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Stable and Unstable Cadherin Dimers: Mechanisms of Formation and Roles in Cell Adhesion

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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 continuous formation of high-affinity cadherin 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 ions.
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 Ec1A(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 molecules (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 Cd2+ 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 Cd2+ or Cd2+ 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 (10 μl) in 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 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 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.

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 pcDNA3. 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 μM) or latrunculin A (Molecular Probes, Eugene, OR; final concentration 0.2 μM) were used.

Antibodies

Mouse antibodies were as follows: anti-E-cadherin (C20820), anti-clathrin heavy chain, anti-α-adaptin, anti-AP50, anti-epidermal growth factor (EGF) receptor (all BD Biosciences, 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)-phallolidin (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; CGG GAA AUA UGA CAA UAC UAC A, HC Oligo 2); the AP-2 subunit α-adaptin siRNA (CCU GGG CCG CUA GUU CUCU CUA U); the AP-2 μα-subunit siRNA (GGU GGU CUA CAA GGC CAA CUA 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 CaCl2 (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 cultured cells or 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 × 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 μg per sample, 1 h) and protein A-Sepharose (100 μl, 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 Cd2+ 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 Cd2+ or Cd2+ 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 (10 μl) in 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 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 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.

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 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.

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 E1C-E2 calcium-binding site (Figure 1B, lanes C1 and C2). 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 mutants, 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
Stable and Unstable Cadherin Dimers

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 Ca\(^{2+}\) (−) or in the presence of 50 μM (50), 100 μM (100), 500 μM (500), or 1 μM (1) Ca\(^{2+}\). The mutant protein was then revealed by anti-myc Western blotting. Note that addition of calcium ions above the 50 μM 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 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 2. Calcium dependency of cadherin dimers. (A) Sepharose beads coated with the Ec1M-C163A/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-C163A/V176C– and Ec1F-C163/V176C–expressing A-431 cells were cocultured overnight and cross-linked. The presence of Ca\(^{2+}\) (1 mM) or EDTA (1 mM) during the preincubation (5 min) and cross-linking (5 min) steps is indicated. The total cell lysates were analyzed by a SHE78– 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.
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 concentrations of cadmium ions (indicated below the lanes in mM). 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.

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).
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 loose 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 (Chitaev 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 (Hirnichen 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 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 (Hirnichen 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 (Hirnichen et al., 2003), clathrin siRNA did not change the surface level of EGF receptors.

**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.
However, it significantly elevated the level of the Ec1Δ(748-882)M mutant. Nevertheless, the surface expression of this mutant was still slightly below the level of Ec1M mutant, as determined by Western blotting. Note that CHC siRNA-treated cells have reduced amount of CHC but increased amount of the Ec1M mutant (Figure 6C). The next question we studied was whether the recruitment of the Ec1Δ(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 Ec1Δ(748-882)M mutant. The resulting Ec1Δ(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 cadherin 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 cadherins, the Ec1Δ(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 Ec1Δ(748-882)M mutant into cell–cell contacts depends upon its adhesive homodimerization, or if it is transported there by some other protein–protein interaction. To selectively abolish adhesive dimerization of the Ec1Δ(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 Ec1Δ(748-882)M-Ca4/5 mutant behaved very similarly to the parental Ec1Δ(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, Ec1Δ(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 Ec1Δ(748-882)M-containing junctions. To this end, the actin cytoskeleton of the
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 Ec1Δ(748-882)M (Figure 8B). Moreover, formation of new Ec1Δ(748-882)M clusters was also independent from 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 Ec1Δ(748-882)M clusters in the presence of latrunculin A.

Taken together, our experiments showed that the mutant Ec1Δ(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 homodimers, 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 βA 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” 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 βA 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 βA 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.
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 βA 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 βA strand. Recent experiments with the desmosomal cadherin 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-denstabilizing 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 diglution in concentrations higher than 0.015% completely blocked the formation of adhesive dimers in diglution-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 βA 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 NH₄Cl, 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(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 target- ing 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 (Troyanovsky 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.

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