Removal of calcium ions triggers a novel type of intercadherin interaction

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SUMMARY

Depletion of Ca^{2+} ions from epithelial cell cultures has been shown to result in the rapid destruction of intercellular junctions. To understand the mechanism of this effect we have examined how removal of calcium ions from the culture medium of A-431 epithelial cells affects complexes incorporating the cell-cell adhesive receptors, E-cadherin, desmoglein or desmocollin. Sedimentation and biochemical analysis demonstrated that calcium removal triggers a rapid formation of a novel type of complex formed via direct lateral E-cadherin-desmoglein, E-cadherin-desmocollin and desmoglein-desmocollin dimerization of the extracellular cadherin regions. Replacement of Trp^{156} and Val^{157} of E-cadherin, that has been shown to abolish lateral and adhesive E-cadherin homodimerization in standard cultures, did not influence the formation of these 'calcium-sensitive' complexes. Furthermore, experiments with this mutant revealed that EGTA induced lateral Trp^{156}/Val^{157}-independent homodimerization of E-cadherin. Deletion mutagenesis of E-cadherin showed that these complexes are mediated by at least two extracellular cadherin domains, EC3 and EC4. Notably, protein kinase inhibitor H-7 which confers EGTA-independence of the adhesive E-cadherin complexes does not block this association. We propose that this novel type of intercadherin interaction is involved in the assembly of adherens junctions and their disassembly in low-calcium medium.

Key words: Cadherin, Catenin, Intercellular adhesion

INTRODUCTION

Classic and desmosomal cadherins are two subfamilies of cadherins serving as structural transmembrane elements in specialized, morphologically distinct intercellular junctions termed adhering junctions (Schäfer et al., 1993; Schmidt et al., 1994, and references therein). These junctions are characterized by a dense cytoplasmic plaque which joins the adhesive transmembrane junctional core with the intracellular cytoskeleton. The group of adhering junctions referred to as an 'adherens junction', incorporates classic cadherins (e.g. E-cadherin) and anchors bundles of microfilaments (reviewed by Geiger and Ayalon, 1992; Kemler, 1992; Takeichi, 1995; Yap et al., 1997). Desmosomes are another type of adhering junctions present in epithelial cells (Schwarz et al., 1990; Garrod et al., 1996; Troyanovsky and Leube, 1998). They contain desmosomal cadherins (e.g. desmoglein, Dsg and desmocollin, Dsc) and are coupled with intermediate filaments. The intracellular domains of classic and desmosomal cadherins contain a characteristic segment that is highly conserved between members of the family and mediates binding of cadherins to cytoplasmic proteins collectively termed catenins (α- and β-catenins, and plakoglobin). The binding to catenins has a critical function in cell-cell adhesion (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989, 1990b; Ozawa and Kemler, 1998; Knudsen et al., 1995; Rimm et al., 1995; Angres et al., 1996; Chitaev and Troyanovsky, 1998). Most molecular details of this process are not yet understood, though some critical clues for its comprehension were evolved in recent years.

Cadherins of both groups are single-pass transmembrane proteins containing four homologous extracellular cadherin domains (EC1-4, numbered from the N terminus). The crystal structure of the EC1 domain of N-cadherin revealed that it is able to form lateral dimers. This interaction is mediated by a Trp residue (Trp^{156} in E-cadherin; here and below the E-cadherin sequence is numbered according to GenBank accession # Z13009, cf. Bussemakers et al., 1993) that is conserved among classic and desmosomal cadherins. In addition, the EC1 domain can form antiparallel dimers which are likely to establish a direct link between opposing cells (Shapiro et al., 1995). In our recent work (Chitaev and Troyanovsky, 1998), we presented substantial evidence demonstrating that E-cadherin forms both lateral and adhesive E-cadherin homodimerization in standard cultures, did not influence the formation of these...
this process the E-cadherin-catenin complex is recruited into a Triton X-100 insoluble pool and associates with microfilaments (McNeill et al., 1993; Adams et al., 1996; Gloushankova et al., 1998).

One of the fundamental features of the cadherin-based adhesion is its sensitivity to the extracellular Ca\(^{2+}\) concentrations (Kartenbeck et al., 1982; Mattey and Garrod, 1986; Volberg et al., 1986; Green et al., 1987). Importantly, the current understanding of cell-cell adhesion is based on experiments with cells dissociated by calcium chelators. The mechanism of such dissociation, however, is still poorly understood. Our experiments (Chitaev and Troyanovsky, 1998) showed that the adhesive E-cadherin complex was stable in the absence of calcium ions in vitro or in cultured cells at 4°C, but immediately disappeared at 37°C. Such a temperature-sensitive, immediate response to changes in the extracellular calcium concentration suggests involvement of specific signal transduction pathways. This point of view is consistent with the observation that the protein kinase inhibitor H-7 prevents dissociation of adherens junctions and desmosomes in low calcium concentration (Citi, 1992; Pasdar et al., 1995). Examination of this effect led authors to propose that dissociation of the adherens junctions upon removal of extracellular calcium ions is caused by contraction of the cortical microfilament cytoskeleton (Citi et al., 1994; Denisenko et al., 1994; Volberg et al., 1994). However, how the decrease in extracellular concentration of Ca\(^{2+}\) ions generates signaling across the plasma membrane and initiates actomyosin-driven contraction is not known.

In an attempt to understand the molecular mechanisms regulating the assembly of desmosomes and adherens junctions, we studied the behavior of the adhesive and lateral E-cadherin complexes in response to shifts in the extracellular calcium level. Surprisingly, we found that removal of calcium ions from the growth medium causes immediate assembly of novel intercadherin complexes in A-431 epithelial cells incorporating either several (two or more) E-cadherin molecules or both E-cadherin and desmosomal cadherins. These complexes are independent of Trp156 of E-cadherin and required the integrity of the EC3 and EC4 domains. This novel type of intercadherin interaction could play an important role in normal junction assembly and in dissociation of the adherens junctions in low-calcium medium.

**MATERIALS AND METHODS**

**DNA constructs, cell culture, DNA transfection and immunofluorescence microscopy**

The construction of the expression plasmids coding for E-cadherin with an internal deletion His\(^{273}\)-Leu\(^{791}\) and tagged C-terminally either by myc (Ec1M) or by flag (Ec1F) epitopes, and for mutants of Ec1M (clone 3.10 and U114, respectively, provided by Dr W. W. Franke); anti-myc (clone 9E10, provided by Dr R. Kopan, Washington University, St Louis, MO); rabbit anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-flag M2 (Sigma, St Louis, MO); anti-\(\alpha\)-catenin, anti-E-cadherin (Mab C20820), anti-\(\beta\)-catenin and anti-EGF receptor (Transduction Laboratories, Lexington, KY); and anti CD44 (Zymed Laboratories Inc., San Francisco, CA).

To remove extracellular calcium, EGTA (Sigma) was added to a final concentration of 10 mM. Experiments performed with a lower concentration of EGTA (5 \(\mu\)M) gave identical results. Treatment with H-7 inhibitor (Sigma) was carried out as described by Citi (1992).

To determine the localization of E-cadherin complexes, cells were digested with 0.05% trypsin in PBS containing 0.02% EDTA for 1 minute at 37°C. After the addition of soybean trypsin inhibitor (final concentration 2 mg/ml), cells were subjected to immunoprecipitation analysis.

**Immunoprecipitation and sedimentation analysis**

For most immunoprecipitation experiments, 2\(\times\)10⁶ cells were cultured in a 10-cm tissue culture dish at 37°C for about 72 hours. In co-culture experiments, 6\(\times\)10⁶ cells producing myc- and flag-tagged forms of E-cadherin were mixed in a 1:1 ratio and were cultured in a 10-cm dish for 24 hours. Immunoprecipitation assay and sucrose gradient centrifugation were described previously (Troyanovsky et al., 1994; Chitaev and Troyanovsky, 1998). In brief, the confluent monolayer (approximately 10⁷ cells) was washed and extracted in 1.5 ml of immunoprecipitation lysis buffer (IP-buffer; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 20 \(\mu\)M p-APMSF, 2 mM EDTA, and 1% NP-40). The lysates were subjected to immunoprecipitation by subsequent incubations with specific antibody and Protein A-Sepharose. For sucrose gradient centrifugation, confluent monolayer cells from three 10 cm dishes were lysed with 2 ml of IP-buffer. Lysates (1 ml) were precleared by centrifugation at 100,000 \(g\) for 1 hour, and then loaded on top of a 12 ml linear 5-20% (wt/wt) sucrose gradient prepared in IP-buffer. Gradients were centrifuged at 200,000 \(g\) for 17 hours in a SW40Ti rotor (Beckman Instruments) at 4°C, fractionated from bottom to top into 12 fractions (1 ml each), and analyzed by co-immunoprecipitation. The following protein standards of known S values were centrifuged on replicate gradients: BSA, 4.5S; IgG, 7.5S; catalase, 11.3S; apoferritin, 17S.

**RESULTS**

**Removal of calcium ions triggers lateral intercadherin association**

We have studied whether E-cadherin may form complexes with desmosomal cadherins in A-431 cells. Total lysates of standard confluent cultures of these cells were immunoprecipitated either with anti-Dsg or anti-E-cadherin antibodies (Fig. 1A,B). Obtained immunoprecipitates were analyzed by western blot for the presence of different cadherins or catenins. Surprisingly, long exposure of these blots demonstrated that two different cadherins, E-cadherin and Dsg, may form a heteromeric complex. Another desmosomal cadherin, Dsc was not found in the anti-E-cadherin or anti-Dsg immunoprecipitates (Fig. 1). Dsc-Dsg and Dsc-E-cadherin complexes also were not detected in the reciprocal experiments in which immunoprecipitates obtained with anti-Dsg antibody were analyzed by anti-E-cadherin or anti-Dsg staining (not shown). These experiments also showed that a minor pool of Dsg associated with \(\beta\)-catenin (Fig. 1B).

To define whether E-cadherin and Dsg molecules in the complex have parallel or antiparallel alignment, we performed
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co-immunoprecipitation experiments with A-431 cells that were dissociated by EGTA treatment prior to lysis. Surprisingly, this treatment strongly increased the amount of the Dsg-Dsc cadherin complexes and resulted in formation of Dsg-Dsc and E-cadherin-Dsc complexes (Fig. 1). The same effect, an increase of the E-cadherin-Dsg complex and an appearance of the E-cadherin-Dsc and the Dsg-Dsc complexes, was also caused by replacement of the standard culture medium for 10 minutes with low calcium medium (not shown). Both treatments did not change amounts of E-cadherin or desmosomal cadherins in the NP-40-soluble or insoluble fractions (not shown). This calcium-sensitive intercadherin association was unaltered upon addition of 0.25% SDS into the IP-lysis buffer and washing solution (Fig. 1C). These conditions, however, completely released β-catenin/plakoglobin from E-cadherin immunoprecipitates

Fig. 1. Formation of the calcium-sensitive heterocadherin complexes. (A-C) Total lysates of A-431 cells before (−) or after incubation with EGTA (+) were immunoprecipitated under standard conditions or in the presence of 0.25% SDS (C, + SDS) with anti-E-cadherin (IP-Ec; A and C) or anti-Dsg (IP-Dsg, B) antibodies. Immunoprecipitates were analyzed by western blot for the presence of E-cadherin (Ec); Dsg (Dsg); Dsc (Dsc) and β-catenin (β-cat). D, wild-type HT-1080 cells (lanes 1 and 2) and HT-1080 cells producing recombinant Ec1M (lanes 3 and 4) were lysed without (−) or after (+) EGTA treatment, immunoprecipitated with anti-Dsg antibody, and immunoprecipitates obtained were analyzed by western blot with antibodies against Dsg (Dsg), myc epitope (Myc), or β-catenin (β-cat). Note that only E-cadherin-producing HT-1080 cells exhibit strong calcium-sensitive Dsg-β-catenin association.

Fig. 2. Sedimentation analysis (A) and dynamics (B and C) of the calcium-sensitive heterocadherin complexes. (A) Total lysate of wild-type A-431 cells treated before lysis for 10 minutes with 10 mM EGTA was subjected to sucrose gradient centrifugation. The collected fractions (numbered 1 to 12, with 1 at the bottom of the gradient) were immunoprecipitated with anti-Dsg mAb, separated by SDS-PAGE and analyzed by immunoblotting with antibodies against Dsg (Dsg), β-catenin (β-cat) or E-cadherin (Ec). The filter stained with anti-Dsg mAb was overexposed to show presence of minor pool of Dsg in fractions 3-6. Arrowhead indicates the position of the lateral and the adhesive E-cadherin complexes described by Chitaev and Troyanovsky (1998). The peak distribution of protein standards of known S values (bovine serum albumin, 4S; rabbit IgG, 7.5S; catalase, 11.4S; apoferritin, 17S) was determined in a parallel gradient and is shown at the bottom. (B) Wild-type A-431 cells growing under standard conditions (0) or after incubation with 10 mM EGTA for 30 seconds (30'), 1, 2, 5, or 10 minutes (1', 2', 5', 10' correspondingly) were immunoprecipitated using anti-E-cadherin antibody. The immunoprecipitates obtained were analyzed by western blotting with E-cadherin (Ec), Dsg (Dsg) or Dsc (Dsc) antibodies. (C) A-431 cells were treated for 10 minutes with EGTA, then washed in PBS and further cultivated in standard growth medium for 10 (10'), 20 (20') minutes, or 1, 2 or 4 hours (1, 2, 4, respectively) and then analyzed as indicated in B.
suggesting that catenins do not mediate association between E-cadherin and desmosomal cadherins. Anti-Dsg staining of the E-cadherin immunoprecipitate of the EGTA-treated cells analyzed under nonreducing conditions yielded only a band of monomeric Dsg (not shown) indicating that the detected calcium-sensitive intercadherin association was not caused by disulfide crosslinking.

We next studied whether β-catenin associates with Dsg via E-cadherin or whether these two proteins form a separate complex. To clarify this, we examined the Dsg-β-catenin interaction in E-cadherin-negative HT-1080 cells. Fig. 1D shows that β-catenin was not co-immunoprecipitated with Dsg in the HT-1080 cells growing under normal conditions and only very weak binding was detected in EGTA-treated HT-1080 cells. In contrast, the Dsg-β-catenin complex was efficiently formed in HT-1080 cells transfected to produce myc tagged E-cadherin (Ec1M) upon addition of EGTA. These experiments strongly support the idea that the Dsg-β-catenin interaction is due to association of the conventional E-cadherin-catenin complex with Dsg. A weak Dsg-β-catenin binding found in the wild-type HT-1080 cells under low calcium conditions (Fig. 1, lane 2) is probably caused by an interaction between Dsg and N-cadherin, an endogenous classic cadherin of HT-1080 cells (Sacco et al., 1995). The lack of an affinity anti-N-cadherin antibody prevented us from testing this hypothesis.

To estimate the overall size of the E-cadherin-Dsg complex, the total lysate of the EGTA-treated A-431 cells was subjected to sucrose gradient centrifugation. Examination of the Dsg distribution along the gradient showed that the major pool of this protein sedimented at 8S and is consistent with our previous observation (Chitaev et al., 1998). In addition to this form of Dsg, overexposure of the same filters demonstrated that Dsg has minor forms sedimenting up to 16S (Fig. 2A). Anti-Dsg immunoprecipitation of each gradient fraction revealed that these heavy forms of Dsg associate with E-cadherin and β-catenin (Fig. 2A). Interestingly, in contrast to the 13S lateral or adhesive E-cadherin complexes that we described recently (Chitaev and Troyanovsky, 1998), this complex has a much broader distribution sedimenting between 11 and 16S. This broad distribution suggests that this complex has either a variable composition and/or a polymorphic conformation.

In a special set of experiments we studied the dynamics of assembly and disassembly of the intercadherin complexes. The results of these experiments (Fig. 2B,C) show that E-cadherin-Dsg or E-cadherin-Dsc complexes appeared very rapidly after removal of calcium ions from the culture medium. Their amount reached a plateau approximately five minutes after cultivation of the cells in medium with EGTA. Addition of normal medium to the EGTA-treated cells completely normalized the amount of these complexes during a few hours. These data suggest that normalization of the extracellular calcium level leads to gradual dissociation of the Dsg-E-cadherin complex and/or incorporation of this complex into the insoluble pool.

To show that E-cadherin-Dsg association takes place on the cell surface, the cells were treated with trypsin after addition of EGTA (Fig. 3). This experiment showed that practically all Dsg-E-cadherin complexes are localized on the cell surface at least up to 30 minutes after reduction of the calcium concentration. Interestingly, the level of the Dsg-E-cadherin complex found in cells cultured in normal medium was insensitive to trypsin treatment, suggesting that in normal cells this complex either has an intracellular localization or has a trypsin-resistant conformation. Staining of total cell lysates of the trypsin-treated cells by antibodies against intracellular epitopes of Dsg and E-cadherin demonstrated generation of the intracellular fragments of these proteins. These fragments were not co-immunoprecipitated (Fig. 3B) suggesting that E-cadherin interacts with Dsg via extracellular segments.

**Calcium removal induces a novel type of the E-cadherin dimers**

The data presented above show that the removal of extracellular Ca²⁺ ions triggers rapid formation of the E-cadherin-Dsg and E-cadherin-Dsc complexes. To delineate the portions of E-cadherin required for formation of such complexes, we studied A-431 cells producing recombinant forms of human E-cadherin, Ec1M or its mutants. Ec1M protein was tagged by myc epitope and contained a 19 amino acid long internal deletion (His773-Leu791) within the intracellular region. This deletion completely abolished binding of this mutant to the anti-E-cadherin C2080 antibody (Chitaev and Troyanovsky, 1998). Thus, endogenous E-cadherin and Ec1M can easily be distinguished by specific antibodies thereby allowing the detection of E-cadherin homoassociation in co-immunoprecipitation experiments. In our recent paper (Chitaev and Troyanovsky, 1998) we showed that Ec1M protein behaves similarly to wild-type E-cadherin. In the present work, using A-431 cells stably expressing Ec1M, we showed that this protein, similar to endogenous E-cadherin, associates with Dsg upon removal of Ca²⁺ ions (Fig. 4A, lanes 1 – and +).
The Ec1Δ(744-879)M mutant of the Ec1M protein lacking the intracellular region responsible for binding to catenins and p120 had the same ability as Ec1M to form a complex with Dsg (Fig. 4, lanes 2 – and +). This observation supports the conclusion made above that Ca2+-sensitive interactions are not mediated by catenins. Inactivation of two of the three Ca2+-binding sites in the E-cadherin EC1/EC2 domains that had been defined by crystallographic and mutational analysis (Ozawa et al., 1990a; Nagar et al., 1996) was done by double amino acid substitution Gln225Ala/Asn256Ala (mutant Ec1QNM, Fig. 4A, lanes 3 – and +). This alteration did also not abolish the association with Dsg. Only deletion of the entire extracellular cadherin-like repeats 1-3 of E-cadherin in the Ec1Δ(155-532)M mutant completely abolished this binding. This suggests that extracellular regions are involved in these interactions (Fig. 4A, lanes 5 – and +).

Notably, double mutation of Trp156Ala/Val157Gly (Ec1WVM mutant) did not inhibit the Ca2+-sensitive association with Dsg (Fig. 4A, lanes 4 – and +). Furthermore, since mutant Ec1WVM did not form lateral or adhesive dimers with endogenous E-cadherin under standard culture conditions (see Chitaev and Troyanovsky, 1998), anti-E-cadherin analysis of the anti-myc immunoprecipitates revealed clearly the strong Ca2+-sensitive interaction of this mutant with endogenous E-cadherin (Fig. 4A, lanes 4 + and –).

Western blot analysis of the anti-myc immunoprecipitates obtained after sucrose gradient centrifugation of the total lysates of Ec1WVM-producing cells showed that addition of EGTA in the culture medium significantly changed the sedimentation characteristics of the Ec1WVM mutant (Fig. 4B). Upon normal culture conditions mutant Ec1WVM was recovered from fractions distributed below of 13.5S value (corresponding to fraction 5), while a significant pool of the mutant obtained from EGTA-treated cells sedimented far above this value. Analysis of the same immunoprecipitates with anti-Dsg and anti-E-cadherin antibodies revealed broad peaks of the Ec1WVM-Dsg and Ec1WVM-E-cadherin complexes, similar to the peak of E-cadherin-Dsg complexes in wild-type A-431 cells. Thus, removal of extracellular calcium ions induced E-cadherin homoassociation. This homoassociation was not seen in cells producing Ec1M since the Trp156-mediated lateral E-cadherin 13S complex prevented us from noticing the formation of the calcium-sensitive association. We noted, however (Chitaev and Troyanovsky, 1998) that the combined amount of the E-cadherin 13S complexes was surprisingly unchanged upon EGTA treatment, despite the dissociation of adhesive complexes. Therefore, the constant amount of 13S complexes suggests that the decrease of adhesive complexes in low calcium is counterbalanced by an increased production of lateral Ca2+-sensitive complexes.

In an attempt to determine more precisely the region of E-cadherin responsible for the formation of the calcium-sensitive complexes, we transfected A-431 cells with Ec1M mutants lacking either one of EC domains. Fig. 5 shows that deletion of EC3 or EC4 domains significantly decreased the amount of Dsg in the anti-myc co-immunoprecipitates. Formation of the complexes was nearly abolished by deletion of both these domains in the mutant Ec1Δ(3+4)M. These data showed for the first time that EC domains, other than aminoterminal EC1 domain, may participate in E-cadherin hetero- or homodimerization. Interestingly, under normal culture

![Fig. 4. Association of Ec1M and its mutants with endogenous E-cadherin and Dsg (A) and sedimentation properties of the calcium-sensitive Ec1WVM/Dsg and Ec1WVM/E-cadherin complexes (B).](image-url)
Extracellular calcium depletion but not concomitant dissociation of the adhesive E-cadherin dimers induces formation of the calcium-sensitive complexes

We have shown (Chitaev, Troyanovsky, 1998) that cultivation of A-431 cells with 10 mM EGTA at 4°C or addition of 10 mM EGTA directly into the lysis buffer (but not in the culture medium) did not dissociate adhesive E-cadherin complexes. These data indicate that calcium ions do not participate in the maintenance of the adhesive dimers. In our attempt to understand the mechanism of formation of the calcium-sensitive Trp156-independent lateral E-cadherin complexes, we studied the association between Ec1WVM protein and endogenous E-cadherin and Dsg under these conditions. These experiments demonstrated that the presence of EGTA in the lysis buffer or in the culture medium at 4°C is not sufficient to induce formation of the calcium-sensitive complexes (Fig. 5A). This suggests that the calcium-sensitive inter-cadherin association might be connected in some way to dissociation of the adhesive E-cadherin complexes. To test this hypothesis, we studied whether the dynamics of the calcium-sensitive association correlates with the rate of disassembly of the adhesive complexes. In order to reveal the dynamics of the adhesive E-cadherin complexes, two A-431 sublines producing either myc-tagged (Ec1M) or flag-tagged (Ec1F) forms of E-cadherin were co-cultivated overnight, treated by EGTA for different time intervals, lysed and then immunoprecipitated with anti-myc antibody. Staining of these immunoprecipitates with anti-flag showed the amount of adhesive dimers (Chitaev and Troyanovsky, 1998) and with anti-Dsg showed calcium-sensitive E-cadherin/Dsg complexes. This experiment demonstrated a striking inverse correlation between these complexes (Fig. 5B).

Dissociation of adherens junctions can be blocked by preincubation of the cells with the protein kinase inhibitor H-7 (Citi, 1992; Citi et al., 1994). While the exact mechanism of this effect remains to be elucidated, the data suggest that the primary effect of H-7 is an inhibition of actomyosin-driven contractility of the cortical cytoskeleton (Volberg et al., 1994). It was suggested that the depletion of calcium ions triggers contraction of the adherens junction-associated microfilaments that, in turn, breaks the adherens junctions. In our attempt to understand the relationship between degradation of the adhesive complexes and formation of the calcium-sensitive E-cadherin complexes we studied the effect of the H-7 inhibitor on the amount of both complexes. For this, co-cultures of the Ec1M- and Ec1F-producing A-431 cells were exposed to EGTA with or without pretreatment with H-7 for 30 minutes. In agreement with published observations (Citi et al., 1994) pretreatment of the cells for 30 minutes with 100 μM H-7 inhibited cell dissociation (not shown). Furthermore, the data presented on Fig. 5C show that H-7 pretreatment completely blocked EGTA-induced dissociation of adhesive E-cadherin complexes. These results confirm on the molecular level the conclusion made by City et al. (1994) that chelator-induced dissociation of adherens junctions is not the result of a decrease in homophilic inter-adherin affinity.

Finally, we compared the amount of the calcium-sensitive E-cadherin complexes in cells treated with EGTA alone and in combination with H-7 (Fig. 5C). These data showed that H-7, completely blocked EGTA-induced dissociation of the adhesive E-cadherin complexes but was unable to prevent formation of the calcium-sensitive lateral E-cadherin complexes. Similar experiments performed with A-431 cells producing the Ec1WVM mutant demonstrated that H-7 pretreatment also did not block formation of calcium-sensitive inter-E-cadherin complexes (data not shown). Thus, our data indicate that the dissociation of adhesive complexes is not a necessary prerequisite for the formation of the calcium-sensitive E-cadherin complexes.
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Fig. 6. Degradation of the adhesive E-cadherin complexes is not essential for assembly of the calcium-sensitive intercadherin complexes. (A) A-431 cells producing Ec1WVM mutant were immunoprecipitated with anti-myc and immunoprecipitates were analyzed for myc (Myc); E-cadherin (Ec), or Dsg (Dsg) by immunoblotting. Before lysis cells were either untreated (1 and 4), or treated at 37°C (2) or at 4°C (3) with 10 mM EGTA for 10 minutes. In lane 4 10 mM EDTA was added to the lysis buffer. (B) To analyze dynamics of the adhesive and calcium-sensitive E-cadherin complexes, A-431 cells stably expressing EC1F were co-cultivated overnight with cells expressing Ec1M. Before lysis EGTA was added to the lysis buffer. (C) By analyzing the myc-tagged (Myc) and Flag-tagged (Flag) forms of E-cadherin, or Dsg (Dsg). Disappearance of the adhesive complexes as assayed by anti-flag staining correlates with assembly of the calcium-sensitive lateral intercadherin complexes. (C) Cells expressing Ec1M were co-cultured as indicated in B with Ec1F-producing cells. In lanes 1 and 3 cells were not treated with EGTA; in lanes 2 and 4 cells were incubated with 10 mM EGTA for 10 minutes at 37°C (+); in lanes 3 and 4 cultures were preincubated with 300 mM of H-7 (H-7). Cell lysates were immunoprecipitated with anti-myc and analyzed by immunoblotting with anti-myc (Myc), anti-cadherin (Ec); anti-flag (Flag); anti-Dsg (Dsg); and anti-Dsc (Dsc) antibodies. Note that pretreatment with H-7 prevents degradation of the adhesive complexes but does not inhibit assembly of the intercadherin complexes.

DISCUSSION

Lateral oligomerization of cell-surface receptors is a widely distributed mechanism regulating receptor activity. Thus, it was suggested that adhesion potential of E-cadherin depends on its lateral homodimerization (Brieher et al., 1996; Tomlschy et al., 1996). Little is known, however, about how many types of lateral E-cadherin-containing complexes can be assembled. Recently we have shown that the homodimerization of E-cadherin depends on its Trp156/Val157 residues (Chitaev and Troyanovsky, 1998), which is consistent with the model proposed by Shapiro et al. (1995). In the present work we show that transferring the cells to low calcium medium induces a rapid assembly of a novel type of lateral E-cadherin complexes.

The major structural feature of the new lateral complexes is their independence of a Trp156 residue. Notably, this Trp156-independent, calcium-sensitive lateral association occurs not only between two E-cadherin molecules, but also between E-cadherin and both desmosomal cadherins, Dsg and Dsc. On the other hand, this association is cadherin-specific, since, using co-immunoprecipitation assays, we were not able to detect a calcium-sensitive association between E-cadherin and other transmembrane molecules of A-431 cells, such as EGF receptor or CD44 (data not shown). The detailed composition of this Trp156-independent lateral complex is not completely clear. It sediments in a much broader peak than Trp156-dependent lateral or adhesive E-cadherin complexes, indicating that it is polymorphic, e.g. contains two or more of the conventional cadherin/catenin complexes aligned in parallel fashion. Our mutagenesis experiments indicate that the calcium-sensitive E-cadherin association requires EC3 and EC4 extracellular domains, but not the intracellular, catenin-binding region of E-cadherin and extracellular EC1 domain mediating adhesive and lateral E-cadherin dimerization under standard culture conditions. Since conformation of the EC domains is calcium-dependent (Takeichi et al., 1981; Volk et al., 1990; Pokutta et al., 1994), it appears reasonable to propose that this interaction is induced by conformational changes in cadherin structure after dissociation of the calcium ions. The fact that assembly of the calcium-sensitive E-cadherin complexes is strongly temperature-dependent, suggests, however, that some additional factors could be involved in this process.

It has been shown that reduction in extracellular calcium concentration causes an immediate splitting of cell-cell junctional structures (Kartenbeck et al., 1982; Mattey and Garrod, 1986; Volberg et al., 1986; Green et al., 1987) and dissociation of the E-cadherin adhesive complex (Chitaev and Troyanovsky, 1998). Notably, pretreatment of epithelial cells with protein kinase inhibitor H-7 confers Ca2+ independence on cell-cell junctions (Citi, 1992). Experiments presented here revealed that, although H-7 completely prevents EGTA-induced dissociation of the adhesive E-cadherin complexes, it does not abolish the induction of the calcium-sensitive complexes. Thus, the calcium-sensitive E-cadherin association is not caused by splitting of the intercellular junctions and dissociation of the adhesive complexes, in particular. It strongly supports the idea that loss of calcium from E-cadherin results albeit through some intermediate steps in activation of the new intercadherin-binding activity.

The present study also shows strong similarities in kinetics of the assembly of the calcium-sensitive and disassembly of the adhesive E-cadherin complexes upon removal of Ca2+ ions. It suggests that both processes might be directly related to each other. How could the intercadherin linkage induced by depletion of the extracellular Ca2+ ions lead to the splitting of cell-cell junctions? Experiments reported by Citi et al. (1994), Denisenko et al. (1994), and Volberg et al. (1994) demonstrated that the H-7 inhibitor affects the contractility of the adherens junction-associated microfilaments. It was suggested that these microfilaments provide pulling forces required for disintegration of the adhesion structures. Experiments reported
in the present work support this point of view. They unambiguously demonstrate that H-7 protein kinase inhibitor renders adhesive E-cadherin complexes insensitive to Ca\(^{2+}\) ions. Furthermore, additional experiments (not shown) showed that cytochalasin D, a potent inhibitor of actin polymerization, also prevents EGTA-induced dissociation of the adhesive E-cadherin dimers. One interpretation of the present results is that formation of the Trp\(^{156}\)-independent lateral complexes triggers the contraction of the cortical cytoskeleton that is mediated either by cadherin-dependent signalling or by direct anchorage of this type of complexes to the cytoskeleton.

Experiments presented also show that the reduction of the extracellular Ca\(^{2+}\) concentration leads to immediate formation of complexes incorporating both desmosomal cadherins, Dsg and Dsc. Complexes with similar compositions were not detected in A-431 cells growing at a normal Ca\(^{2+}\) concentration. This oligomerization of desmosomal cadherins could explain the assembly of half-desmosomal structures in epithelial cells cultured in low calcium medium (Demlehner et al., 1995). This possibility is supported by our previous observation that assembly of the desmosome-like plaques can be promoted by clustering of the intracellular Dsc region (Troyanovsky et al., 1993).

Another open question is whether the Trp\(^{156}\)-independent, calcium-sensitive intercadherin interaction is involved in normal adherens junction assembly or its formation is only a consequence of the experimentally induced decrease of calcium concentration. Our data do not provide direct and unequivocal evidence for the existence of such an interaction in cells under normal Ca\(^{2+}\) conditions. It is possible, however, that the Trp\(^{156}\)-independent lateral intercadherin interaction is responsible for the formation of the small, but detectable amount of Dsg-E-cadherin dimers revealed in standard cultures of A-431 cells. Such a complex could be responsible for the Dsg-\(\beta\)-catenin co-immunoprecipitation reported by Norvell and Green (1998). The exact role of this Dsg-E-cadherin complex, which could be a structural element in the proposed cross-talk between desmosomes and adherens junctions (Wheelock and Jensen, 1992; Lewis et al., 1994, 1997; Amagai et al., 1995), remains to be elucidated.

Identification of the calcium-sensitive type of inter-E-cadherin lateral dimerization in normal cells is complicated by the presence of other forms of E-cadherin complexes with similar protein composition. Additionally, complexes formed by the Trp\(^{156}\)-independent mechanism during normal adhesion could be tightly anchored to the cytoskeleton and would therefore be insoluble. Experiments that will define the precise determinants involved in such interactions are essential for understanding their functioning in normal cells. It is attractive to speculate that lateral Trp\(^{156}\)-independent self-association of E-cadherin molecules has a critical role in the biogenesis of adherens junctions. It was shown that E-cadherin is recruited into a Triton X-100 insoluble pool approximately 10 minutes after the formation of the stable intercellular contact (McNeill et al., 1993). Since the adhesive E-cadherin complexes are soluble in Triton X-100 (Chitaev and Troyanovsky, 1998), one may propose that in the next step of assembly they enter a Triton X-100 insoluble pool that is accompanied by critical changes in their structure. Such a step could be the Trp\(^{156}\)-independent lateral association of the adhesive dimers. In turn, this association may trigger anchorage of the adhesive complexes to the cortical cytoskeleton. In theory Trp\(^{156}\)-independent association could be induced by changes in occupancy of the calcium-binding sites of E-cadherin. The significance of the divalent cation occupancy is widely accepted in integrin-mediated transmembrane signal transduction (see for recent review, Humphries, 1996).

In summary, our data show that E-cadherin has at least two alternative pathways to form lateral complexes. Although much more needs to be done to gain further insight into the molecular mechanisms of E-cadherin-mediated adhesion, the results of this study suggest a complex order of transitional stages in the organization of the E-cadherin/catenin complex during its integration into the mature adherens junctions. We also show how depletion of calcium ions may activate one of these stages.

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