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Prototypical Type I E-cadherin and Type II Cadherin-7 Mediate Very Distinct Adhesiveness through Their Extracellular Domains*[S]

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Using a dual pipette assay that measures the force required to separate adherent cell doublets, we have quantitatively compared intercellular adhesiveness mediated by Type I (E- or N-cadherin) or Type II (cadherin-7 or -11) cadherins. At similar cadherin expression levels, cells expressing Type I cadherins adhered much more rapidly and strongly than cells expressing Type II cadherins. Using chimeric cadherins, we found that the extracellular domain exerts by far the dominant effect on cell adhesivity, that of E-cadherin conferring high adhesivity, and that of cadherin-7 conferring low adhesivity. Type I cadherins were incorporated to a greater extent into detergent-insoluble cytoskeletal complexes, and their cytoplasmic tails were much more effective in disrupting strong adherent junctions, suggesting that Type II cadherins form less stable complexes with β-catenin. The present study demonstrates compellingly, for the first time, that cadherins are dramatically different in their ability to promote intercellular adhesiveness, a finding that has profound implications for the regulation of tissue morphogenesis.

Adhesive interactions, so essential to multicellular life, are mediated by a diversity of cell surface receptors. Prominent among them are the cadherins, calcium-dependent adhesion molecules central to tissue development and morphogenesis (1–3). The growing superfamily of cadherins is subdivided into five families: classical Type I cadherins, atypical Type II cadherins, desmosomal cadherins, protocadherins, and seven-pass transmembrane cadherins (4, 5). Classical Type I and desmosomal cadherins are found primarily in tissues where a high degree of cell cohesion is required for tissue integrity. Other types of cadherins are expressed in situations where cells are more motile, and intercellular interactions are more transitory (6–9). It is increasingly clear that cadherins contribute to other cellular functions, including cell signaling, proliferation, differentiation, segregation, and migration (10–17).

Predictably, the pattern of cadherin expression during development is complex. For example, development of the neural crest involves epithelial to mesenchymal transitions, cell migration, cell aggregation, and cell differentiation (18), each of which is associated with tightly regulated, differential expression of Type I and II cadherins. Premigratory cells of the avian neural crest express first N-cadherin, and then they down-regulate N-cadherin and express Type II cadherin-6B, but later down-regulate it and induce expression of the Type II cadherin-7 as they migrate throughout the embryo (19, 20). Another Type II cadherin, cadherin-11, is similarly induced in migrating neural crest cells of rat and *Xenopus* embryos (21–23). Cell grafting experiments *in vivo* verify that expression of cadherin-7 correlates with cell dispersion and migration along migratory pathways, whereas that of N-cadherin fosters strong intercellular cohesivity and failure to migrate (6).

Regulation of cellular adhesion can be achieved in a variety of ways (for review see Ref. 24). The simplest, altering the number of cadherins on the cell surface, has been clearly shown to dictate the pattern of cell sorting *in vitro* (25–27). Changes in the cadherins expressed on cells during morphogenesis and under pathological conditions are also well documented (1–3). Such differential expression would be even more effective if each cadherin promoted intercellular adhesion that is qualitatively or quantitatively unique.

To test this possibility, we developed a dual pipette assay that quantifies precisely and reproducibly the force required to separate pairs of adherent cells (28, 29). In earlier work we focused on E-cadherin that mediates rapid initial adhesion subsequently strengthened by cytoskeletal remodeling and nectin modulation (28, 30). Here we compare four different cadherins. The results indicate that Type I cadherins (E and N) promote much stronger adhesiveness than Type II cadherins (7 and 11), the difference being attributable primarily to the extracellular domain of the molecules.

MATERIALS AND METHODS

Antibodies and Reagents—NCD-2 and CCD7–1 mAbs[6] were a generous gift from M. Takeichi and S. Nakagawa (Kyoto University, Japan). The polyclonal antibody recognizing an epitope common to N- and E-cadherin and the ECCD-2 mAb were from Takara Biomedicals. mAb against cadherin-11 (clone 5B2H5) was obtained from Zymed Laboratories. ACAM and DECMA-1 mAb directed against N- and E-cadherin, respectively, were from Sigma. 7D6 mAb directed against chicken E-cadherin was from Developmental Studies Hybridoma Bank. mAbs

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[6] The abbreviations used are: mAb, monoclonal antibody; SF, separation force(s); GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; MDCK, Madin-Darby canine kidney.

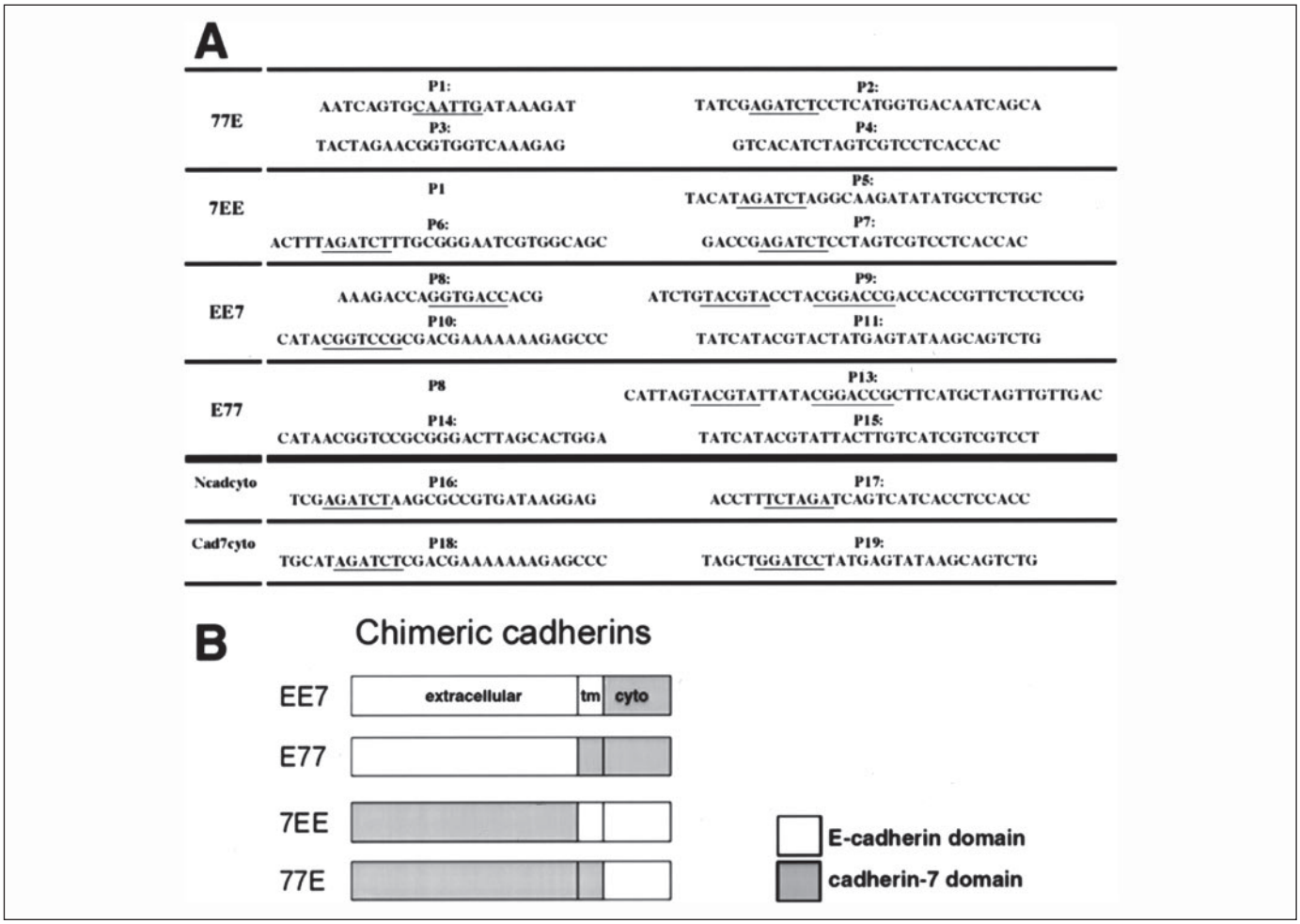

FIGURE 1. **Wild type and chimeric cadherins used in this study.** *A*, primers used for the construction of sequences encoding the chimeric cadherins and the GFP-tagged cytoplasmic domain of cadherins. The positions of restriction sites are *underlined*. *B*, schematic representation of the wild type cadherins and chimeras carrying the extracellular, transmembrane, or cytoplasmic domain exchanged between E-cadherin and cadherin-7.

against α-catenin, β-catenin, or phosphorylated tyrosine (clone PY20) were from Transduction Laboratories. mAb against α-tubulin and secondary antibodies coupled to horseradish peroxidase were from Amersham Biosciences. Secondary antibodies coupled to Texas Red, Cy3, and fluorescein isothiocyanate were from Jackson ImmunoResearch Laboratories.

Cell Lines and Stable Transfections—The Ecad and Ncad are S180 transfected clones expressing chicken E-cadherin and N-cadherin, respectively, and were previously described (6, 26). The E14, E38, E41, and E58 clones, named according to their level of E-cadherin expression, were previously described by Chu *et al.* (28). Clones with different levels of cadherin-7 or cadherin-11 were obtained by stable transfection of S180 cells with pMiwccad7 or pMiwmcad11, respectively (20, 31), together with pAG60, as previously described (28). Transient transfections of MDCK cells with constructs containing the cytoplasmic tails of cadherins were carried out using Lipofectamine (Invitrogen).

Construction of Chimeric Cadherins and Cytoplasmic Tails of Cadherins Fused to GFP—pMiwNcad, pMiwcad7, and pBSSKcad7 were a generous gift from M. Takeichi. The pCE2 and pCE-Ecad plasmids were described by Boyer *et al.* (32). We produced plasmids coding for chimeric EE7, E77, 7EE, and 77E cadherins (Fig. 1*B*), in which the extracellular, transmembrane, and cytoplasmic domains of E-cadherin and cadherin-7 were exchanged, as described in supplemental Fig. S2.

Constructs coding for GFP fused in frame with the cytoplasmic tails of N-cadherin and cadherin-7 (GFP-Ncadcyto and GFP-cad7cyto, respectively) were produced as indicated below. The list of primers used is shown in Fig. 1*A*. The cytoplasmic domain of N-cadherin was amplified by PCR from pMiwcNcad using P16 and P17 primers. Amplification of the cadherin-7 cytoplasmic domain was performed from pMiwcad7 using P18 and P19 primers. The corresponding inserts were digested with either BglII and XbaI or BglII and BamHI and subcloned into pEGFPC1 (Clontech). The integrity of each construct was verified by analysis of the DNA sequence.

Cell Dissociation and Aggregation Assays—The cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum, and confluent cultures were routinely passaged by treatment with 0.05% trypsin + 0.02% EDTA in phosphate-buffered saline. For separation force (SF) measurements and cell aggregation assay, cell dissociation was performed in TC buffer (0.01% trypsin + 10 mM calcium) (20). Prior to SF measurements, the cells were resuspended in working medium (CO_2-independent medium; Invitrogen) supplemented with 1% fetal calf serum and used immediately. Pre-existing doublets (doublets never disrupted by the dissociation procedure used to prepare cell suspensions) were allowed to sit for one additional hour in the assay dish before use to be certain that they were not mitotic pairs. Cadherin-dependent aggregation assays were performed as described elsewhere (6).

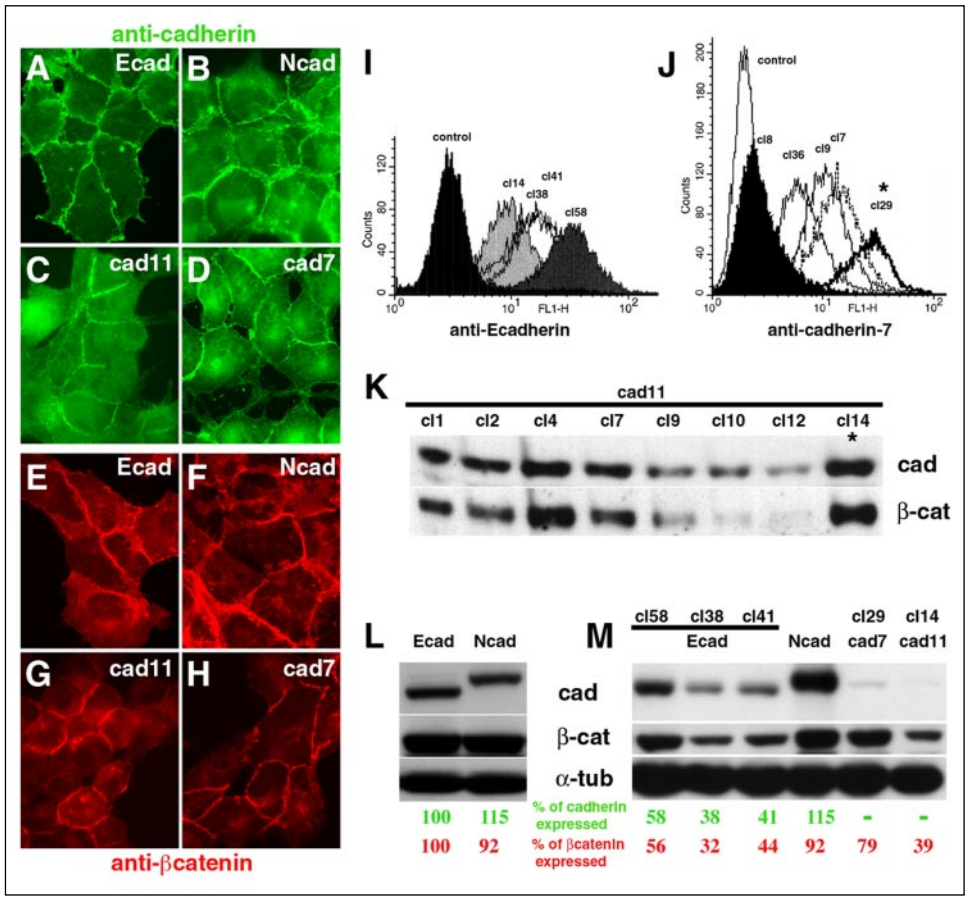

FIGURE 2. **Characterization of the different cadherin-expressing clones.** *A–H*, immunolocalization of cadherin (*A–D*) and β-catenin (*E* and *F*) in Ecad (*A* and *E*), Ncad (*B* and *F*), cad11 (*C* and *G*), and cad7 (*D* and *H*) cells. Cadherins and β-catenin are localized at cell-cell contacts. *I* and *J*, FACS of cadherin expressed at cell surface for various E-cadherin-expressing (*I*) or cadherin-7-expressing (*J*) clones using DECMA-1 and CCD7–1 antibodies, respectively. Clone cad7-cl29 (labeled with *asterisk*) expressing the higher levels of cadherin-7 was selected for further analysis. *K–M*, Western blot analysis of β-catenin, α-tubulin, and cadherin levels in cell extracts of Ecad and Ncad cells (*L*) and various clones expressing cadherin-11 (*K*), E-cadherin, cadherin-11, or cadherin-7 (*M*). In *K*, the membranes were incubated with anti-cadherin-11 and anti-β-catenin antibodies. Clone cad11-cl14 (labeled with *asterisk*) expressing the higher levels of cadherin was selected for further analysis. In *L* and *M*, the membranes were incubated with an antibody directed against a common cytoplasmic epitope of E- and N-cadherin or an antibody against β-catenin together with an antibody against α-tubulin. Ncad and Ecad cells expressed similar levels of cadherins.

Preparation of Cell Extracts and Immunoblotting—Extraction of cell monolayers and Western blot analysis were performed as described previously (6) with a mixture of antibodies directed against β-catenin and α-tubulin or cadherin, followed by ECL detection (Amersham Biosciences). Quantitative analysis was done by the ImageQuant program on a representative Western blot from three independent experiments. The α-tubulin content was used to normalize for the protein level of each lane. The levels of β-catenin and E-cadherin of Ecad cells were taken as the 100% standard for comparison with all other transfected clones. For Western blot analysis of tyrosine-phosphorylated proteins, the cell extracts were prepared in the presence of 100 μM sodium orthovanadate.

Analysis of cadherin distribution between the detergent-soluble and -insoluble fractions was carried out as described by Bauer *et al.* (33). Briefly, the cell monolayers were extracted using CSK buffer to obtain the Triton X-100-soluble and -insoluble fractions. Identical volumes of the detergent-soluble and -insoluble fractions were subjected to Western blot analysis as described above with cadherin-specific antibodies.

Metabolic labeling of cells was performed by an overnight incubation of subconfluent monolayers with 250 μCi of [^{35}S]methionine (*in vivo* labeling grade; Amersham Biosciences). Co-immunoprecipitation of catenins with cadherins were performed as previously described (34). Quantitative analysis was done using the ImageQuant program on a representative autoradiograph displaying the results from three independent experiments. The methionine content was taken into account to normalize the band intensity calculated for each cadherin.

Immunofluorescence Microscopy—Immunodetection of cadherins in cultured cells was performed as previously described (6). The cells were viewed by epifluorescence using a Leica DMRBE microscope and a cooled CCD camera (Hamamatsu C5985). Image acquisitions were controlled by a Power Macintosh work station operating IP-Lab software. Cadherin expression was analyzed by flow cytometry, as previously described (28).

Measurement of Separation Forces between Cells—The micromanipulation technique was previously described in detail by Chu *et al.* (28, 29). Briefly, SF were measured on the heating stage of a Leica inverted epifluorescence microscope equipped with a cooled CCD Hamamatsu C5985 or Nikon Coolpix 5000 camera. Image acquisition was controlled by a Power Macintosh work station operating IP-Lab software. Two cells were gently aspirated, one on the tip of each pipette, and were brought into contact for a designated period of time through the use of micromanipulators. At the end of the designated time, the pipettes were moved apart in an effort to detach the adherent cells from one another. A doublet pulled intact from the left pipette was moved back to the orifice of that pipette, where the aspiration was then increased. The cycle was repeated with discrete increments in aspiration, the magnitudes of which were measured with a pressure sensor (Validyne; model DP103–38; 0–50000 Pa), until the level reached in the left pipette was sufficient to pull the doublet apart. Aspiration values recorded for each of the last two cycles in the series (P_{n-1} and P_n) were used to calculate the SF for each doublet using the equation: $SF = \pi(d/2)^2 (P_{n-1} + P_n)/2$ where d is the internal diameter of left pipette. The results for 30–50 measurements were used to obtain the mean SF for a specific contact time in at least three independent experiments. The mean SF is referred to as the SF values indicated in the figures presented.

RESULTS

Cadherin and β-Catenin Expression in Transfected Cells—Parental S180 cells do not express detectable levels of cadherins or β-catenin and

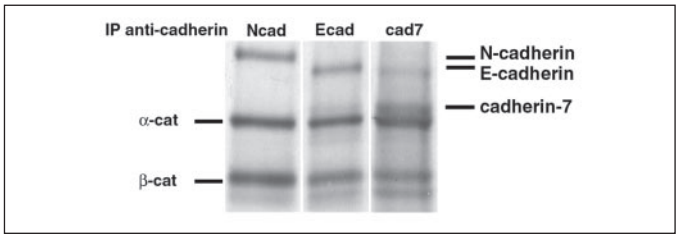

FIGURE 3. **Co-immunoprecipitation of α-catenin and β-catenin together with different cadherins.** Autoradiogram showing the cadherin-catenin immunocomplexes prepared from metabolically labeled Ncad, Ecad, and cad7 cell extracts. *IP*, immunoprecipitation.

exhibit minimal cell-cell adhesion in tissue culture (6, 26). S180 cells stably transfected to express E-cadherin (Ecad cells), N-cadherin (Ncad cells), cadherin-11 (cad11 cells), or cadherin-7 (cad7 cells) formed characteristic intercellular adhesions in tissue culture with the respective cadherin and β-catenin (Fig. 2, *A–H*) localized at cell-cell contacts. Several transfected clones expressing varying levels of E-cadherin (Fig. 2, *I* and *M*), cadherin-7 (Fig. 2, *J* and *M*), or cadherin-11 (Fig. 2, *K* and *M*) were compared for their cadherin and β-catenin contents. The highest expressor clones for cadherin-11 and cadherin-7 (cad11-cl14 and cad7-cl29, respectively) were selected for further analysis and referred to simply as cad11 and cad7 cells.

The cell lines were analyzed by Western blot analysis using an antibody directed against a common epitope in cadherin cytoplasmic domain. Ncad cells and three E-cadherin clones, E58, E41, and E38 (28), expressed 115, 58, 41, and 38%, respectively, of the 100% Ecad reference (Fig. 2, *L* and *M*). Type II cadherins could not be quantified with this because of a lack of the common epitope. However, the level of β-catenin expression was proportional to that of cadherin in all the clones (Fig. 2, *K–M*, and data not shown). Moreover, E-cadherin, N-cadherin, and cadherin-7 co-precipitated at a comparable level with β- and α-catenin (Fig. 3). β-Catenin expression was therefore used to estimate the cadherin content in Type I and Type II cadherin-expressing clones.

E- and N-cadherin and cadherin-7 are not removed from the cell surface by TC treatment (see "Materials and Methods") as shown by FACS (Figs. 2, *I* and *J*, and 4*A*). The lack of an appropriate antibody prevented comparable visualization of cadherin-11 at the surface of TC-treated cad11 cells. However, cad11 cells, like Ecad, Ncad, and cad7 cells, formed aggregates in short term gyratory aggregation assays (supplementary Fig. S1), implying that cadherin-11, like the other cadherins, is present and functional after TC treatment.

FACS analysis showed that Ecad, Ncad, and cad7 cells expressed high levels of cadherin at their surfaces (Fig. 4*A*). The mean intensity values for the FACS profile peaks were 71.19, 72.21, and 51.39 for Ecad, Ncad and cad7 cells, respectively. Thus, Ecad and Ncad cells expressed similar levels of cadherin at their surface, and cad7 cells expressed 72% of the Ecad level (taken as the 100% reference level). These results correlate with the percentage of cadherin evaluated from the Western blot analyses (Fig. 2, *L–M*) of cell extracts. They were also in agreement with the fact that cadherins are mostly located at the surfaces of cell monolayers, as shown in Fig. 2 (*A–H*).

Kinetics of the SF Measured for Different Cadherin-expressing Cells—Using our dual pipette assay, we first determined the time course of SF for cells expressing the four different cadherins. We had previously observed that Ecad cells adhere rapidly and then strengthen their adhesion with time of contact (Ref. 28 and Fig. 4*B*). Ncad cells (Fig. 4*C*) showed nearly identical kinetics but with lower overall adhesivity. After 4 and 30 min of contact, we measured a mean SF of 8 and 73 nanonewtons (nN), respectively, for Ncad doublets compared with 52 and 180 nN, respectively, for Ecad doublets.

Adhesions between cells expressing Type II cadherins formed much more slowly and were very weak. Cad7 adhesion displayed kinetics seemingly similar to those of Type I cadherins, but on axes with very different scales (Fig. 4*D*). A SF of 17 nN developed after 60 min of contact was comparable with values for Ecad cells (20 nN) and Ncad cells (27 nN) developed after only 30 s and 8 min of contact, respectively. The results for Cad11 cells were even more striking; they displayed no measurable SF for contact times of less than 30 min (not shown). The cad11 doublets were so susceptible to vibrations that SF measurements were impractical at early times. The SF of 3.5 and 7.4 nN measured at 30 and 60 min, respectively, were far below those obtained even for cad7 cells.

Measurement of SF in Long Term Adherent Doublets—The maximal SF was determined using doublets never disrupted by the TC dissociation procedure (pre-existing doublets). In every case, the SF for pre-existing doublets was significantly higher than that obtained for doublets formed after 60 min of contact (Fig. 4*E*). For cells expressing Type I cadherins, the SF for pre-existing doublets were much more similar (SF Ncad/SF Ecad = 0.86) than those of 60-min doublets (SF Ncad/SF Ecad = 0.40). SF for pre-existing doublets expressing Type II cadherins (cadherin-7 or cadherin-11) were much weaker than those of Type I cadherins (about 50 nN) and were indistinguishable from those of pre-existing doublets of parental S180 cells lacking cadherins.

Fixed Time Point Comparison of SF for Cells Expressing Different Cadherins—A fixed time point comparison of cells expressing different cadherins yielded corroborating results (Table 1). The SF at 30 min for Ecad doublets (194 nN) was much greater than that obtained for Ncad doublets (74 nN), even though cadherin expression in the latter was ~115% that in the former. Similar results were obtained with a second, comparable N-cadherin expressing clone (not shown).

The difference between Type I and Type II cadherins was even more dramatic. Although cad7 and cad11 cells expressed cadherin at 79 and 39% of the Ecad reference level, they exhibited SF of only 8.8 and 3.5 nN, respectively. These values were either comparable with or less than those measured for the E14 clone expressing the lowest level (14%; 8.5 nN) of Type I E-cadherin.

Incorporation of Cadherins into Junctional Complexes—We next investigated the subcellular distribution of cadherins by analyzing detergent-soluble and -insoluble fractions extracted from confluent monolayers of Ecad, Ncad, and cad7 cells. All three cadherins were found in both fractions (Fig. 5*A*), but their distribution between the two fractions varied considerably with the type of cadherin: 34 and 33% of the E- and N-cadherin, respectively, but only about 6% of cadherin 7, was found in the detergent-insoluble fraction of the corresponding cell extracts. The results for cadherin-11 were similar to those for cadherin-7 (not shown). Post-extraction immunolabeling of cells with anti-β-catenin revealed that cell-cell junctions of Ncad cells were much more resistant to detergent treatment than those of cad7 cells. The majority of junctional staining remained in Ncad cells after detergent treatment (Fig. 5, *B* and *D*), whereas most staining was lost in cad7 cells (Fig. 5, *C* and *E*). These results suggest that Type II cadherin-7 is incorporated much less readily than Type I E- or N-cadherin into the detergent-insoluble cytoskeleton complex.

E-cadherin, N-cadherin and cadherin-7 co-precipitated with β- and α-catenin (Fig. 3). Tyrosine phosphorylation of β-catenin is known to modulate cadherin function (35, 36), but Ecad, cad7, and Ncad cells displayed similar levels of tyrosine-phosphorylated β-catenin (Fig. 5*F*), suggesting that the reduced adhesiveness of cad7 cells does not involve changes in the tyrosine phosphorylation of β-catenin.

Transient expression of Type I cadherin cytoplasmic tails can disrupt adherent junctions in highly cohesive cells and interfere with β-catenin localization (37). We have therefore compared the ability of the cytoplasmic domain of the different cadherins to affect intercellular adhesion. Constructs containing GFP fused to the cytoplasmic tails of each

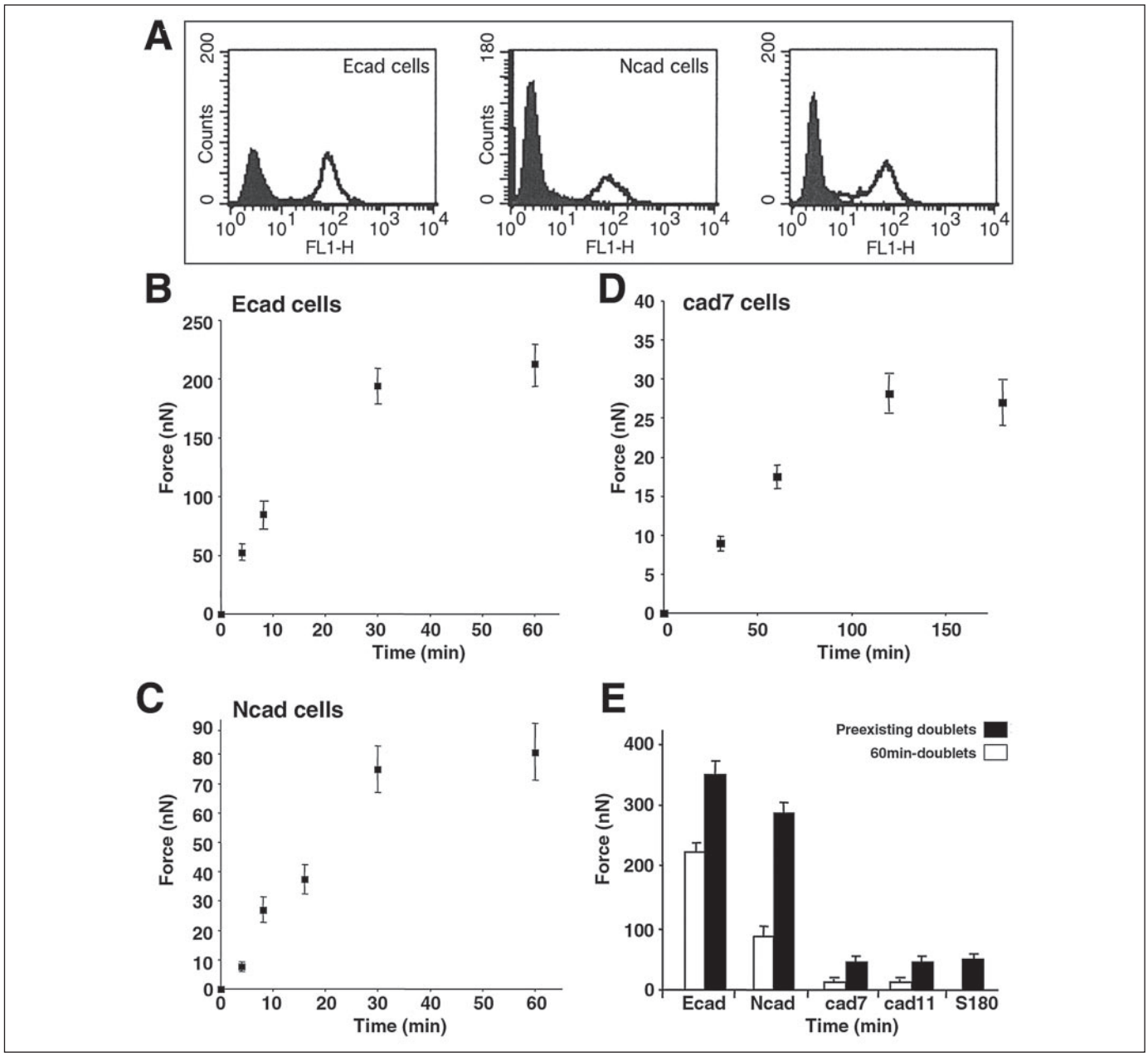

FIGURE 4. **Expression of cadherins at the surface and kinetics of SF for cells expressing Type I and Type II cadherins.** *A*, FACS of cadherin expressed at surface for Ecad (*left panel*), Ncad (*middle panel*), and cad7 (*right panel*) cells using 7D6, ACAM, and CCD7–1 mAbs, respectively. *B–D*, the mean force required for separating formed doublets of Ecad (*B*), Ncad (*C*), and cad7 (*D*) cells at different times of contact. The cells were held in contact for 4–60 min (Ecad and Ncad cells) or for 30–180 min (cad7 cells) before separation. The *x* axis represents the time of contact. The *y* axis represents the mean SF (in nN) necessary to separate adherent cells. *E*, the mean SF necessary to separate pre-existing doublets (*black bars*) and 60-min doublets (*white bars*).

TABLE 1
Comparison between the mean SF measured for 30-min doublets expressing different cadherins

Cells	Estimated cadherin content	SF at 30 min
	%	*nN*
Ncad	115	74.9 ± 7.8
Cad7	79	8.8 ± 0.9
Cad11	39	3.5 ± 0.5
Ecad	100	194.1 ± 14.9
E58	58	53.4 ± 1.3
E38	38	19.9 ± 2.4
E14	14	8.5 ± 1.4

cadherin were transiently expressed in MDCK cells, and their effect on the organization of adherens junctions was determined (Fig. 5, *G* and *J*). Expression of a Type I cadherin tail (E- or N-) resulted in an increased cytoplasmic pool of β-catenin and a significant decrease in the localization of β-catenin at cell-cell contacts (Fig. 5, *G* and *H*, *arrows*, and Ref. 37). Of the 110 cells examined, more than 90% displayed accumulation of β-catenin in the cytoplasm, and at least 55% showed much reduced staining for β-catenin at cell-cell junctions (Fig. 5*J*). By contrast, expression of the Type II cadherin-7 cytoplasmic tail affected neither junctional organization nor β-catenin distribution. β-Catenin was localized at cell-cell contacts in more than 70% of the cells (Fig. 5*I*), and only about 50% of them displayed accumulation of β-catenin in the cytoplasm (Fig. 5*J*).

SF Measurements on Cell Doublets Expressing Chimeric Cadherins—We constructed expression vectors coding for chimeric cadherins where the extracellular, transmembrane or cytoplasmic domain were

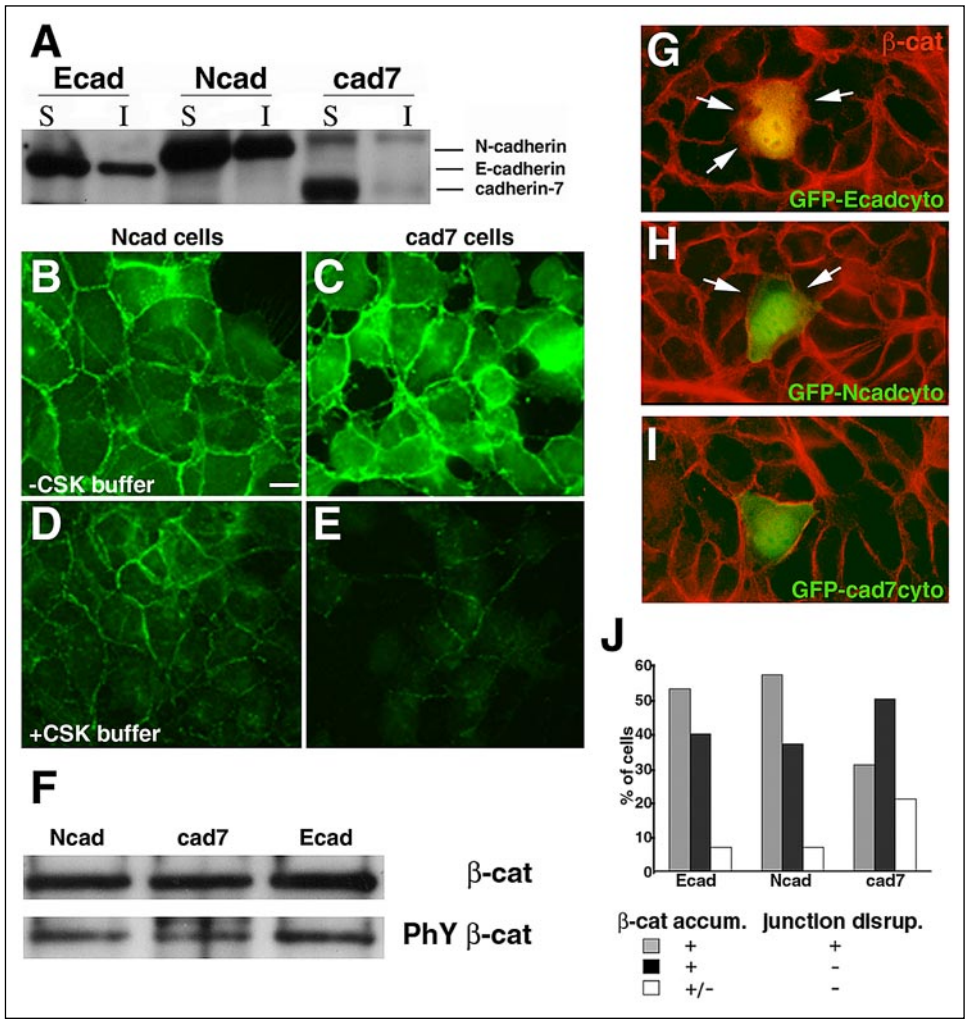

FIGURE 5. **Fractionation of cadherins between Triton X-100-soluble and -insoluble fractions, level of tyrosine-phosphorylated β-catenin, and disruption of MDCK cell junctions by overexpression of cytoplasmic tails of cadherins.** *A*, distribution of cadherins into the Triton X-100-soluble and -insoluble fractions of Ecad, Ncad, and cad7 cell extracts. *B–E*, the effect of detergent treatment on adherens junctions. Monolayers of Ncad cells (*B* and *D*) or cad7 cells (*C* and *E*) were either untreated (*B* and *C*) or treated (*D* and *E*) with CSK buffer for 10 min on ice, fixed, and then labeled with an antibody directed against β-catenin (in *green*). The *bar* represents 20 μm. *F*, Western blot analysis of total and tyrosine-phosphorylated β-catenin (*PhY*) levels in Ecad, Ncad, and cad7 cell extracts. *G–J*, effect of the cytoplasmic domains of different cadherins on the organization and level of β-catenin in MDCK cells. GFP-tagged cadherin cytoplasmic tails of E-cadherin (*G*), N-cadherin (*H*), and cadherin-7 (*I*) were transfected into MDCK cells and the cell cultures immunostained for β-catenin (in *red*). The *arrows* in *G* and *H* point to a decrease in β-catenin staining at cell-cell junctions in cells transfected with N-cad and E-cad tails but not in cells transfected with the cad7 tail (*I*). *J*, quantitative analysis of the effect of cadherin tail expression on the organization of junctional complexes in MDCK cells and the staining intensity of β-catenin. The patterns of β-catenin and GFP co-staining were used to distinguish three categories of cells: those showing a clear increase in the intensity of β-catenin staining in the cytoplasm, either with (*gray bars*), or without (*black bars*) a concomitant decrease in junctional staining, and those showing a weak increase in β-catenin staining and no effect on junctional staining (*white bars*).

exchanged between E-cadherin (prototypical Type I cadherin) and cadherin-7 (prototypical Type II cadherin). These two cadherins were chosen because they fostered the greatest measurable difference in SF in our assay. Stably transfected S180 cells expressing EE7, E77, 77E, or 7EE chimeric cadherins (Figs. 6 and 7) were selected and compared with clones producing either wild type E-cadherin (EEE) at different levels or cadherin-7 (777) (Table 1).

Western blot and FACS (Fig. 6, *B* and *D*, respectively) analysis revealed that E77-cl10 and EE7-clK1 expressed cadherin at levels slightly higher than EEE-E58 cells. 7EE-cl4 and 77E-cl1 cells expressed cadherin levels similar to EEE-E38 cells (Fig. 7, *B*, *asterisks*, and *D*). All chimeric cadherins localized at cell-cell contacts, indistinguishable from the wild type cadherin (Figs. 6*C* and 7*C*).

SF measurements were performed on doublets of cells expressing each of the chimeric cadherins (Figs. 6, *E* and *F*, and 7*E*). 30-min doublets of EEE-E58, EE7-clK1, and E77-cl10 cells (SF of 52.5, 65, and 44 nN, respectively) all exhibited SF significantly higher than that of cad7 cells (777-cl29; Fig. 6*E*), although cad7 cells expressed higher levels of cadherin than of those clones (Table 1). The kinetics of SF measured for EE7 and E77 clones exhibited strong initial SF that increased in intensity with time (Fig. 6*F*), similar to those displayed by E58 cells expressing wild type E-cadherin. By contrast, the SF for 7EE-cl4 and 77E-cl1 doublets, at 30 min, were under the lowest measurement limit of our technique (<1 nN). After 90 min of contact, 7EE-cl4 and 77E-cl1 doublets displayed the very weak SF of 3.1 and 2.0 nN, respectively, as compared with the 40.2 nN obtained for 90 min E38 doublets (Fig. 7*E*). Interestingly, E77-cl10 and 77E-cl1 expressing the cadherin-7 transmembrane domain displayed slightly lower SF (44 and 2.0 nN, respectively) than the corresponding clones EE7clk1 and 7EE-cl4 (65 and 3.1 nN, respectively) expressing the E-cadherin transmembrane domain.

DISCUSSION

Cells Expressing Different Cadherins Differ in Their Adhesiveness—We previously studied the strength, kinetics, and regulation of E-cadherin-mediated intercellular adhesion (28, 30). Here, we compare the adhesivity conferred upon cells by four different cadherins: E- and N-cadherin, (prototypical Type I cadherins) and cadherin-7 and -11 (prototypical Type II cadherins). All four cadherins supported intercellular adhesion in gyratory aggregation assays (supplementary Fig. S1). In the dual pipette assay Ecad, Ncad, and cad7 cells showed a similar kinetic curve: rapid initial adhesion, an early phase showing increased in force with time of contact, and a later plateau phase (Fig. 4). Despite the comparable levels of cadherin expression and the similar shape of the curve generated by both Type I cadherins, the values for Ecad cells were significantly higher than those for Ncad cells. The differences between Type I and Type II cadherins were even greater. For cad7 cells, the early linear phase took much longer than for cells expressing Type I cadherins, and the maximum force level reached was much lower. Only the lowest expressor of E-cadherin (E14) yielded an SF in the range of those displayed by cad7 and cad11 cells.

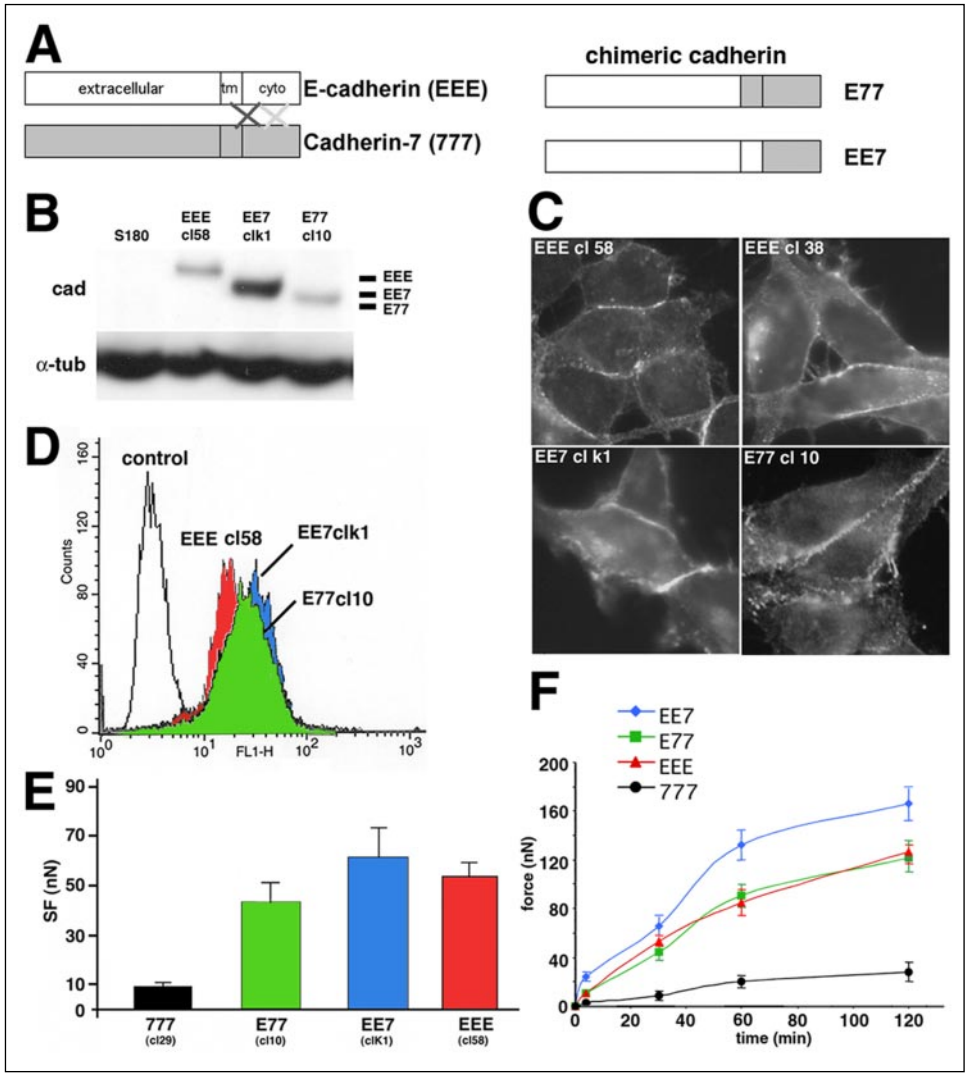

FIGURE 6. **Characterization of clones expressing EE7 and E77 chimeric cadherins and determination of their SF.** *A*, schematic representation of the wild type and chimeric cadherins where the cytoplasmic and transmembrane domains were exchanged between cadherin-7 and E-cadherin. *B*, Western blot analysis of cadherin and α-tubulin contents in cell extracts of S180, E58 cells, and clones expressing the highest levels of EE7 (clone K1) and E77 chimeras (clone 10). *C*, immunolocalization of the wild type (E58 and E38 cells) and chimeric cadherins (clK1 and cl10) at intercellular contacts. *D*, FACS of cadherins presented at the cell surface of clones expressing wild type E-cadherin (EEE-cl58, *red peak*), chimeric cadherins EE7-clk1 (*blue peak*), and E77-cl10 (*green peak*). *E*, SF measurements on 30 min doublets. *F*, kinetics of SF for clones expressing wild type E- (*red*), cadherin-7 (*black*), EE7 (*blue*), and E77 (*green*) chimeric cadherins.

Taken together, the data indicate that different cadherins are intrinsically different in their ability to confer adhesive properties on S180 cells, and, in particular, Type I cadherins confer much greater cell adhesiveness than Type II cadherins. Additional support for this view was provided by the results obtained with pre-existing doublets where the forces measured for doublets transfected with Type II cadherins were much weaker (approximately one seventh) than those for doublets transfected with Type I cadherins.

It has been previously reported that fibroblastic L cells transfected with different Type II cadherins (cadherin-6 to -12 or cadherin-14) formed aggregates in suspension similar in size to those formed with cells expressing E-cadherin (38). In that study, the authors used a long term aggregation assay (48 h) because the trypsin sensitivity of Type II cadherin-6 and -14 rendered the short term gyratory assays useless. By contrast, the Type II cadherins used in our study (cadherin-7 and -11) are resistant to trypsin proteolysis (6, 20, 39), and suspensions of Ecad, Ncad, cad7, and cad11 cells can all form aggregates in short term gyratory assays (supplementary Fig. S1). Even so, aggregates formed by Ecad or by Ncad cells after 2 h of gyratory culture were characteristically of a larger size and more cohesive than those formed with cad7 or cad11 cells (not shown).

The dual pipette assay and aggregation assay yield mutually supportive results, but they are not equally sensitive. In the aggregation assay, Ecad cells and cad11 cells were, respectively, 95 and 39% aggregated after 1 h, whereas the dual pipette assay yielded SF of 200 and 7.4 nN for 60-min Ecad and cad11 doublets. Similarly, although EEE-cl58, EE7clk1, E77cl10, and cad7 clones expressing approximately the same level of cadherin formed aggregates of similar size after 24 h in suspension,[7] the kinetics of their adhesiveness revealed by the dual pipette assay showed that cad7 cells were much less adhesive than the other clones (Fig. 6*F*). These results therefore indicate that the two assays give different, yet complementary information characterizing intercellular adhesiveness.

A Possible Role for the Cadherin Extracellular Domain in Controlling the Differences in Cell Adhesiveness by Various Cadherins—We show here that chimeric cadherins possessing the extracellular domain of cadherin-7 confer low adhesivity to cells, whereas those possessing the extracellular domain of E-cadherin confer high adhesivity. This suggests that the inefficiency of cadherin-7 to promote high cellular adhesivity results from intrinsic properties of the cadherin-7 extracellular domain, not its intracellular domain, a view consistent with the immunoprecipitation data showing that, similar to E- and N-cadherin, cadherin-7 co-precipitated with β- and α-catenin (Fig. 3).

The cadherin-7 extracellular domain exerts its effect on cell adhesiveness probably by preventing cadherins from forming stable homophilic

[7] O. Eder, unpublished data.

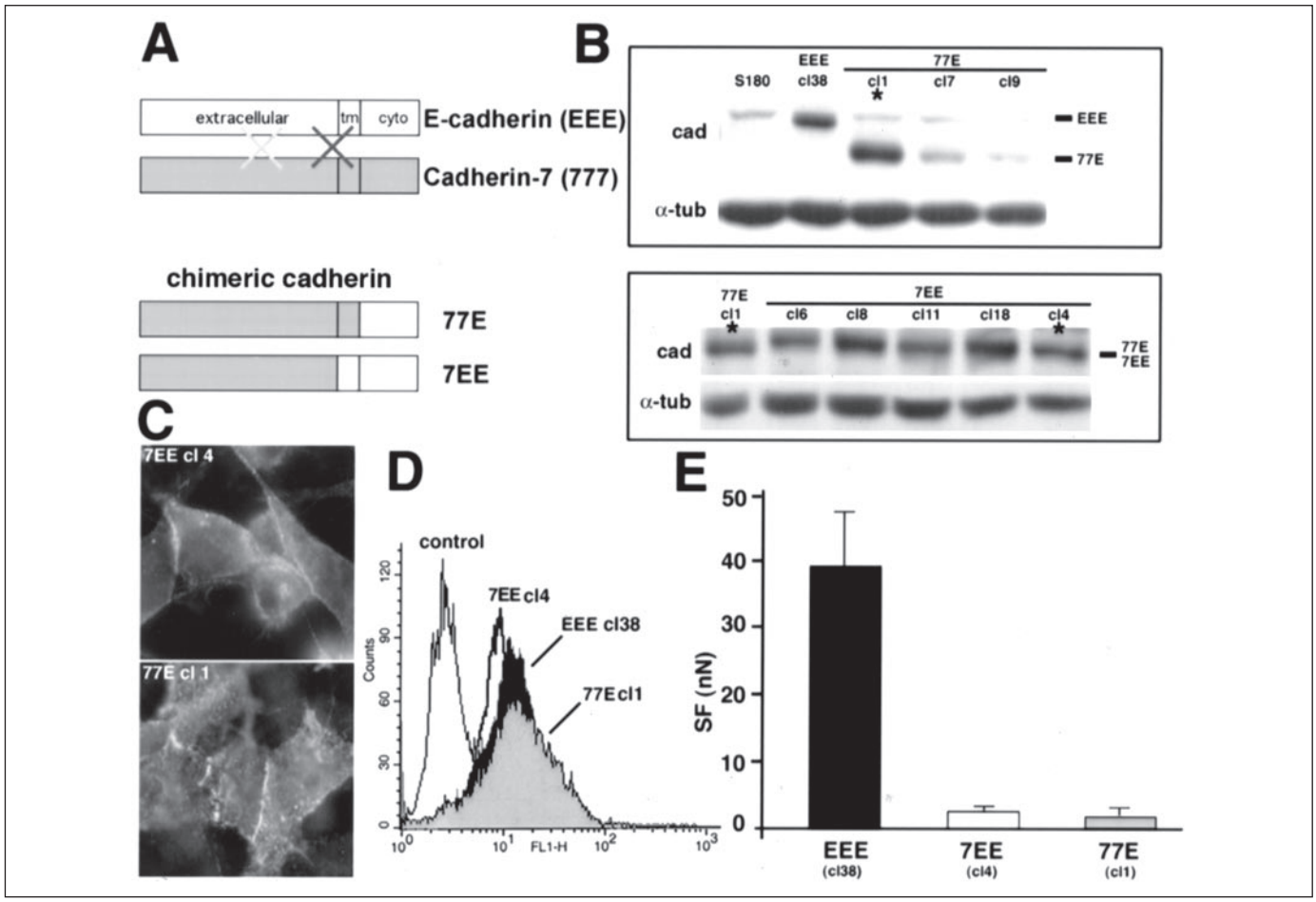

FIGURE 7. **Characterization of clones expressing 77E and 7EE chimeric cadherins and determination of their SF.** *A*, schematic representation of wild type and chimeric cadherins in which the extracellular domains were exchanged between E-cadherin and cadherin-7. *B*, Western blot analysis of cadherin and α-tubulin contents in cell extracts of S180, EEE-cl38 cells, and various clones expressing 77E (*upper panels*; with a polyclonal antibody directed against the cytoplasmic domain of E-cadherin) and 7EE (*lower panel*; with mAb CCD7 directed against the extracellular domain of cadherin-7) chimeric cadherins. The clones labeled by *asterisks*, 77E-cl1 and 7EE-cl4, expressed a level of cadherin similar to that of the clone EEE-cl38 and were selected for further analysis. *C*, immunolocalization of the chimeric cadherins at intercellular contacts. *D*, FACS of cadherins presented at the cell surface of EEE cl38 (*black peak*), 77E cl1 (*gray peak*), and 7EE cl4 (*white peak*). These clones expressed a similar level of cadherin at their surface. *E*, mean SF measured on 90-min doublets expressing EEE (*black bar*), 7EE (*white bar*), and 77E (*gray bar*) cadherins.

adhesions (40, 41). This view gains support from results showing that cell-cell contacts mediated by cadherin-7 have a higher turnover rate than those mediated by N-cadherin (6). A failure to dimerize or oligomerize could also prevent cadherin-7 from being rapidly incorporated into higher order molecular complexes strongly connected to the cytoskeleton, as suggested by the reduced incorporation of this cadherin into the detergent-insoluble fraction (Fig. 5). Because cytoskeleton recruitment and remodeling are essential for the evolution of the strong adhesions mediated by E-cadherin (28), a defect in the ability of cadherin-7 to contribute to larger molecular complexes could account for its much weaker ability to confer cellular adhesiveness. This may prove to be a consistent difference between Type I and Type II cadherins.

Studies using chimeric or mutated cadherins (42–44), x-ray crystallography (45), and EM analysis (46, 47) of cadherin extracellular domains have identified the EC1 domain as the one that supports cadherin binding and specificity. Studies applying other methods of analysis (surface force apparatus, flow assays, and surface plasmon resonance) (48–50) indicate that the EC1–EC5 domains of cadherin are all involved in the adhesive function. Nevertheless, Trp^2 and the histidine-alanine-valine (HAV) pocket are essential for the formation of adhesive Type I cadherin trans-dimers. Type II cadherins possess Trp^2 in their EC1 domain, but they exhibit a conserved Trp at position 4, not found in other cadherin families, and their hydrophobic pocket differs from that of Type I cadherins (38, 51). Analysis of the adhesive interface of Type II cadherins may provide insight into the differences in adhesivity conferred by Type I and Type II cadherins.

Possible Mechanisms Controlling the Differences in Cell Adhesiveness Modulated by Different Cadherins: the Role of the Transmembrane and Cytoplasmic Domains—We observed that the presence of the cadherin-7 transmembrane domain in chimeric cadherins slightly reduced cell adhesivity relative to that observed with chimeric cadherins containing the E-cadherin transmembrane domain (Figs. 6 and 7). Huber and co-workers (52) have shown that the transmembrane domain plays a role in lateral associations of E-cadherin molecules. Our results suggest that the transmembrane domain of cadherin-7 is less effective in this function, contributing to a reduced *cis*-dimerization and, in turn, reduced adhesiveness (as discussed above for the extracellular domain).

Cadherins transduce adhesion-dependent signals through catenin-mediated association with the cytoskeleton (53–55). The differential partitioning of Type I and Type II cadherins into the detergent-soluble and -insoluble cell-cell contacts (Fig. 5) indicates that Type I cadherins may be more readily stabilized by association with the cytoskeleton than their Type II counterparts. Previous results employing transient trans-

fection (37, 56) showed that cytoplasmic expression of E- and N-cadherin tails can interfere with β-catenin localization and the stability of the very robust MDCK cell-cell junctions. MDCK cells express E-cadherin as the only type I cadherin along with a low amount of cadherin-6 (type II cadherin) (57). In transfected cells, the cadherin cytoplasmic tails that are free and not bound by the membrane can act as "dominant negative" competitors with the membrane-bound endogenous cadherins by complexing β-catenin. This conclusion is supported by results showing that cadherin tails co-precipitate with β-catenin (37, 56). Here we show that the effect of cadherin cytoplasmic tails on the junctions of MDCK cells varies greatly with the type of cadherin tail expressed. Although cadherin-7 tails can interact and co-precipitate with β-catenin in cell extracts (data not shown and Fig. 3), they were significantly less efficient than tails of either E- or N-cadherin in disrupting MDCK junctions and in reducing the association of β-catenin with cell-cell contacts (Fig. 5). These results underscore a notable difference in the biological activity of Type II cadherin-7 tail compared with those of Type I (E- or N-) cadherin which might be due to a reduced binding affinity of cad7 tails for β-catenin or additionally to their inability to interact with yet unknown cytoplasmic partners playing a role in the maintenance of epithelial cell-cell junctions. Because chimeric cadherins possessing the cytoplasmic domain of cadherin-7 confer high adhesivity to cells, this difference appears to play no significant role in the control of cad7 cell adhesiveness.

Biological Significance of the Differences in Cell Adhesiveness Modulated by Different Cadherins—The dynamics of cellular adhesions are important for a variety of morphogenetic events, and cadherins are unquestionably major players in many of these processes (58). It remains to be determined how cadherins are regulated in time and space to orchestrate the observed morphogenetic events. We show here that cells expressing Type I cadherins (E-and N-) develop SF much faster and to a substantially higher level than cells expressing Type II cadherins (cadherin-7 and -11). Our results further suggest differential ability of cadherins to become organized at the cell surface prior to or during contact formation and strengthening. Any or all of these differences could be of critical importance in regulating adhesion-mediated biological processes.

Previous studies have reported that forces of several hundreds of nanonewtons are necessary to detach epithelial and fibroblastic cells from an extracellular matrix-coated surface (59–62). In the present study, we show that the SF measured for adherent cells varies (with cadherin Type and duration of contact) from a few nanonewtons to 400 nN. This range appears sufficient for regulating the differential adhesive interactions necessary to influence cell migration in response to environmental cues *in vivo*, especially when one considers the time frame over which such forces are likely to act.

In summary, the results of this study demonstrate clearly, for the first time, that Type I cadherins (E- and N-) are intrinsically different from Type II cadherins (cadherins-7 and -11) in the intercellular adhesions they generate, the time frame over which they work, and the way in which they are regulated; Type I cadherins produce stronger adhesions more rapidly, whereas Type II cadherins form much weaker adhesions and more slowly. These findings may explain, at least in part, the observation that Type I cadherins are primarily detected in compact colonies of cells, whereas Type II cadherins are preferentially expressed on the surface of migrating dispersed cells. These results may also provide insights into the regulation of other, as yet poorly understood, morphogenetic processes.

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