

Amino-terminal domain of classic cadherins determines the specificity of the adhesive interactions

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SUMMARY

Classic cadherins are transmembrane receptors involved in cell type-specific calcium-dependent intercellular adhesion. The specificity of adhesion is mediated by homophilic interactions between cadherins extending from opposing cell surfaces. In addition, classic cadherins can self-associate forming lateral dimers. Whereas it is widely expected that lateral dimerization of cadherins is critical for adhesion, details of this process are not known. Yet, no evidence for physical association between different classic cadherins in cells expressing complex cadherin patterns has been reported. To study lateral and adhesive intercadherin interactions, we examined interactions between two classic cadherins, E- and P-cadherins, in epithelial A-431 cells co-producing both proteins. We showed that these cells

exhibited heterocomplexes consisting of laterally assembled E- and P-cadherins. These complexes were formed by a mechanism involving Trp¹⁵⁶ of E-cadherin. Removal of calcium ions from the culture medium triggered a novel Trp¹⁵⁶-independent type of lateral E-cadherin-P-cadherin association. Notably, an antiparallel (adhesive) mode of interaction between these cadherins was negligible. The specificity of adhesive interaction was localized to the amino-terminal (EC1) domain of both cadherins. Thus, EC1 domain of classic cadherins exposes two determinants responsible for nonspecific lateral and cadherin type-specific adhesive dimerization.

Key words: Cadherin, Catenin, Intercellular adhesion

INTRODUCTION

Classic cadherins, which are single transmembrane domain glycoproteins, have been identified as major receptors mediating cell-type specific intercellular adhesion. There is overwhelming evidence that these molecules function in a wide range of cell-cell recognition processes during morphogenesis. Further, it has been demonstrated that specific defects in cadherin-based adhesion promote tumor invasion and metastasis (Geiger and Ayalon, 1992; Takeichi, 1995; Peifer, 1995; Klymkowsky and Parr, 1995; Gumbiner, 1996). It was shown that cells exposing different cadherins such as E- and P-cadherins or E- and N-cadherins in the presence of Ca²⁺ ions form separate aggregates (Volk et al., 1987; Nose et al., 1988; Friedlander et al., 1989). These classic experiments evaluated the homophilic properties of the cadherin-mediated intercellular adhesion and suggested that adhesion is based on formation of homomeric Ca²⁺-dependent cadherin complexes. Although the precise structure of such complexes and the molecular principals of their assembly have not been established, some important clues for understanding the cadherin-based adhesion machinery have entered the limelight.

It was shown that the extracellular cadherin region consists of five homologous domains (EC1-EC5, numbered from the N terminus) of approximately 110 amino acids. Together they coordinate several calcium ions which maintain the rod-like conformation of the extracellular cadherins region (Pokutta et

al., 1994; Shapiro et al., 1995; Nagar et al., 1996). Experiments with cadherin mutants and chimeric molecules showed that the EC1 domain determines the adhesive specificity of cadherins (Nose et al., 1990). Crystallography data (Shapiro et al., 1995; Nagar et al., 1996) not only showed the precise structure of the EC domains but also revealed, for the first time, an ability of the EC1 domain to form lateral and antiparallel homodimers. In our recent work (Chitaev and Troyanovsky, 1998), we presented substantial evidence demonstrating that E-cadherin forms both lateral and adhesive complexes in vivo. Formation of the adhesive, but not the lateral, complex requires interaction of E-cadherin with catenins, important intracellular regulators of cadherin adhesive activity (for references see Geiger and Ayalon, 1992; Gumbiner, 1996; Troyanovsky, 1999). Some biochemical properties of the lateral complex identified were consistent with the structural model proposed by Shapiro et al. (1995) in which lateral dimers are formed via reciprocal interactions between Trp¹⁵⁶ (here and below the E-cadherin sequence is numbered according to GenBank accession # Z13009, cf. Bussemakers et al., 1993) of one subunit and a hydrophobic pocket provided by the other subunit. Formation of this Trp¹⁵⁶-mediated lateral complex was not dependent on the presence of Ca²⁺-binding sites between EC1 and EC2 domains. In addition to these two complexes, recently we have found that removal of extracellular calcium ions from epithelial A-431 cells causes immediate assembly of a novel form of intercadherin complexes incorporating either several (two or

more) E-cadherin molecules or both E-cadherin and desmosomal cadherins (Trojanovsky et al., 1999). These complexes are independent of Trp¹⁵⁶ of E-cadherin. The mechanism of assembly of any of these three types of E-cadherin complexes is not known. Furthermore, it is not clear whether these complexes represent consecutive steps in the cell-cell adhesion process or whether they fulfill different functions.

Interestingly, Trp¹⁵⁶, which drives the formation of the E-cadherin lateral homodimers, is absolutely conserved among all known types I and II classic cadherins. This suggests that distinct species among this group of cadherins either are able to form lateral Trp¹⁵⁶-dependent heterodimers or there is a special mechanism protecting them from heterodimerization. In an attempt to address this issue, we have studied whether A-431 cells co-expressing E- and P-cadherins may form heteromeric complexes. This work presents clear data showing that under normal culture conditions A-431 cells expose lateral heterodimers consisting of these two cadherins. Notably, adhesive dimerization of E-cadherin, while also depends from Trp¹⁵⁶ residue is cadherin type-specific.

MATERIALS AND METHODS

DNA constructs, cell culture, DNA transfection and immunofluorescence microscopy

The construction of the expression plasmids coding for the E-cadherin with an internal deletion, His⁷⁷³-Leu⁷⁹¹, and tagged COOH-terminally either by myc (Ec1M) or by flag (Ec1F) epitopes, as well as mutants Ec1WVM, Ec1QNM, Ec1Δ(748-882)M, and Ec1Δ(159-536)M was described recently (Chitaev and Trojanovsky, 1998). A new E-cadherin point mutant Ec1M-W156A was produced using site-directed mutagenesis. As other E-cadherin mutants, it was constructed in the eukaryotic expression vector pRcCMV (Invitrogen, Carlsbad, CA) containing a neomycin resistance gene. A human P-cadherin cDNA was generated from two fragments which were amplified by RT-PCR from human skin cDNA (Invitrogen, Carlsbad, CA) using the following pairs of primers: 5'-AAA AAA GCT TCA CCC CTC TCT CTG CAG CCA TGG GGC-3' (primer #1) and 5'-TTT TGG ATC CAT GGC TAG CCA CCC TGC TGG G-3'; 5'-CCC AGC AGG GTG GCT AGC CAT GG-3' and 5'-TTT TGG ATC CGT CGT CCT CCC CGC CAC CGT AC-3'. The first fragment was cut by *Hind*III and *Bam*HI and subcloned in Bluescript II KS1 vector (Stratagene, La Jolla, CA). The resulting plasmid was cleaved by *Nhe*I/*Bam*HI and ligated with the *Nhe*I/*Bam*HI-digested second fragment that generates a plasmid, pBI-hPc. This plasmid encodes the entire human P-cadherin in which the stop codon is replaced with an unique *Bam*HI site. Sequencing analyses of this plasmid showed that the cloned sequence was identical to the published 31-2541 nucleotide sequence of P-cadherin cDNA (Shimoyama et al., 1989), the GenBank/EMBL/DBJ accession number X63629) with two exceptions: adenine substituted for guanine at position 774 and for cytosine at position 867 (the nucleotide and the amino acid sequences are numbered accordingly to the hP-cadherin clone X63629). Neither substitution, however, changed the amino acid composition of P-cadherin. The *Bam*HI/blunt-ended *Hind*III insert of pBI-hPc was ligated with *Bam*HI/blunt ended *Sac*I pBEHpac18-Dsg1M plasmid containing the puromycin resistance gene and a sequence encoding 6×myc epitope (Chitaev et al., 1998). The resulting plasmid named pBEHPcadM, encodes intact P-cadherin tagged COOH terminally by the 6×myc epitope. To construct CMVP1Ec1M and CMVPIEc1F plasmids, the encoding region for leader peptide and EC1 domain of P-cadherin (Met¹-Pro²¹³) was amplified by PCR from plasmid pBI-

hPc using the primer #1 and primer 5'-AAA GAA TTC GGG CTT GTG GTC ATT CTG GTC-3' and resulted product was inserted between *Hind*III and *Eco*RI sites of the plasmids CMVEc1M or CMVEc1F.

Transfection of human epidermoid carcinoma A-431 cells (ATCC, CRL1555) and selection, growth, and immunofluorescence microscopy were done as described (Trojanovsky et al., 1994). HaCat human keratinocytes (Boukamp et al., 1988) were provided by Dr W. W. Franke (German Cancer Research Center, Heidelberg, Germany). The following mouse monoclonal antibodies were used: anti-E-cadherin, clone SHE 78-7, and anti-plakoglobin, clone 11E4 (Zymed laboratories, San Francisco, CA); anti-myc (clone 9E10, provided by Dr R. Kopan, Washington University Medical School, St Louis, MO); anti-flag M2 (Sigma, St Louis, MO); anti-α-catenin, anti-E-cadherin (Mabs C20820), anti-P-cadherin, anti-N-cadherin, anti-β-catenin (Transduction Laboratories, Lexington, KY), and rabbit anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation and sedimentation analysis

For most immunoprecipitation experiments, 2×10⁶ cells were cultured in a 10 cm tissue culture dish at 37°C for about 72 hours. In co-culture experiments, 6×10⁶ 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 (Chitaev and Trojanovsky, 1998). In brief, the confluent monolayer (approximately 10⁷ cells) was washed and extracted at 4°C with 1.5 ml of immunoprecipitation lysis buffer (IP-buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 20 μM p-APMSF, 2 mM EDTA, and 1% NP-40). The cells detached from the tissue culture dish after a few minutes in IP-buffer and then were transferred into 1.5 ml microcentrifuge tubes (Sarstedt, Newton, NC) and agitated for 10 minutes at 4°C. NP-40 insoluble material was removed by centrifugation at 100,000 g for 1 hour. The lysates were subjected to immunoprecipitation by subsequent incubations with specific antibody and Protein A-Sepharose.

In some experiments, cells were dissociated into single cell suspension before lysis. To accomplish this, EGTA (Sigma) was added to a final concentration of 10 mM. After 10 minutes at 37°C, the cells were dissociated by gentle pipetting and then centrifuged. Our previous experiments (Trojanovsky et al., 1999) showed that this EGTA treatment did not result in cadherin internalization and did not change amounts of E-cadherin in the cell lysates. In either case approximately 90% of E-cadherin was released into the IP-buffer. To determine whether the E-/P-cadherin complexes are exposed on the cell surface, 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 0.4 mg/ml), cells were subjected to immunoprecipitation analysis.

For sucrose gradient centrifugation, confluent monolayer cells from three 10 cm dishes were lysed with 2 ml of IP-buffer. Lysates (1 ml) were pre-cleaned 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, 11.35S; apoferritin, 17S.

RESULTS

E-cadherin-P-cadherin heteroassociation

To determine the complexity of intercadherin interactions, we have studied whether E-cadherin can form heterocomplexes with other members of the classic cadherins family. Good

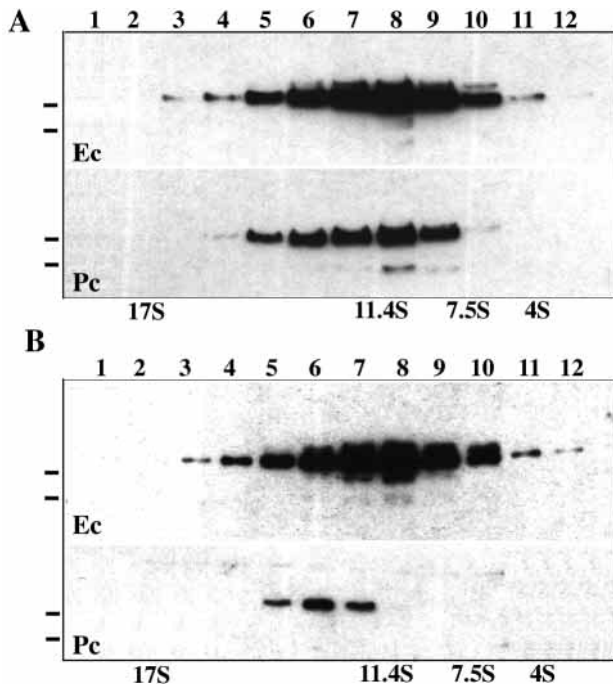


Fig. 1. Sedimentation analysis of the E-cadherin-P-cadherin complexes. Total lysate of A-431 cells was subjected to sucrose gradient centrifugation. The collect fractions (numbered 1 to 12, with 1 at the bottom of the gradient) were analyzed for the presence of E-cadherin or P-cadherin by immunoblotting (A) or were at first immunoprecipitated with anti-E-cadherin mAb and then analyzed by immunoblotting (B) with antibodies against E-cadherin (Ec) or P-cadherin (Pc). Note that the complex containing both E- and P-cadherins appears in fractions 5-7. 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. Bars (left) denote the relative positions of coelectrophoresed reference proteins (from top to bottom: β -galactosidase, 116,000; phosphorylase b, 97,400).

candidates for this study are A-431 cells and HaCat keratinocytes co-producing E- and P-cadherins (Johnson et al., 1993). To determine whether E-cadherin interacts with P-cadherin, total lysates of these cells were immunoprecipitated with anti-E-cadherin monoclonal antibodies C20820 or SHE 78-7 and the immunoprecipitates were stained by western blot with P-cadherin-specific antibodies. In a set of independent experiments, we found that the anti-E-cadherin immunoprecipitates contain a 120 kDa protein that cross-reacts with anti-P-cadherin antibody (not shown, see also Fig. 1). Since 120 kDa is the approximate molecular mass of human E- and P-cadherins (Shimoyama et al., 1989; Johnson et al., 1993), one possible explanation for our results was that both proteins are incorporated into the mutual complex.

This observation prompted us to study the overall size of this presumed E-cadherin-P-cadherin complex using sucrose gradient centrifugation of the total A-431 cells lysate (Fig. 1). Examination of the E- and P-cadherins in gradient fractions by western blot analysis showed that both proteins have similar sedimentation characteristics (Fig. 1A). Their major pools sedimented at 9 S, while a significant amount of these proteins sedimented far above this value (up to 14 S). However, only in

the 13 S fractions (fractions 5-7), not in the 9 S fractions, anti-E-cadherin antibody co-immunoprecipitated a P-cadherin-positive 120 kDa band (Fig. 1B). As we had shown previously, 13 S fractions contain complexes incorporating E-cadherin homodimers, while the 9 S form of E-cadherin originates from a complex consisting of one E-cadherin molecule and a set of catenins (Chitaev and Troyanovsky, 1998). Furthermore, the P-cadherin positive 120 kDa band was also detected in 13 S complexes when A-431 cells producing the myc-tagged version of E-cadherin (Ec1M protein, see description of these cells in Chitaev and Troyanovsky, 1998) were immunoprecipitated with anti-myc antibody (not shown, see also below). These data confirmed that E- and P-cadherins were present in the same complex. We were not able, however, to discount the possibility that the monoclonal antibody against P-cadherin used in our experiments cross-reacts with a specific 'dimeric' conformation of E-cadherin present in the 13 S complex.

To clarify this question, we cloned P-cadherin cDNA using PCR amplification, tagged it C-terminally by the sequence encoding a 6 \times myc epitope (PcM protein, see Fig. 2 for details) and expressed the resulting construct in A-431 cells stably producing the flag-tagged version of E-cadherin lacking the epitope for C20820 anti-E-cadherin antibody (Ec1F protein, Fig. 2). The resulting stably transfected cells produced two forms of E-cadherin and two forms of P-cadherin. Both forms of each cadherin could be easily distinguished by western blot analysis allowing us to monitor (i) the complexes incorporating E-cadherin or P-cadherin dimers and (ii) the presumed heterocadherin complexes incorporating E- and P-cadherins using myc-flag co-immunoprecipitation. Double immunofluorescent staining of these cells with anti-rabbit myc and monoclonal anti-flag or C20820 anti-E-cadherin antibodies showed that both recombinant proteins, as well endogenous E-cadherin, co-distributed in adherens junction-like structures along the cell-cell contact regions (Fig. 3 and not shown). Moreover, the myc-tagged version of P-cadherin associates with α -, and β -catenins and plakoglobin, as was shown by co-immunoprecipitation assay (not shown). Taken together, these results demonstrated that the addition of the 6 \times myc tag does not influence subcellular distribution and major binding partners of P-cadherin.

To study the interactions between Ec1F and PcM proteins, each fraction recovered from the sucrose gradient was divided into two equal portions which were subjected either to anti-myc or anti-E-cadherin immunoprecipitation. Western blot analysis of the myc immunoprecipitates with anti-flag and anti-P-cadherin antibodies showed that anti-myc antibody, recognizing PcM protein, co-immunoprecipitated both flag-tagged E-cadherin and endogenous P-cadherin exclusively in the 13 S fractions (Fig. 2B). Parallel anti-E-cadherin immunoprecipitation of the same gradient using C20820 antibody against endogenous E-cadherin and staining of the resulting immunoprecipitates with anti-flag antibody reacting with Ec1F verified that 13S fractions also contained E-cadherin homodimeric complexes (Fig. 2C). Taken together, these data unambiguously demonstrated that A-431 cells, under standard culture conditions, could assemble complexes containing either E-cadherin or P-cadherin homodimers or E-cadherin-P-cadherin heterodimer. Treatment of cells co-expressing Ec1F and PcM proteins with trypsin/EDTA prior lysis nearly

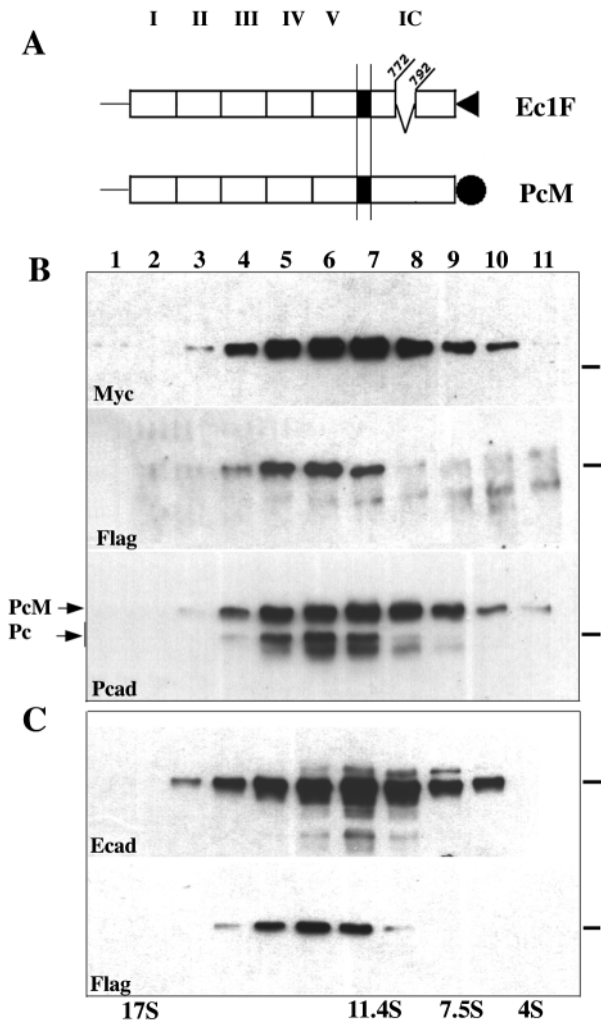


Fig. 2. Sedimentation analysis of the A-431 cell coproducing Ec1M and PcF recombinant proteins. A-431 cells were co-transfected to produce Ec1F and PcM proteins, schematically represented in A. Both proteins consist of a leader peptide (solid line), five extracellular cadherin-like repeats (numbered I-V), a transmembrane domain (solid box), and an intracellular region (IC). C-terminally, E-cadherin was tagged by a single flag epitope (filled triangle) and P-cadherin by a 6×myc epitope (filled circle). Ec1M also contains a small internal deletion (His⁷⁷³-Leu⁷⁹¹) abolishing binding of E-cadherin to the anti-E-cadherin monoclonal antibody used in this study. Numbers show the positions of the corresponding amino acids. To examine the interactions between both proteins, fractions of the total lysate of these cells were separated in a sucrose gradient, then each fraction was split into two equal portions and co-immunoprecipitated with antibodies either against myc epitope (B) or against E-cadherin (C). Co-immunoprecipitates were then analyzed by immunoblotting for the presence PcM using anti-myc (Myc); Ec1F using anti-flag (Flag); PcM and endogenous P-cadherin using anti-P-cadherin (Pcad) staining. The last two proteins are shown by arrows (PcM and Pc, respectively). Note that the sedimentation of the PcM-EcF complexes visualized by anti-flag antibody in B exactly corresponds to the sedimentation of the PcM-P-cadherin complex indicated by distribution of the endogenous P-cadherin in B and the E-cadherin-Ec1F complex revealed by anti-flag antibody in C. The molecular mass marker at 116 kDa is indicated by bars at the right.

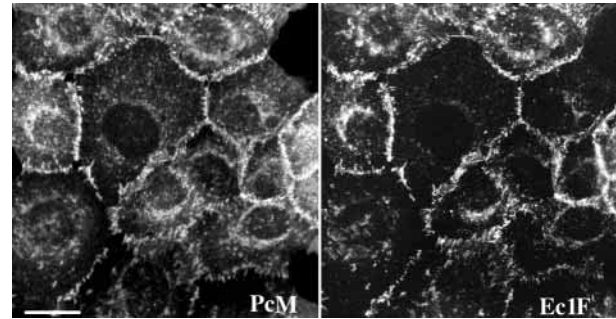


Fig. 3. Double immunofluorescence microscopy of A-431 cells stably producing Ec1F and PcM proteins. The left hand picture (PcM) shows the distribution of PcM as visualized by rabbit anti-myc antibody in comparison with the localization of Ec1F as detected by anti-flag monoclonal antibody. Bar, 40 µm.

abolished their co-immunoprecipitation (data not shown). The high sensitivity of this complex to trypsin treatment indicates its surface localization. One of the possible explanations for the failure to detect E-cadherin-P-cadherin heterocomplexes in previous co-immunoprecipitation experiments (Johnson et al., 1993) was the use of a low-sensitive immunostaining of the western blots in that study.

Two distinct Trp¹⁵⁶dependent and Trp¹⁵⁶independent mechanisms of E-cadherin – P-cadherins heteroassociation

To determine whether E-cadherin and P-cadherin molecules in the heterocadherin 13 S complex have lateral or adhesive alignment, A-431 cells stably expressing myc-tagged recombinant E-cadherin (Ec1M, see Chitaev and Troyanovsky, 1998, for details) were dissociated into single-cell suspension using 10 mM EGTA and subjected to anti-myc immunoprecipitation (Fig. 4B, lanes Ec1M). This experiment showed that EGTA treatment slightly increased the amount of P-cadherin co-immunoprecipitated with anti-myc antibody, suggesting that E- and P-cadherins in the heterocomplex at least after Ca²⁺ depletion extend from the same cell surface. To characterize the requirement of different portions of the E-cadherin molecule for formation of E-/P-cadherin complex, we studied the interaction between endogenous P-cadherin and different Ec1M mutants constructed and described previously (Chitaev and Troyanovsky, 1998, see also Fig. 4A). The Ec1Δ(748-882)M mutant lacking the intracellular region responsible for binding of E-cadherin to catenins and p120 had the same ability as Ec1M to form a complex with P-cadherin (Fig. 4B). This observation suggested that heterocadherin interactions are not mediated by catenins. Inactivation of two of the three presumable Ca²⁺-binding sites (Nagar et al., 1996) in the E-cadherin EC1/EC2 domains in the mutant Ec1QNM also did not abolish the association with P-cadherin. In contrast, double mutation Trp156Ala/Val157Gly (Ec1WVM mutant) or point mutation Trp156Ala (Ec1M-W156A mutant, not shown), strongly inhibited the association of the Ec1M protein with P-cadherin. Taken together, these data suggested that structurally the P-cadherin-E-cadherin complex is very similar to the recently described E-cadherin homodimeric lateral complex (Chitaev and Troyanovsky, 1998). As it was

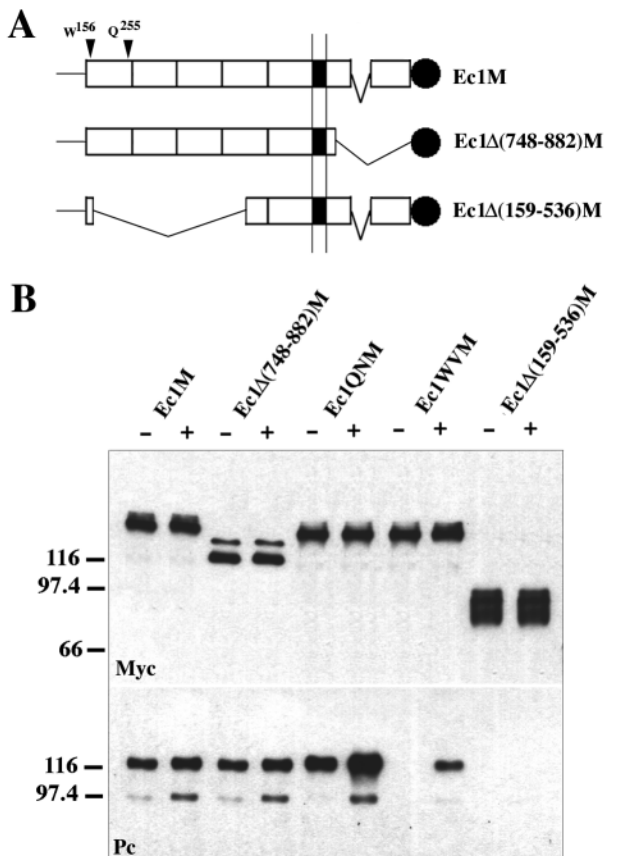


Fig. 4. Association of Ec1M and its mutants with P-cadherin. (A) Schematic representation of Ec1M mutants. Arrowheads show positions of the Trp¹⁵⁶ and Gln²⁵⁵ mutated in the Ec1WVM and Ec1QNM mutants, respectively. Other abbreviations as in Fig. 2. (B) Untreated (-) or EGTA treated (+), A-431 cells stably producing Ec1M; Ec1Δ(748-882)M; Ec1QNM; Ec1WVM; Ec1Δ(159-536)M were immunoprecipitated with anti-myc antibody. Immunoblot analysis of these immunoprecipitates with anti-myc (Myc) and anti-P-cadherin (Pc) antibodies show that all mutants except Ec1Δ(159-536)M establish complexes with P-cadherin upon removal of extracellular calcium. Molecular mass standards are shown in kDa. The nature of the weak low molecular mass band visible after anti-P-cadherin staining is unknown.

proposed, this complex is established by Trp¹⁵⁶-dependent hydrophobic interactions between two E-cadherin molecules.

We have recently demonstrated that removal of calcium ions from culture medium triggers another type of the lateral intercadherin association (Trojanovsky et al., 1999). Formation of such 'calcium-sensitive' complexes was independent of Trp¹⁵⁶. Importantly, this interaction leads to formation not only of the E-cadherin dimeric complexes, but also heterocomplexes incorporating E-cadherin and desmosomal cadherins. Removal of the calcium ions from the medium of Ec1WVM-expressing cells demonstrated that this type of interactions also mediates the formation of the calcium-sensitive, Trp¹⁵⁶-independent complexes incorporating E- and P-cadherins (Fig. 4B). This calcium-sensitive association could account for the increase in the amount of the P-cadherin-E-cadherin heterocomplexes in cells treated with EGTA described above (see Fig. 4, lanes Ec1M). Deletion of the EC1

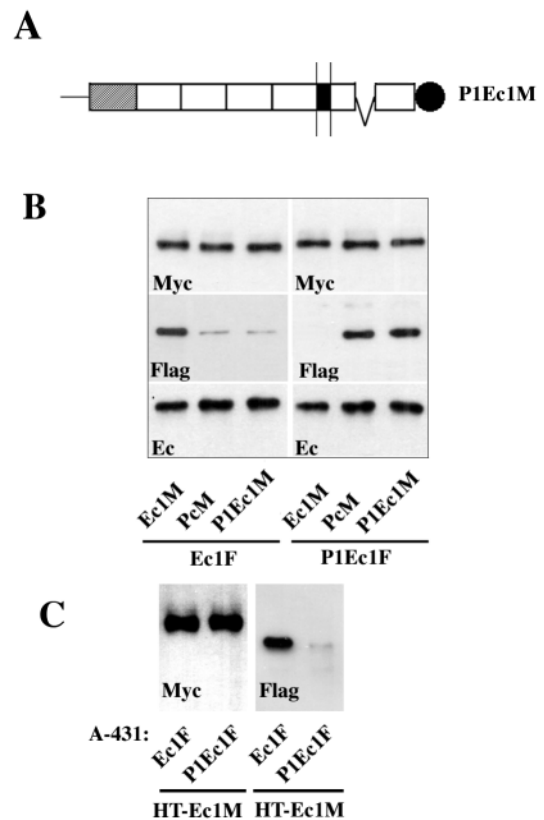


Fig. 5. Specificity of the adhesive interaction is determined by EC1 domain. (A) Schematic representation of the P1Ec1M mutant. Hatched box designates the EC1 domain derived from P-cadherin in the chimeras P1Ec1M. Other abbreviations as in Fig. 2. (B) Cells expressing either Ec1F (left panel) or P1Ec1F (right panel) were co-cultivated with cells producing Ec1M, PcM, or P1Ec1M and then were co-immunoprecipitated with anti-myc antibody and assayed for presence of the myc-tagged (Myc), flag-tagged (Flag) forms of cadherins and endogenous E-cadherin (Ec). Note that adhesive complexes detected by anti-flag staining are formed only between cadherins containing identical EC1 domains. (C) A-431 cells producing either Ec1F or P1Ec1F were co-cultivated with HT-1080 cells producing Ec1M were immunoprecipitated with anti-myc antibody. Immunoblot analysis of these immunoprecipitates with anti-myc (Myc) and anti-flag (Flag) antibodies show that Ec1M is not able to form adhesive dimers with P1Ec1F in this system.

to EC4 domains of E-cadherin in the Ec1Δ(159-536)M mutant completely abolished any type of P-cadherin-E-cadherin interaction.

EC1 domain determines the adhesion specificity of E-cadherin

Experiments reported above show that E- and P-cadherins may form two types of heteromeric complexes, in which both molecules extend from the surface of the same cells and thus, are likely to have a lateral arrangement. In the next experiments, we have investigated whether P-cadherin might form adhesive complexes with E-cadherin. Cells expressing myc-tagged P-cadherin (PcM) or E-cadherin (Ec1M) were co-cultivated overnight with cells stably producing the flag-tagged form of E-cadherin. In this system, anti-myc antibody reacting either with PcM or Ec1M would co-immunoprecipitate Ec1F

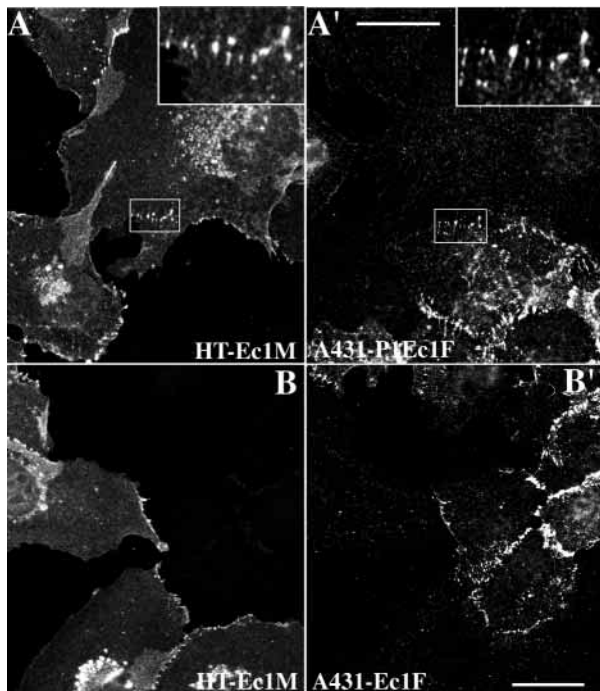


Fig. 6. Unengaged in adhesive dimerization P1Ec1F chimera is recruited into adherens junctions. Double-label immunofluorescence microscopy of HT-1080 cells expressing Ec1M and A-431 cells expressing either P1Ec1F (A,A') or Ec1F (B,B') in coculture. The distribution of the Ec1M visualized by immunostaining with rabbit anti-myc antibody (A,B). This is compared with the localization of the P1Ec1F (A') or Ec1F (B') detected by anti-flag mAb. Both proteins, although present in different cells, form clusters located at the same areas of cell-cell contacts. Higher magnification of the selected regions are shown in the inserts. Bars, 40 μ m.

only if myc- and flag-tagged cadherins interact in the antiparallel 'adhesive' fashion. This experiment showed that Ec1F efficiently co-immunoprecipitated with anti-myc antibody only from protein lysates obtained from Ec1M-Ec1F, but not PcM-Ec1F co-culture (Fig. 5B). These data present a compelling evidence that P- and E-cadherin could not establish adhesive heterocomplexes soluble in NP-40 despite their ability to produce lateral heterocomplexes.

To verify the pivotal role of EC1 domain in the determination of the specificity of adhesive interactions, we constructed and produced in A-431 cells the P1Ec1M and P1Ec1F chimeric proteins in which EC1 domain of E-cadherin in the Ec1M and Ec1F proteins was replaced with the EC1 domain derived from P-cadherin. Next, we studied whether such replacement shifted the adhesive specificity of E-cadherin. These experiments are presented in Fig. 5. In complete agreement with the published data obtained by the co-aggregation approach (Nose et al., 1990), these experiments demonstrated that adhesive dimers formed only between cadherins containing identical EC1 domains. For example, P1Ec1F mutant could be efficiently co-immunoprecipitated by myc antibody in co-culture with PcM and P1Ec1M expressing cells, but not with cells expressing Ec1M (Fig. 5B). The absence of heterodimerization between Ec1F and PcM (or P1Ec1M) in this co-culture experiment also indicates that the formation of the E-/P-cadherin heterodimers described above

were not driven by detergent solubilization. Furthermore, staining of the anti-myc co-immunoprecipitates obtained from Ec1M-, PcM- or P1Ec1M-expressing cells with the antibody C20820 against endogenous E-cadherin (Fig. 5B) revealed that they contained similar amount of E-cadherin. Importantly, endogenous E-cadherin in anti-myc immunoprecipitates obtained from Ec1M-expressing cells was derived from both adhesive and lateral complexes, whereas it originated predominantly from lateral heterodimers in anti-myc immunoprecipitates obtained from PcM (or P1Ec1M) expressing cells. This observation suggests that the level of the lateral E-/P-cadherin heterodimers on the surface of A-431 cells may be similar or even higher than that of the lateral E-cadherin homodimers.

Incorporation of the chimeric P1Ec1F cadherin into adhesive structure is independent to its adhesive dimerization

While experiments reported above suggest that the E-cadherin and P-cadherin adhesive homodimers may represent critical structural elements of cell-cell adhesion, they can not exclude that adherens junctions also recruit either cadherin monomers or lateral cadherin dimers uninvolved in adhesion interactions. This is also suggested by the observation of Katz et al. (1998) who showed that the junctional plaque recruited cadherins lacking their extracellular region. Such a specific delivery of the cadherins unengaged in adhesion dimerization into junctional structures may explain strong discrepancy between our biochemical data showing relatively low amount of the adhesive cadherin dimers and immunomorphological data demonstrating that nearly all epithelial cadherins are located in cell-cell contacts. Thus, we sought to understand whether full-size cadherins were able to be targeted into cell-cell contact structures in which they could not form adhesive dimers detected by our co-immunoprecipitation assay.

To address this question, we have studied incorporation of the P1Ec1F chimeric protein expressing in A-431 cells (A-P1F cells) into the cell-cell contact structures established between these cells and HT-1080 cells expressing Ec1M and lacking endogenous P- or E-cadherins (HT-EcM cells). Double immunofluorescence microscopy of the co-cultures containing these two cell types using monoclonal anti-flag and polyclonal anti-myc antibodies showed that P1Ec1F protein provided by A-431 cells co-localized precisely with Ec1M belonging to HT-1080 cells along the contacts between these cells (Fig. 6A,A'). Well organized junctional structures between these two types of cells were revealed also by staining of the same co-cultures with a monoclonal anti-E-cadherin antibody exclusively recognizing in this system endogenous E-cadherin of the A-P1F cells and with a polyclonal anti-myc antibody (data not shown). These experiments demonstrated that A-P1F and HT-EcM cells were able to form E-cadherin-containing junctions and these junctions also recruited P1Ec1F protein. Incorporation of P1Ec1F protein into these junctional structures occurred despite the fact that this protein could not be co-immunoprecipitated using anti-myc antibody from such co-cultures (Fig. 5C). In control experiments (Fig. 6B,B'), the HT-EcM cells were co-cultured with A-431 cells stably producing Ec1F (A-EcF). In this co-culture, myc- and flag-tagged cadherins formed mutual adhesive dimers (Fig. 5C). Interestingly, the efficiency of incorporation of Ec1F into the

junctional structures arising between these two types of cells was indistinguishable from that of P1Ec1F protein in the A-PIF/HT-EcM co-cultures.

DISCUSSION

The results reported in this study demonstrate that lateral heteroassociation between E-cadherin and another classic cadherin, P-cadherin results in formation of at least two types of heterocadherin complexes. In the first type, two cadherins interact together in a Trp¹⁵⁶-dependent manner. It is very likely that the structure of the resulting complex corresponds to the model of a N-cadherin lateral homodimer predicted by Shapiro et al (1995). Lateral Trp¹⁵⁶-dependent homodimerization of E-cadherin was described in our recent work (Chitaev and Troyanovsky, 1998). Furthermore, in additional experiments with HT-EcM cells (data not shown), we have revealed lateral heterodimerization between Ec1M and another classic cadherin, N-cadherin. Similarly, these interactions were completely abolished by point mutation Trp156Ala in E-cadherin. Taken together, these observations suggest that the lateral heterodimerization between different members of classic cadherins upon co-expression could be a common phenomenon involved in adhesion and/or cell signaling. In contrast, we were not able to detect Trp¹⁵⁶-dependent heterodimers between E-cadherin and any of desmosomal cadherins expressing in A-431 cells. Importantly, our data suggest that the amount of the E-/P-cadherin Trp¹⁵⁶-dependent heterodimers on the surface of A-431 cells may even exceed the amount of the E-cadherin lateral homodimers of similar structure.

A second type of E-cadherin/P-cadherin heterocomplexes is the Trp¹⁵⁶-independent lateral complex. It is formed immediately after removal of calcium ions from the cell culture medium. Recently, we have shown the formation of similar 'calcium-sensitive' heterocomplexes between E-cadherin and desmosomal cadherins (Troyanovsky et al., 1999). Although the existence of such heterocadherin interactions in standard calcium concentrations was not demonstrated, in theory, they may be involved in the formation of the tightly packed clusters of the adhesive cadherin dimers in the adherens junction.

Our finding that E-cadherin forms stable lateral complexes with P-cadherin does not contradict the concept that classic cadherins mediate homophilic cell-cell adhesion. In fact, the properties of the intercadherin interactions detected in the adhesive dimers completely correspond to those shown to mediate cell-cell adhesion. The interactions are dependent on catenins and calcium ions (Chitaev and Troyanovsky, 1998). They have the same kinetics of formation after elevation of calcium concentration as do cell-cell adhesion interactions (Troyanovsky et al., 1999). The data presented here show that they are cadherin type-specific and this specificity is a function of EC1 domain. Taken together, these data confirm that the adhesive interaction detected in our co-immunoprecipitation assay actually mediates cadherin-based adhesion *in vivo*.

Our data are consistent with two alternative ideas: either the Trp¹⁵⁶-dependent lateral heterocomplex is not involved in cell-cell adhesion, or it dissociates before (or immediately after) the establishment of the adhesive interaction. The last point of

view is supported by our recent experiments (Chitaev and Troyanovsky, 1998). We have found that the adhesive complex soluble in Triton X-100 cannot be formed by head-to-head association of two lateral complexes since the adhesive E-cadherin complex is likely to contain two, but not four, E-cadherin molecules. Taken together with our mutagenesis data showing that the ablation of the Trp¹⁵⁶ of E-cadherin abolished both lateral and adhesive complexes, and with some published evidence (Shapiro et al., 1995; Briehner et al., 1996; Tomschy et al., 1996) suggesting an involvement of the lateral dimers in cell-cell adhesion, we have proposed that the lateral complex is unstable. It dissociates giving rise to two monomeric (in respect of cadherin) complexes, containing E-cadherin able to participate in adhesive association. If so, the function of the hetero- and homo-cadherin complexes could be the same; they are temporary formed on the cell surface as a source of the cadherins able to participate in cell-cell adhesion.

Another important observation made in our study is that the A-431 cells recombinantly expressing Ec1F or P1Ec1F establish junctional structures with HT1080 cells expressing Ec1M. Previous work (Sacco et al., 1995) and our unpublished data showed that the HT-1080 cells express N-cadherin, but did not have P-cadherin, whereas A-431 cells exhibited E- and P-cadherins. Thus, cells expressing different sets of classic cadherins at least in some cases can form heterojunctions. Furthermore, P1Ec1F protein which was not able to form adhesive dimers neither with Ec1M nor with N-cadherin (data not shown), was recruited into the intercellular junctions formed with HT-1080 cells expressing Ec1M. These data demonstrate that the classic cadherin unengaged into adhesive dimerization can be efficiently clustered into junctional structures. This suggests that only a pool of cadherins within adherens junctions is actually involved in adhesive dimerization. This conclusion is also supported by the fact that the amount of the adhesive dimers detected in A-431 cells is surprisingly low relatively to the total amount of E-cadherin (Chitaev and Troyanovsky, 1998). Whether this minor portion is sufficient to establish cell-cell adhesion or there is alternative mechanism for adhesive interaction is not known. The clustering of the unengaged cadherins can be mediated through catenins and cortical cytoskeleton, as was suggested by Katz et al. (1998).

In summary, our data showed that lateral Trp¹⁵⁶-dependent dimerization of classic cadherins is not cadherin type-specific. Adhesive dimerization, in contrast, could efficiently proceed only between cadherins encompassing identical EC1 domains.

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REFERENCES

- Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A. and Fusenig, N. E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell Biol.* **106**, 761-771.
- Briehner, W. M., Yap, A. S. and Gumbiner, B. M. (1996). Lateral dimerization

- is required for the homophilic binding activity of C-cadherin. *J. Cell Biol.* **135**, 487-496.
- Bussemakers, M. J., van Bokhoven, A., Mees, S. G., Kemler, R. and Schalken, J. A.** (1993). Molecular cloning and characterization of the human E-cadherin cDNA. *Mol. Biol. Rep.* **17**, 123-128.
- Chitaev, N. A. and Troyanovsky, S. M.** (1998). Adhesive but not lateral E-cadherin complexes require calcium and catenins for their formation. *J. Cell Biol.* **142**, 837-846.
- Chitaev, N. A., Averbakh, A. Z., Troyanovsky, R. B. and Troyanovsky S. M.** (1998). Molecular organization of the desmoglein-plakoglobin complex. *J. Cell Sci.* **111**, 1941-1949.
- Friedlander, D. R., Mege, R. M., Cunningham, B. A. and Edelman, G. M.** (1989). Cell sorting-out is modulated by both the specificity and amount of different cell adhesion molecules (CAMs) expressed on cell surfaces. *Proc. Nat. Acad. Sci. USA* **86**, 7043-7047.
- Geiger, B. and Ayalon, O.** (1992). Cadherins. *Annu. Rev. Cell Biol.* **8**, 307-332.
- Gumbiner, G. M.** (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345-357.
- Johnson, K. R., Lewis, J. E., Li, D., Wahl, J., Soler, A. P., Knudsen, K. A. and Wheelock, M. J.** (1993). P- and E-cadherin are in separate complexes in cells expressing both cadherins. *Exp. Cell Res.* **207**, 252-260.
- Katz, B.-Z., Levenberg, S., Yamada, K. M. and Geiger, B.** (1998). Modulation of cell-cell adherens junctions by surface clustering of the N-cadherin cytoplasmic tail. *Exp. Cell Res.* **243**, 415-424.
- Klymkowsky, M. and Parr, B.** (1995). The body language of cells: the intimate connection between cell adhesion and behavior. *Cell* **83**, 5-8.
- Nagar, B., Overduin, M., Ikura, M. and Rini, J. M.** (1996). Structural basis of calcium-induced E-cadherin rigidification and dimerization. *Nature* **380**, 360-364.
- Nose, A., Nagafuchi, A. and Takeichi, M.** (1988). Expressed recombinant cadherins mediate cell sorting in model systems. *Cell* **54**, 993-1001.
- Nose, A., Tsuji, K. and Takeichi, M.** (1990). Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell* **61**, 147-155.
- Peifer, M.** (1995). Cell adhesion and signal transduction: The Armadillo connection. *Trends Cell Biol.* **5**, 224-229.
- Pokutta, S., Herrenknecht, K., Kemler, R. and Engel, J.** (1994). Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding. *Eur. J. Biochem.* **223**, 1019-1026.
- Sacco, P. A., McGranahan, T. M., Wheelock, M. J. and Johnson, K. R.** (1995). Identification of plakoglobin domains required for association with N-cadherin and α -catenin. *J. Biol. Chem.* **270**, 20201-20206.
- Shapiro, L., Fannon, A. M., Kwong, P. D., Thompson, A., Lehmann, M. S., Grubel, G., Legrand, J.-F., Als-Neilsen, J., Colman, D. R. and Hendrickson, W. A.** (1995). Structural basis of cell-cell adhesion by cadherins. *Nature* **374**, 327-337.
- Shimoyama, Y., Yoshida, T., Terada, M., Shimosato, Y., Abe, O. and Hirohashi, S.** (1989). Molecular cloning of a human Ca^{2+} -dependent cell-cell adhesion molecule homologous to mouse placental cadherin: its low expression in human placental tissues. *J. Cell Biol.* **109**, 1787-1794.
- Takeichi, M.** (1995). Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* **7**, 619-627.
- Tomschy, A., Fauser, C., Landwehr, R. and Engel, J.** (1996). Homophilic adhesion of E-cadherin occurs by a co-operative two-step interaction of N-terminal domains. *EMBO J.* **15**, 3507-3514.
- Troyanovsky, S. M., Troyanovsky R. B., Eshkind L. G., Krutovskikh V. A., Leube R. L. and Franke W. W.** (1994). Identification of the plakoglobin-binding domain in desmoglein and its role in plaque assembly and intermediate filament anchorage. *J. Cell Biol.* **127**, 151-160.
- Troyanovsky, S. M.** (1999). Mechanism of cell-cell adhesion complex assembly. *Curr. Opin. Cell Biol.* **11**, 561-566.
- Troyanovsky, R. B., Klingelhofer, J. and Troyanovsky, S. M.** (1999). Removal of calcium ions triggers a novel type of intercadherin interaction. *J. Cell Sci.* **112**, 4379-4387.
- Volk, T., Cohen, O. and Geiger, B.** (1987). Formation of heterotypic adherens-type junctions between L-CAM-containing liver cells and A-CAM-containing lens cells. *Cell* **50**, 987-994.