

# Lateral Dimerization is Required for the Homophilic Binding Activity of C-Cadherin

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**Abstract.** Regulation of cadherin-mediated adhesion can occur rapidly at the cell surface. To understand the mechanism underlying cadherin regulation, it is essential to elucidate the homophilic binding mechanism that underlies all cadherin-mediated functions. Therefore, we have investigated the structural and functional properties of the extracellular segment of *Xenopus* C-cadherin using a purified, recombinant protein (CEC 1-5). CEC 1-5 supported adhesion of CHO cells expressing C-cadherin. The extracellular segment was also capable of mediating aggregation of microspheres. Chemical cross-linking and gel filtration revealed that CEC 1-5 formed dimers in the presence as well as absence of calcium. Analysis of the functional activity of purified dimers and monomers demonstrated that dimers retained substantially greater homophilic binding activity than monomers. These results demonstrate that lateral dimerization is necessary for homophilic binding between cadherin extracellular segments and suggest multiple potential mechanisms for the regulation of cadherin activity.

Since the extracellular segment alone possessed sig-

nificant homophilic binding activity, the adhesive activity of the extracellular segment in a cellular context was analyzed. The adhesion of CHO cells expressing a truncated version of C-cadherin lacking the cytoplasmic tail was compared to cells expressing the wild-type C-cadherin using a laminar flow assay on substrates coated with CEC 1-5. CHO cells expressing the truncated C-cadherin were able to attach to CEC 1-5 and to resist detachment by low shear forces, demonstrating that tailless C-cadherin can mediate basic, weak adhesion of CHO cells. However, cells expressing the truncated C-cadherin did not exhibit the complete adhesive activity of cells expressing wild-type C-cadherin. Cells expressing wild-type C-cadherin remained attached to CEC 1-5 at high shear forces, while cells expressing the tailless C-cadherin did not adhere well at high shear forces. These results suggest that there may be two states of cadherin-mediated adhesion. The first, relatively weak state can be mediated through interactions between the extracellular segments alone. The second strong adhesive state is critically dependent on the cytoplasmic tail.

**T**HE establishment, regulation, and maintenance of cadherin-based cell-cell adhesion is known to be crucial for the formation of tissue structure and the proper physiological functioning of tissues (Gumbiner, 1996). Cadherin-mediated adhesion requires the calcium-dependent, homophilic ligation between extracellular segments of cadherins from opposing cells. Additionally, cadherin activity is regulated by cytoplasmic signaling events that ultimately control the function of the extracellular domain, presumably through modulation of its intrinsic bond strength or controlling the extent to which it is clustered (Gumbiner, 1996). Thus, a complete comprehension of the structure and functional activity of the extracellular segment of cadherins is fundamental to understanding

the mechanism of cadherin-based adhesion, the regulation of cadherin activity, and their broader roles in morphogenesis.

Recent investigations of extracellular domains of cadherins have provided descriptions of their structure and models of the possible mechanism underlying their functional activity. The extracellular segment of cadherins consists of five domains, which, in the case of E-cadherin, were shown by EM to form an elongated structure in the presence of calcium (Pokutta et al., 1994). In the absence of calcium, the extracellular segment undergoes a dramatic conformational change and loses its elongated structure. Equilibrium sedimentation analysis as well as visualization of the entire extracellular segment of E-cadherin by EM detected a monomeric structure in the presence and absence of calcium (Pokutta et al., 1994). Two different x-ray crystallographic analyses of cadherin extracellular domains, however, have revealed dimeric structures,

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although the interfaces linking subunits of the dimers have differed. The crystal structure of the NH<sub>2</sub>-terminal domain of N-cadherin (NCD-1) revealed a parallel dimer (Shapiro et al., 1995). The dimers are predicted to form through the lateral interaction of two cadherins extending from the same cell surface. In this structure, the lateral dimer interface formed through hydrophobic interactions between the two protomers. Determination of the crystal structure of the first two NH<sub>2</sub>-terminal domains of E-cadherin also revealed a lateral dimer, but, in this case, the subunits were linked together by mutually coordinating calcium ions (Nagar et al., 1996). Which of these structures represents the active form of the extracellular segment is not known.

The amount of adhesive activity intrinsic to the extracellular segment is not well understood. Proteolytic fragments of extracellular segments of cadherins have been shown to disrupt cell-cell contacts (Wheeler et al., 1987). Furthermore, N-cadherin-expressing cells were shown to preferentially attach to a purified, extracellular, proteolytic fragment of N-cadherin (Paradies and Grunwald, 1993). Finally, neurite outgrowth (Bixby and Zhang, 1990) and astrocyte spreading (Payne and Lemmon, 1993) on purified N-cadherin have been observed. These results demonstrate that isolated cadherin proteins, and even the extracellular segment alone, retain some degree of functional activity.

On the other hand, classical cadherins contain a conserved cytoplasmic tail that associates with cytoplasmic proteins known as catenins (Ozawa et al., 1989) and is critical for strong adhesion (Fujimori and Takeichi, 1993; Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). Cells expressing mutant forms of cadherins lacking the cytoplasmic tail do not aggregate. Additionally, cells expressing normal E-cadherin but lacking  $\alpha$ -catenin do not aggregate (Hirano et al., 1992). These observations have led to the conclusion that the extracellular segment alone is insufficient to mediate detectable homophilic adhesion.

In this report we have analyzed the quaternary structure and functional activity of the entire extracellular segment of *Xenopus* C-cadherin and assessed how the quaternary structure influences the activity of the extracellular segment. The results demonstrate that the extracellular segment is sufficient for homophilic binding and that a lateral, dimeric structure is central to the intrinsic functional activity of the extracellular segment of cadherins.

## Materials and Methods

### Plasmid Constructions

For expression of the wild-type C-cadherin in CHO cells, the cDNA encoding the full-length *Xenopus* C-cadherin (Levine et al., 1994) was excised from Bluescript (Stratagene, La Jolla, CA) with EcoRI and subcloned into the expression vector pEE14 at the EcoRI site. pEE14 encodes the glutamine synthase minigene as a selectable marker for CHO K1 cells expressing the minigene in the absence of glutamine and in the presence of the glutamine synthase inhibitor, methionine sulfoximine (Davis et al., 1990). CHO K1 cells can normally grow in the absence of glutamine, but growth in the absence of glutamine and the presence of methionine sulfoximine requires expression of the glutamine synthase minigene.

To generate a soluble, secreted extracellular segment of C-cadherin with a COOH-terminal six-histidine tag, a synthetic oligonucleotide en-

coding six histidines and a stop codon was inserted into pEE14 between the HindIII/BclI site generating pEE14.6His. The HindIII fragment of C-cadherin encoding the signal peptide, precursor region, and the entire extracellular segment up to the predicted first amino acid of the transmembrane domain was ligated in frame with the six-histidine tag into the HindIII site of pEE14.6His.

To construct a cadherin mutant lacking the cytoplasmic tail, PCR was used to isolate the transmembrane region of C-cadherin from the HindIII site to the predicted junction of the transmembrane and cytoplasmic domains. The 3' primer included NdeI and EcoRI for use as cloning sites and included the first, cytoplasmic, juxtamembrane lysine residue followed by two additional amino acids, histidine and methionine. The PCR fragment was ligated into Bluescript using HindIII and EcoRI, and the signal peptide, precursor region, and extracellular segment of C-cadherin were restored by directional cloning with KpnI and HindIII. Synthetic oligonucleotides were then used to introduce a myc tag at the COOH terminus of the molecule between NdeI and EcoRI sites. Subsequently, the construct was subcloned into the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) between EcoRV and NotI.

The nucleotide sequence of junctional regions and regions generated by PCR was determined in all of the constructs.

### Cell Lines

CHO K1 cells were grown in complete Glasgow MEM with 10% dialyzed FCS. cDNA constructs encoding the wild-type C-cadherin or CEC 1-5 in pEE14 were transfected into CHO K1 cells by lipofection in serum-free Glasgow MEM using Lipofectin (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. Cells containing the transfected plasmid were selected by culturing the cells in the presence of 25  $\mu$ M methionine sulfoximine (Sigma Chemical Co., St. Louis, MO). Expression of CEC 1-5 by methionine sulfoximine-resistant cell lines was determined by Western blotting conditioned media with the anti-C-cadherin mAb 6B6 as described previously (Briehner and Gumbiner, 1994). Expression of wild-type C-cadherin by CHO cells was determined by Western blotting detergent extracts of methionine sulfoximine-resistant cell lines.

The cDNA construct encoding the truncated C-cadherin lacking the cytoplasmic tail in pcDNA3 was transfected by lipofection as described above. G418-resistant cell lines were analyzed for the expression of the truncated C-cadherin by blotting detergent extracts of cells as described above.

### Protein Purification

Conditioned media of cells expressing CEC 1-5 was centrifuged at 25,000 g for 30 min. The protein containing the six-histidine tag was first purified by applying the supernatant to a metal chelating column charged with nickel (Pharmacia Fine Chemicals, Piscataway, NJ). The protein was eluted with a 20-column volume gradient of 0 to 250 mM Imidazole in 20 mM Tris, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, pH 7.2. Fractions containing CEC 1-5 were combined, diluted fivefold with 20 mM Tris, pH 7.2, and applied to an ion exchange column (Mono Q; Pharmacia Fine Chemicals). CEC 1-5 was eluted with a 20-column volume gradient from 0 to 300 mM NaCl in 20 mM Tris, pH 7.2. CaCl<sub>2</sub> was immediately added to the eluted fractions to a final concentration of 1 mM. Fractions containing CEC 1-5 were applied to a gel filtration column (Sephacryl S-300; Pharmacia Fine Chemicals), and the column was eluted with 20 mM Hepes, 100 mM NaCl, and 1 mM CaCl<sub>2</sub>. Fractions containing CEC 1-5 were concentrated in a Centri-con 50 (Amicon Corp., Danvers, MA).

### Adhesion Assays

Laminar flow adhesion assays in glass capillaries are an adaptation of a method initially described for integrins (Berlin et al., 1995). 1.0-mm internal diameter glass capillary tubes (Sutter Instruments Co., Novato, CA) were coated overnight in a humidified chamber at 4°C with 10  $\mu$ g/ml CEC 1-5 in the presence of calcium. The capillary was subsequently blocked with 10 mg/ml BSA for 2 h at room temperature. The coated capillary was connected to a 60-cc syringe attached to a syringe pump (Harvard Instruments, S. Natick, MA) and mounted on an inverted microscope (Carl Zeiss, Inc., Thornwood, NY).

Cells were harvested using a procedure that leaves cadherins intact as described previously. The concentration of cells was adjusted to  $2 \times 10^5$  cells per ml and either calcium or EDTA was added to a final concentration of 1 mM. In some experiments, cells were incubated either with Fab fragments of the anti-C-cadherin mAb 6B6 or Fab fragments of nonim-

mune mouse antibodies at 150  $\mu\text{g/ml}$  for 15 min before the addition of calcium.

The adhesion assay was performed at room temperature. Cells were drawn into the coated capillary from a reservoir using the pump. After 1 min, the flow was stopped and the cells were allowed to bind to the surface under static conditions for 10 min. The number of cells in a  $\times 20$  field was then counted. Flow was initiated and the number of cells remaining in the field was counted after 30 s. Subsequently, the flow was doubled every 30 s, and the number of cells remaining in the field was counted at the end of each time point.

### Bead Aggregation Assays

CEC 1-5 at 1 mg/ml in 20 mM Hepes, 100 mM NaCl, 1 mM  $\text{CaCl}_2$ , pH 7.2, was coupled via amine groups to 0.5- $\mu\text{m}$  red fluorescent beads (Covaspheres MX; Duke Scientific Corp., Palo Alto, CA) at a ratio of 1  $\mu\text{g}$  of protein per 1  $\mu\text{l}$  of bead suspension for 60 min at room temperature. Unbound sites were blocked by adding Tris, pH 7.2, and BSA to final concentrations of 20 mM and 2 mg/ml, respectively, for 30 min at room temperature. The coated beads were pelleted at 10,000  $g$  for 10 min and resuspended at 10 times their original volume in 20 mM Hepes, 100 mM NaCl, pH 7.2. The suspension was briefly sonicated to obtain single particles as determined by fluorescence microscopy before the addition of either 1 mM  $\text{CaCl}_2$  or 1 mM EDTA as appropriate. When appropriate, Fab fragments of nonimmune mouse IgG or Fab fragments of the anti-C-cadherin Ab 6B6 were also added at this time to a final concentration of 100  $\mu\text{g/ml}$ . The samples were incubated at room temperature, and at various time points, 10- $\mu\text{l}$  aliquots were removed. The number of particles large enough to be detected by a Coulter counter (Hialeah, FL) was determined as described previously for N-CAM (Grumet et al., 1993).

For experiments requiring binding of CEC 1-5 via the six-histidine tag, iminodiacetic acid-derivatized Covaspheres were made by combining equal volumes of bead suspension and 250 mM iminodiacetic acid (Sigma Chemical Co.) in 0.5 M  $\text{NaHCO}_3$ . The suspension was incubated for 30 min at 50°C and periodically vortexed. The suspension was diluted five-fold in 20 mM Hepes, pH 7.2, and washed three times with 20 mM Hepes, pH 7.2, by centrifugation. After the final wash, the beads were suspended in their original volume by sonication in 100 mM  $\text{NiSO}_4$  and incubated for 15 min. After three washes, the beads were suspended in their original volume in 20 mM Hepes, pH 7.2.

Fractions from gel filtration columns run in EDTA were desalted on a Sephadex G-25 column (Pharmacia Fine Chemicals) into 20 mM Hepes and 100 mM NaCl to remove the EDTA. 50  $\mu\text{l}$  of the desalted fractions were bound to 25  $\mu\text{l}$  of the derivatized beads for 15 min at room temperature. In each experiment, multiple protein concentrations ranging from 5–75  $\mu\text{g/ml}$  were mixed with the beads to empirically obtain a coating density that was low enough to prevent reformation of dimers from monomers on the beads and to obtain beads with similar amounts of bound protein. The coated beads were then separated from the remaining protein by centrifugation and resuspended at their original volume in 20 mM Hepes, 100 mM NaCl, pH 7.2. 5- $\mu\text{l}$  aliquots of this bead suspension were added to 100  $\mu\text{l}$  of the same buffer with or without 1 mM  $\text{CaCl}_2$  and containing either nonimmune mouse IgG Fab fragments or anti-C-cadherin monoclonal Fab fragments at a final concentration of 100  $\mu\text{g/ml}$ . In experiments testing the reversibility of the monomeric fraction, calcium was added to the protein sample to a final concentration of 1 mM for one hour before coupling the protein to the beads as described above.

The amount of protein coupled to the derivatized beads was determined by adding EDTA to the washed protein-coated beads to a final concentration of 50 mM. The beads were subsequently pelleted, and the supernatant was blotted with anti-C-cadherin mAb after SDS-PAGE.

### Chemical Cross-linking

CEC 1-5 at 50  $\mu\text{g/ml}$  in 20 mM Hepes and 100 mM NaCl, pH 8.0, containing either 1 mM  $\text{CaCl}_2$  or 1 mM EDTA was incubated with different amounts of ethylene glycolbis(succinimidylsuccinate) (EGS)<sup>1</sup> (Pierce Chemical Co., Rockford, IL) for 30 min at room temperature. The reaction was quenched by the addition of glycine, pH 8.0, to 50 mM for 15 min. The reaction products were separated by SDS-PAGE and blotted with mAb 6B6.

1. *Abbreviations used in this paper:* C-CHO, CHO cells expressing C-cadherin; CT-CHO, CHO cells expressing mutant C-cadherin lacking the cytoplasmic tail; EGS, ethylene glycolbis(succinimidylsuccinate).

Fractions from gel filtration columns were cross-linked with 1 mM EGS, as described above, before or after the addition of  $\text{CaCl}_2$  to a final concentration of 1 mM. Cross-linked products were detected as described above.

CEC 1-5 bound to iminodiacetic acid-derivatized beads was cross-linked in the presence of calcium with 1 mM EGS and quenched as described above. CEC 1-5 was eluted from the beads with 50 mM EDTA, and the cross-linked products were detected by Western blotting as described above.

### Gel Filtration

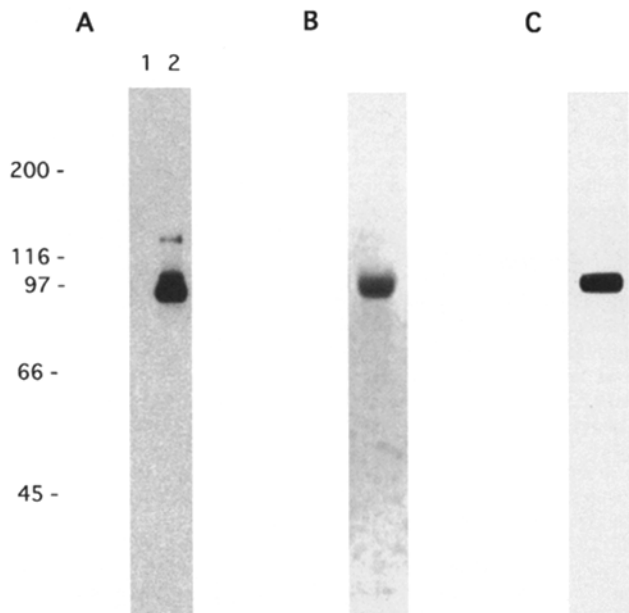
200  $\mu\text{l}$  of 1 mg/ml CEC 1-5 was applied to a Superose 12 column (Pharmacia Fine Chemicals) equilibrated in 20 mM Hepes, 100 mM NaCl, pH 7.2, containing either 1 mM  $\text{CaCl}_2$  or 0.2 mM EDTA. When the column was run in the presence of calcium, the protein was injected in the same buffer used to equilibrate the column. When the column was run in the absence of calcium, EDTA was added to the protein solution to a final concentration of 10 mM before applying the protein to the column. Columns were run at 4°C.

To test the stability of the high molecular weight peak, a fraction of the high molecular weight peak was directly reapplied to the gel filtration column. To test the stability of the low molecular weight peak, fractions from the low molecular weight peak were concentrated in a Centricon 50 (Amicon Corp., Danvers, MA) to 400–500  $\mu\text{g/ml}$  in the absence of calcium, and the protein was reapplied to the column as described above.

## Results

### Expression and Purification of CEC 1-5

To generate a soluble version of C-cadherin, CHO cells were transfected with a construct encoding a truncated C-cadherin lacking the cytoplasmic tail and transmembrane domain. Conditioned media of CHO cells stably transfected with the construct encoding CEC 1-5 contained a 75-kD polypeptide recognized by an anti-C-cadherin mAb (Fig. 1 A). No bands were detected in conditioned media from parental CHO cells. The predicted molecular weight of the mature, nonglycosylated extracellular segment of C-cadherin is 59,000. Approximately 20% of the molecular weight of the full-length C-cadherin in *Xenopus* or in L cells appears to result from glycosylation or other post-translational modifications (Lee and Gumbiner, 1995). Assuming CEC 1-5 is modified to a similar extent as the full-length C-cadherin, 75,000 is a reasonable molecular weight to expect for the mature secreted protein. Consistent with this finding, release of the extracellular segment of C-cadherin from L cells and CHO cells transfected with the full-length C-cadherin by trypsinization produces a 75-kD band recognized by anti-C-cadherin antibodies (data not shown). Additionally, CEC 1-5 binds to Con A Sepharose, demonstrating that CEC 1-5 is glycosylated (data not shown). Cadherins are synthesized with a large pro region that is normally proteolytically cleaved to give the mature cadherin. The cDNA encoding CEC 1-5 includes the pro region of the molecule, but CHO cells processed CEC 1-5 to the mature form. Western blots with anti-C-cadherin antibodies of detergent extracts of CEC 1-5 expressing CHO cells revealed that the majority of intracellular CEC 1-5 was a 100-kD species consistent with the molecular weight of CEC 1-5 with the pro region. The majority of CEC 1-5 that accumulated in the media, however, had been processed to the 75-kD form (data not shown). Therefore, CHO cells transfected with a construct encoding a soluble version of C-cadherin specifically express and secrete a protein of the expected molecular weight and im-



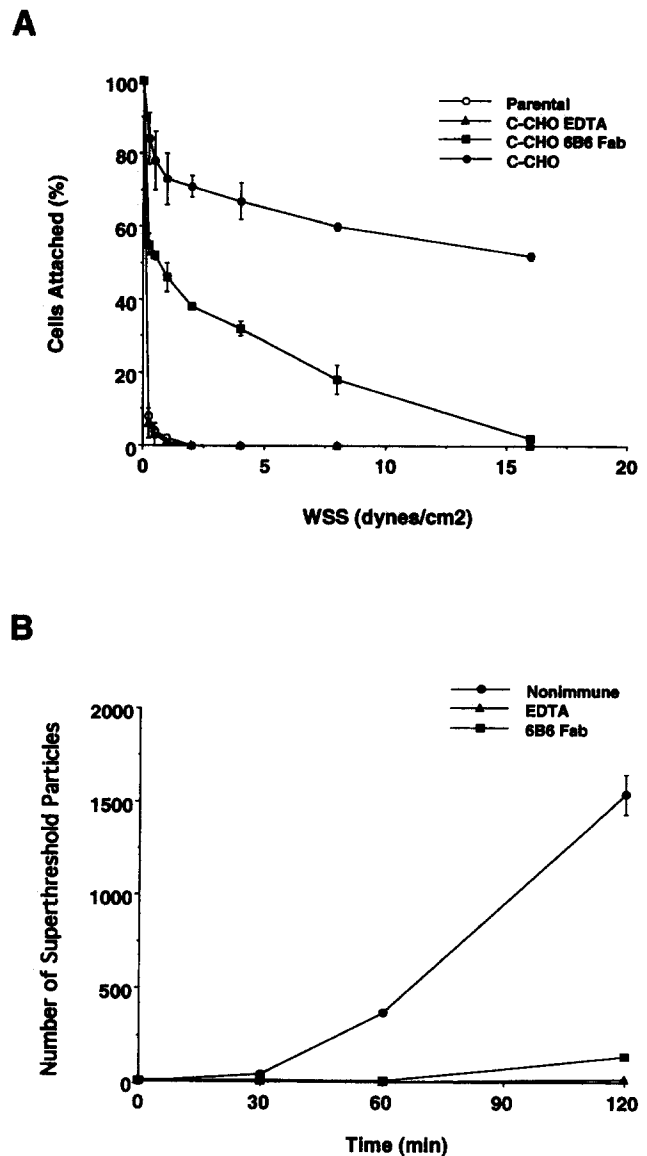
**Figure 1.** Expression and purification of CEC 1-5. (A) Western blots with anti-C-cadherin antibody 6B6 of conditioned media from untransfected CHO cells (lane 1) or from CHO cells expressing CEC 1-5 (lane 2). (B) Coomassie-stained gel of 5  $\mu$ g of purified CEC 1-5. (C) Western blot with anti-C-cadherin antibody 6B6 of purified CEC 1-5.

munoreactivity of a processed, mature extracellular segment of C-cadherin.

CEC 1-5 was purified to apparent homogeneity by a series of chromatographic separations. The final purified material appeared as a single band of  $\sim$ 75 kD on SDS gels (Fig. 1 B), and this band was recognized by anti-C-cadherin mAbs (Fig. 1 C).

### Functional Activity of CEC 1-5

The functional activity of CEC 1-5 was analyzed by measuring the capacity of C-cadherin-expressing CHO cells (C-CHO cells) to adhere to surfaces coated with CEC 1-5. As expected, C-cadherin-expressing CHO cells aggregated in suspension in the presence, but not in the absence, of calcium (data not shown). Adhesion of C-cadherin-expressing CHO cells to CEC 1-5 was determined by quantitating the number of cells that bound to CEC 1-5-coated substrates and resisted detachment by increasing shear forces. As shown in Fig. 2, C-CHO cells adhered to glass tubes coated with CEC 1-5 in the presence of calcium and resisted increasing detachment forces. Adhesion of C-CHO cells to CEC 1-5 required calcium because only 10% of the cells remained adherent to the substrate at the lowest shear force applied (0.125 dynes/cm<sup>2</sup>) when the assay was performed in the presence of EDTA. Only 10% of CHO cells not expressing C-cadherin remained adherent at the lowest shear stress, demonstrating that the adhesive interaction of cells with CEC 1-5 required cellular expression of C-cadherin. Additionally, adhesion of C-CHO cells to CEC 1-5 was inhibited by incubating the cells with Fab fragments of an anti-C-cadherin mAb, confirming that the adhesive interaction between C-CHO cells and CEC 1-5



**Figure 2.** Functional activity of CEC 1-5. (A) Adhesion of CHO cells expressing C-cadherin (C-CHO cells) to CEC 1-5. Cells were allowed to bind to the substrate for 10 min under static conditions (no flow). The flow was subsequently increased every 30 s, and the number of cells remaining within the field of view was counted. Assays were performed either in the presence of calcium (Parental, C-CHO, C-CHO 6B6 Fab) or absence of calcium (C-CHO EDTA). Some assays were performed either in the presence of nonimmune mouse Fab fragments (C-CHO) or anti-C-cadherin Fab fragments (C-CHO 6B6 Fab). The experiment was performed in triplicate, and the mean  $\pm$  SEM is shown. (B) Aggregation of CEC 1-5-coated microspheres. The number of particles large enough to be detected by a Coulter counter (superthreshold particles) that formed by aggregation of CEC 1-5-coated microspheres is plotted. Samples were incubated in the absence of calcium (EDTA) or the presence of calcium and either nonimmune mouse Fab fragments (Nonimmune) or Fab fragments of anti-C-cadherin mAb 6B6 (6B6 Fab). The experiment was performed in triplicate, and the mean  $\pm$  SEM is shown.

was C-cadherin specific. These results demonstrate that purified CEC 1-5 is a functional protein mediating specific, calcium-dependent, homophilic associations with C-cadherin.

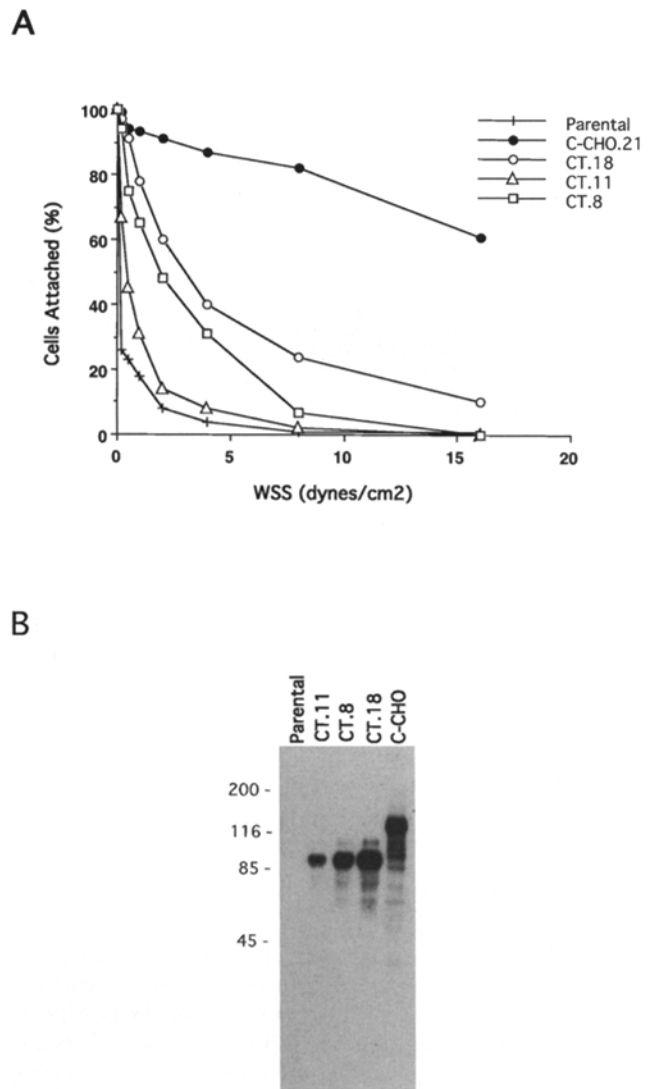
Because C-CHO cells express the full-length C-cadherin with complete activity, it is possible that CEC 1-5 shows activity only because it provides an acceptor site to which the wild-type C-cadherin can bind. Alternatively, CEC 1-5 may by itself be capable of homophilic binding. To test this possibility, the ability of CEC 1-5 to cause the calcium-dependent aggregation of beads coated with CEC 1-5 was tested (Fig. 2 B). CEC 1-5 induced aggregation of beads as demonstrated by the appearance of particles large enough to be detected by a Coulter counter (superthreshold particles). Aggregation of the beads was calcium dependent because CEC 1-5-coated beads failed to aggregate in the presence of EDTA. Aggregation of the coated beads was inhibited by incubation of the beads with Fab fragments of the anti-C-cadherin antibody 6B6, demonstrating that aggregation required the specific functional activity of CEC 1-5. Thus, CEC 1-5 contains all the information required for specific, calcium-dependent, homophilic binding activity in the absence of additional cellular components.

In previous studies using cell aggregation assays, the cytoplasmic tail of cadherins has been found to be essential for adhesive function (Fujimori and Takeichi, 1993; Nagafuchi and Takeichi, 1988; Ozawa et al., 1990). The results with CEC 1-5 described above, however, demonstrate that the extracellular segment can act alone to mediate cell attachment or bead aggregation. Therefore, we reexamined the activity of tailless cadherins expressed in cells using the flow assay described above, which yields more sensitive and quantitative measurements of adhesive forces than cell aggregation assays.

Three cell lines expressing different amounts of a mutant version of C-cadherin lacking the cytoplasmic tail (CT-CHO cells) were compared to C-cadherin-expressing CHO cells for their ability to adhere to CEC 1-5-coated substrates (Fig. 3). Again, C-CHO cells adhered tightly to CEC 1-5 and resisted detachment by increasing shear forces. Only 20% of the parental CHO cells remained attached at the lowest shear force (0.25 dynes/cm<sup>2</sup>), confirming that CHO cells do not bind to CEC 1-5. In contrast with parental CHO cells, a significant number of CHO cells expressing tailless C-cadherin bound to CEC 1-5 and resisted detachment by increasing shear forces. Thus, a mutant version of C-cadherin that lacks the cytoplasmic tail retains a demonstrable amount of adhesive activity in cells. The truncated C-cadherin, however, does not mediate as strong adhesion as the wild-type C-cadherin. This was most clearly demonstrated by the adhesive activity of the CT-CHO.18 cell line. Despite greater than threefold higher levels of the truncated C-cadherin in this cell line relative to wild-type C-cadherin levels in C-CHO cells, CT-CHO.18 cells consistently detached at lower shear stresses than C-CHO cells.

### Dimerization of CEC 1-5

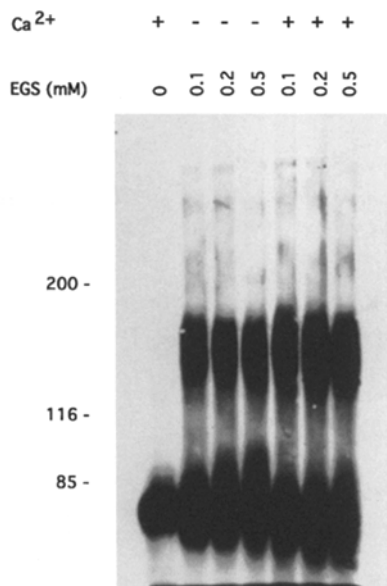
The oligomeric state of CEC 1-5 was analyzed with chemical cross-linkers. Incubation of purified CEC 1-5 with the amine-reactive cross-linker EGS resulted in the formation



**Figure 3.** Comparison of the functional activity of a truncated C-cadherin lacking the cytoplasmic tail with the full-length C-cadherin in CHO cells. (A) Adhesion to CEC 1-5 and resistance to increasing detachment forces of C-CHO cells, CHO cells expressing a tailless mutant of C-cadherin (CT.8, CT.11, and CT.18 cells), and parental CHO cells. (B) Western blot with anti-C-cadherin antibody 6B6, comparing the levels of full-length C-cadherin and the tailless version of C-cadherin in CHO cells after trypsinizing the cells using the procedure for harvesting the cells for the adhesion assay.

of two bands recognized by anti-C-cadherin antibodies (Fig. 4). The lower band ran at a molecular weight of 75,000, consistent with the molecular weight of a monomer of CEC 1-5. The upper band ran at a molecular weight of 150,000, consistent with the molecular weight of a dimer. Only the monomeric species was detected in the absence of the crosslinker. Strikingly, the 150-kD species formed in the absence of calcium as well as in the presence of calcium.

We have not been able to obtain compelling evidence that C-cadherin can be cross-linked to a dimer on the surface of cells. This is most likely due to inaccessibility of the cross-linker to the proper residues on C-cadherin required



**Figure 4.** Chemical cross-linking of CEC 1-5 with EGS. Purified CEC 1-5 was cross-linked in the presence or absence of calcium. The products of the reaction were separated by SDS-PAGE and detected by Western blotting with an anti-C-cadherin antibody.

to cross-link the molecule. Cross-linking proteins with known oligomeric structure on the cell surface, such as Rous sarcoma virus envelope glycoprotein, has also proven to be difficult even though they can be efficiently cross-linked in solution after partial purification or on the surface of virus particles (White, J.M., personal communication).

The oligomeric nature of CEC 1-5 was also analyzed by gel filtration chromatography. In the absence of calcium, purified CEC 1-5 eluted from the column as two peaks (Fig. 5 A), suggesting that two different oligomeric species had been separated. This was confirmed by cross-linking each fraction across the column in the absence of calcium. The protein in the high molecular weight peak could be cross-linked to a dimer, whereas protein eluting in the lower molecular weight peak was not cross-linked to a dimer (Fig. 5 B). Addition of calcium to the fractions, however, promoted dimerization (Fig. 5 E). Thus, the monomeric form of CEC 1-5, isolated in the absence of calcium, is a reversible species that can reform dimers in the presence of calcium. Together, these results demonstrate that CEC 1-5 forms dimers in the presence and absence of calcium.

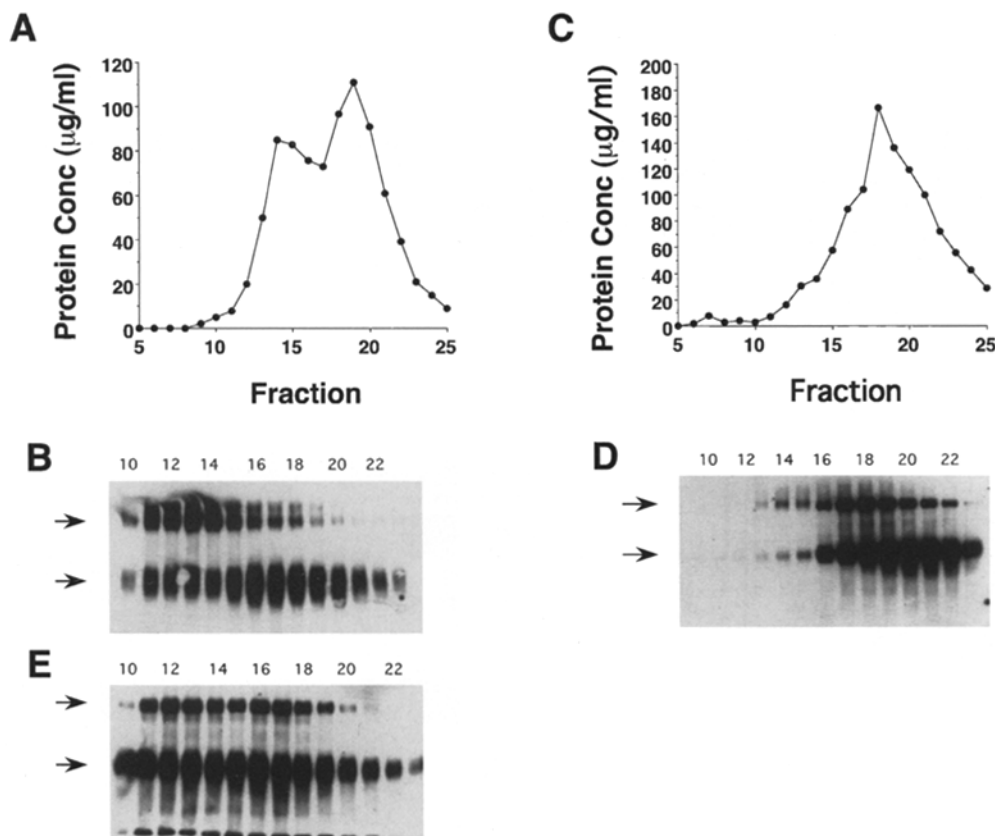
When analyzed by gel filtration in the presence of calcium, CEC 1-5 eluted from the column as a single peak (Fig. 5 C). Cross-linking of all the fractions eluting from the column in the presence of calcium revealed that CEC 1-5 can be cross-linked to a dimer in every fraction (Fig. 5 D). It is possible that nearly all of CEC 1-5 is present in the dimeric form in the presence of calcium, resulting in the appearance of a single peak in gel filtration. Alternatively, monomers and dimers may both be present in the presence of calcium but inseparable. In the presence of calcium, the extracellular segment of cadherins forms an elongated, rodlike shape (Pokutta et al., 1994), which may have a dominant effect on the behavior of the molecule in gel filtration relative to molecular weight.

The stability of the monomeric and dimeric states of CEC 1-5 in the absence of calcium was assessed by gel filtration chromatography. Since monomers and dimers can be separated only in the absence of calcium, all gel filtration columns in the following experiments were run in the absence of calcium. (For this reason, a similar analysis of dimer and monomer stability in the presence of calcium could not be performed.) The dimeric form of CEC 1-5 is a stable species in the absence of calcium. When a fraction from the high molecular weight peak was reapplied directly to the gel filtration column, it eluted in the position of the high molecular weight peak (Fig. 6 A). The low molecular weight peak isolated in the absence of calcium is also a stable species when kept in the absence of calcium. When fractions corresponding to the low molecular weight peak were reapplied to the column, almost all of the material eluted in the low molecular weight peak (Fig. 6 B), even when the low molecular weight fractions were reconcentrated to 500  $\mu$ g/ml. Thus, monomers and dimers of CEC 1-5 can be isolated and maintained as stable species in the absence of calcium.

#### **Functional Activity of Dimers and Monomers of CEC 1-5**

The ability to separate monomers from dimers by gel filtration in the absence of calcium and maintain them as stable species allowed us to test the functional activity of each of these two species independently. To do this, however, required that the monomers be captured and held in a monomeric state before the addition of calcium. Otherwise, addition of calcium necessary for binding activity would promote dimerization. Fractions containing dimers or monomers were trapped in the absence of calcium by binding the protein to nickel-derivatized beads via the COOH-terminal, six-histidine tag. Concentrations of CEC 1-5 from dimer and monomer fractions that yielded similar amounts of protein bound to the nickel-derivatized beads (Fig. 7 A) were empirically chosen. The ability to trap and hold CEC 1-5 in the monomeric form by this technique was demonstrated by cross-linking the protein bound to the beads (Fig. 7 B). Purified dimers bound to the beads were efficiently cross-linked to a dimer (lane 2). Purified monomers trapped on the beads, however, were not cross-linked to a dimer even after calcium was added to the beads (lane 1). If monomers were allowed to redimerize in solution by adding calcium to the fraction before binding the protein to beads, the protein was efficiently cross-linked to a dimer (lane 3). Thus, monomers or dimers can be selectively bound to beads and their activities compared.

Upon addition of calcium, the dimer species mediated bead aggregation to a five- to sixfold greater extent than the monomer fraction (Fig. 7 C). The aggregating activity of the monomer fraction could be restored to the level of the dimer fraction by reverting the monomers to dimers in solution before binding the protein to the beads. The aggregating activity was specific because it was inhibited by anti-C-cadherin Fab fragments and calcium dependent. Because similar amounts of protein were bound to the beads in each case (Fig. 7 A), the difference in aggregation detected between CEC 1-5 from the low molecular weight peak compared with the high molecular weight peak is due



**Figure 5.** Gel filtration analysis of CEC 1-5 dimerization. (A) Elution profile of 1 mg/ml CEC 1-5 from a Superose 12 gel filtration column run in the absence of calcium. (B) Chemical cross-linking of fractions 10–23 from gel filtration run in the absence of calcium. CEC 1-5 was detected by Western blotting with an anti-C-cadherin antibody. (C) Elution profile of 1 mg/ml CEC 1-5 from a Superose 12 gel filtration column run in the presence of calcium. (D) Chemical cross-linking of fractions 10–23 from gel filtration run in the presence of calcium. CEC 1-5 was detected by Western blotting with an anti-C-cadherin antibody. (E) Chemical cross-linking of fractions 10–23 from gel filtration run in the absence of calcium cross-linked after the addition of calcium to each fraction. CEC 1-5 was detected by Western blotting with an anti-C-cadherin antibody. Arrows in B, D, and E mark the positions of monomers and dimers of CEC 1-5.

to their monomeric vs dimeric structure. These results demonstrate that the dimeric structure of CEC 1-5 substantially increases its intrinsic homophilic binding activity relative to monomers.

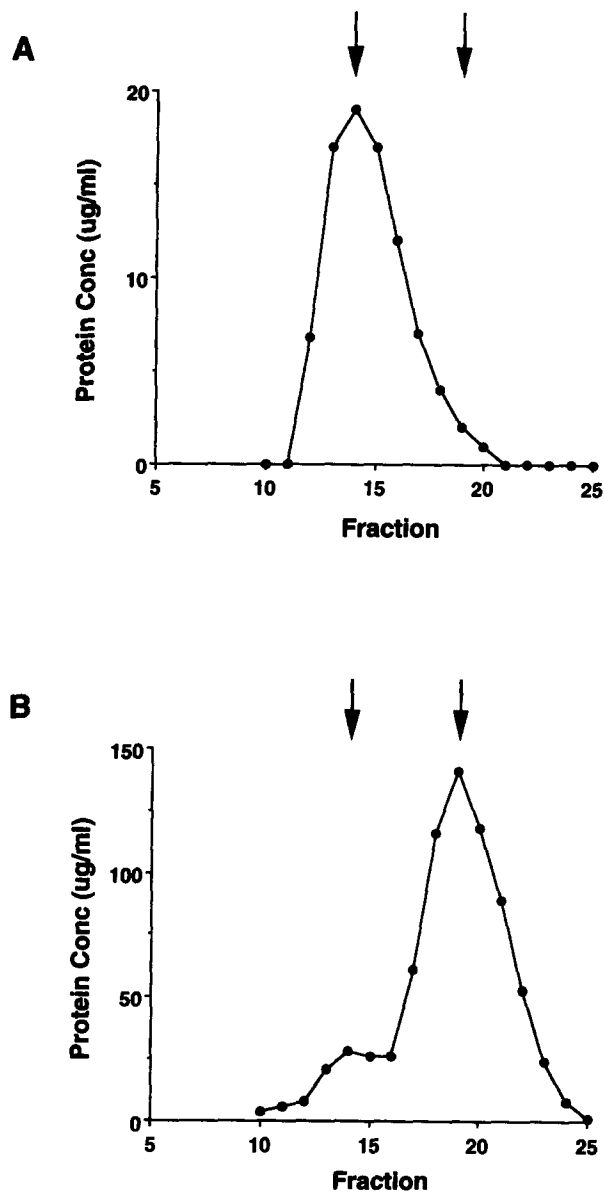
## Discussion

We have analyzed the quaternary structure and functional activity of the entire extracellular segment of C-cadherin and assessed how its quaternary structure influences its activity. The results show that CEC 1-5 is a functional molecule that can support adhesion of C-cadherin-expressing cells. Moreover, CEC 1-5 by itself is sufficient for calcium-dependent, homophilic binding activity. The activity of CEC 1-5 was dramatically influenced by its quaternary structure. A dimeric structure of CEC 1-5 was required for full expression of its intrinsic homophilic binding activity and even may be essential for homophilic binding to occur.

Proteolytic fragments of cadherin extracellular segments have been shown to retain a demonstrable degree of activity (Wheelock et al., 1987). Cells expressing truncated cadherins lacking the cytoplasmic tail, however, do not aggregate (Fujimori and Takeichi, 1993; Nagafuchi and Takeichi, 1988; Ozawa et al., 1990), which raises questions as to how much adhesive activity exists without the cytoplasmic tail and its associated proteins. Functional assays of the activity of CEC 1-5 demonstrate that a significant amount of cadherin activity resides in the extracellular segment. CEC 1-5 supported the adhesion of C-cadherin-

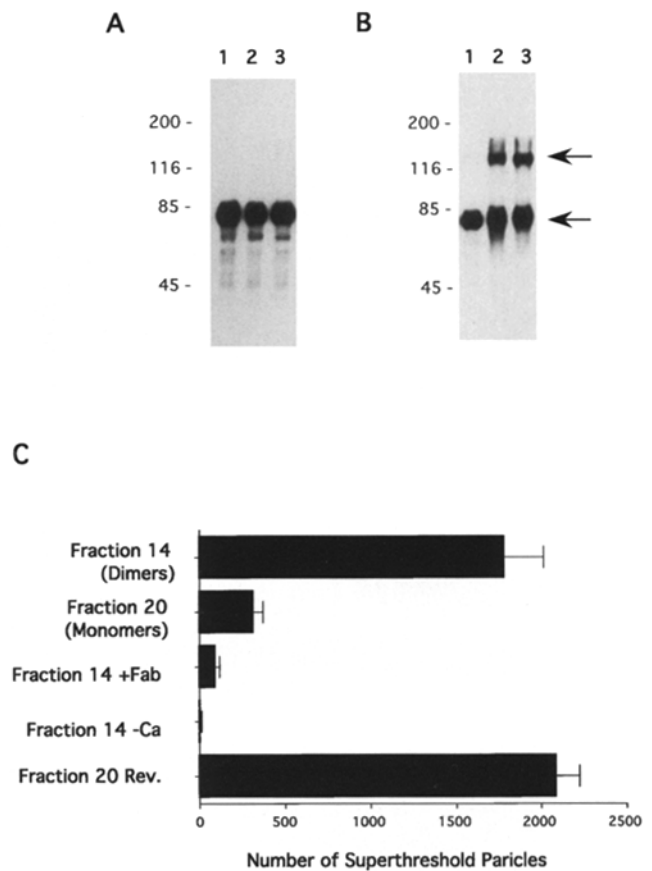
expressing cells. Furthermore, CEC 1-5 by itself mediated bead aggregation, demonstrating that the extracellular segment is sufficient for homophilic binding activity. Finally, using a new laminar flow assay for measuring the adhesion of cells to substrates coated with CEC 1-5, we analyzed the activity of a truncated version of C-cadherin lacking the cytoplasmic tail. This assay appears to provide more quantitative and sensitive measurements of adhesive forces than aggregation assays. Standard aggregation assays measure cadherin activity under constant shear. In contrast, the laminar flow assay allows cells to bind to immobilized CEC 1-5 under static conditions, allowing sufficient time for enough bonds to form between the substrate and cell to detect even weak adhesive interactions that would be sensitive to high shear. Using this assay, a truncated version of C-cadherin lacking the cytoplasmic tail was demonstrated to retain significant adhesive activity. Cells expressing the truncated cadherin, however, did not adhere as strongly to CEC 1-5 as cells expressing full-length C-cadherin. We propose, therefore, that there are two states of cadherin-mediated adhesion. The first, relatively weak state of adhesion is mediated by the extracellular segment alone. The second, strong adhesive state requires the cytoplasmic tail, and presumably, the catenins and linkage to the cytoskeleton.

CEC 1-5 was found to form dimers that could, in theory, form either through homophilic, head-to-head interactions or lateral interactions. Several findings indicate that the dimer species of CEC 1-5 detected in our experiments



**Figure 6.** Stability of the high and low molecular weight species of CEC 1-5 in the absence of calcium. The elution profiles of different fractions of CEC 1-5 originally obtained by gel filtration in the absence of calcium are shown. All columns were run in the absence of calcium. Arrows mark the original positions of the peak fractions of the high and low molecular weight peaks. (A) A high molecular weight fraction (fraction 14) rerun on a gel filtration column in the absence of calcium. (B) The low molecular weight peak (fractions 18–22) concentrated to 500  $\mu\text{g/ml}$  in the absence of calcium before running the sample on the column.

forms through lateral interactions similar to those identified in the crystal structures of extracellular fragments of N- and E-cadherin (Nagar et al., 1996; Shapiro et al., 1995). First, dimers of CEC 1-5 were detected in the presence as well as absence of calcium, whereas adhesive, head-to-head, homophilic binding interactions might be expected to show strict calcium dependence. Second, dimers of CEC 1-5 isolated in the absence of calcium appear to be stable, and yet mediate effective calcium-dependent adhesion. If stable dimers in the absence of calcium



**Figure 7.** Homophilic binding activity of monomeric vs dimeric forms of CEC 1-5. (A) Comparison of the amount of CEC 1-5 monomers and dimers bound to nickel-derivatized beads via the COOH-terminal six-histidine tag. CEC 1-5 was stripped from the beads with EDTA and blotted with an anti-C-cadherin antibody. (Lane 1) Gel filtration fraction 20 (monomers); (lane 2) fraction 14 (dimers); (lane 3) fraction 20 redimerized in solution with 1 mM  $\text{CaCl}_2$  before binding to beads. (B) Chemical cross-linking of CEC 1-5 monomers and dimers bound to nickel-derivatized beads via the COOH-terminal six-histidine tag. Samples were cross-linked on the beads in the presence of calcium. CEC 1-5 was eluted from the beads, and the reaction products were detected by Western blotting with an anti-C-cadherin antibody. Arrows mark the positions of monomers and dimers. (Lane 1) Gel filtration fraction 20 (monomers) bound to beads; (lane 2) fraction 14 (dimers) bound to beads; (Lane 3) fraction 20 redimerized in solution with 1 mM  $\text{CaCl}_2$  before binding to the beads. (C) Aggregation of beads coated with monomeric or dimeric forms of CEC 1-5. The number of superthreshold particles formed by nickel-derivatized beads with selectively trapped monomers (Fraction 20), dimers (Fraction 14), or monomers redimerized in solution before binding to the beads (Fraction 20 Rev.) is plotted. Some of the samples contained either Fab fragments of nonimmune mouse IgG (Fraction 14, Fraction 20, Fraction 20 Rev.) or Fab fragments of anti-C-cadherin antibody 6B6 (fraction 14 6B6 Fab). Calcium was added to the samples (except for fraction 14-Ca) to initiate aggregation, and the number of superthreshold particles that formed in 60 min was determined on a Coulter counter. The experiment was performed in triplicate, and the mean  $\pm$  SEM is shown.



formed via homophilic, head-to-head interactions, CEC 1-5 would not be expected to show functional activity. This was tested directly by trapping stable dimers isolated in the absence of calcium on beads via the COOH-terminal, six-histidine tag. Dimers trapped in this manner mediated bead aggregation after the addition of calcium. Thus, we conclude that the dimeric structure of CEC 1-5 forms through lateral interactions. In this configuration, the extracellular segment of C-cadherin would be predicted to extend from the cell surface as a dimer as suggested previously from the crystallographic data.

Our results provide evidence for both calcium-dependent and -independent dimerization of CEC 1-5. While dimers of CEC 1-5 were detected in the absence of calcium, dimer formation was not completely calcium independent. Calcium promoted the reformation of dimers from isolated monomers. Interestingly, both calcium-dependent and -independent dimerization interactions have been described in two crystal structures, although each interaction was exclusively detected in each of the different crystals. Lateral dimers of the NH<sub>2</sub>-terminal domain of N-cadherin appeared to be mediated by a calcium-independent, hydrophobic interaction in which the side chain of Trp2 was buried in a hydrophobic pocket provided by the other subunit of the dimer (Shapiro et al., 1995). Since the second residue of C-cadherin is also a tryptophan, it is possible that the subunits of the stable, lateral dimers of C-cadherin, detected in the absence of calcium, are linked together by a similar hydrophobic interaction. In the x-ray crystal structure of the first two domains of E-cadherin, however, the two subunits of the lateral dimer were linked together at the region between the first and second domains by mutually coordinating calcium ions (Nagar et al., 1996). Perhaps mutual coordination of calcium by individual subunits of lateral dimers is the mechanism by which calcium promotes dimer formation of CEC 1-5. However, it is not clear whether the two kinds of dimer interactions can be accommodated into a single model, since dimerization by calcium coordination appeared to preclude the tryptophan-dependent dimerization interface (Nagar et al., 1996). It is difficult to ascertain which features of the two crystals could account for our biochemical evidence for dimerization of CEC 1-5, because the functional activity of the crystallized proteins was not analyzed or reported. Determining whether lateral dimers of an active extracellular segment are supported exclusively by calcium-dependent or -independent interactions, or both interactions will require high resolution, structural analysis of cadherin extracellular domains of known functional activity.

Comparison of the functional activities of CEC 1-5 monomers and dimers demonstrated that the homophilic binding activity of CEC 1-5 is critically dependent on a dimeric structure. Interesting in this regard is the finding that a recombinant extracellular segment of E-cadherin expressed in baculovirus, which was monomeric, was not able to inhibit the aggregation of E-cadherin-expressing cells (Herrenknecht and Kemler, 1993), suggesting that monomeric forms of cadherins may not have functional activity. Although we detected low, residual activity of the CEC 1-5 monomer in bead aggregation assays, it is not clear whether it is due to some intrinsic activity of the

monomer or to the presence of a small amount of dimers reformed during the assay. Nonetheless, our findings suggest that the functional activity of the extracellular segment of all classical cadherins may require the formation of lateral dimers.

Several mechanisms could be invoked to explain why dimers have greater activity than monomers. Dimers may have higher effective homophilic binding activity simply because they are multivalent. Alternatively, dimerization may create unique binding sites at the dimerization interface that allow or increase homophilic, head-to-head binding. Finally, a self-assembling adhesive zipper model has been proposed based on the packing of the crystal of NCD-1 in which lateral dimers interact in a head-to-head fashion (Shapiro et al., 1995). A variation of this model would be assembly of dimers into a two-dimensional, adhesive lattice (Gumbiner, 1996). In either case, if such a supramolecular structure is necessary for adhesion, lateral dimers would be crucial for activity because formation of the zipper or lattice requires interdigitation of molecules from opposing cell surfaces to allow multivalent binding.

Cadherin-mediated adhesion has been shown to be a regulated process during tissue morphogenesis (Brieher and Gumbiner, 1994; Fleming and Johnson, 1988) and in some pathophysiological states (Behrens et al., 1993; Hama-guchi et al., 1993; Williams et al., 1993). The fact that the extracellular segment possesses intrinsic, homophilic binding activity and that its activity depends on lateral dimers provides possible mechanisms through which regulation could occur. Regulatory signals probably influence the contribution of the cytoplasmic tail to the strength of adhesion. Adhesive activity could range from a weak state attainable by the intrinsic activity of the extracellular segment when the cytoplasmic tail is inactive to a strong state when the contribution of the cytoplasmic tail is maximal. It could also be possible to regulate cadherin activity by controlling the number of lateral dimers vs monomers on the cell surface. Finally, a dimeric structure could allow for conformational changes in the extracellular segment resulting in changes in the homophilic binding affinity. Affinity modulation is unlikely to occur with a monomeric structure because conformational changes on the cytoplasmic side are unlikely to propagate through the protein and across the membrane to the extracellular segment. A dimeric structure, however, could allow for affinity modulation by mechanisms similar to those invoked for affinity modulation of integrins that form heterodimers (Schwartz et al., 1995). The spatial relationship between subunits of lateral, cadherin dimers on the cytoplasmic side could be altered in response to cytoplasmic signals. These spatial changes could be propagated through the membrane, thereby altering the spatial relationships between subunits of lateral dimers on the extracellular side and resulting in modulation of the homophilic binding affinity. Therefore, the identification of lateral dimers of the extracellular segment C-cadherin and their influence on homophilic binding activity has implications for the regulation of cadherin-mediated adhesion as well as the mechanism of cadherin-mediated adhesion.

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