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Multiple cadherin extracellular repeats mediate homophilic binding and adhesion

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The extracellular homophilic-binding domain of the cadherins consists of 5 cadherin repeats (EC1–EC5). Studies on cadherin specificity have implicated the NH_2-terminal EC1 domain in the homophilic binding interaction, but the roles of the other extracellular cadherin (EC) domains have not been evaluated. We have undertaken a systematic analysis of the binding properties of the entire cadherin extracellular domain and the contributions of the other EC domains to homophilic binding.

Lateral (cis) dimerization of the extracellular domain is thought to be required for adhesive function. Sedimentation analysis of the soluble extracellular segment of C-cadherin revealed that it exists in a monomer–dimer equilibrium with an affinity constant of ~64 μM. No higher order oligomers were detected, indicating that homophilic binding between cis-dimers is of significantly lower affinity.

The homophilic binding properties of a series of deletion constructs, lacking successive or individual EC domains fused at the COOH terminus to an Fc domain, were analyzed using a bead aggregation assay and a cell attachment–based adhesion assay. A protein with only the first two NH_2-terminal EC domains (CEC1-2Fc) exhibited very low activity compared with the entire extracellular domain (CEC1-5Fc), demonstrating that EC1 alone is not sufficient for effective homophilic binding. CEC1-3Fc exhibited high activity, but not as much as CEC1-4Fc or CEC1-5Fc. EC3 is not required for homophilic binding, however, since CEC1-2-4Fc and CEC1-2-4-5Fc exhibited high activity in both assays. These and experiments using additional EC combinations show that many, if not all, the EC domains contribute to the formation of the cadherin homophilic bond, and specific one-to-one interaction between particular EC domains may not be required. These conclusions are consistent with a previous study on direct molecular force measurements between cadherin ectodomains demonstrating multiple adhesive interactions (Sivasankar, S., W. Brieher, N. Lavrik, B. Gumbiner, and D. Leckband. 1999. *Proc. Natl. Acad. Sci. USA.* 96:11820–11824; Sivasankar, S., B. Gumbiner, and D. Leckband. 2001. *Biophys J.* 80:1758–68). We propose new models for how the cadherin extracellular repeats may contribute to adhesive specificity and function.

Introduction

Cadherin-mediated cell–cell adhesion is essential for the morphogenesis of tissues and the maintenance of tissue function (Takeichi, 1995; Gumbiner, 1996). Adhesion results from the homophilic binding between extracellular domains of cadherins, which is controlled by the cytoplasmic domain and associated catenin polypeptides and the actin cytoskeleton. Cadherins make up a family of adhesion molecules, and the type of cadherin expressed in a cell can affect the specificity (Nose et al., 1990; Takeichi, 1995; Gumbiner, 1996) as well as the physiological properties (Levine et al., 1994; Kim et al., 2000) of cell interactions. Cadherin adhesive activity is also regulated by cytoplasmic signaling events, via the catenins and cytoplasmic domain. Ultimately, regulation of adhesion is mediated through the homophilic binding function of the extracellular domain, by modulation of either its binding strength or by its clustering (Yap et al., 1997). Therefore, an understanding of the molecular structure of the cadherin homophilic bond is fundamental to understanding the mechanism of cadherin-mediated adhesion, the specificity of adhesion, and the regulation of adhesion during tissue morphogenesis.

Recent findings about the structure of the cadherin extracellular domain have provided important clues about the molecular nature of the homophilic bond. Particularly important are the findings that the cadherin ectodomain forms a parallel, or cis, dimer that is required for homophilic bind-

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ing and cell adhesion (Shapiro et al., 1995; Brieher et al., 1996; Chitaev and Troyanovsky, 1998; Takeda et al., 1999; Shan et al., 2000). The extracellular domain of classical cadherins consists of five cadherin repeats, or extracellular cadherin (EC)* domains. The three-dimensional structure of the NH_2-terminal EC domain (EC1) of N-cadherin determined by x-ray crystallography revealed an important element of dimerization, called the strand dimer (Shapiro et al., 1995). This parallel cis-dimer forms by reciprocal binding of the trp2 residue of each subunit in a hydrophobic pocket on the other subunit of the dimer, and the trp2 residue is crucial for cis-dimerization and adhesive function (Chitaev and Troyanovsky, 1998; Tamura et al., 1998; Shan et al., 2000). (An alternative model for cis-dimerization between EC1-2 domains has also been proposed [Nagar et al., 1996; Pertz et al., 1999].) Ca^{2+} is also required for cadherin function, and in the presence of Ca^{2+}, the cadherins form protease-resistant elongated rod structures (Hyafil et al., 1981; Takeichi, 1991; Pokutta et al., 1994; Sivasankar et al., 1999). The three-dimensional x-ray structures of fragments containing EC domains 1 and 2 reveal that Ca^{2+}-binding sites link successive domains together in a fixed orientation (Nagar et al., 1996; Tamura et al., 1998). Thus, the basic structural unit capable of making a homophilic bond between cells appears to be a parallel dimer, mediated by EC1, of two rigid rod-like cadherin ectodomains.

The molecular structure of the homophilic bond is much less well understood. In particular, the identity of the actual binding site(s) for the homophilic interaction remains uncertain. Most studies have focused exclusively on the EC1 domain, mostly because of an elegant early study that attributed the specificity of adhesion to this domain (Nose et al., 1990). However, direct attempts to identify a specific homophilic binding site in EC1 have not been conclusive. The HAV sequence conserved in many cadherin EC1 domains was initially proposed to be a critical part of the binding site (Blaschuk et al., 1990; Nose et al., 1990; Williams et al., 2000), analogous to the role of the RGD sequence integrin-binding substrates. However, unlike the RGD sequence, the HAV sequence does not form a specific loop or pocket typical for a binding site; indeed, the ala residue is not even on the surface of EC1 (Shapiro et al., 1995). Moreover, type II cadherins have a different sequence at this site, QAI, and mutation of either the HAV or QAI residues does not affect either homophilic binding or cadherin specificity (Shimoyama et al., 1999; Kitagawa et al., 2000). The x-ray analysis of N-cadherin EC1 did reveal an antiparallel crystal packing interaction between subunits, which was interpreted to represent homophilic binding (Shapiro et al., 1995). However, the antiparallel packing interaction was quite different for crystals of the two domain fragment, EC1-2, of N-cadherin (Tamura et al., 1998). Moreover, mutagenesis of many residues at the surface of EC1 has failed to reveal a role in cell adhesion (Kitagawa et al., 2000; Shimoyama et al., 1999), in contrast to the striking effects of mutating the trp2 that forms the parallel cis-dimer (Chitaev and Troyanovsky, 1998; Tamura et al., 1998; Shan et al., 2000). Thus, direct evidence for a specific homophilic binding site in EC1 remains elusive, and the role of EC1 in determining cadherin specificity needs to be reconsidered, especially in light of the importance of EC1 in establishing the lateral/cis-dimers required for adhesion.

Indeed, several findings in the literature provide evidence that the rest of the cadherin EC domain has a function in adhesion beyond serving as a simple spacer region. Although some adhesion blocking antibodies have been found to bind to EC1 (Nose et al., 1990; Amagai et al., 1992), many adhesion blocking antibodies and an adhesion activating antibody have been found to recognize other EC domains, including EC5 (Ozawa et al., 1990b; Zhong et al., 1999) and EC3 (mAb 6B6; unpublished data). In addition, naturally occurring missense mutations in EC2 and EC3 domains of E-cadherin have been found in several human tumors (Berx et al., 1998a,b), and mutations in one Ca^{2+}-binding site of E-cadherin between EC domains abolish adhesive function (Ozawa et al., 1990a). Furthermore, a biophysical study measuring direct molecular forces between cadherin ectodomains found evidence for multiple adhesive interactions, with maximal adhesive force developing when the ectodomains domains overlap entirely (Sivasankar et al., 1999, 2001). Although none of these studies identified specific binding sites, they do suggest that cadherin EC domains other than EC1 play important roles in the homophilic binding interactions between cadherin cis-dimers.

In a previous study, we were able to express and analyze the biochemical and homophilic binding properties of the entire soluble ectodomain of *Xenopus* C-cadherin, CEC1-5, which exhibited functional activity only when dimeric (Brieher et al., 1996). This provided a starting point to begin to analyze the roles of all the cadherin EC domains in the homophilic binding function of the cadherin ectodomain. We have undertaken a systematic structure–function analysis of CEC1-5 using deletions of specific EC domains and assays for homophilic binding activity.

Results

Analytical centrifugation

In a previous study of the purified soluble ectodomain of C-cadherin, CEC1-5, lateral dimerization was shown to be required for the homophilic binding activity (Brieher et al., 1996). However, the conditions for CEC1-5 dimerization were not well defined. Moreover, it has not always been possible to detect dimers of other soluble cadherin ectodomains (Pokutta et al., 1994; Tamura et al., 1998). Therefore, we wished to determine whether dimers of CEC1-5 exist in dynamic equilibrium with monomers and to measure the affinity of the dimer interaction. Furthermore, we wished to determine whether the formation of higher order oligomeric species of CEC1-5, which would result from homophilic adhesive binding interactions between dimers, could be detected. To measure these interactions in solution, equilibrium sedimentation analysis was performed using the analytical ultracentrifuge.

The stock CEC1-5 solution had an absorbance of 1.4975 at 280 nm and a concentration determined by fringe count of 2.12 mg/ml, resulting in a calculated $E_{280nm}^{1\,cm}$ of 0.706 mg/ml.

Calculated apparent weight average molecular weights

*Abbreviation used in this paper: EC, extracellular cadherin.

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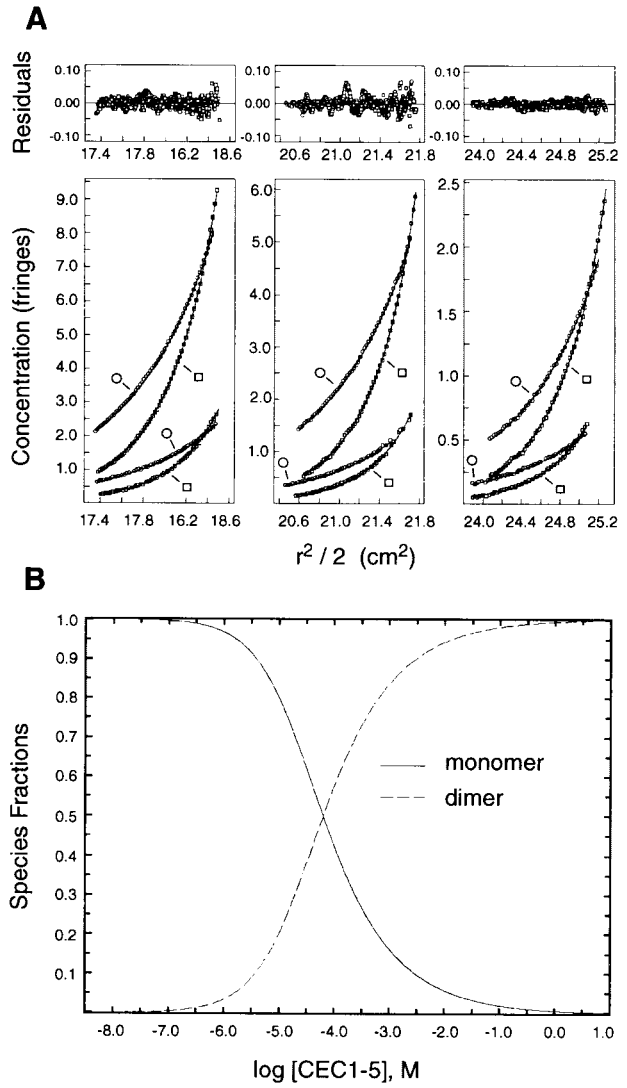

Figure 1. **Sedimentation equilibrium analysis of the soluble C-cadherin ectodomain (CEC1-5).** (A) Sedimentation equilibrium data. (Bottom) Show global fit of data collected at six loading concentrations ranging from 2–25 μM and rotor speeds of 10,000 rpm (○) and 14,000 rpm (□) to a monomer–dimer self association model. Symbols represent measured data points, and solid lines represent theoretical fits to the model. (Top) Illustrates the deviations of the measured points from the theoretical fit lines. (B) Plots illustrating expected fractions of monomer (solid line) and dimer (dashed line) at different CEC1-5 concentrations, calculated using the *Kd*(1-2) of 64 μM determined by the global fit of the sedimentation equilibrium data to a monomer–dimer association model.

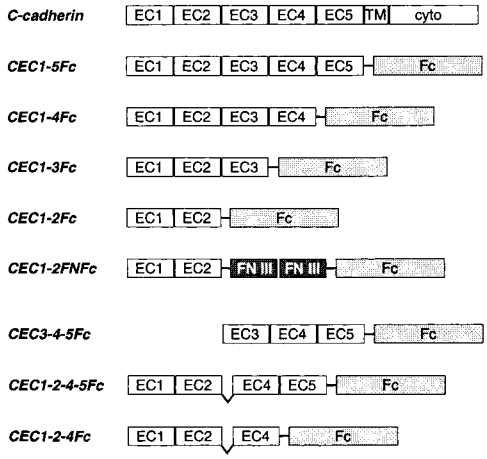

Figure 2. **Schematic representation of *Xenopus* C-cadherin and the chimeric Fc fusion proteins.** Full-length C-cadherin molecule consists of the ectodomain (five EC domains), transmembrane region (TM) and the cytoplasmic tail (CP). CEC1-5Fc consists of the ectodomain fused to the Fc part of the human IgG (Fc). Domains were expressed as Fc chimaeras to force dimerization, since dimerization of C-cadherin was shown to be crucial for adhesive function. CEC1-4Fc, CEC1-3Fc, and CEC1-2Fc consist of successively fewer number of cadherin repeats fused to Fc at the COOH terminus. CEC3-4-5Fc consists of the ectodomain deleted from the NH_2-terminal region fused to Fc at the COOH terminus. CEC1-2FNFc consists of the first two domains fused to the two fibronectin type III repeats of the chicken N-CAM (FN III), still having Fc as the COOH-terminal region. CEC1-2-4Fc consists of successively domains 1-2 and 4 fused to Fc at the COOH terminus and on the same scheme CEC1-2-4-5Fc of domains 1-2 and 4-5.

from individual sedimentation equilibrium data sets collected over a loading concentration range of 2–25 μM varied from 78,570 to 102,660, whereas an apparent weight average molecular weight of 88,850 was determined from a global fit of all the data sets to a single species model. The best global fit was obtained for a monomer–dimer self-association model, using an assumed value of 75,000 for the monomer molecular weight, and allowing the *Ka* for each data set to float (Fig. 1 A). There was no obvious concentration-dependent trend in the determined Kas for the various data sets, which would have indicated possible heterogeneity or non-specific aggregation. Averaging all the individual raw *Ka* values resulted in a calculated Molar *Kd*(1-2) of 64 μM. Using this value, the CEC1-5 appears to consist of ~5–30% dimer in the concentration range at which the measurements were performed (Fig. 1 B).

The lack of evidence for any higher oligomeric species, which might result from homophilic binding between dimers, at the concentrations of protein used indicates that any potential binding between dimers could only occur with a significantly lower affinity than the monomer–dimer affinity (i.e., with a $Kd \gg 64$ μM). Thus, the formation of adhesive bonds between cadherin dimers may involve multivalent low affinity interactions (see Discussion), and an analysis of the homophilic binding properties of CEC1-5 or domains of CEC1-5 requires the use of techniques that can assay this multivalent binding activity.

Expression and purification of cadherin–Fc fusion proteins

To examine the contribution of the different extracellular (EC) cadherin domains of C-cadherin, a series of C-cadherin mutants were designed (Fig. 2). First, we sequentially deleted the EC domains from the COOH terminus according to the described sequence of C-cadherin (Lee and Gumbiner, 1995) and the structures of the cadherin repeats observed by x-ray crystallography (Shapiro et al., 1995; Nagar et al., 1996; Tamura et al., 1998). After analyzing the first constructs, we decided to make additional deletion constructs also shown in Fig. 2. Previous studies on the soluble

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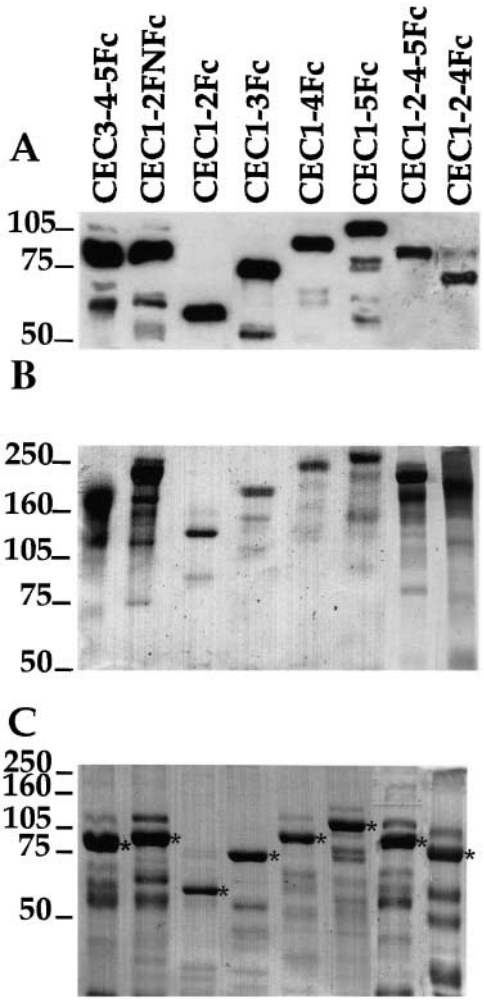

Figure 3. **Expression and purification of the mutant cadherin proteins secreted from transfected CHO cells.** (A) Western blotting of purified proteins with an anti–human Fc; (B) Coomassie staining of purified proteins on a non reducing gel; (C) Coomassie staining of purified proteins separated by an 8% reducing gel. *Indicate processed mature full-length protein as confirmed by NH_2-terminal sequencing.

C-cadherin ectodomain showed that dimerization was necessary for adhesive function (Brieher et al., 1996). During initial attempts to express C-cadherin with EC domain deletions, it was difficult to obtain active dimeric forms (not shown); therefore, chimeras having the IgG Fc domain (Fc) fused to the COOH terminus were constructed to force dimerization (the IgFc domain forms parallel stable disulfide-linked dimers). A similar approach has been used to produce functional soluble dimers of N-cadherin and VE–cadherin (Baumgartner et al., 2000; Lambert et al., 2000) and human E-cadherin (unpublished data). We also made a construct in which a linker was inserted between the COOH terminus of EC1-2 and the IgFc domains, CEC1-2FNFc (Fig. 2). This linker consists of the two fibronectin-like domains founded in the extracellular domain of the chicken N-CAM, each of which is similar in size and in shape to the EC domains and have not been found to have any kind of adhesive activity (Cunningham et al., 1987; Ranheim et al., 1996). Additionally, constructs were also made with deletions of the either the first two NH_2-terminal domains (CEC3-4-5Fc) or a deletion of domain 3 (CEC1-2-4Fc and CEC1-2-4-5Fc). We also tried to make a construct having only the EC1 domain fused to Fc (CEC1Fc), but it was poorly expressed and could not be recovered in reasonable quantities.

Proteins were stably expressed in CHO cells and were purified from conditioned media on Protein A column. These polypeptides are recognized by an anti–human Fc antibody, demonstrating that the Fc part of the IgG is present (Fig. 3 A), and by anti–C-cadherin antibody (not shown). Assuming these proteins are modified by glycosylation or other posttranslational modifications (Lee and Gumbiner, 1995), the molecular weights are the expected sizes for the mature secreted proteins. We also made sure that those proteins were dimeric by running them on a nonreducing gel (Fig. 3 B). Between 1 and 2 mg of each purified protein were obtained from two liters of conditioned media as determined by Coomassie staining (Fig. 3 C). Minor bands of higher and lower molecular weight than mature full-length protein probably correspond to precursor forms and breakdown products, respectively, since they are recognized by anti–human Fc and by anti–C-cadherin antibodies.

Cadherins are synthesized with a large proregion that is normally proteolytically cleaved to yield the mature cadherin, and functional activity depends on the precise cleavage at the correct amino acid (Ozawa and Kemler, 1990). To make sure that CHO cells processed these proteins to the correct mature form, NH_2-terminal sequencing of each purified protein was performed. The majority of the chimeric proteins (CEC1-5Fc, CEC1-4Fc, CEC1-3Fc, CEC1-2Fc, CEC1-2FNFc, CEC1-2-4Fc, and CEC1-2-4-5Fc) were processed correctly to yield the appropriate NH_2-terminal residue of EC1. However, the CEC3-4-5Fc protein contained a mixture of three proteins; one was cleaved at the proper site, and two were cleaved at different sites within the proregion. This construct is the only one lacking the NH_2-terminal EC1 domain; presumably, the proper connection of the proregion to this domain is important for effective processing of the protein.

In all of the following experiments, we tested proteins produced by at least two different clones of secreting cells for each chimera and several protein preparations from each clone.

Analysis of the EC domain deletions by bead aggregation and cell adhesion assays

The low affinity of the homophilic binding interaction between cadherin dimers requires that assays for multivalent interactions are used to analyze the binding properties of deletion mutants. One such assay that has been frequently used for the analysis of cell adhesion molecules is a bead aggregation assay, which provides an in vitro mimic of cell aggregation assays for adhesion using purified proteins (Grumet and Edelman, 1988; Grumet et al., 1993; Ranheim et al., 1996; Retzler et al., 1996; Lambert et al., 2000). Indeed, bead aggregation can be used as a specific measure of calcium-dependent homophilic binding activity of the extracellular domain of C-cadherin (CEC1-5) (Brieher et al., 1996). Therefore, this assay was used to test the capacity of various deletion mutant proteins to mediate homophilic binding.

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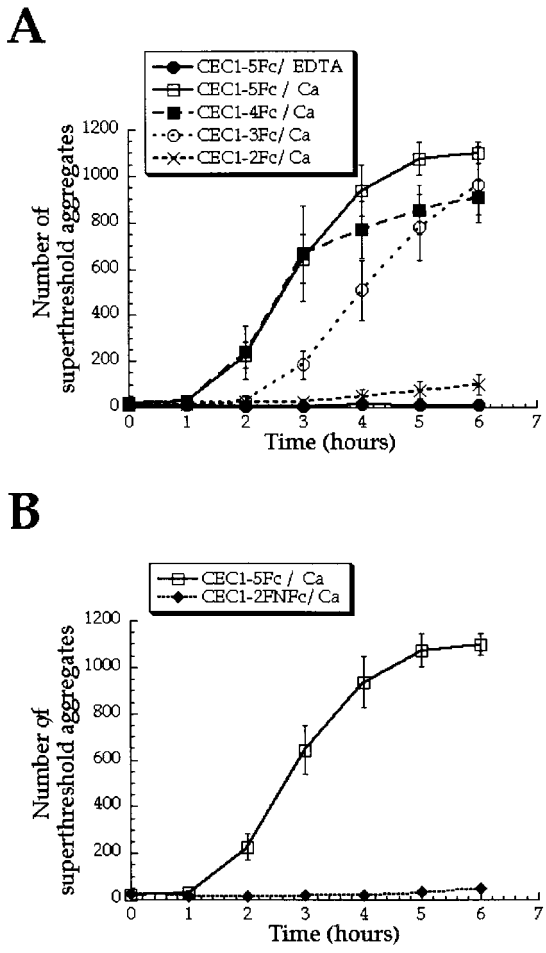

Figure 4. **Basic homophilic binding activity of cadherin mutants assessed by bead aggregation assay.** (A) Full-length and COOH-terminal EC domain deletions: CEC1-5Fc, CEC1-4Fc, CEC1-3Fc, and CEC1-2Fc. (B) Analysis of CEC1-2 with spacers inserted: CEC1-2FNFc compared to CEC1-5Fc. The number of aggregates of coated microspheres large enough to be detected by a Coulter counter is plotted as function of time. Samples were incubated in the absence of calcium (EDTA) or in the presence of calcium (Ca). The experiment was performed with at least three different batches of protein and the mean ± SEM is shown.

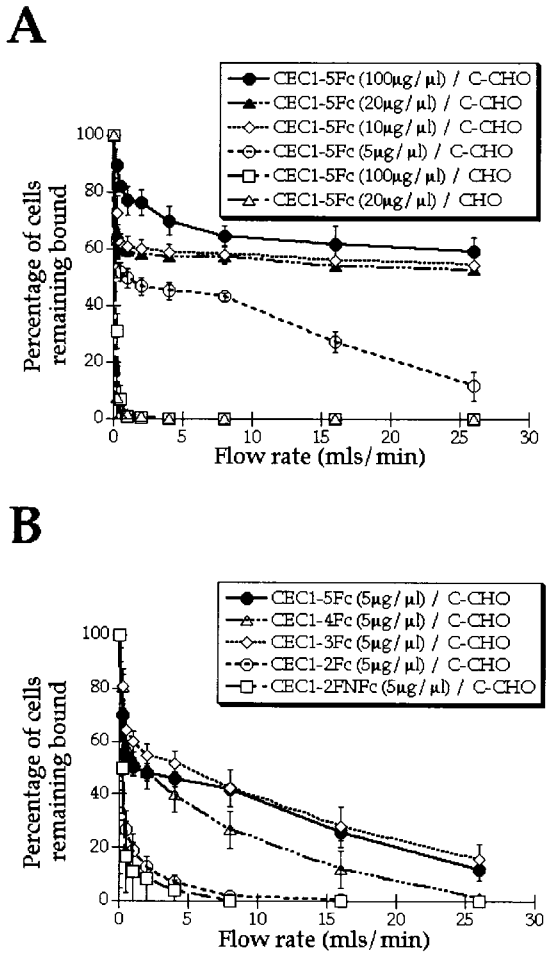

Figure 5. **Adhesive activity of C-cadherin mutants assessed by a cell detachment assay.** The adhesive strength is measured by the resistance of cell detachment under a laminar flow from surfaces coated with chimeric proteins. (A) Adhesion of CHO cells expressing C-cadherin (C-CHO cells) to CEC1-5Fc at different concentrations of CEC1-5Fc (100, 20, 10, and 5 μg/μl). The construct was attached to the tube surface via protein A, and the cells were allowed to bind to the substrate under static conditions. The flow was subsequently increased every 30 s, and the number of cells remaining within the field of view was counted. Assays were performed in the presence of calcium using C-CHO cells or control CHO cells. (B) Adhesion of C-CHO cells to surfaces coated with CEC1-5Fc, CEC1-4Fc, CEC1-3Fc, CEC1-2Fc (two different clones), and CEC1-2FNFc, all at 5 μg/μl. The experiments were performed in triplicate and the mean ± SEM is shown.

The COOH-terminal IgFc domain allowed us to use protein A–coated beads in order to orient the chimeric proteins on the beads. The full-length cadherin ectodomain, CEC1-5Fc, induced aggregation of beads (Fig. 4 A), similar to CEC1-5, as described previously (Brieher et al., 1996). Thus, addition of Fc to the COOH-terminal EC domain did not interfere with adhesive function of full-length C-cadherin. Aggregation of the coated beads was specific and dependent on cadherin activity, because CEC1-5Fc–coated beads failed to aggregate in the absence of calcium (Fig. 4 A, +EDTA) and was specifically inhibited by anti–C-cadherin mAb 6B6 (not shown).

We then tested the other chimeric proteins. CEC1-4Fc and CEC1-3Fc induced calcium-dependent aggregation of beads quite effectively (Fig. 4 A) and aggregation was specifically inhibited by anti–C-cadherin mAb 6B6 (not shown). Therefore, domains 4 and 5 do not seem to be essential for basic homophilic binding. They may enhance aggregation activity somewhat because aggregation mediated by CEC1-3Fc was not quite as effective as CEC1-5Fc. In contrast, CEC1-2Fc did not stimulate high rates of bead aggregation compared with CEC1-3Fc, CEC1-4Fc, and CEC1-5Fc, although it did over background levels (Fig. 4 A), suggesting that domains EC1 and EC2 are not sufficient for effective aggregation activity.

It was possible that the loss of bead aggregation activity by CEC1-2Fc was simply due to the lack of a spacer region necessary to provide sufficient distance from the bead surface or due to conformational constraints on the normal dimerization of domains 1 and 2 forced by a proximal Fc dimer. Therefore, a spacer consisting of the two fibronectin-like do-

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mains of the chicken N-CAM (similar in size and folding to two EC domains) was inserted in frame between the COOH-terminal part of domain 2 and the Fc part of the IgG. Like CEC1-2Fc, CEC1-2FNFc failed to induce strong aggregation of beads (Fig. 4 B). Therefore, EC1 and EC2 domains alone do not seem to be sufficient for effective homophilic binding activity.

The in vitro bead aggregation assay analyzes the basic binding activity of each of the dimeric proteins. We also wished to determine the abilities of these proteins to mediate cell adhesion. We have previously described a flow assay that measures the strength of cell attachment under shear forces. This assay measures the capacity of CHO cells expressing full-length wild-type C-cadherin (C-CHO cells) to adhere to surfaces coated with different chimeric proteins. Similar to the CEC1-5 protein described previously (Brieher et al., 1996), the full-length Fc chimera (CEC1-5Fc) mediated strong adhesion of C-CHO cells (Fig. 5 A). Adhesion to CEC1-5Fc was specific because it required calcium (not shown) and because CHO cells not expressing C-cadherin did not adhere, even at the lowest shear stress (Fig. 5 A). Additionally, adhesion of C-CHO cells to CEC1-5Fc was inhibited by incubating the cells with Fab fragments of an anti–C-cadherin mAb, 6B6 (data not shown), confirming that the adhesive interaction between C-CHO cells and these substrates are C-cadherin specific.

The conditions of the flow assay were optimized to make the measured range of adhesion strengths sensitive to the adhesive activity of the chimeric protein coated on the substrate. The resistance of cell detachment to increasing shear force was determined as a function of the concentration of CEC1-5Fc coated on the substrate (Fig. 5 A; see Materials and methods). At high concentrations of CEC1-5Fc (100, 20, and 10 μg/ml) cells remained strongly attached over the entire range of shear forces used. Only at 5 μg/ml did cells exhibit sensitivity to detachment at high shear force; therefore, a concentration of 5 μg/ml was used for all of the chimeric proteins to test their cell adhesion activities.

CEC1-4Fc and CEC1-3Fc exhibited similar adhesive activity as CEC1-5Fc in the flow assay, having a similar resistance of cell detachment as a function of increasing shear force (Fig. 5 B). This suggests that domains 4 and 5 are not essential for strong adhesion, similar to the bead aggregation assay. In contrast, CEC1-2Fc exhibited significantly weaker adhesive activity compared with the longer proteins, suggesting that EC1 and EC2 are not sufficient for full adhesive activity. Addition of the spacer domain in CEC1-2FNFc did not increase the adhesive activity of EC1-2. Thus, although domains 1 and 2 retain low levels of adhesive activity, they are not sufficient to mediate the high level of cell adhesion activity exhibited by the full-length protein.

The significantly greater bead aggregation and cell adhesion activities of CEC1-3Fc compared with CEC1-2Fc or CEC1-2FNFc could have several different explanations. EC3 alone may possesses significant homophilic binding activity; three EC domains could be required for high binding activity; EC1 or EC2 (or both) of one cadherin in the pair might need to bind to EC3, EC4, or EC5 of the other cadherin. Several experiments were designed to try to distinguish between these possibilities.

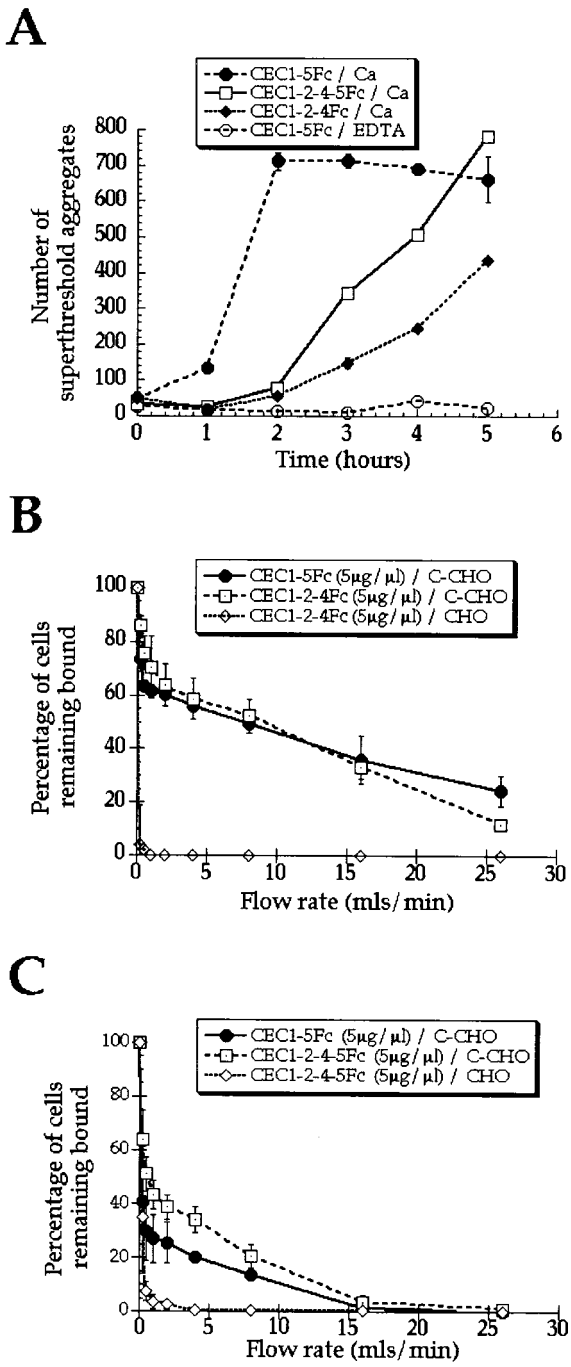

Figure 6. **Homophilic binding and cell attachment activities of constructs lacking the EC3 domain: CEC1-2-4Fc and CEC1-2-4-5Fc.** (A) Bead aggregation assay using a Coulter counter as described in the legend to Fig. 4. (B and C) Analysis of adhesion to chimaeric proteins using the laminar flow assay as described in Fig. 5. Adhesion of C-CHO cells to CEC1-2-4Fc (B) and CEC1-2-4-5Fc (C) compared with CEC1-5Fc, all at 5 μg/μl. The experiments were performed in triplicate and the mean ± SEM is shown.

To determine whether EC3 is specifically required for effective homophilic binding, we made two constructs with EC3 deleted: CEC1-2-4-5Fc and CEC1-2-4Fc. Both were able to induce a high rate of bead aggregation (Fig. 6 A), which was inhibited by anti–C-cadherin Fab (not shown). The rates of aggregation were similar to the activity of

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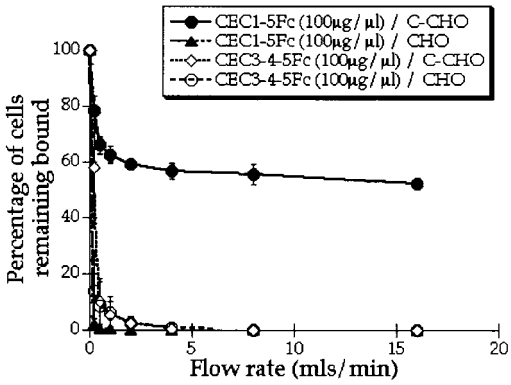

Figure 7. **Lack of adhesion activity of a construct lacking EC domains 1 and 2 (CEC3-4-5Fc) by laminar flow assay.** Attachment of C-CHO cells to high concentrations of CEC3-4-5Fc (100 μg/μl) compared with CEC1-5Fc. The experiment was performed in triplicate and the mean ± SEM is shown.

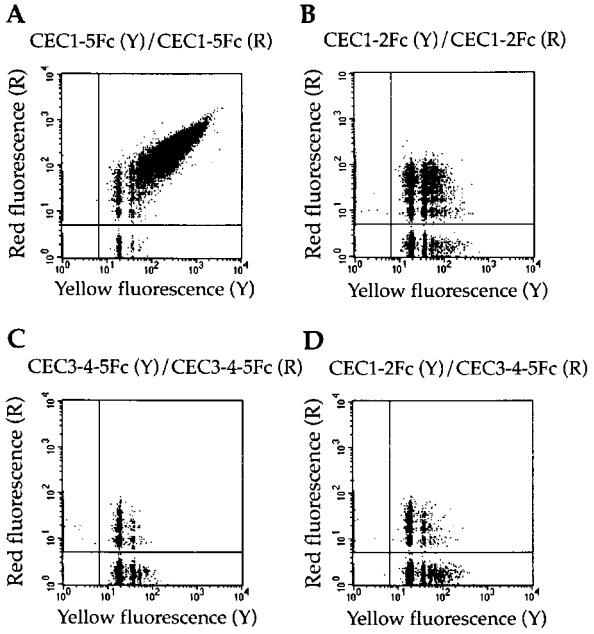

Figure 8. **Mixed bead aggregation assay to assess homophilic binding activity between different cadherin mutants.** Flow cytometry was used to detect and quantify mixed aggregates formed between yellow fluorescent beads (Y) and red fluorescent beads (R) coated with different cadherin EC constructs. Mixed aggregates appear in the region to the right of and above the lines drawn on the graph. (Yellow only singlets and small aggregates appear in the lower right region, but red only singlets and small aggregates do not appear on the graph because they lie on the y axis.) (A) Analysis of aggregation between CEC1-5Fc on both sets of beads. (B) Analysis of aggregation between CEC1-2Fc on both sets of beads. (C) Analysis of aggregation between CEC3-4-5FC on both sets of beads. (D) Analysis of aggregation between CEC1-2Fc–coated yellow beads and CEC3-4-5FC–coated red beads.

CEC1-3Fc and significantly greater than aggregation due to CEC1-2Fc. Also, both CEC1-2-4-5Fc and CEC1-2-4Fc exhibited high cell adhesion activity in the laminar flow assay, similar to the activity of CEC1-5Fc (Fig. 6, B and C) and much better than CEC1-2Fc. Therefore, EC3 is not specifically required for effective homophilic binding or cell adhesion. Furthermore, the high binding and adhesion activity of CEC1-2-4Fc demonstrates that EC4 is interchangeable with EC3. Thus, there may not be any defined specificity to the binding interactions between EC domains, raising the possibility that multiple interactions occur in the homophilic bond.

Although EC1 and EC2 are not sufficient for effective binding activity, we wanted to test whether they are required. Therefore, we analyzed whether a construct lacking domains 1 and 2, CEC3-4-5Fc, retains bead aggregation and cell adhesion activity. Most preparations of CEC3-4-5Fc (70%) failed to induce detectable bead aggregation (not shown). In ~30% of the preparations, there was some evidence of aggregation, but it was highly variable and irreproducible from day to day. Furthermore, CEC3-4-5Fc never exhibited detectable cell adhesion activity in the flow assay (Fig. 7), irrespective of the preparation or day of experiment. The lack of activity in the cell adhesion assay probably cannot be attributed to the variability in the NH_2-terminal propeptide cleavage that we observed because there was no detectable adhesion even at very high concentrations of the protein (100 μg/μl), which is 20 times more than needed for strong adhesion to CEC1-3Fc or for low but detectable adhesion to CEC1-2Fc. Thus, even if domains EC3, EC4, and EC5 possess some binding activity, domains 1 and 2 appear to be required for effective homophilic binding and cell adhesion. The requirement for EC1 and EC2 could be due either to the presence of a critical binding site in one or both of these two domains or to the requirement for the EC1 domain in the formation of normal cadherin cis-dimers (Shan et al., 2000). Although CEC3-4-5Fc dimerize through the COOH-terminal Fc domain in the absence of EC1, it may be ineffective in creating the proper protein conformation and/or dimeric binding interface.

Since domains 1 and 2 are required but not sufficient for homophilic binding activity, it is possible that domains 1 and 2 need to bind to EC domains 3, 4, or 5 in the full C-cadherin ectodomain. To try to test this possibility, bead mixing experiments were performed. A flow cytometry assay with different color fluorescent beads (yellow and red) was used to determine whether CEC1-2Fc–coated beads and CEC3-4-5Fc–coated beads aggregate better with each other than they do by themselves (Fig. 8; Table I). A positive control for the assay is shown by an analysis of mixed aggregates formed by two sets of beads coated with full-length C-cadherin (CEC1-5Fc) in Fig. 8 A. Aggregates containing both fluorescent colors (yellow and red) appear along the diagonal of the fluorescence intensity graph. The formation of mixed aggregates was quite extensive at this time in the assay, since each point on the graph is a single fluorescent event that corresponds to a single aggregate, each of which can contain a large number of beads. For CEC1-5Fc, >90% of the detected events and ~790,000 beads are present in mixed aggregates (Table I). As expected, mixed aggregates between two sets of beads, which both contained CEC1-2Fc, were smaller and fewer (Fig. 8 B). Only 27% of the events contained mixed aggregates (i.e., 73% were either single beads or small unmixed aggregates), with only ~28,000 beads present in mixed aggregates (Table I). The negative control (i.e., background) is shown by analysis of CEC3-4-5Fc by itself, which formed even fewer and smaller mixed aggregates

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Table I. **Quantitation of mixed bead aggregation assay assessed by flow cytometry**

	Percentage events that are mixed aggregates	Total no. of beads in the mixed aggregates
CEC1-5Fc (Y)/CEC1-5Fc (R)	91.36	793,474
CEC1-2Fc (Y)/CEC1-2Fc (R)	27.11	28,145
CEC3-4-5Fc (Y)/CEC3-4-5Fc (R)	9.52	4,540
CEC1-2Fc (Y)/CEC3-4-5Fc (R)	7.68	4,951
CEC3-4-5Fc (Y)/CEC1-2Fc (R)	9.62	6,749
CEC1-2Fc (Y)/CEC1-2Fc (R)	27.11	28,145

Data were derived from the experiment shown in Fig. 8. The percentage of fluorescent events present in the mixed aggregate region (Fig. 8) and the total number of beads present in the mixed aggregates was calculated.

(Fig. 8 C), with <10% of events in mixed aggregates (i.e., >90% single or small unmixed) and <5,000 beads present in mixed aggregates (Table I). The experimental analysis of the mixing between CEC1-2Fc beads and CEC3-4-5Fc beads is shown in Fig. 8 D (one case) and Table I (both the case in Fig. 8 D and the reciprocal mixture). These samples formed mixed aggregates no better than CEC3-4-5Fc alone, and worse than CEC1-2Fc alone. Therefore, using this assay for mixed aggregation, it was not possible to detect a direct binding interaction between EC domains 1-2 and domains 3, 4, and 5.

At face value, the above finding may seem to show that EC domains 1 and 2 do not interact with EC domains 3, 4, and 5 in the C-cadherin homophilic bond. However, there are alternative explanations for the absence of mixed aggregates between beads coated with these deletion mutant proteins. It is possible that the lack of dimerization at the NH_2 terminus, which is normally provided by EC1, renders CEC3-4-5Fc aggregation incompetent towards all potential binding partners. Alternatively, effective and strong homophilic binding may require complete reciprocal binding interactions between binding partners (e.g., for the full-length protein, EC1-2 domains of molecule A binds EC3-4-5 domains of molecule B, plus EC1-2 domains of molecule B binds EC3-4-5 domains of molecule A). In contrast, the potential bond between CEC1-2Fc and CEC3-4-5Fc could only engage one half of the reciprocal binding interaction (although, EC1-2 of molecule A could bind EC3-4-5 of molecule B, there would be no EC1-2 of molecule B or EC3-4-5 of molecule A available to interact). Although the contribution of binding reciprocity to the rate of bead aggregation is not known, reciprocal binding interactions between proteins in solution are known to result in a highly synergistic increase in affinity. Unfortunately, therefore, for homophilic binding proteins there is a theoretical limitation to interpreting mixing experiments between proteins with different binding site deletions.

Discussion

A thorough structure–function analysis of the homophilic binding properties of the soluble C-cadherin ectodomain reveals that multiple cadherin EC repeats contribute to a low affinity interaction between cadherin cis-dimers. Although the EC1 domain appears to be required for the formation of an effective adhesive bond, perhaps due to its role in cis-dimerization, it cannot account for the entire homophilic

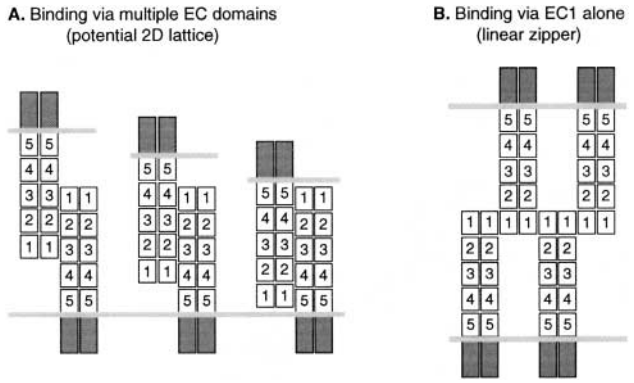

Figure 9. **Model of adhesive bond formation via the extracellular cadherin repeats.** (A) Binding via multiple EC domains, as demonstrated in this study. If binding sites have different orientations, cadherin dimers could form two-dimensional lattices (not shown). (B) Linear zipper model for the homophilic bond via the EC1 domains alone, as proposed in Shapiro et al. (1995).

binding interaction as has been previously believed. A minimum of three of the EC domains is required for effective homophilic binding and adhesion, since domains EC1-2 are not sufficient. Although domains EC4 and EC5 do not seem to be absolutely required, they can contribute to the binding interaction. CEC1-4Fc and CEC1-5Fc do exhibit a somewhat better binding activity than CEC1-3Fc. Moreover, EC3 is not specifically required for binding, and EC4 is able to substitute for EC3, since CEC1-2-4-5Fc and CEC1-2-4Fc have high binding and adhesion activity. Together, these findings suggest that the homophilic bond formed between cadherins involves extensive overlap between the extracellular domains and may arise from multiple interactions or different combinations of interactions between EC domains (Fig. 9 A).

The homophilic binding interaction between individual cadherin cis-dimers appears to be of very low affinity, supporting the notion that multivalent interactions via a large number of cadherin dimers is required for the formation of the adhesive bond. Sedimentation analysis of purified CEC1-5 reveals only a monomer-to-dimer interaction. Of course, sedimentation analysis by itself cannot distinguish between cis- or trans-dimer interactions. However, previous work on CEC1-5 showed that this same dimer is required to mediate strong bead aggregation and adhesion; the monomeric species has no or little activity (Brieher et al., 1996). Moreover, we find that forcing parallel cis-dimerization through the COOH-terminal Fc domain results in molecules with similar adhesion activity as CEC1-5, and Fc-mediated dimerization was required for the adhesive activity of deletion constructs. The measured affinity of the monomer–dimer equilibrium of 64 μM should not be taken too literally, since anchorage of normal cadherins in the plasma membrane is likely to increase the effective affinity of cis-dimerization. Nonetheless, the lack of any detectable higher oligomeric species indicates that any interaction between cis-dimers will have a *Kd* significantly higher than 64 μM.

The concept that cadherin-mediated cell adhesion involves multivalent low affinity interactions is supported by other observations. Deletion of the cytoplasmic domain results in a

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cadherin with very poor adhesive activity even when it is expressed at high levels at the cell surface. Forced clustering of the cadherin into patches through an artificial oligomerization domain independent of any interactions with the actin cytoskeleton resulted in significant strengthening of adhesion (Yap et al., 1997). Also, the measurement of the trans-interaction between dimers of VE–cadherin by atomic force microscopy suggested a low affinity reaction ($Kd = 10^{-3}$–10^{-5} M) (Baumgartner et al., 2000). Similarly, induction of integrin clustering, resulting from enhanced membrane mobility, is thought to underlie integrin activation in lymphocytes (Dransfield et al., 1992; Stewart and Hogg, 1996; Yauch et al., 1997; Bazzoni and Hemler, 1998). There are, however, some reports of potentially higher affinity binding interactions between cadherins, including electron microscopic detection of interactions between pentameric forms of E-cadherin (Tomschy et al., 1996) and the detection of interactions between cadherins present in neighboring cells by immunoprecipitation (Chitaev and Troyanovsky, 1998; Shan et al., 2000). The reason for this difference is not clear, but the actual molecular nature of the interacting cadherin pentamers or the coimmunoprecipitated cadherins is not yet well established. Our direct analysis of the interactions between functionally active purified C-cadherin ectodomains, along with the demonstrated contribution of clustering to adhesion (Yap et al., 1997), lead us to favor a model for the cadherin adhesive bond involving multivalent low affinity homophilic interactions.

Our findings that the homophilic bond forms through the interactions of multiple EC domains is in agreement with a previous biophysical study of the adhesive forces that develop between opposing C-cadherin (CEC1-5)–covered lipid bilayers (Sivasankar et al., 1999, 2001). The surface force apparatus that was used allowed the measurement of both the magnitude of the forces that develop and the distance dependence of the forces between the full-length cadherin extracellular segments. The strongest interaction was detected when the antiparallel proteins were fully interdigitated, corresponding to extensive overlap involving multiple EC domains. Interestingly, two other weaker adhesive interactions were detected when the interdigitated proteins were separated by greater distances corresponding to additional EC domain lengths. The authors proposed a model for the cadherin adhesive bond in which successive rupture of distinct interactions along the length of the cadherin molecule occurs to impede the abrupt failure of cadherin-mediated contacts under the forces arising between cells.

Although our structure–function analysis demonstrates that the homophilic bond forms by the overlap/interaction of multiple EC domains, it has not been possible to discern exactly which specific EC domain interacts with which other EC domain in the bond, or even whether there are specific one-to-one domain interactions. Because EC1-2 is required for adhesive activity and exhibits only low levels of adhesive activity alone, it is possible that EC1 and EC2 preferentially bind to EC3, EC4, or EC5. We were not able to detect such preferential binding in bead mixing experiments, but this analysis may be limited by having only 1 of 2 complete binding partners in the assay. The fact that EC4 and EC5 are not essential for binding and adhesion might be taken to suggest that they do not participate in binding. However, both the somewhat higher aggregation activity when EC4 (or EC4 and EC5) is present, and the ability of EC4 to substitute for EC3 indicates that EC4 (and perhaps EC5) can participate in the formation of the bond. Indeed, the interchangeability of EC3 and EC4 suggests that the interactions between EC domains may not be entirely specific, and that the cadherins may be able to interact at multiple different sites or degrees of overlap (Fig. 9 A). Interactions at multiple sites would be consistent with the biophysical measurements by Sivasankar et al. (1999, 2001), showing that adhesive forces developed at multiple extents of overlap between cadherin on two surfaces.

Our findings challenge the prevailing model for the structure of the cadherin homophilic bond, which entails a direct interaction between EC1 domains at the distal tips of the cadherin molecules (Fig. 9 B) (Takeichi, 1995; Shapiro and Colman, 1998; Koch et al., 1999; Shan et al., 1999). In fact, direct binding between EC1 domains has never been demonstrated, nor has it ever been shown that EC1 alone is sufficient to form the homophilic binding site. Moreover, mutations in other EC domains of E-cadherin have been found associated with cancers and to affect adhesion (Ozawa et al., 1990a; Berx et al., 1998a,b), consistent with our findings of a requirement for additional EC domains in binding. Furthermore, the measurement of the adhesive force distance profile with the surface force apparatus did not reveal a detectable interaction when the distal EC1 domains were brought into proximity (Sivasankar et al., 1999, 2001). All of these findings together with our structure–function analysis of the C-cadherin ectodomain argue strongly against the prevailing model of adhesive binding exclusively via the EC1 domain.

The x-ray crystal structure of the EC1 domain of N-cadherin led to a very attractive model of the homophilic bond, called the zipper model (Fig. 9 B), which relies on the direct antiparallel adhesive interactions between EC1 domains (Shapiro et al., 1995). However, this putative adhesive interaction could have resulted from simple crystal packing interactions rather than true adhesive interactions, and other potential adhesive interactions could not have been observed, since the other EC domains were not present in the crystallized protein. Nonetheless, one important concept from the zipper model may still be important for the structure of the homophilic bond; the idea that cis-dimerization could endow the cadherin on one cell with more than one adhesive binding site. Indeed, there is now considerable evidence that cis-dimers form the basic adhesive unit. With multiple EC domains, the binding interactions of each cis-dimer could potentially occur in multiple orientations, leading to the formation of a two-dimensional lattice instead of a linear zipper. Such a two-dimensional lattice might be a more reasonable structure for a zone of adhesive contact or cell junction, and would be consistent with the concept of a multivalent low affinity interactions between cadherin dimers.

Until now, the strongest evidence that the homophilic binding site resides in EC1 came from the finding that the adhesion specificity is determined by EC1. Cells expressing either E-cadherin or P-cadherin sort out from each other in aggregation assays, and the analysis of E-cadherin/P-cadherin chimeras showed that sorting out was determined en-

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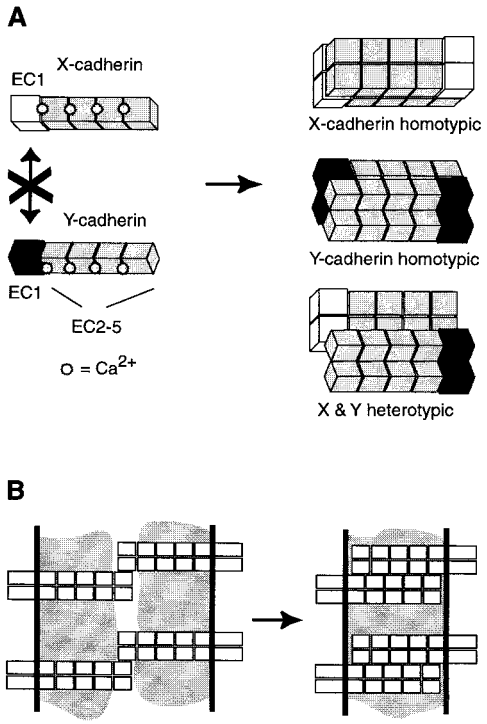

Figure 10. **Two hypothetical models for role of EC1 domain in determining cadherin binding specificity.** (A) Cis-dimerization specificity could influence adhesive binding specificity. Calcium binding causes the ectodomain to behave as a single structural unit. Therefore, alterations in the orientation of the EC1 domain dimerization interface are propagated to the binding sites throughout the rest of the EC domains. (B) An initial cadherin specific interaction between EC1 domains could precede the formation of the final homophilic bonds between the other EC domains. A repulsive barrier between cells would be postulated to prevent interactions between EC2-5 from occurring directly, and an initial weak binding between EC1 domains would lower the energy barrier leading to the final binding state.

tirely by the EC1 domain (Nose et al., 1988), and similar findings have been obtained more recently for E-cadherin and N-cadherin (Shan et al., 2000). However, alternate models for the role of EC1 in cadherin specificity are possible in light of more recent findings on cadherin structure and function. First, it should be recognized that many different pairs of cadherins fail to exhibit adhesion specificity, including some that have fairly different amino acid sequences (Volk et al., 1987; Steinberg and McNutt, 1999; Shimoyama et al., 2000; unpublished data), and the level of expression of a single cadherin may be a more important determinant of cell sorting specificity (Steinberg and Takeichi, 1994). In these cases, there is no need to postulate a significant specificity determining site in EC1. When specificity between cadherins is observed, the role of EC1 in determining specificity could be due to its role in the formation of cis-dimers. Indeed, in a recent study of E-cadherin/R-cadherin chimeras, EC1 was found to determine the specificity of cis-dimer formation (Shan et al., 2000)

One theoretical model for which cis-dimerization specificity could lead to adhesive binding specificity is shown in Fig. 10 A. The model also depends on another documented structural feature of cadherins, the linking of successive EC domains together via calcium binding sites to form a rigid rod-like protein. Because of this property, we postulate that the entire ectodomain behaves as a single structural unit, and any alterations in the orientations of the EC1 domain dimer interface will be propagated throughout the rest of the EC domains. Thus small differences in the relative orientations of the EC1 dimerization interfaces for different cadherins would alter the orientations of other putative adhesive binding sites in the other EC domains (shown as large changes for emphasis), resulting in less compatible binding and/or in a reduced ability to form an extended two-dimensional lattice. Other models to explain how EC1 could determine adhesion specificity when other EC domains contribute to homophilic binding are also possible. For example, in the model shown in Fig. 10 B, an initial cadherin-specific interaction between EC1 domains could precede the formation of the final homophilic bonds between the other EC domains. For this to make sense physically, there would have to be some sort of repulsive barrier between cells to prevent interactions between EC2-5 from occurring directly, and an initial weak binding between EC1 domains would lower the energy barrier leading to the final binding state. For either of these models, there would be no cadherin-type specificity in the homophilic binding interactions between EC domains 2–5, which is consistent with the low adhesion specificity observed for many pairs of different cadherins. Irrespective of whether either of these two theoretical models is correct, this theoretical exercise demonstrates that determination of cadherin adhesion specificity by EC1 can be compatible with the participation of EC domains 2–5 in the homophilic binding interactions.

We favor a new model for the structure of the cadherin homophilic bond entailing the overlap of cadherin ectodomains and the interactions between multiple EC domains. We proposed that multivalent interactions between large numbers of individual low affinity and low specificity bonds lead to the formation of a two-dimensional lattice at the sites of cell–cell contact. Future studies will be required to determine the exact structural basis of the molecular interactions that contribute to the homophilic bond and to understand how catenins and cytoplasmic signals regulate the formation and strength of the adhesive bond between cells.

Materials and methods

Plasmid construction

Because dimerization is crucial for adhesive function (Brieher et al., 1996) but not always obtained when soluble cadherins are expressed, we generated chimeric constructs having an IgG Fc domain (Fc) fused to the COOH terminus of one of the cadherin ectodomain in order to force dimerization through the stable parallel interaction of the Fc domains. The IgG1 Fc domain was excised from the plg plus vector (Novagen) by digestion with HindIII and BclI and subcloned into the expression vector pEE14. The vector pEE14 encodes the glutamine synthase minigene as a selectable marker for CHOK1 cells expressing the minigene in the absence of glutamine and in the presence of the glutamine synthase inhibitor, methionine sulfoximine (Davis et al., 1990).

DNA sequences containing the C-cadherin signal sequence (amino acids 1–155; sequence is numbered according to EMBL/GenBank/DDBJ accession no. UO4707; Levine et al., 1994), followed by either EC domains 1–5 (amino acids 1–697), EC domains 1–4 (amino acids 1–593), EC domains 1–3 (amino acids 1–487), or EC domains 1 and 2 (amino acids 1–376) were isolated by PCR (Roche Expand high-fidelity PCR System: Taq

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DNA and two DNA polymerases) using the cDNA encoding the full-length *Xenopus* C-cadherin (Levine et al., 1994) as a template.

The PCR primers were, for CEC1-5Fc, (SF1-5A) 5′-ccaagcttgggcaccatgggggcaccaggcttaga and (SF1-5B) 3′-tagtctagacttttcctggcattttattgc; for CEC1-4Fc, (97.7) 5′-ccaagcttgggcaccatgggggcaccaggcttaga and (CECf) 3′-atgggggcaccaggcttagaaac; for CEC1-3Fc, (SF1-5A) 5′-ccaagcttgggcaccatgggggcaccaggcttaga and (SF1-3B) 3′-tagtctagaaaagaaggggcttcattgac; for CEC1-2Fc, (SF1-5A) 5′-aagcttcaccatgggggcaccaggcttaga and (SF1-2R2) 3′-tagtctagaaaaaattggagcattgtcgtttgc.

For cloning purposes, an HindIII cloning site was introduced at the 5′ end of the PCR fragment and an XbaI cloning site at the 3′ end. These different PCR products were then cloned by insertion in Fc-pEE14 digested by HindIII/XbaI.

The three other chimeric cDNAs were constructed using the same pattern (CEC3-4-5Fc, amino acids 377–697; CEC1-2-4Fc, amino acids 488–593; and CEC1-2-4-5Fc, amino acids 488–697), by overlap extension (Horton et al., 1989) using the following primers: for CEC3-4-5Fc, (SF1-5A) 5′-ccaagcttgggcaccatgggggcaccaggcttaga and (SS3R1) 3′-tgctgtataagttttggatctctcttcttccttttgaggcc; (SS3F1) 5′-aggaagaagagagatccaaaaacttatacagcactg and (SF 1-5B2) 3′-tagtctagacttttcctggcattttattgctttcc; for CEC1-2-4Fc, (SF1-5A) 5′-ccaagcttgggcaccatgggggcaccaggcttaga and (97.7) 3′-gactcgagggcttctagaaggaccatt; (SF2-4f) 5′-gacaatgctccaatttttgtaccagctgtcagtaga and (SF4B) 3′-tctagaaggaccattgtcatttac; for CEC1-2-4-5Fc, (SF1-5A) 5′-ccaagcttgggcaccatgggggcaccaggcttaga and (97.7) 3′-gactcgagggcttctagaaggaccatt; (SF2-4f) 5′-gacaatgctccaatttttgtaccagctgtcagtaga and (SF4B2) 3′-tagtctagacttttcctggcattttattgcttttcc.

For the cDNA construct CEC1-2FNFc, the two fibronectin-like domains of the chicken N-CAM (Cunningham et al., 1987; Ranheim et al., 1996) were inserted in frame between the COOH-terminal part of domain 2 and the Fc part of the IgG. For this construct, we also used an overlapping PCR method using a chicken N-CAM cDNA (provided by Urs Rutihauser, Memorial Sloan-Kettering Cancer Center, New York, NY) and the cDNA encoding the full-length *Xenopus* C-cadherin (Levine et al., 1994) as templates with the following primers: (SF1-5A) 5′-ccaagcttgggcaccatgggggcaccaggcttaga and (SF1-2FN) 3′-ctccactctgtcaatagaaggtcctccactaaaaattggagcattgtcgtttgc, (SF2FNf) 5′-aatgctccaatttttagtggaggaccttctattgacagagtggagccctac and (SFFNr) 3′-tagtctagagacagtaggctgagcagatgtccg. All the cDNA constructs were verified by sequencing in their entirety.

Cell lines

We used the mammalian CHO cell line expression–secretion system for the production of all our recombinant proteins (Davis et al., 1990) to ensure proper folding and posttranslational processing (misfolded proteins in the secretory pathway are usually degraded). CHOK1 cells were grown in complete Glasgow glutamine-free MEM with 10% dialyzed FCS. cDNA constructs encoding the different combinations of the five cadherin domains of *Xenopus* C-cadherin in the pEE14 expression vector, containing the glutamine synthase minigene, were transfected into CHOK1 cells by lipofection in serum-free Glasgow MEM using lipofectin (GIBCO BRL). Cells containing the transfected plasmid were selected by culturing the cells in the presence of 25 μM methionine sulfoximine (Sigma-Aldrich). CHOK1 cells can normally grow in the absence of glutamine, but growth in the absence of glutamine and in the presence of methionine sulfoximine requires expression of the glutamine synthase minigene.

Expression and secretion of the desired protein by CHO cells was determined by Western blotting–conditioned media of methionine sulfoximine–resistant cell lines. The C-CHO cells used for adhesion assays are CHO cells expressing the wild-type C-cadherin (Brieher et al., 1996).

Antibodies

Cadherin constructs were detected using either a polyclonal antibody (Yap et al., 1997) or mAb 6B6 (Brieher et al., 1996) directed against the ectodomain of C-cadherin (EC3 domain; unpublished data) or an anti–human IgG–HRP conjugate to detect the Fc domain (1 mg/ml; Promega).

Protein purification

CEC1-5 was purified from CHO cells in conditioned medium as described previously (Brieher et al., 1996). The Fc-containing chimeras were purified differently. The supernatant was harvested when cells became confluent to the point where they are no longer adherent (~10–14 d after seeding cells initially at 1.104 cells/ml). Conditioned media was filtered through a cellulose acetate low protein binding 0.45-μm pore membrane. The protein containing the IgFc domain was purified by applying the filtrate to a protein–A column (1 ml bed volume; Pharmacia Fine Chemicals) at a drop rate of 1 ml/min at 4°C. The column was then washed with 100–150 ml of 20 mM Hepes, 50 mM NaCl, 1 mM $CaCl_2$. The protein was eluted with 150 mM glycine, pH 2.0. Fractions of 1 ml were collected and buffered with 1 mM $CaCl_2$, 1 M Tris, pH 8.0. Fractions containing the protein were combined, desalted on a PD-10 column (Pharmacia Fine Chemicals) and concentrated in a Microcon 10 (Amicon Corp.).

For sequence analysis purified recombinant proteins were separated by SDS-PAGE and transferred to a polyvinylidine difluoride (Bio-Rad Laboratories) membrane. The membrane was first stained with Coomassie blue and then destained with 50% methanol. The desired proteins bands were cut out and subjected to NH_2-terminal Edman degradation by the microchemistry core facility at the Memorial Sloan-Kettering Cancer Center, New York, NY.

Bead aggregation assays

The beads used in this assay were Protein-A–coated polystyrene beads (carboxylate modified), fluorescent yellow, 0.9 μM (Bangs Laboratories Inc.). Before using them they were washed once in sodium acetate 100 mM, pH 3.9 (pH at which any impurities coupled to protein A will be eluted) and twice in 10 mM Hepes, 50 mM NaCl, pH 7.2. Then, the Fc–cadherin protein was bound to the beads at a ratio of 40 μg of protein per 40 μl of beads suspension (2.1010 beads/ml) in 10 mM Hepes, 50 mM NaCl, pH 7.2, 1 mM $CaCl_2$ for 90 min at 4°C on an Eppendorf shaker (1,400 rpm). The coated beads were pelleted, washed twice, and resuspended in 400 μl of 10 mM Hepes, 50 mM NaCl, pH 7.2. The suspension was briefly sonicated to obtain single beads, as determined by microscopy, before the addition of either 1 mM $CaCl_2$ to initiate aggregation or 1 mM EDTA as appropriate. The samples were incubated at room temperature, and at various time points 10 μl aliquots were removed. The number of particles large enough to be detected by a Beckman Coulter counter (parameters: aperture 100 μm, threshold 5–15 μm, count above 5 μm) was determined.

The amount of protein coupled to the beads was determined by taking an aliquot and pelleting it and resuspending in 2 × SDS sample buffer containing 1 mM EDTA. The beads were subsequently pelleted, and the supernatant was immunoblotted with the anti–human IgG HRP conjugate (1 mg/ml; Promega) after SDS-PAGE.

To study the aggregation between different sets of beads coated with different cadherin EC constructs, a flow cytometry assay was developed with the help of the Memorial Sloan-Kettering Cancer Center flow cytometry facility. For this purpose, we also used an other type of protein A–coated beads with a red fluorochrome to easily distinguish the two sets of beads. Two different Fc–cadherin proteins were coupled to two different fluorescent beads at a ratio of 120 μg of protein per 30 μl of beads suspension (2.1010 beads/ml) overnight at 4°C on an Eppendorf shaker (1,400 rpm). The coated beads were pelleted, washed twice, and resuspended in 150 μl of Fc 1 mg/ml in PBS for 15 min (in order to block all the protein A empty sites) after a brief sonication. The final volume was brought up to 300 μl with 10 mM Hepes, 50 mM NaCl, pH 7.2. The suspension was sonicated to obtain single beads as determined by microscopy. Red beads and yellow beads were then mixed (equal volume of each) as appropriate for a final volume of 300 μl and 1 mM $CaCl_2$ was added to initiate aggregation. The samples were incubated at 4°C on an Eppendorf shaker (1,400 rpm), and at various time points, 10 μl aliquots was removed. The appearance of mixed aggregates as a function of time was determined.

Laminar flow cell adhesion assay

The modified laminar flow adhesion assay performed was a modification of the one described previously (Brieher et al., 1996). In brief, borosilicate glass capillaries (1.1 mm internal diameter) (Sutter Instrument) were precoated with protein A (Amersham Pharmacia Biotech) 100 μg/ml in PBS^{++} during 5 h at 4°C, and nonspecific binding sites were then blocked with 0.5% casein hydrolysate enzymatic (ICN Biochemicals, Cleveland, OH) in PBS++ for 2 h at 4°C. Then these capillaries were coated overnight at 4°C with the Fc–cadherin fusion proteins at various concentrations (100, 20, 10, and 5 μg/ml). To control the amount of Fc–cadherin fusion protein bound to protein A on the surface, the total Fc-containing protein concentration is maintained at 100 μg/ml by adding the appropriate amount of 1 mg/ml Fc (Human IgG, Fc Fragment, Plasma, Calbiochem) in PBS. Nonspecific binding sites were then blocked with 5% milk (nonfat dry milk; Carnation, Nestle) in HBSS containing 1 mM $CaCl_2$. CHO cells or the stable cell line C-CHO were grown under standard conditions then harvested by a method that leaves cell surface cadherins intact (incubation with crystalline trypsine [0.01% wt/vol] in PBS^{++}), washed, and resuspended in HBSS/1 mM $CaCl_2$ or HBSS/1 mM EDTA. At that point, cells were infused into the coated capillary from a reservoir using a pump. After 1 min, the flow was stopped, and the cells were allowed to bind to the surface under static conditions for 10 min. Capillaries were observed with a phase microscope, and the number of cells attached to the substrate in a 20× field was counted. Flow was initiated, and the number of cells remaining in the field

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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. Data were normalized to the number of cells present in the field before starting the flow.

Analytical ultracentrifugation

Protein concentration and extinction coefficient determinations were performed using a Beckman XLI analytical ultracentrifuge and a double sector capillary synthetic boundary sample cell after the fringe count procedures described by Babul and Stellwagen (1969). Before running in the ultracentrifuge, the sample was equilibrated with the buffer solution using a Microsep microconcentrator. The absorbance of the sample was then measured in a Perkin-Elmer Lambda 5 spectrophotometer. 150 ul of stock sample was then loaded into one sector of the sample cell, and 400 ul of buffer solution were loaded into the other sector. The run was performed at 8,000 rpm, and scans were taken when fringes could be resolved across the boundary region between the protein solution and buffer solution. The number of fringes produced across the boundary was then measured and converted to concentration using an average refractive increment of 3.31 fringes/mg/ml.

Sedimentation equilibrium experiments were carried out at 20°C in a Beckman XLI analytical ultracentrifuge using both Interference and Absorbance optics following the procedures described by Laue and Stafford (1999). 110 μl aliquots of sample solution, with loading concentrations ranging from 2–25 μM, was loaded into two six-sector CFE sample cells, allowing six concentrations of sample to be run simultaneously. Runs were performed at 10,000 and 14,000 rpm, and each speed was maintained until there was no significant difference in scans taken 2 h apart to ensure that equilibrium was achieved.

The sedimentation equilibrium data was evaluated using the program NONLIN, which incorporates a nonlinear least-squares curve-fitting algorithm described by Johnson et al. (1981). This program allows the analysis of both single and multiple data files. Data can be fit to either a single ideal species model or models containing up to four associating species, depending on which parameters are permitted to vary during the fitting routine. To fit all the data sets globally, the data collected with the absorbance optical system was converted from absorbance to fringe displacement using the extinction coefficient determined from the fringe count. To convert the raw *Ka* in fringes, determined from fitting to a self association model, to a molar *Ka*, the following equation was used:

$$K_{\text{conc}} = K_{\text{fringe}} \times \left(\frac{\frac{dn}{dc} \times l}{\lambda} \right)^{n-1} \times \frac{(M_1)^{n-1}}{n}.$$

Where K_{conc} is the association constant in molar concentration terms, K_{fringe} is the signal association constant, $\frac{dn}{dc}$ is the specific refractive increment, l is the pathlength of the centerpiece in cm, λ is the lightsource wavelength in cm, M_1 is the monomer molecular weight, and n is the stoichiometry of the larger association species.

Assuming 20% glycosylation, an estimated value of 0.706 was used for the partial specific volume, and a monomer molecular weight of 75,000 was assumed for the fitting to a monomer–dimer model. The buffer solution density was estimated using the program SEDNTERP, which incorporates calculations detailed by Laue et al. (1991).

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