adhesive dimerization (Chitaev and Troyanovsky, 1998; Troyanovsky *et al.*, 2003). Both control experiments suggested that Ec1M-C163A/V176C cross-linking is caused by specific cadherin dimerization but does not result from calcium-dependent changes of cadherin molecules bound to the anti-myc antibody.

Second, we compared the cross-linking efficiency of different cadherin cysteine mutants. We had previously shown that among a large set of cysteine mutations, the mutation V176C resulted in the most efficient cross-linking of cadherin-adhesive dimers on the cell surface (Troyanovsky *et al.*, 2003). When we cross-linked the same set of cadherin mutants on beads, we found the same phenomenon: the

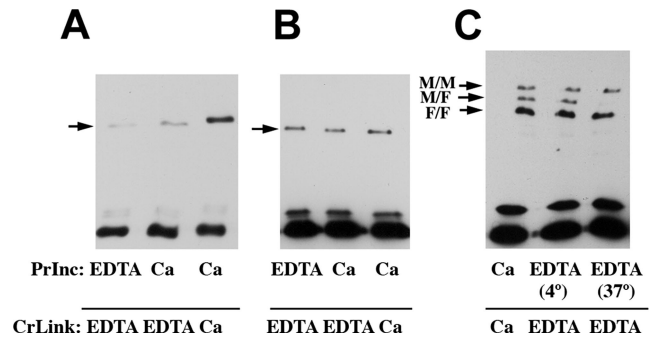


**Figure 1.** E-cadherin dimerization in vitro. (A) The Ec1M-C163A/V176C mutant was cross-linked by BM[PEO]3 on the protein A-Sepharose beads in the absence of  $\text{Ca}^{2+}$  (-) or in the presence of 50  $\mu\text{M}$  (50), 100  $\mu\text{M}$  (100), 500  $\mu\text{M}$  (500), or 1  $\mu\text{M}$  (1)  $\text{Ca}^{2+}$ . The mutant protein was then revealed by anti-myc Western blotting. Note that addition of calcium ions above the 50  $\mu\text{M}$  level induces cadherin cross-linking. (B) Two mutants of Ec1M-C163A/V176C containing additional point mutations—W156A (lanes W156A) or the point mutation E165A in the Ca1/2-binding site (lanes Ca1/2)—were cross-linked as in A in the absence (-) or in the presence of 1 mM (+) of calcium ions. (C) Different cysteine mutants Ec1M-C163A/L175C (lane 175), Ec1M-C163A/V176C (lane 176), Ec1M-C163A/Q177C (lane 177), Ec1M-C163A/K179C (lane 179), Ec1M-C163A/T229C (lane 229), and Ec1M-C163A/L311C (lane 311) were cross-linked in the presence of 1 mM of calcium ions. Note that the mutation V176C facilitates the highest efficiency of dimer cross-linking. Arrows indicate cross-linked cadherin dimers.

Ec1M-C163A/V176C mutant was the most efficient of all the mutants (Figure 1C). These data imply that dimers produced on the Sepharose beads and on the cell surface are similar: they are both formed via the EC1 domain in a Trp156-dependent manner, and in both dimers the V176C mutation generates a cysteine pair that is most favorable for cross-linking.

The unique feature of cadherin dimers detected in cell culture using coimmunoprecipitation or cross-linking assays is that once formed, they become calcium independent (Chitaev and Troyanovsky, 1998; Shan *et al.*, 2000; Ozawa, 2002; Troyanovsky *et al.*, 2003). For example, cross-linking of the Ec1M-C163A/V176C dimers on the cell surface was the same as after their extraction and immunoprecipitation, regardless of the presence or the absence of calcium ions (Troyanovsky *et al.*, 2003). Therefore, we studied whether the Ec1M-C163A/V176C dimers, once they formed on agarose beads, also acquired calcium independence. To this end, cadherin-loaded beads were first preincubated with calcium-containing buffer that allows dimers to form. Then, beads were cross-linked in the presence of EDTA or calcium ions (Figure 2A). Surprisingly, and in a sharp difference from cross-linking in vivo, the addition of EDTA nearly completely abolished the cross-linking of the preformed cadherin dimers. This experiment compellingly demonstrated that calcium is required for both the formation and the integrity of the in vitro-formed dimers.

The unexpected calcium dependency of the in vitro-formed dimers prompted us to re-examine this feature of cadherin dimers using intact cells. In particular, it was unclear whether all or just some of the populations of cell



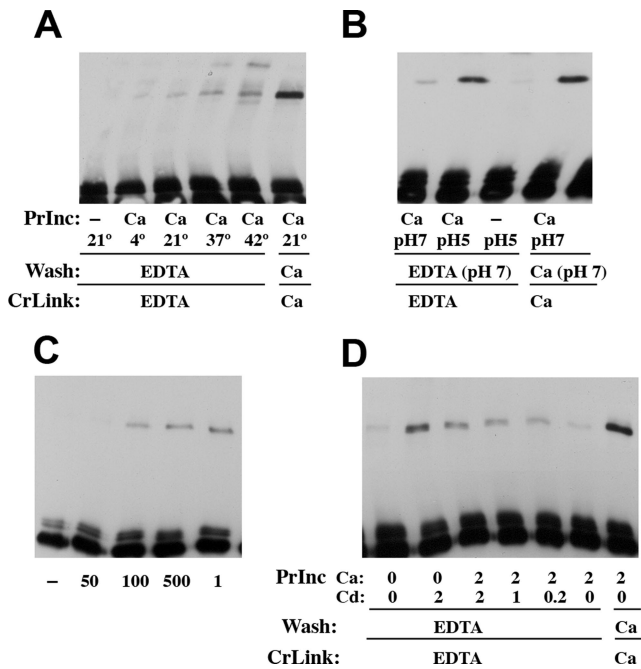
**Figure 2.** Calcium dependency of cadherin dimers. (A) Sepharose beads coated with the Ec1M-C163/V176C mutant were first preincubated (lane PrInc) for 10 min with PBS containing 2 mM EDTA (EDTA) or 1 mM calcium ions (Ca). The beads were then cross-linked (lane CrLink) either in the presence of EDTA or calcium. Note that the majority of the dimers, which are formed during the 10-min-long preincubation with calcium, dissociate upon addition of EDTA. (B) Exactly the same experiment as in A, but performed with living cells. After cross-linking the total cell lysates were analyzed by anti-myc. Note that it produced very different results: 1) dimers do not dissociate in the presence of EDTA and 2) no new dimers form upon calcium addition. (C) Ec1M-C163/V176C- and Ec1F-C163/V176C-expressing A-431 cells were cultured overnight and cross-linked. The presence of  $\text{Ca}^{2+}$  (1 mM) or EDTA (1 mM) during the preincubation (5 min) and cross-linking (5 min) steps are indicated. The total cell lysates were analyzed by a SHE78-7 anti-E-cadherin antibody recognizing both mutants. The adhesive dimer containing both myc- and flag-tagged mutants (arrow labeled M/F) is easily distinguished by its molecular mass from the lateral dimers containing either two myc-tagged (M/M) or two flag-tagged (F/F) cadherin molecules. Note that in the absence of calcium ions adhesive dimers are stable at 4°C, but in contrast to lateral dimers, rapidly dissociate at 37°C. M and F, the myc- or flag-tagged monomers.

surface cadherin dimers are calcium independent. We, therefore, compared the amounts of the Ec1M-C163A/V176C dimers cross-linked on the cell surface in the presence and in the absence of calcium ions. This experiment (Figure 2B) demonstrated that the presence of calcium in a cross-linking solution has no effect on the yield of the cross-linked dimers. Because these dimers derived from both adhesive and lateral cadherin dimers, we redesigned our experiment to test the calcium dependency of adhesive dimers exclusively. To accomplish this, we used cocultures of A-431 cells producing either myc- or flag-tagged versions of the same C163A/V176C mutant, as it had been described previously (Troyanovsky *et al.*, 2003). In this approach cross-linked adhesive dimers, which contain both myc- and flag-tagged mutants, are clearly distinct from lateral dimers by molecular mass. Figure 2C shows that the yield of the cross-linked adhesive dimers was also independent of the presence of calcium in the cross-linking solution even when cells had been washed before cross-linking for several minutes with the ice-cold PBS-EDTA. Adhesive dimers disappeared only when cocultures were maintained in the absence of calcium ions at 37°C for several minutes.

#### Stable Cadherin Dimers

Experiments with the cadherin mutants described above showed that in contrast to dimers produced in vivo, in vitro-formed cadherin dimers are unstable: they immediately dissociate after the depletion of calcium ions. Despite this clear difference, both types of dimers have a similar dimerization interface located at the EC1 domain. The sim-

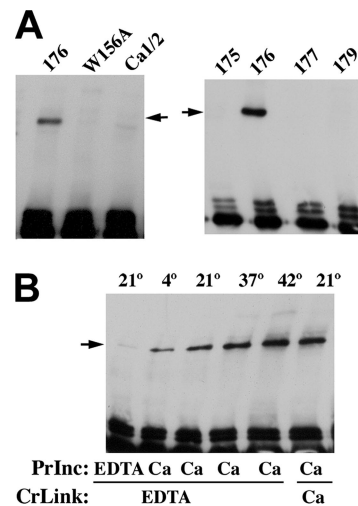




**Figure 3.** Mild denaturants facilitate formation of stable dimers. (A) Before cross-linking the beads coated with the Ec1M-C163A/V176C mutant were preincubated with PBS-Ca at different temperatures (from 4 to 42°C, indicated below the lanes.) Note that the yield of stable dimers rises with an increase in preincubation temperature. (B) The beads coated with Ec1M-C163A/V176C were preincubated with PBS-Ca adjusted to pH 7 (Ca pH 7) or pH 5 (Ca pH 5). A control sample was preincubated with PBS without Ca at pH 5 (PBS pH 5). Then, the samples were washed in PBS-EDTA (pH 7) and cross-linked in the presence of EDTA. Note that after preincubation with PBS-Ca at pH 5, the dimers became calcium-independent. (C) The beads coated with Ec1M-C163A/V176C were cross-linked at different concentration of cadmium ions (indicated below the lanes in  $\mu\text{M}$ ). Note, cadmium ions replace calcium ions in the reaction of cadherin dimerization (compare with Figure 1A). (D) Before cross-linking in the presence of EDTA, the beads were preincubated with different concentrations of calcium and cadmium ions (indicated in mM). Note that cadmium induces the formation of stable dimers.

ilarity is suggested by the fact that the V176C cysteine mutant was the most efficient in the cross-linking reaction both on the cell and on agarose surfaces. The best explanation for this phenomenon is that small conformational changes within the same interface are responsible for the observed differences in calcium dependency of the dimers. If so, the formation of dimers that are stable at low calcium could be facilitated *in vitro* by conditions increasing the conformational plasticity of protein structures.

Several different possibilities to promote formation of cadherin stable dimers *in vitro* were explored. First, cadherin-coated beads were preincubated with calcium-containing buffer at different temperatures and then cross-linked at room temperature in the presence of EDTA. Indeed, the yield of the stable dimers (able to be cross-linked in the absence of calcium ions) increased slightly, but consistently, upon the raising the temperature of the preincubation step (Figure 3A). A much stronger effect was obtained when cadherin-coated beads were preincubated with calcium ions at pH 5 (Figure 3B). After such preincubation, the amount of dimers subsequently cross-linked at the neutral pH was the same in the presence or in the absence of calcium ions. Notably, the dimers that formed at pH 5 were very stable; no



**Figure 4.** Stable and unstable dimers are slightly different. (A) A set of cysteine mutants, the same as in Figure 1, was cross-linked in the presence of EDTA after preincubation with PBS-Ca, pH 5. Note that the mutant Ec1M-C163A/K179C (lane 179) is completely unable to facilitate dimer cross-linking. (B) The beads coated with the Ec1M-C163A/V176C/D155A mutant were preincubated at different temperatures (from 4 to 42°C, indicated above the lanes) with PBS-Ca and then cross-linked in the presence of EDTA. This mutant forms strong dimers much more efficient than parental Ec1M-C163A/V176C mutant (see Figure 3A).

apparent dissociation was noticed even after several hours of incubation with EDTA (up to 10 mM in PBS). Similar to unstable dimers, the formation of strong dimers at pH 5 was also calcium-dependent (Figure 3B).

Finally, we tested whether stable dimers are formed upon replacing calcium with cadmium ions. It has been shown that some divalent cations, including  $\text{Cd}^{2+}$ , can substitute  $\text{Ca}^{2+}$  in the cadherin calcium-binding sites and affect cadherin function (Waisberg *et al.*, 2003). Figure 3C shows that cadmium ions (at room temperature and at pH 7) triggered the formation of cadherin dimers on the bead surface, just as calcium ions do. The majority of the resulting dimers, however, were stable, as shown by their resistance to the subsequent EDTA treatment (Figure 3D). Interestingly, even relatively small (200  $\mu\text{M}$ ) concentration of cadmium ions at the presence of the physiological calcium concentration (2 mM) was sufficient to promote the formation of stable dimers (Figure 3D).

#### Structures of Stable and Unstable Dimers Are Slightly Different

To compare some structural characteristics of the stable dimers produced at pH 5 with unstable dimers produced at the neutral pH, we used the same set of cysteine mutants as indicated above. These experiments showed that the W156A point mutation as well as a mutation of the EC1/EC2 calcium-binding site completely abolished formation of stable dimers (Figure 4A). Similar to unstable dimers, stable dimer cross-linking is most efficient in the case of cysteine mutation V176C (Figure 4). But the mutation K179C revealed some minor structural differences between these two types of dimers—in contrast to unstable dimers, in stable dimers this mutation produced no detectable level of cross-linking.

#### Point Mutation D155A Facilitates Formation of Stable Dimers

Among a number of E-cadherin point mutations we tested for the production of adhesive dimers using the coimmuno-

precipitation assay, only one mutation—D155A—significantly increased the level of adhesive dimers (Laur *et al.*, 2002). This mutation also increased the amount of E-cadherin recruitment into adherens junctions. Thus, we have tested whether this mutation similarly elevates the efficiency of cadherin dimerization *in vitro*. To study this, the D155A mutation was introduced into the Ec1M-C163A/V176C mutant, and the cells expressing the resulting mutant were studied first by immunofluorescent microscopy and coimmunoprecipitation assay. These data showed that by the subcellular distribution and by the efficiency of adhesive dimer formation, this mutant was indistinguishable from Ec1M-D155A (see Supplementary Figure S1, A and B, in Supplementary Materials).

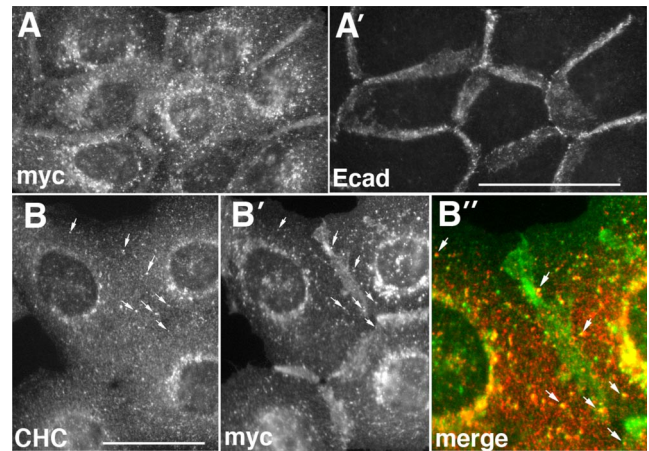
Next we compared the Ec1M-C163A/V176C/D155A mutant with the control Ec1M-C163A/V176C mutant in our cross-linking *in vitro* assay. No differences between them were revealed when they were cross-linked in the presence or in the absence of calcium ions at room temperature and at neutral pH (Figure 4B). However, the test on calcium independence showed that at physiological conditions in which the Ec1M-C163A/V176C mutant formed only unstable dimers, the mutant Ec1M-C163A/V176C/D155A predominantly produced stable dimers. Thus the D155A mutation promoted the formation of stable dimers on both the cell and bead surfaces.

#### Intracellular Cadherin Clustering Is Not Essential for Junction Formation

A number of previous experiments showed that tailless cadherin mutants, which cannot interact and, hence, cannot be clustered by cadherin-associated intracellular proteins, completely lose their adhesive potential (Yap *et al.*, 1998). However, Ozawa and Kemler (1998) found that, at least in some particular cases, such mutants are able to produce strong adhesion. Although the reasons for these conflicting data are still unclear, the observation that cadherin adhesion can be independent from cytoplasmic interactions strongly supports the hypothesis that high-affinity extracellular cadherin–cadherin interactions are involved in cell–cell adhesion. Therefore, to further validate the role of high-affinity cadherin–cadherin interactions in junction formation, we sought to clarify the conditions upon which the tailless E-cadherin mutant Ec1Δ(748–882)M can be recruited into the adherens junctions of A-431 cells. Previously we had shown that this mutant, retaining only a 17-amino-acid-long juxtamembrane region of E-cadherin intracellular tail, does not interact with any known cytoplasmic cadherin partners (Chitaya and Troyanovsky, 1998). Therefore, the behavior of this mutant on the cell surface appears to be independent of cytoplasmic interactions, including interactions with the actin cytoskeleton.

In agreement with previous data, we found that the Ec1Δ(748–882)M mutant was only weakly recruited into the intercellular junctions of stably transfected A-431 cells (Figure 5, A and A'). Instead its majority appeared in the small patches outside the cell–cell contact areas. Double staining of these cells with anti-myc and anti-clathrin antibodies revealed that many of these patches colocalized to clathrin-containing structures (Figure 5, B and B'). This observation suggested that after being delivered to the plasma membrane, the Ec1Δ(748–882)M mutant internalized very rapidly, preventing its clustering into adherens junctions.

To test this hypothesis, we depleted clathrin using siRNA. This approach had been shown to be very effective in blocking clathrin-dependent endocytosis (Hinrichsen *et al.*, 2003). Two different CHC siRNAs were used (oligos 1 and 2) in our experiments. Because both oligos produced indistinguishable results, only experiments with oligo 1 are presented

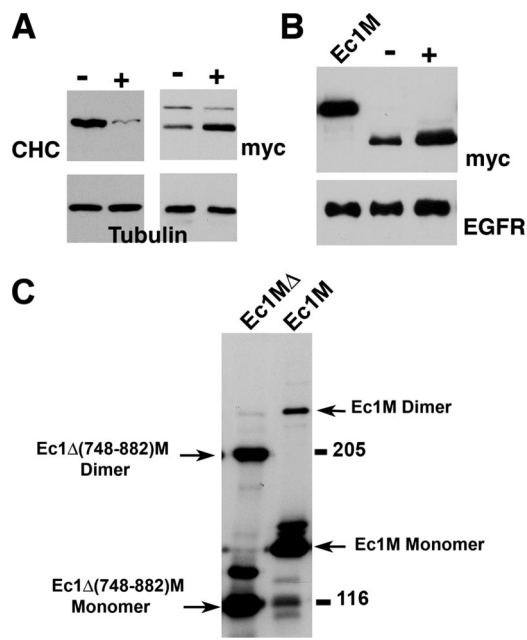


**Figure 5.** Tailless cadherin mutant Ec1Δ(748–882)M associates with cadherin endocytic machinery in A-431 cells. Double immunofluorescence microscopy detecting distributions of Ec1Δ(748–882)M (A and B', myc) versus endogenous E-cadherin (A', Ecad) or clathrin heavy chain (B, CHC) using rabbit anti-myc and mouse anti-E-cadherin or anti-CHC, respectively. Note that only a limited amount of the mutant is recruited into E-cadherin-containing junctions (A and A'). However, numerous dot-like clathrin-positive structures (presumably clathrin-coated pits, some indicated by arrows) completely colocalize with Ec1Δ(748–882)M (B and B'). (B'') Higher magnification of the overlaid selected region. The arrows in B'' indicate the same structures as in B and B'. Bar, 40 μm.

below (see data with oligo 2 in Supplementary Material.) Treatment of Ec1Δ(748–882)M-expressing A-431 cells with the clathrin siRNAs resulted in a significant decrease in clathrin level measured by either Western blotting (Figure 6A) or immunofluorescence microscopy (Supplementary Figure S2, A, and B). In addition, clathrin depletion blocked the uptake of FITC-conjugated transferrin nearly completely (Supplementary Figure S2, C and D); this process is known to be mediated by clathrin (Hinrichsen *et al.*, 2003). These control experiments confirmed that our approach efficiently inactivated clathrin-mediated endocytosis in A-431 cells. The clathrin-siRNA-treated cells exhibited remarkable changes in Ec1Δ(748–882)M amounts and distribution. The total level of the Ec1Δ(748–882)M mutant was notably increased (Figure 6A). In contrast, the level of the endogenous E-cadherin was unchanged. Furthermore, nearly all of the Ec1Δ(748–882)M mutant was redistributed toward the cell–cell contacts, in which it was colocalized to endogenous E-cadherin (Figure 7A and A'). Exactly the same data were obtained when we suppressed clathrin-mediated endocytosis using AP-2 alpha-adaptin or mu2-subunit siRNAs (Supplementary Figures S3 and S4).

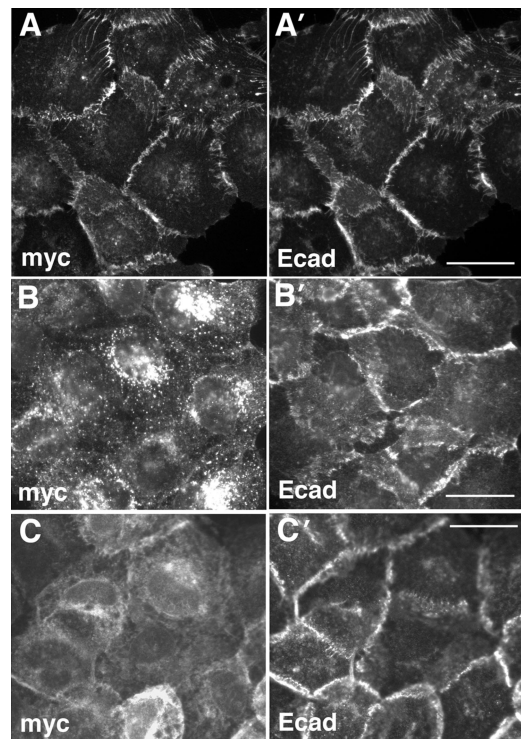
We then sought to exclude the possibility that the efficient recruitment of the cadherin mutant into the junction was caused by overexpression of Ec1Δ(748–882)M after the inactivation of clathrin endocytosis. The surface amount of this mutant was compared with that of Ec1M, which is a full-size version of Ec1Δ(748–882)M. We had previously shown that the subcellular distribution and expression level of Ec1M in our A-431 subclones are indistinguishable from those of endogenous E-cadherin (Klingelhofer *et al.*, 2002). To assess the surface expression of myc-tagged proteins, cells were surface biotinylated and precipitated with streptavidin-agarose. The precipitated proteins were then analyzed by anti-myc and anti-EGF receptor antibodies (Figure 6B). Consistent with published data (Hinrichsen *et al.*, 2003), clathrin siRNA did not change the surface level of EGF receptors.





**Figure 6.** Blockage of clathrin-mediated endocytosis stabilizes Ec1 $\Delta$ (748-882)M mutant. A-431 cells stably producing Ec1 $\Delta$ (748-882)M mutant were treated by clathrin heavy-chain (CHC) siRNA and analyzed after 72 h. (A) Total lysates of the cells transfected with control medium GC (lanes -) and CHC oligo 1 (lanes +) siRNAs were probed for CHC (CHC), Ec1 $\Delta$ (748-882)M mutant (myc), and tubulin (as loading control) by Western blotting. Note that CHC siRNA-transfected cells have reduced amount of CHC but increased amount of the E-cadherin mutant. (B) Cells were surface-biotinylated, precipitated by streptavidin-agarose, and the surface expression of myc-tagged proteins (myc) versus EGF receptor (EGFR, as loading control) was determined. Note that the surface expression of Ec1 $\Delta$ (748-882)M mutant dramatically increased in the siRNA-treated cells (compare lanes - and +), but it is not overexpressed relative to Ec1M (lane Ec1M) on the surface of untreated cells. (C) A-431 cells stably producing Ec1 $\Delta$ (748-882)M-C163A/V176C mutant were treated by CHC oligo 1 siRNA (Ec1M $\Delta$ , left lane) or control untreated cells producing Ec1M-C163A/V176C (Ec1M, right lane) were cross-linked in PBS-EDTA and then analyzed by Western blotting using anti-myc. Corresponding monomeric and cross-linked dimeric forms of both cadherin mutants are indicated. Bars (right) denote the relative positions of protein markers myosin,  $M_r$  205,000 (205), and galactosidase,  $M_r$  116,000 (116). Note that the truncated Ec1 $\Delta$ (748-882)M-C163A/V176C mutant cross-linked into dimers much more efficiently than the parental Ec1M mutant.

However, it significantly elevated the level of the Ec1 $\Delta$ (748-882)M mutant. Nevertheless, the surface expression of this mutant was still slightly below the level of Ec1M. Thus, efficient recruitment of Ec1 $\Delta$ (748-882)M into junctions cannot be explained by the abnormally high expression level. Another explanation for the very efficient recruitment of the Ec1 $\Delta$ (748-882)M mutant into the intercellular contacts is its abnormally high dimerization level in cells lacking clathrin-mediated endocytosis. To test this possibility the combinatorial mutation C163A/V176C that produced the highest yield of cadherin dimers upon cysteine-specific cross-linking was inserted into the Ec1 $\Delta$ (748-882)M mutant. The resulting Ec1 $\Delta$ (748-882)M-C163A/V176C mutant was stably expressed in A-431 cells. Then, using a cysteine-specific cross-linking assay, we compared the amount of homodimers produced by this mutant upon clathrin depletion with that of the full-size Ec1M-C163A/V176C cadherin in control (nontransfected) cells. Although these two cell cultures expressed approximately the same amounts of the myc-tagged

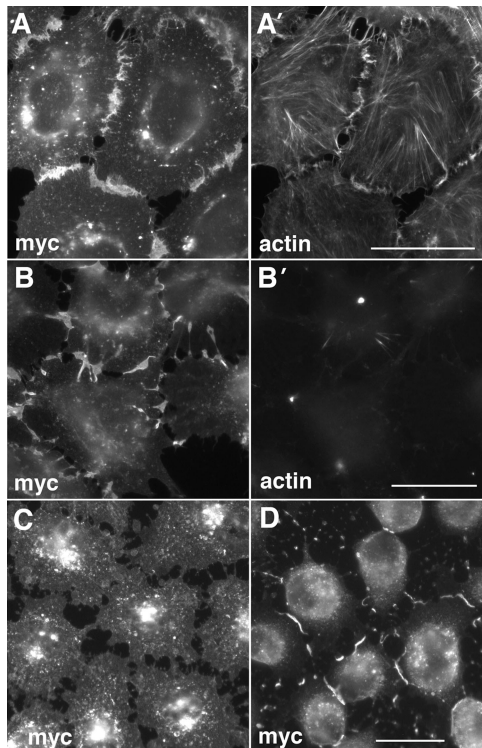


**Figure 7.** Impact of clathrin depletion on distribution of cadherin mutants. (A and A') A-431 cells expressing Ec1 $\Delta$ (748-882)M were depleted for clathrin using oligo 1 and stained by double immunofluorescence for Ec1 $\Delta$ (748-882)M using anti-myc (A, myc) and anti-endogenous E-cadherin (A', Ecad). Note that in stark contrast to the control cells (see Figure 5, A and A') the cells depleted for clathrin exhibit a complete colocalization of two proteins. (B and B') A-431 cells expressing Ec1 $\Delta$ (748-882)M-Ca4/5 mutant were transfected either with the control medium GC oligo (B and B') or CHC siRNA (C and C') and stained as above. Note, that although clathrin depletion results in the translocation of the cadherin mutant to the cell surface, only a negligible amount of the mutant is recruited into cell-cell junctions. Bar, 40  $\mu$ M.

cadherins, the Ec1 $\Delta$ (748-882)M-C163A/V176C mutant in the clathrin-depleted cells produced significantly larger amounts of stable dimers relative to the parental Ec1M-C163A/V176C mutant (Figure 6C).

The next question we studied was whether the recruitment of the Ec1 $\Delta$ (748-882)M mutant into cell-cell contacts depends upon its adhesive homodimerization, or if it is transported there by some other protein-protein interactions. To selectively abolish adhesive dimerization of the Ec1 $\Delta$ (748-882)M mutant, we additionally mutated its calcium-binding sites located between the EC4/EC5 domains. We had shown that such Ca4/5 mutation specifically inactivates adhesive (not lateral) mode of cadherin dimerization *in vivo* (Klingelhofer *et al.*, 2002). The resulting Ec1 $\Delta$ (748-882)M-Ca4/5 mutant behaved very similarly to the parental Ec1 $\Delta$ (748-882)M mutant in the control A-431 cells (Figure 7, B and B'); the majority of the mutant was present in patches along the entire cells. Clathrin siRNA treatment resulted in accumulation of the mutant on the cell surface. But in stark contrast to the parental mutant, Ec1 $\Delta$ (748-882)M-Ca4/5 was completely unable to form clusters within cell-cell junctions (Figure 7, C and C').

We also sought to discard any possibility that the actin cytoskeleton is required to maintain Ec1 $\Delta$ (748-882)M-containing junctions. To this end, the actin cytoskeleton of the



**Figure 8.** Maintenance and assembly of Ec1 $\Delta$ (748-882)M clusters is independent from the actin cytoskeleton. (A and A') Clathrin-depleted cells in control culture (A and A') or after a 20-min-long treatment with latrunculin A (B and B') were double-stained for Ec1 $\Delta$ (748-882)M using anti-myc (A and B, Myc) and for actin using FITC-phalloidin (A' and B', actin). Note that the nearly complete disappearance of actin filaments does not abolish Ec1 $\Delta$ (748-882)M clustering. (C and D) The cells were incubated for 20 min with latrunculin A in low-calcium medium and stained for myc (C). This treatment completely disrupted Ec1 $\Delta$ (748-882)M clusters. The cells were then transferred into high-calcium/latrunculin-containing medium for additional 10 min (D). Note a complete recovery of cadherin clusters, regardless to the presence of latrunculin. Bar, 40  $\mu$ M.

clathrin siRNA-pretreated cells was depolymerized by either latrunculin A or cytochalasin D. Although such treatments completely destroyed the actin cytoskeleton (Figure 8B'), they had little effect on the cell-cell junctional clustering of Ec1 $\Delta$ (748-882)M (Figure 8B). Moreover, formation of new Ec1 $\Delta$ (748-882)M clusters was also independent from the state of the actin cytoskeleton. Figures 8, C and D, show that cells whose contacts had been dissociated by pretreatment with low-calcium medium rapidly reconstituted their Ec1 $\Delta$ (748-882)M clusters in the presence of latrunculin A.

Taken together, our experiments showed that the mutant Ec1 $\Delta$ (748-882)M cannot be recruited into adherens junctions because of its continuous and efficient internalization from the surface of the control A-431 cells. Once this process was blocked, the tailless cadherin mutant formed well-organized intercellular clusters regardless of its disconnection from the actin cytoskeleton.

## DISCUSSION

### *Stable and Unstable Cadherin Dimers Are Similar in Structure*

The *in vitro*-binding experiments presented in this work show that E-cadherin is able to form two types of ho-

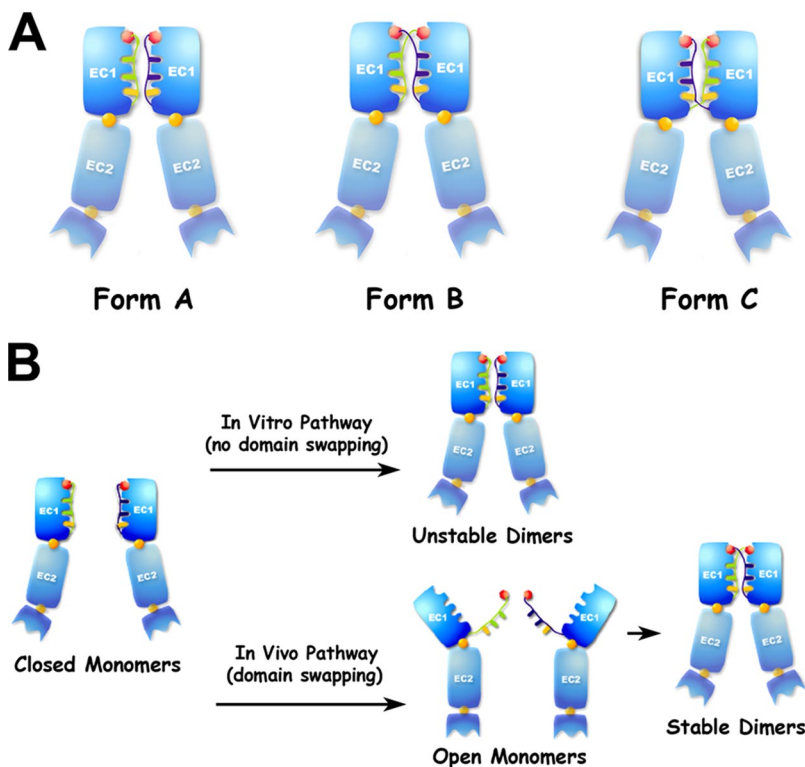
modimers, referred to here as unstable and stable dimers. At physiological conditions E-cadherin forms the unstable dimer, which immediately dissociates upon depletion of calcium ions. The stable dimer forms when E-cadherin dimerization is performed at destabilizing conditions, such as at high temperature, low pH, or in the presence of cadmium ions. These dimers are similar in many parameters: they have a similar dimerization interface and their formation is Trp156- and calcium-dependent. In sharp contrast to the unstable dimer, however, the strong dimer, once formed, is locked in a calcium-independent state.

What are the structural differences between stable and unstable dimers? The high structural plasticity of cadherin dimers is a well-known phenomenon. In part, it is based on the conformational instability of the N-terminal portion of the EC1 domain  $\beta$ A strand. The hallmark residue of this strand, Trp156, can be inserted into the hydrophobic pocket of its own domain. On this "closed" conformation, E-cadherin dimerization proceeds through EC1/EC2 calcium-binding sites (Nagar *et al.*, 1996; Pertz *et al.*, 1999; Haussinger *et al.*, 2004). Consequently, such "calcium site" dimers require calcium not only for their formation but also for their maintenance. In another type of cadherin dimer, the strand dimer, the same part of the  $\beta$ A strand (including Trp156) of one monomer, substitutes for its own counterpart derived from the paired molecule (Shapiro *et al.*, 1995; Boggon *et al.*, 2002). Because calcium-binding sites are not directly involved in this type of dimerization, the resulting strand dimer can be calcium-independent. Thus, an obvious possibility is that these two dimers are the prototypes for the unstable and stable dimers detected in our work. However, this simple possibility is very unlikely. The unstable dimer is Trp156-dependent; the calcium-binding site dimer is not (Nagar *et al.*, 1996; Pertz *et al.*, 1999; Haussinger *et al.*, 2004). Our data show that the stable dimer is very stable; the NMR data show that the lifetime of the strand dimer is less than a second (Haussinger *et al.*, 2004).

The formation of the strand dimer is an example of a 3D domain swapping process (Liu and Eisenberg, 2002; Rousseau *et al.*, 2003). During this process, two molecules form a dimer by exchanging an identical structural element. In the strand cadherin dimer this structural element is the  $\beta$ A strand including Trp156. A unique feature of the 3D domain-swapping dimerization is that monomeric and dimeric species are separated by a high-energy barrier that is required to unfold the protein and release the exchanging element. This barrier, which must be overcome for both dimer assembly and disassembly, kinetically traps domain-swapped proteins in monomeric or dimeric states. Consequently, swapped dimers often have a remarkably long lifetime of several months (Hakansson *et al.*, 2001; Rousseau *et al.*, 2001; Barrientos *et al.*, 2002). The kinetic barrier can be reduced, however, under conditions that promote protein unfolding, e.g., low pH, high temperature, or interaction with different ligands. Such destabilizing conditions trigger 3D domain-swapping dimerization.

Our study provides compelling evidence that 3D domain swapping is involved in the formation of stable dimers. First, monomeric E-cadherin does not spontaneously form stable dimers at neutral pH, even upon clustering on the bead surface. This indicates the existence of a kinetic barrier for stable dimerization. Second, destabilizing conditions rapidly convert clustered cadherin molecules to stable dimers. Third, stable dimers, once formed, are very stable. The long lifetime of stable dimers is indicated by their survival at low calcium, when no new dimers can be formed.





**Figure 9.** (A) Hypothetical structures of cadherin dimers. Only the EC1 and EC2 domains of E-cadherin are shown. The yellow circles are EC1/EC2 calcium-binding sites.  $\beta$ A strand is shown separately from the rest of the EC1 domain. The Trp156 residue located at the tip of  $\beta$ A strand is indicated by red hexagon. The overall structures of all dimers are similar, because in all cases dimerization proceeds along  $\beta$ A strands. In form A, however, Trp 156 residues are inside of the own pockets; in the form B (strand dimer) the same residues are completely inserted within paired molecules; and in the form C the entire  $\beta$ A strands are reciprocally exchanged. See text for details. (B) Two pathways of cadherin dimerization. At physiological in vitro conditions EC1 domain has a stable “closed” conformation (closed monomers) and the Trp156 swapping process is not achievable. At these conditions cadherin forms only unstable low-affinity dimers (in vitro pathway). Within intercellular contacts, however, some unidentified mechanism facilitates a release of the  $\beta$ A strand that produces open monomers. These monomers, in turn, efficiently form stable dimers by a 3D domain-swapping mechanism (in vivo pathway.)

The long lifetime of stable cadherin dimers suggests that they are separated from monomers by a high-energy barrier.

Because both unstable and stable dimers depend on Trp156, one may suggest two possibilities (Figure 9A). First, our stable dimer may correspond exactly to the strand dimer (form B, Figure 9A). If so, the cadherin dimer described in the NMR spectroscopy study by Haussinger *et al.* (2004) would be similar to our unstable dimer and could not be the strand dimer. Its hypothetical structure might feature some of the interactions present in strand dimers but lack complete Trp156 exchange (Form A, Figure 9A). One of such “intermediate” structures, in which the Trp aromatic rings are located at the pocket entrances, was suggested by steered molecular dynamics simulations of C-cadherin (Bayas *et al.*, 2004). It is more likely, however, that both dimers—the cadherin dimer detected in the NMR study and the unstable dimer detected in our work—correspond to the strand dimer. The instability of the strand dimer has been explained by competition between intramolecular and intermolecular docking of Trp156 in conjunction with the relatively low energy barrier for Trp156 exchange (Chen *et al.*, 2005, Harrison *et al.*, 2005). The high-energy barrier, which provides stability for the strand dimer, must be based on much more extensive swapping. For example, the stable dimer might have the entire  $\beta$ A strand exchange, similar to that found in a strand dimer of type II cadherins (Patel *et al.*, 2006; form C, Figure 9A).

Protonation of the Glu243 residue and the consequent disruption of the salt bridge between this residue and N-terminal amino group (such a bond was detected in the crystals of C-cadherin strand dimer, cf. Boggon *et al.*, 2002) might be one of the mechanisms lowering the energy barrier of stable dimerization at low pH. An alternative possibility is that a low pH alters, but does not inactivate, the EC1/EC2 calcium-binding sites, and that this modification releases the  $\beta$ A strand. Recent experiments with the desmosomal cad-

herin Dsg1, the calcium-binding sites of which are nearly identical to those of E-cadherin, strongly support such a hypothesis. The experiments showed that pH 5 detectably decreased, but did not abolish, the binding of Dsg1 to calcium ions (Hanakawa *et al.*, 2003). Our experiments with cadmium ions provided additional support to the possibility that some changes in calcium-binding sites facilitate stable cadherin dimerization.

#### Stable Cadherin Dimerization and Cell–Cell Adhesion

The complete lack of unstable cadherin dimers on the cell surface compellingly shows that low-affinity cadherin–cadherin interactions, at least those which can be detected between EC1 cadherin domains in vitro (in vitro pathway, Figure 9B), have no role in cell–cell adhesion. Stable dimers were the only type of cadherin–cadherin interactions (in vivo pathway, Figure 9B) detected on the surface of epithelial cells using both cross-linking (Troyanovsky *et al.*, 2003) and coimmunoprecipitation assays (Chitaev and Troyanovsky, 1998; Shan *et al.*, 2000; Ozawa, 2002). Furthermore, point mutation D155A, which facilitates production of cadherin dimers in vivo, also increases the yield of stable dimers in our in vitro assay.

Despite the fact that the formation of stable dimers in vitro requires protein-destabilizing conditions, these dimers are continuously produced on the cell surface in physiological media (Klingelhofer *et al.*, 2002; Troyanovsky *et al.*, 2006). This suggests that cells have specific mechanisms decreasing the activation energy of cadherin dimerization. The mechanism facilitating cadherin dimerization in vivo was also evident in our previous work, which showed that digitonin in concentrations higher than 0.015% completely blocked the formation of adhesive dimers in digitonin-permeabilized cells (Klingelhofer *et al.*, 2002). The nature of this mechanism, however, is obscure. As a matter of fact, no clear cellular mechanisms involved in the formation of any 3D domain-

swapped dimers have been evaluated (reviewed in Liu and Eisenberg, 2002; Rousseau *et al.*, 2003). One of the often-regarded possibilities is the partial unfolding of swapping proteins at low pH in late endosomes. In light of this idea, one may propose that cadherin releases its  $\beta$ A strand in late endosomes during recycling; this “active” form of cadherin is then delivered to the cell surface, where it either forms dimers or refolds. However, all our attempts to inhibit the formation of cadherin dimers in cells by increasing the pH of late endosomes using  $\text{NH}_4\text{Cl}$ , monensin, or bafilomycin A1 were unsuccessful (data not shown). Thus, cells appear to have another mechanism responsible for stable dimer formation. This mechanism may play an important role in the regulation of cell–cell adhesion. Its defects may abolish cadherin-based adhesion regardless of the high amount of cadherin on the cell surface. Cells with such phenotype have been identified in normal development and tumor progression (reviewed in Gumbiner, 2005).

The efficient production of stable cadherin dimers on cell surface suggests that cadherin molecules can be recruited into the cell–cell contact site simply by the diffusion-trapping mechanism. This possibility is supported by our experiments with the tailless cadherin mutants. They show that the cadherin mutant Ec1 $\Delta$ (748–882)M, which lacks nearly the entire intracellular region, efficiently forms junctions in cells in which the actin cytoskeleton has been destroyed completely by latrunculin A. The reason why in many previous experiments similar tailless mutants have been unable to form contacts is their fast uptake from the cell surface by clathrin-mediated endocytosis. However, in some particular cell models tailless cadherin mutants produced robust cell–cell aggregation (Ozawa and Kemler, 1998). The authors interpreted these data as implying that some mechanisms negatively regulate intrinsic cadherin adhesion activity. Our findings strongly support this point of view. Clathrin-mediated endocytosis, which, as we show here, prevents targeting cadherin tailless mutants into cell–cell contacts, is clearly one such negative mechanism. Endocytosis was also shown to regulate adhesion of the full-size cadherin. For example, a total arrest of cadherin internalization rapidly converts nearly all monomeric cadherin molecules into dimeric form (Trojanovsky *et al.*, 2006).

In summary, our data convincingly show that the EC1 domain of E-cadherin can produce two types of dimers: unstable and stable. The stable dimers, which by their properties completely correspond to adhesive dimers found *in vivo*, are potent candidates for being the minimal structural unit of cadherin-based adhesion. Taken together our results point compellingly to the conclusion that cadherin is more than low-affinity glue; it is a sophisticated device that contains a hidden, highly adhesive site whose function is under strict cellular control.

## ACKNOWLEDGMENTS

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