

Cadherin-mediated cell sorting not determined by binding or adhesion specificity

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Cadherin adhesion molecules play important roles in the establishment of tissue boundaries. Cells expressing different cadherins sort out from each other in cell aggregation assays. To determine the contribution of cadherin binding and adhesion specificity to the sorting process, we examined the adhesion of cells to different purified cadherin proteins. Chinese hamster ovary cell lines expressing one of four different cadherins were allowed to bind to the purified cadherin extracellular domains of either human E-cadherin or *Xenopus* C-cadherin, and the specificity of adhesion was compared with cell-sorting assays. None of the different cadherin-expressing cells exhibited any adhesive specificity toward either of the two

purified cadherin substrates, even though these cadherins differ considerably in their primary sequence. In addition, all cells exhibited similar strengthening of adhesion on both substrates. However, this lack of adhesive specificity did not determine whether different cadherin-expressing cells would sort from each other, and the tendency to sort was not predictable by the extent of sequence diversity in their extracellular domains. These results show that cadherins are far more promiscuous in their adhesive-binding capacity than had been expected and that the ability to sort out must be determined by mechanisms other than simple adhesive-binding specificity.

Introduction

The cadherin family of Ca^{2+} -dependent cell–cell adhesion molecules play important roles in the formation and maintenance of contacts between cells and tissues in development (Takeichi, 1994; Gumbiner, 1996; Tepass et al., 2000). It is a large and diverse family of different adhesion molecules that exhibits spatial and temporal expression patterns during development. Cadherins are homophilic binding molecules, and the specificity of their interactions is thought to underlie the sorting out or segregation of cells into specific tissue layers and the formation of tissue boundaries (Takeichi, 1995; Redies, 2000).

The classical cadherins each have five extracellular cadherin (EC)* repeats (1–5), whereas their cytoplasmic domains

interact with p120^{cas} and β -catenin, which binds to α -catenin (Yap et al., 1997a). The cytoplasmic tail has the capacity to cluster cadherin molecules (Yap et al., 1998) and provide a link to the cytoskeleton via α -catenin (Rimm et al., 1995). These functions allow the cytoplasmic domain to confer adhesive strengthening on the intrinsic adhesive activity of the extracellular domain (Briehner et al., 1996; Ozawa and Kemler, 1998). Homophilic binding specificity has been attributed to the NH_2 -terminal EC1 domain because exchange of this domain between two different cadherins determined cell aggregation and sorting specificity (Nose et al., 1990). However, several recent studies have shown that binding and adhesion of cadherins involves other EC repeats besides EC1 (Sivasankar et al., 1999, 2001; Chappuis-Flament et al., 2001). EC1 is also essential for the specification of lateral dimer formation (Shan et al., 2000), a prerequisite for functional cadherin molecules (Briehner et al., 1996; Tamura et al., 1998), and this function may contribute to its role in determining cadherin specificity (Chappuis-Flament et al., 2001).

The conclusion that cadherins are homophilic adhesion molecules came from aggregation experiments in which cells expressing different types of classical type I cadherins were observed to sort out and form distinct aggregates (Nose et al., 1988). There is also evidence supporting the notion that expression of different cadherins is required to maintain

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*Abbreviations used in this paper: C, *Xenopus* C-cadherin; diO, diOC₁₈(3) 3,3'-dioctadecyloxycarbocyanine perchlorate; diI, diIC₁₈(3) 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; EC, extracellular cadherin; HE, human E-cadherin; HN, human N-cadherin; XE, *Xenopus* E-cadherin.

Key words: cadherins; adhesion; cell sorting; heterophilic; specificity

tissue boundaries *in vivo*. Exogenous N-cadherin expression in early *Xenopus* embryos resulted in the perturbation of tissue boundaries in the ectoderm and perturbed histogenesis (Detrick et al., 1990; Fujimori et al., 1990). Also, overexpression of cadherins blocked the movement of neural crest cells migrating away from the neural tube, a morphogenetic movement that coincides with a switch in cadherin type (Nakagawa and Takeichi, 1995, 1998).

Several observations suggest, however, that cadherins can interact in a heterophilic fashion. A-CAM- (N-cadherin) expressing cells and L-CAM- (E-cadherin) expressing cells were found to form heterotypic junctions (Volk et al., 1987). N-cadherin-expressing cells can interact and mix with R-cadherin cells (Matsunami et al., 1993; Shapiro et al., 1995), but their extracellular domains are rather similar (69%). B-cadherin-expressing cells can mix with cells expressing L-CAM, even though they have greater sequence differences in their EC1–5 domains (58% similarity) than N- and R-cadherin (Murphy-Erdosh et al., 1995). Both these cadherins are, however, expressed in the liver. The type II cadherins exhibit even less specificity in the cell aggregation sorting assay (Shimoyama et al., 2000), even though they exhibit quite variable sequences. Furthermore, it was shown recently that E-cadherin could substitute for N-cadherin in the development of the heart in mice, showing that cadherin mediated adhesion, but not tissue-specific cadherin expression, was important for heart development (Luo et al., 2001).

Cadherins may also contribute to cell segregation by differential levels of expression of a single cadherin type (Steinberg, 1970). Indeed, cells expressing high levels of cadherin will sort out from cells expressing low levels of the same cadherin (Friedlander et al., 1989; Steinberg and Takeichi, 1994). That different cadherin levels can mediate morphogenetic processes *in vivo* was recently shown in the *Drosophila* egg chamber, where differential expression of DE-cadherin on the follicle and nursing cells position the oocyte, thereby contributing to the anterior–posterior axis (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998).

The different types of cadherins and different patterns of cadherin expression may have biological functions other than cell sorting and tissue segregation, such as the qualitative type of adhesive state, including the tendency for motile versus stable adhesions. For example, N-cadherin confers a more motile phenotype than E-cadherin when expressed on cells (Kim et al., 2000). Overexpression of N- or C-cadherin in *Xenopus* led to disruptions of the ectoderm, suggesting that only E-cadherin, which is the endogenous cadherin in this tissue, is specifically required to maintain the integrity of this tissue (Detrick et al., 1990; Levine et al., 1994). Different cadherins may also generate different signals. For ex-

ample, E-cadherin–negative embryonic stem cells seem to differentiate along different paths after exogenous expression of either N-cadherin or E-cadherin (Larue et al., 1996). Also, E-cadherin but not N-cadherin can act as a tumor suppressor (Islam et al., 1996; Christofori and Semb, 1999). Thus, different cadherins may have different physiological functions that are required for different cell types.

In light of the observations that expression of different cadherins may have other biological roles than cell sorting and that cadherin interactions may be less specific than previously envisioned, we decided to address the question of cadherin specificity in more detail. The sorting process consists of a complex set of events, requiring both initial recognition and binding of cadherins and the strengthening of this interaction over time. Although it is very clear that cadherins are essential players in the aggregation and sorting process, it is not easy to distinguish between the role of cadherin binding specificity, downstream events that can potentially occur after two cells are brought into close proximity, or the possible interplay between cadherins and other cell surface or signaling molecules upon cell–cell contact. To address the role of cadherin binding and adhesion specificity we used an adhesion flow assay, which allows one to study both the initial adhesion of cadherin-expressing cells with purified cadherin extracellular domain proteins, as well as the strengthening of adhesion to these purified proteins over time (Brieher et al., 1996; Yap et al., 1998). This assay was used in conjunction with traditional aggregation/sorting assays to address the role of cadherin adhesive activity in the cell-sorting process.

Results

To examine the specificity of the primary homophilic binding and adhesion event, we started with two different purified cadherin substrates and cells expressing these two wild-type cadherins. Human E-cadherin and *Xenopus* C-cadherin are 57% similar in their EC1–5 domains (Table I), which is comparable to, for example, human P-cadherin and human E-cadherin (55%), two cadherins known to mediate sorting. We therefore chose initially to use these two cadherins as substrates and compare their adhesive properties in the flow assay.

To make purified cadherin substrates we expressed the extracellular domains of both the human E-cadherin (HEEC1–5Fc) and *Xenopus* C-cadherin (CEC1–5Fc) as fusion proteins with the Fc part of human IgG in CHO cells. This insures that the protein is secreted as a dimer, which is important because functional cadherins require dimerization of the protein (Brieher et al., 1996; Tamura et al., 1998).

Table I. Percentage of sequence similarities of the classical cadherin EC1–5 domains or EC1 domain used in these experiments

	<i>Xenopus</i> C-cadherin	<i>Xenopus</i> E-cadherin	Human E-cadherin	Human N-cadherin
	%	%	%	%
<i>Xenopus</i> C-cadherin	100/100	59/72	57/71	44/53
<i>Xenopus</i> E-cadherin		100/100	56/75	46/51
Human E-cadherin			100/100	48/58
Human N-cadherin				100/100

Results are shown as EC1–5/EC1.

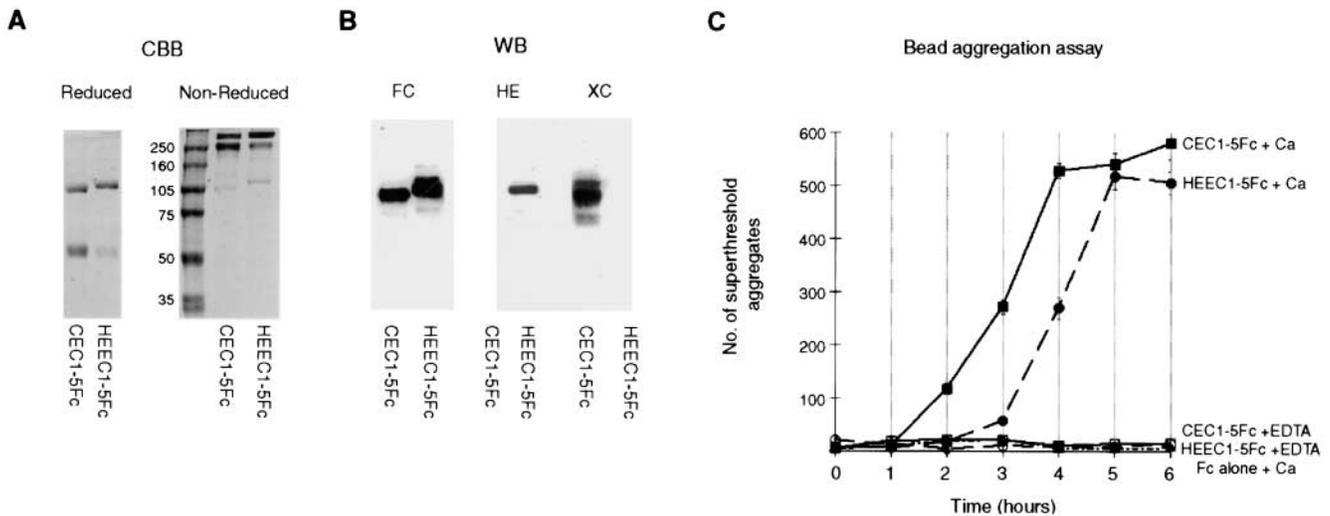


Figure 1. Purification and characterization of recombinant extracellular cadherin proteins. The extracellular domains of HE-cadherin and C-cadherin were both expressed in CHO cells as a COOH-terminal fusion proteins with the Fc part of human IgG. Recombinant proteins were purified from the media on a Protein A column. (A) Coomassie staining of HEEC1-5Fc and CEC1-5Fc run under reducing and nonreducing conditions. (B) Immunoblot analysis of the recombinant HEEC1-5Fc and CEC1-5Fc proteins using anti-human Fc, anti-HE cadherin, or anti-C-cadherin antibodies. (C) Bead aggregation assay: protein-A-coated beads coupled to HEEC1-5Fc or CEC1-5Fc were allowed to aggregate in the presence of Ca^{2+} or EDTA for the indicated time period.

The adhesive properties of CEC1-5Fc already have been well characterized (Chappuis-Flament et al., 2001). The HEEC1-5Fc and CEC1-5Fc proteins were isolated from conditioned medium on protein A columns. Both proteins were isolated to near purity since the size of the most abundant bands as observed by Coomassie brilliant blue staining (Fig. 1 A) corresponded to the correct molecular weights of either HEEC1-5Fc or CEC1-5Fc. Western blot analysis confirmed the identity of both proteins (Fig. 1 B). In the following experiments, we tested proteins produced by at least two different clones of secreting cells for each cadherin EC1-5Fc chimera and several protein preparations from each clone.

We then evaluated whether HEEC1-5Fc could function as an adhesion molecule in vitro by performing bead aggregation assays. The CEC1-5Fc protein was used as a positive control because it was shown previously to mediate strong aggregation when coupled to beads (Chappuis-Flament et al., 2001). Indeed, HEEC1-5Fc was able to aggregate beads to a similar extent as the CEC1-5Fc protein, whereas Fc protein alone was unable to aggregate beads (Fig. 1 C). For both cadherin fusion proteins, aggregation of the beads was sensitive to EDTA, showing that aggregation was calcium dependent, a well-known feature of cadherin-mediated binding. The apparent difference in lag time between HEEC1-5Fc beads and CEC1-5Fc beads was not consistently observed and varied from experiment to experiment.

Adhesion flow assays were done to test if HEEC1-5Fc could also support adhesion of human E-cadherin (HE)-expressing CHO cells. HE-CHO cells could indeed bind to the HEEC1-5Fc protein, whereas control CHO cells did not show any adhesive activity (Fig. 2 A). As was shown before (Chappuis-Flament et al., 2001), *Xenopus* C-cadherin (C)-expressing CHO cells adhered to the CEC1-5Fc protein (Fig. 2 A). Therefore, similar to CEC1-5Fc, HEEC1-

5Fc has functional activity by the two criteria of mediating bead aggregation and supporting strong adhesion of cadherin-expressing cells.

C-cadherin- and E-cadherin-expressing cells can adhere equally well to either the HEEC1-5Fc or the CEC1-5Fc proteins

To determine the specificity of cell adhesion to either HEEC1-5Fc or CEC1-5Fc, heterotypic flow assays were performed. C-CHO cells were allowed to bind to HEEC1-5Fc and HE-CHO cells were allowed to adhere to CEC1-5Fc. To our surprise, we found that C-CHO cells bound equally well to the human E-cadherin protein as it did to the *Xenopus* C-cadherin protein (Fig. 2 A). A similar result was found with HE-CHO cells; they bound equally well to CEC1-5Fc as to HEEC1-5Fc (Fig. 2 A). In addition, adhesion of HE-CHO and C-CHO cells to both cadherin Fc substrates was similar to binding of both cell lines to the CEC1-5 substrate, the well-characterized C-cadherin EC1-5 protein without the Fc fusion (Briehar et al., 1996). These findings were unexpected in light of the presumed homophilic interaction nature of classical cadherins and the substantial sequence difference between EC1-5 of C-cadherin and HE-cadherin (57% similarity; Table I). The observed adhesion was nevertheless a genuine cadherin interaction since it was abrogated by EDTA, and thus Ca^{2+} sensitive and required the expression of the cadherin, since parental CHO cells did not adhere (Fig. 2 A). Importantly, it was found that adhesion of both cell lines to either substrate strengthened as a function of time (Fig. 2 B). This suggests that both E- and C-cadherin are able to somehow reorganize, cluster, or assemble junctional contacts on the cell membrane upon binding to either a homotypic or heterotypic substrate (Yap et al., 1998).

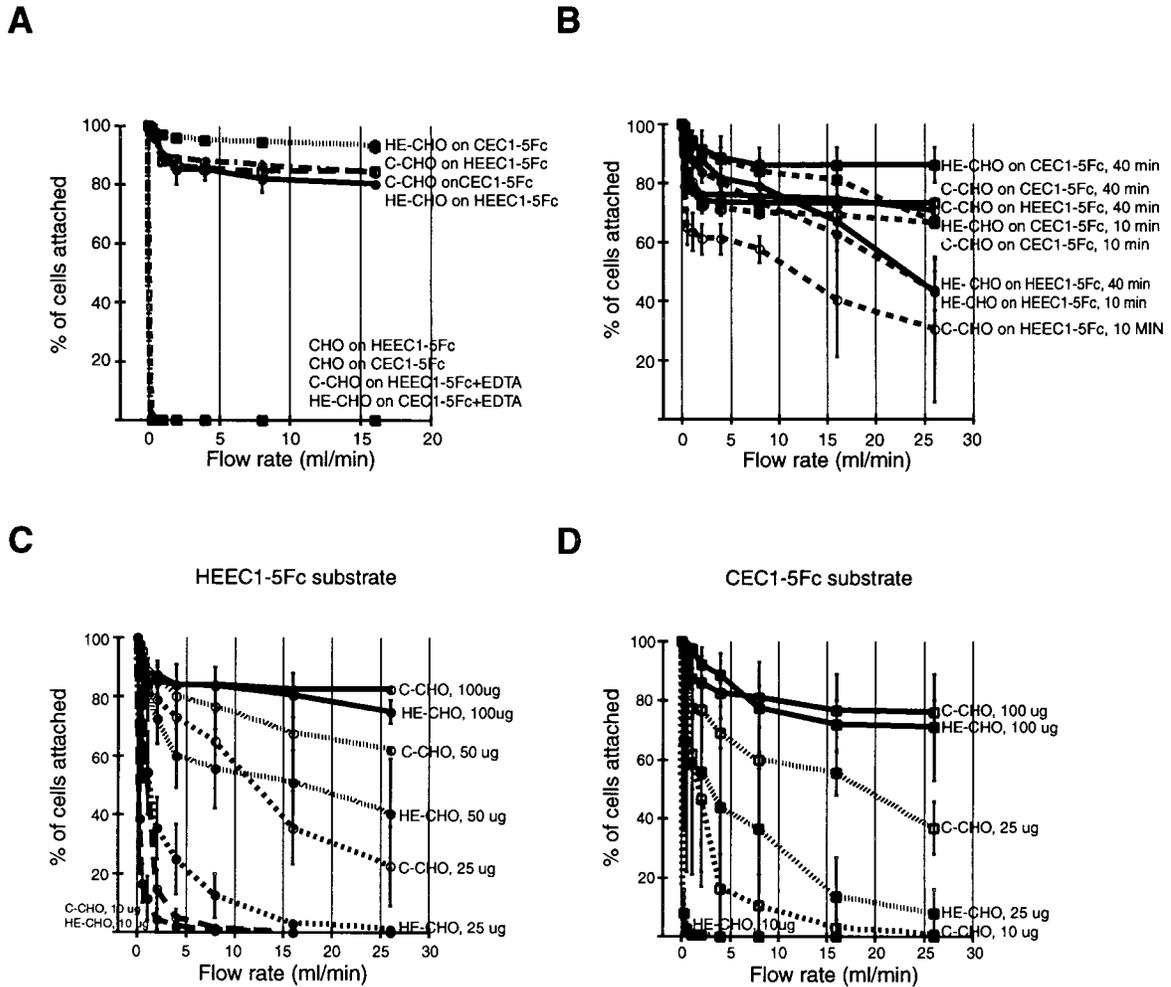


Figure 2. **C-cadherin- or HE-cadherin-expressing CHO cells bind equally well to either HEEC1-5Fc or CEC1-5Fc in an adhesion flow assay.** (A) Adhesion flow assay using 100 μ g of either HEEC1-5Fc or CEC1-5Fc as substrates. Note that the curves representing CHO cells on either substrate or any samples in the presence of EDTA are all collapsed on the X-axis. (B) Strengthening of adhesion. Cells were allowed to attach to HEEC1-5Fc (75 μ g/ml) or CEC1-5Fc (50 μ g/ml) substrates for either 10 or 40 min before they were subjected to increasing flow rates. (C) Different concentrations of HEEC1-5Fc substrate in the adhesion flow assay using both C-CHO and HE-CHO cells. (D) Different concentrations of CEC1-5Fc substrates in the adhesion flow assay using both C-CHO cells and HE-CHO cells.

Thus far, flow assays were done under saturating amounts of substrate protein, which could potentially mask differences in adhesive strength, and therefore specificity, for either HE- or C-CHO cells. Therefore, decreasing concentrations of specific cadherin proteins were used in the adhesion flow assay to sensitize the assay to the quantity of substrate protein. Adhesion of C-CHO cells or HE-CHO cells were similarly sensitive to lower amounts of either HEEC1-5Fc (Fig. 2 C) or CEC1-5Fc (Fig. 2 D). This demonstrates that even under nonsaturating conditions, neither C-cadherin nor E-cadherin shows a preference for its own homophilic substrate. Together, the results show that *Xenopus* C-cadherin can adhere similarly well to human E-cadherin as to itself, and vice versa.

***Xenopus* blastomere cells adhere similarly well to CEC1-5Fc and HEEC1-5Fc**

CHO cells normally do not express any significant amount of cadherin. The observed heterotypic cadherin

interaction could potentially be attributed to the non-physiological expression of cadherins in CHO cells. *Xenopus* blastomere cells, isolated from animal cap tissue explants, express C-cadherin as their major cadherin, and were used to test if physiological cadherin expression would also result in heterophilic adhesion. Using HEEC1-5Fc and CEC1-5Fc as substrates, blastomeres were allowed to bind for a period, after which they were subjected to detachment forces by rotating the dish shortly. Adhesion was measured by counting attached blastomeres before and after rotation (Zhong et al., 1999). Blastomeres adhere just about as well to HEEC1-5Fc as to CEC1-5Fc, and this adhesion showed a similar concentration dependency for both substrates (Fig. 3). Blastomeres did not adhere to BSA (Fig. 3) or either cadherin substrate in the presence of EDTA (unpublished data). Thus, when expressed under normal physiological circumstances, C-cadherin still mediates a strong heterotypic interaction with human E-cadherin protein.

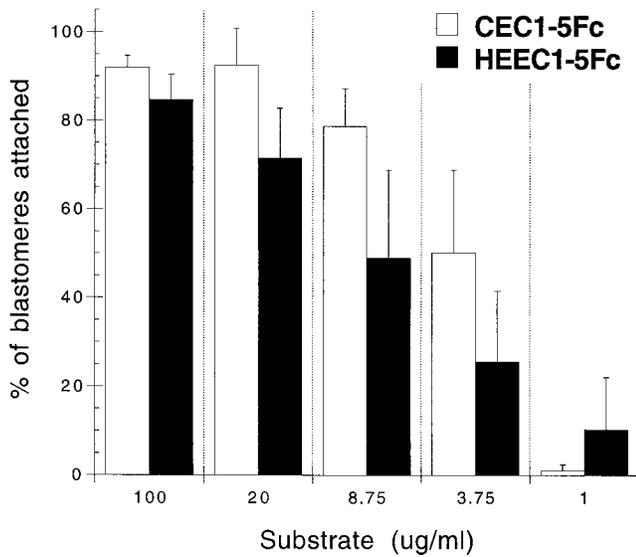


Figure 3. *Xenopus* blastomeres adhere similarly well to both CEC1-5Fc and HEEC1-5Fc proteins. Blastomeres isolated from *Xenopus* animal cap tissue explants were allowed to adhere to different amounts of HEEC1-5Fc, CEC1-5Fc, or BSA. The results shown are the average of four independent experiments. Black bars, HEEC1-5Fc protein; white bars, CEC1-5Fc protein. Note that adherence of blastomeres to BSA alone is negligible, and therefore does not appear in the graph.

Multiple cadherins exhibit low binding and adhesive specificity

Xenopus C-cadherin and human E-cadherin will never interact under physiological conditions since they are of different species origin. Therefore, we wanted to extend our analysis of cadherin specificity to other cadherins, and we chose to include both *Xenopus* E-cadherin and human N-cadherin. It must be noted that *Xenopus* E-cadherin is no more similar to human E-cadherin (56%) in its EC1-5 domains than it is to *Xenopus* C-cadherin (59%). In fact, *Xenopus* E-cadherin is only considered to be an E-cadherin on the basis of its expression pattern rather than because of its sequence homology to other E-cadherins. *Xenopus* C-cadherin has a very broad expression pattern in the embryo, whereas *Xenopus* E-cadherin is mostly confined to epithelial tissues, where it is often localized to the junctional complex (Levi et al., 1991). Human N-cadherin was also chosen since it has already been described to sort out from E-cadherin in aggregation assays (Shan et al., 2000), and we wanted to compare this property directly to its basic binding to an E-cadherin substrate.

Both human N-cadherin and *Xenopus* E-cadherin were stably expressed in CHO cells. Immunofluorescence analysis of the cell lines showed that all four different cadherin lines express the cadherin at the plasma membrane, together with β -catenin and p120^{ctn} (Fig. 4 A). It has been shown that sorting of cells can occur when cells with different levels of the same cadherin are mixed (Friedlander et al., 1989; Steinberg and Takeichi, 1994). Therefore, we used cell lines that showed similar levels of the different cadherins. Cadherin expression levels were assessed by immunoblotting with either β -catenin antibodies or a pan-cadherin antiserum that recognizes a conserved peptide in the cytoplasmic domain of

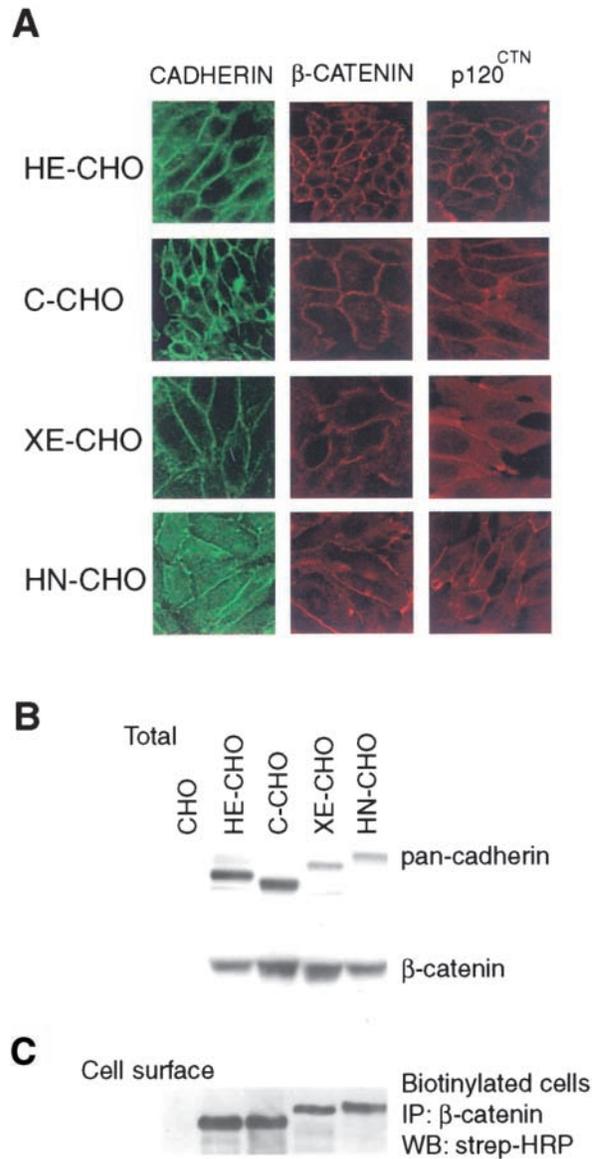


Figure 4. Characterization of CHO cells expressing different classical type I cadherins. (A) Immunofluorescence staining of different cadherin-expressing CHO cell lines, using antibodies to either the specific cadherin as indicated, β -catenin, or p120^{ctn}. (B) Western blot analysis of expression levels of cadherins or β -catenin in the different cadherin CHO cell lines using equal micrograms of total protein. The same membrane was incubated with a β -catenin antibody and a pan-cadherin antibody (PEP-1). (C) Cell surface expression of different cadherins on CHO cells. Intact cells were biotinylated, lysed, and equal amounts of protein were immunoprecipitated with a β -catenin antiserum and immunoprecipitates were Western blotted, after which the biotinylated proteins were recognized by streptavidin-HRP.

cadherins (Levine et al., 1994). CHO cells express only very low levels of β -catenin, which is upregulated upon transfection of the cells with a cadherin. Since the stoichiometry between β -catenin and cadherins is 1:1 (Ozawa and Kemler, 1992; Huber et al., 2001), the cadherin levels can be directly correlated to those of β -catenin levels. Similar amounts of total β -catenin were detected by an anti- β -catenin antiserum in lysates of *Xenopus* E-cadherin (XE)-expressing CHO

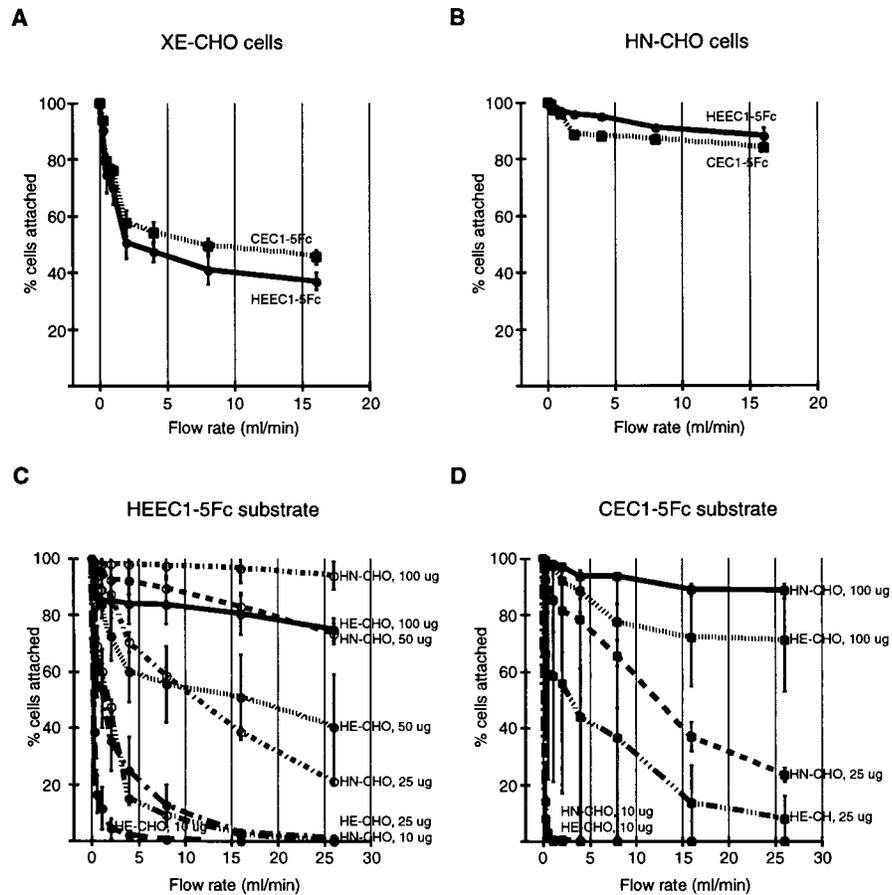


Figure 5. **XE-cadherin CHO cells and HN-cadherin CHO cells show no adhesive specificity.** (A) Adhesion flow assay of XE-CHO cells attached to either CEC1-5Fc or HEEC1-5Fc substrates (100 μ g/ml). (B) Adhesion flow assay of HN-CHO attached to either CEC1-5Fc or HEEC1-5 (100 μ g/ml). (C) Different concentrations of HEEC1-5Fc substrate in the adhesion flow assay using both HN-CHO and HE-CHO cells. (D) Different concentrations of CEC1-5Fc substrates in the adhesion flow assay using both HN-CHO and HE-CHO cells.

cells, human N-cadherin (HN)-expressing CHO cells, HE-CHO, and C-CHO cells (Fig. 4 B). A similar result was found with the pan-cadherin antibody, although here the difference between the C-CHO or HE-CHO cells compared with the XE- or HN-CHO cells seemed more pronounced than when compared with the β -catenin result (Fig. 4 B). Since both antibody incubations were done on the same blot, the difference cannot be explained by loading differences. Although we do not know why we see a difference in reaction intensity with the two antibodies, it might be due to differences in pan-cadherin affinity for the cadherins used. We therefore sought to also examine the cell surface expression of the cadherins, since cadherins can only function in adhesion when present on the cell surface. No major differences were found in surface expression of the different cadherins on CHO cells, as judged by accessibility to cell surface biotinylation (Fig. 4 B). Therefore, the different cadherins were all expressed at similar levels, facilitating comparison between cell lines.

The adhesion of XE-CHO cells to either the human HEEC1-5Fc protein or to *Xenopus* CEC1-5Fc was measured. XE-CHO cells bind equally well to either CEC1-5Fc or HEEC1-5Fc in the adhesion flow assay (Fig. 5 A), again showing no specificity in adhesion to *Xenopus* C- or human

E-cadherin. Similar to both HE-CHO cells and C-CHO cells, adhesion of XE-cadherin-expressing cells also strengthened on both cadherin substrates (unpublished data). N-cadherin is even more divergent from human E-cadherin (48% similarity; Table I) and *Xenopus* C-cadherin (46% similarity) and therefore might be expected to interact less well with those substrates. However, N-cadherin-expressing cells also adhered equally well to both HEEC1-5Fc and CEC1-5Fc (Fig. 5 B), and they adhered to these substrates to a similar extent as the human E-cadherin cells (Fig. 2 A). More importantly, HN-CHO and HE-CHO cells were similarly sensitive to dilution of the HEEC1-5Fc or CEC1-5Fc substrate (Fig. 5, C and D), demonstrating that differences were not being obscured by saturating levels of the substrate. In conclusion, we found that no type I classical cadherin tested thus far mediated any specificity in binding and adhesion to different purified cadherin protein substrates.

Different cadherins exhibit variable sorting properties

Even though the different cadherin expressing CHO cells failed to reveal any specificity in the basic adhesion assay, we decided to evaluate cell sorting behaviors between the different cadherin expressing CHO cells using a coaggregation as-

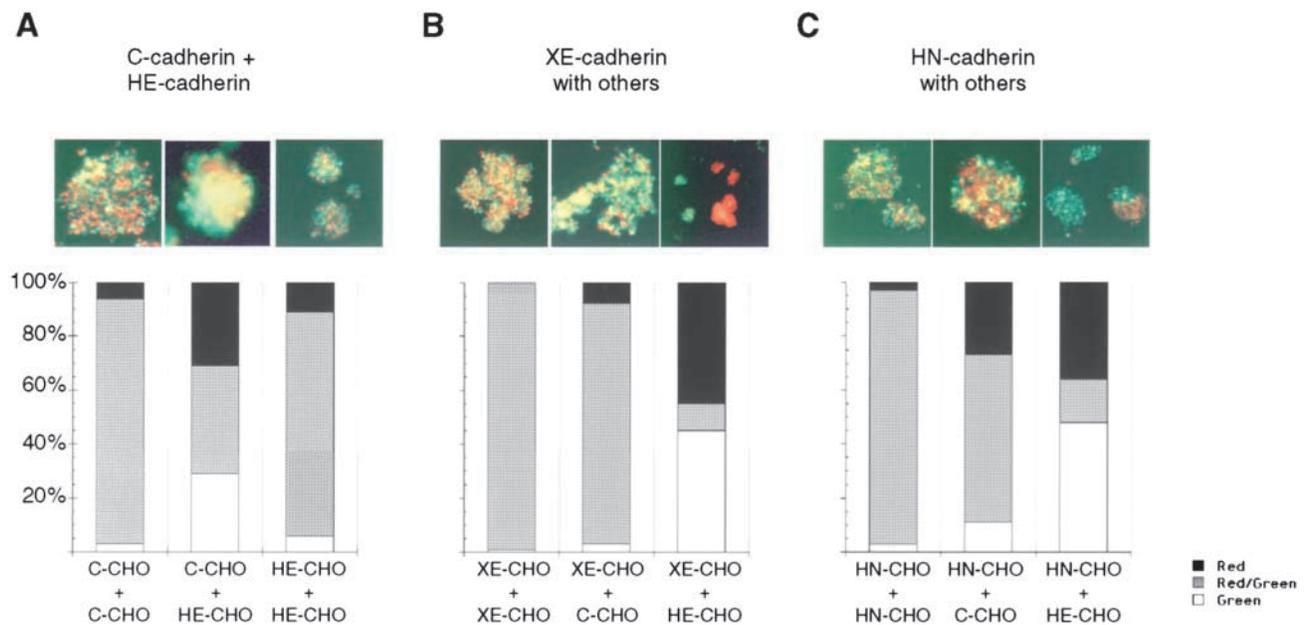


Figure 6. Variable sorting specificities for different cadherin-expressing CHO cells. Cell aggregation assays using cells labeled with fluorescent dyes, either diI (red) or diO (green). Cells were allowed to aggregate for 3 h. For A–C, examples of fluorescence are shown in top panels, with quantification of sorting versus mixing shown in the graphs below. (A) diI-labeled C-CHO cells mix completely with diO-labeled C-CHO cells, and diI-labeled HE-CHO cells also mix completely with diO-labeled HE-CHO cells, showing that the fluorescent label does not cause cells to sort artifactually. Middle, C-CHO cells (red) also mix to a large extent with HE-CHO cells (green). (B) XE-CHO cells (red in all cases) mix with XE-CHO cells (green) or with C-CHO cells (green), but sort out from HE-CHO cells (green). (C) HN-CHO cells (red in all cases) mix with HN-CHO cells (green) or C-CHO cells (green), but sort out from HE-CHO cells (green). Total number of counted aggregates is set at 100%. Black bars, diI labeled aggregates (red); striped bars, diI- and diO-labeled mixed aggregates; white bars, diO-labeled aggregates (green).

say. *Xenopus* C-CHO– and HE-CHO–expressing cells were examined first. Cells were labeled with either diI or diO and allowed to aggregate for different periods of time. Labeled untransfected CHO cells were used as a control and did not show much nonspecific aggregation, even after overnight incubation (3–5 cell aggregates at the most; unpublished data). In addition, no aggregation was observed for either C-CHO or HE-CHO cells in the presence of EDTA. As expected, diI-labeled C-cadherin cells were totally intermixed with diO-labeled C-cadherin cells (Fig. 6 A), showing that the fluorescent label did not influence the formation of mixed aggregates. Similarly, the diI- and diO-labeled HE-CHO cells were mixed (Fig. 6 A). Surprisingly, diI-labeled C-cadherin cells also mixed significantly with diO-labeled HE-cadherin cells, albeit less completely (Fig. 6 A). Quantification showed that >40% of counted aggregates consisted of both C-CHO and HE-CHO cells (Fig. 6 A). Therefore, C-cadherin and human E-cadherin cells did not strongly sort out from each other.

Since *Xenopus* E-cadherin is equally similar to either human E-cadherin (56%) or *Xenopus* C-cadherin (59%) in its EC1–5 domain (Table I) and binds equally well to both these substrates, it was predicted that *Xenopus* E-cadherin cells would mix with both *Xenopus* C-cadherin cells and human E-cadherin cells in aggregation assays. Indeed, when XE-CHO cells and C-CHO cells were incubated together, most cell aggregates consisted of mixed populations of cells (Fig. 6 B). In contrast, however, not much mixing of HE-CHO and XE-CHO cells was observed and most aggregates

consisted of only one cell population (Fig. 6 B). Thus, sorting behavior was not predicted by the extent of sequence similarity nor was it predicted by the results of the basic adhesion assay, suggesting that sorting may be mediated by something other than adhesive specificity.

Previous studies have shown that mouse N-cadherin–expressing L-cells sort out from mouse E-cadherin L-cells (Shan et al., 2000). We confirm that observation with human N-cadherin and human E-cadherin in CHO cells, as HN-CHO cells sorted out from HE-CHO cells (Fig. 6 C). Again, this is an example of sorting that is not dictated by adhesive specificity, since both cell lines adhere equally well to HEEC1–5Fc (Figs. 2 A and 5, B–D). In contrast, HN-CHO cells did not sort from C-CHO cells, (Fig. 6 C). This was unexpected in light of the lower similarity of N-cadherin to C-cadherin (44% similarity; Table I) compared with that of human N-cadherin and human E-cadherin (48% similarity). Therefore, cell-sorting behavior by differential cadherin expression occurred independently of any relatedness in sequence of the extracellular domain.

Discussion

To determine the role of direct cadherin-mediated binding and adhesion in cell–cell sorting and mixing relative to downstream events or interactions that may occur upon cadherin binding, we compared cadherin function in an adhesion flow assay with a cell aggregation/sorting assay. The former assay uses purified cadherin proteins and thus solely

probes cadherin–cadherin binding and adhesion, whereas the latter analyzes a more complex behavior of cells in response to initial cadherin-dependent cell–cell interactions. We show here that type I classical cadherins display a much wider range of binding and adhesive specificities than previously was assumed on the basis of their cell mixing and sorting properties. Moreover, cadherin relatedness at the primary sequence level does not necessarily predict the sorting properties of cadherins toward other cadherins expressed on different cells. Our results indicate that other mechanisms besides basic binding and adhesive activity contribute to the segregation of cells.

The notion of cadherins as homophilic adhesion receptors is based on the fact that cells expressing different cadherins form separate aggregates in cell mixing experiments. However, results from several groups have already shown that several different cadherin pairs do not necessarily mediate sorting when expressed on different cells (Inuzuka et al., 1991; Murphy-Erdosh et al., 1995; Shimoyama et al., 1999, 2000; Shan et al., 2000). Our results also demonstrate a range of sorting activities in the aggregation assay. For example, any combination of C-cadherin–expressing cells with any of the other cadherin-expressing cells will result in considerable mixing, whereas other cell combinations lead to sorting out. Importantly, the extent of sequence similarity between cadherins does not predict whether the two different cadherin-expressing cells will mix or sort out from each other. In addition, we have found that sorting behavior cannot be explained by any readily distinguishable differences in binding and adhesion specificity. Thus, many cadherins can clearly function as heterophilic adhesion molecules, both in the adhesion flow assay and in the aggregation sorting assay. It is important to note that cadherins do not interact with all protein substrates containing cadherin repeats in the adhesion flow assay, since no adhesion for any of the cadherin cell lines to a protocadherin PAPC extracellular domain Fc chimera was observed (unpublished data).

Although we cannot rule out that the small binding differences observed between cadherins in the substrate dilution experiment represent small differences in binding affinities between two different cadherins, this cannot easily explain the sorting behavior. For example, HN-cadherin cells bind better to HECC1–5Fc than HE-cadherin cells, but the two cell types still sort out from each other. How do we reconcile the fact that apparently similar adhesive activity toward two different cadherins can result in sorting out behavior when these cadherins are expressed on cells? One factor known to play a role in the sorting of cells is the level of cadherin expression on the cell surface (Friedlander et al., 1989; Steinberg and Takeichi, 1994). Thus, cells expressing low levels of a certain cadherin will sort out from cells expressing high levels of the same molecule. Our results, however, cannot simply be explained by expression levels of the cadherin alone. First of all, only cell lines that had comparable levels of expression were used, as judged by β -catenin levels, immunoblotting with a pan-cadherin antibody, and cell surface biotinylation of cadherins. Second, no major differences in adhesion in the flow assay were revealed when either the HECC1–5Fc or CEC1–5Fc substrates were serially diluted,

indicating that the cell lines did not differ much in their quantitative levels of adhesion. In addition, if levels are the sole determinant for sorting, XE-cadherin cells should sort out from both C-cadherin and HE-cadherin cells, since the latter two cell lines exhibit somewhat higher expression levels. Instead, the XE-cadherin cells mixed with the C-cadherin cells but sorted out from the HE-cadherin cells. Thus, although cadherin expression levels can contribute to adhesive activity and cell sorting, it is not the sole determinant of the sorting process.

Another factor that could potentially play a role in cell-sorting behavior is a possible difference in the affinity state of the cadherins at the cell surface. Integrins have been shown to undergo affinity modulation at the cell surface (Ginsberg et al., 1992). It is conceivable that cadherins undergo a similar affinity modulation and that only the high affinity state exhibits binding specificity. If affinity modulation plays a role in cadherin specificity, we would have to postulate that the adhesion flow assay does not measure the high affinity adhesive state. Unlike integrins, however, no example of cadherin affinity modulation has been documented so far. Regulation of the adhesive strength of C-cadherin occurs in the *Xenopus* embryo, but this is probably due to changes in higher order organization rather than binding affinity (Zhong et al., 1999). Furthermore, an antibody that activates C-cadherin adhesion in the *Xenopus* embryo, fails to further stimulate adhesive activity of C-cadherin on CHO cells in the adhesion flow assay (Zhong et al., 1999), suggesting that CHO cells express cadherins already in the activated state.

The formation of higher order cadherin structures in the plane of the membrane upon ligand binding could also potentially influence the sorting behavior of cells. Differences in the ability to form higher order clusters or associate with the cytoskeleton for each of the cadherins could result in a mismatch and thus favor the homophilic interaction in the aggregation assay. The different cadherin-expressing cells used in this study all exhibit time-dependent strengthening of adhesion in the flow assay on either substrate, suggesting that they do not differ in their capacity to form clusters on heterotypic substrates (Yap et al., 1998). Moreover, the time frame for strengthening found in the adhesion flow assay is similar to that for which one can observe sorting of cells (45 min to 1 h). Nonetheless, it is important to remember that the clustering of cadherins and strengthening of adhesion in the flow assay is not dependent on the β -catenin binding domain of the cadherin (Yap et al., 1998). Therefore, it is conceivable that sorting behavior could result from the activities of β - and α -catenin; for example, through their interaction with actin and actin binding proteins or signaling proteins. However, there are no obvious differences in the association of catenins with the various cadherins in our CHO cells (unpublished data). Also, sorting specificity does not appear to be determined by the different cadherin cytoplasmic domains, because exchanging the cytoplasmic domain of cadherins did not alter sorting specificity (Nose et al., 1990).

Rather, sorting specificity seems to be determined by the EC1 domain (Nose et al., 1990; Shan et al., 2000). If sorting specificity is determined by EC1, it cannot simply be ex-

plained by the extent of similarity within this domain (Table I). For example, human E-cadherin and *Xenopus* E-cadherin share 76% sequence similarity in EC1 but do sort out when expressed on cells, whereas *Xenopus* C-cadherin cells and human N-cadherin cells mix even though their EC1 domains are only 53% similar. In addition, we could not identify any local sequence differences within the EC1 domain that may easily explain the sorting results observed here. One should keep in mind that the crystallography data on cadherin EC1 and EC1–2 domains have not yet provided a detailed picture of the atomic interactions in the adhesive interface. Because we observe no specificity in the basic binding and adhesion steps, our findings suggest that EC1 does not mediate adhesion/binding specificity. EC1 has been shown to be responsible for the specificity of cadherin cis-dimerization (Shan et al., 2000). Perhaps EC1 specifies sorting because it is more important for the formation of dimers or higher order cadherin structures or other subsequent events that lead to sorting.

The lack of adhesive specificity for cadherins may not be totally surprising in light of the fact that factors other than cadherin subtype specificity can result in sorting of cells. Different levels of cadherin expression on cells will result in sorting (Friedlander et al., 1989; Steinberg and Takeichi, 1994). In fact, one of the best examples in vivo is the position of the oocyte in *Drosophila* embryos, which is driven by differential levels of expression of E-cadherin on the nurse cells (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). Regulation of cadherin adhesive activity also appears to be another mechanism that cells use to segregate tissues. The adhesive activity of *Xenopus* C-cadherin is down regulated in response to the mesoderm-inducing factor activin (Briehner and Gumbiner, 1994). During gastrulation in *Xenopus*, ingressing mesoderm cells separate from the overlying ectodermal layer as a result of their response to mesoderm-inducing factors like activin or related factors, and this segregation behavior was found to be mediated by C-cadherin (Wacker et al., 2000). Similarly, elongation of *Xenopus* animal cap tissue explants, which requires rearrangements and perhaps changes in sorting behavior, also requires the regulation of cadherin activity (Zhong et al., 1999). Nonetheless, changing the subtype of cadherin expression is still likely to be an important mechanism to achieve cell sorting and tissue separation, since there are numerous reports showing that tissue boundaries correlate with different cadherin expression patterns (Takeichi, 1995; Redies, 2000).

The lack of cell sorting behavior in many instances when cells express distinct cadherins raises the question of how generally important differential cadherin expression is for specifying cell sorting and maintenance of tissue boundaries. This question is relevant to morphogenesis in vivo as well as in in vitro–sorting assays. Perhaps the best example during tissue morphogenesis is in the case of heart development, in which the regulation of the pattern of N-cadherin expression was suggested to be essential for cardiac cell compartmentalization (Linask et al., 1997). Heart development is indeed disturbed in N-cadherin–negative mouse embryos (Radice et al., 1997), but this phenotype can be rescued to a great extent by expression of E-cadherin, indicating that cadherin-

mediated adhesion, but not the specificity of adhesion, is required (Luo et al., 2001).

Other groups of molecules may be as important as cadherins for cell sorting, rearrangements of tissues, and maintenance of tissue borders. The best examples are perhaps the ephrins and their receptors that are implicated in repulsion mechanisms in neuronal guidance and blood vessel remodeling (Mellitzer et al., 2000). In *Zebrafish*, rhombomere boundary formation is dependent on the alternate expression of ephrins and their receptors (Xu et al., 1999) and the restriction of cell intermingling requires their bidirectional signaling (Mellitzer et al., 1999). It is possible that these molecules exert part of their sorting effects by regulating cadherin adhesive strength or sorting activity. This is supported by the observation that overexpression of the EphA4 receptor in early *Xenopus* embryos disrupts cadherin dependent cell-cell adhesion (Winning et al., 1996).

Our findings demonstrate that cells expressing distinct cadherins can sort out from each other despite having similar basic binding properties for both cadherins. Future studies will be needed to determine the mechanisms that underlie cell-sorting behavior subsequent to the less specific binding and adhesion event.

Materials and methods

Cell lines and antibodies

CHO cells were obtained from American Type Culture Collection and cultured in F12 medium containing 10% bovine fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% glutamine. The CHO cells stably transfected with full-length *Xenopus* C-cadherin (Briehner et al., 1996) were cultured in MEM Glasgow medium containing 10% dialyzed fetal calf serum, penicillin, and streptomycin. Other stable, cadherin-expressing CHO cells were generated by transfecting cells with the appropriate plasmid using Lipofectamine (GIBCO BRL) and G418 (800 µg/ml) as selection. Single clones were assessed for clonal expression of the protein of interest after which positives were subjected to at least one round of subcloning by limited dilution. The plasmid encoding the extracellular domain of human E-cadherin fused to the Fc domain of human IgG was transfected into CHO cells and selected in 10% FCS/MEM Glasgow without glutamine and in the presence of 25 µM sulfoximine (Sigma-Aldrich). The following antibodies were used: a rabbit serum against *Xenopus* C-cadherin (Yap et al., 1997b), mAbs 9A10, and 5E3 directed against XE-cadherin (Choi and Gumbiner, 1989). The anti-human E-cadherin mAb HECD and the mAb directed against human N-cadherin were purchased from Zymed Laboratories. β-Catenin was detected using either a polyclonal antibody raised against its NH₂ terminus (McCrea et al., 1993) or an mAb (Transduction Laboratories). A monoclonal antibody recognizing all major isoforms of p120^{cas} was purchased from Transduction Laboratories. Fc fusion proteins were detected with an anti-human IgG-HRP conjugate (Promega).

Constructs

HE-cadherin cDNA was made as described (Gottardi et al., 2001). HN-cadherin cDNA was obtained from J. Hemperley (Becton Dickinson) and XE-cadherin cDNA was obtained from Dr. C. Kintner (Levine et al., 1994). The chimeric construct encoding the extracellular domain of C-cadherin fused to the Fc domain of human IgG is described elsewhere (Chappuis-Flament et al., 2001). To generate the human E-cadherin extracellular domain Fc fusion, the HE-cadherin cDNA/pBluescript plasmid was digested with HIII and KpnI, which cuts at position 1,472 in human E-cadherin (Bussemakers et al., 1993) and the resulting fragment was subcloned in the HIII-KpnI sites of pBluescript (pBS/HE1–1472). The polymerase chain reaction was used to generate a cDNA fragment of human E-cadherin (position 1472–2214) and adding a HIII and KpnI site at its 3' end terminus, which was subsequently digested with KpnI and inserted into the KpnI site of the pBS/HE1–1472. This plasmid was partially digested with HIII, filled in, and ligated to a Xba-I 8 mer. Subsequently, the DNA was digested with HIII and Xba-I and inserted into the HIII and Xba-I sites of the pEE14/Fc vector, resulting in a chimeric cDNA that encodes the E-cadherin extracellular do-

main fused at its COOH terminus to the Fc domain of human IgG. The PCR product and the final plasmid were sequenced to verify the presence of wild-type human E-cadherin cDNA and the in frame transition to the cDNA encoding the Fc part of human IgG.

Isolation of recombinant cadherin extracellular domains

CHO cells expressing secreted cadherins were grown for 2 wk to high density, after which the media was collected and filtered through a 0.45- μ m filter. Recombinant cadherin-Fc fusion proteins were isolated as described previously (Chappuis-Flament et al., 2001). Protein concentration was determined by Bradford assay (Bio-Rad Laboratories) and by running the proteins out on SDS-PAGE and staining with Coomassie brilliant blue. In general, a liter of medium yielded \sim 1 mg of recombinant protein.

Blastomere adhesion assay and laminar flow assays

The blastomere adhesion assay was performed as described previously (Zhong et al., 1999). Blastomeres were allowed to adhere to the proteins for 10 min, after which the 6-cm plates were rotated for 1 min at 80 rpm. Adhesion was quantified by counting the blastomeres before and after rotation. Independent experiments were repeated four times and results were averaged.

Adhesion flow assays were done essentially as described (Chappuis-Flament et al., 2001). Cells were allowed to adhere to the substrate (100 μ g/ml unless otherwise indicated) for a period of 10 or 40 min, after which increasing flow rates were applied. To vary specific cadherin concentrations, capillary tubes were first coated with 1 mg/ml protein A in HBSS for 5 h, subsequently blocked with 0.5% casein hydrolysate enzymatic (ICN Bio-medicals) in HBSS for 2 h, and incubated overnight with the appropriate dilution of cadherin-Fc protein mixed with human IgG Fc fragment (Calbiochem) to a concentration of total protein of 100 μ g/ml. Each assay was videotaped and adhesion was quantified by counting cells that remained at each flow rate and expressed as a percentage of total number of cells at start of the assay. In 40-min assays, cells were incubated with RGD peptide (1 μ g/ml; Sigma-Aldrich) 5 min before the assay to prevent nonspecific binding via integrins. All adhesion flow assays were performed at room temperature.

Bead aggregation and cell aggregation assays

Bead aggregation assays were performed as described (Chappuis-Flament et al., 2001). Aggregation assays were performed as described previously (Nose et al., 1988). For mixing assays, cells were labeled with fluorescent probes, either diO (10 μ g/ml or dil (2.5 μ g/ml; Molecular Probes), for 1 h at 37°C in HBSS without Ca^{2+} . After three washes with HBSS, cells were resuspended at a concentration of $4 \cdot 10^5$ /ml in HBSS/2% FCS. A total of 1 ml of cell suspension was added to each well of a 24-well plate, previously coated with 1% agarose. For mixing experiments, 0.5 ml of each cell line suspension was added. Plates were rotated at 80 rpm for 30 min to overnight, after which the cells were fixed in 1% paraformaldehyde. Mixed aggregates were counted by detecting the color of the aggregates and scoring them as either green (diO), red (dil), or containing both types of color, expressed as the percentage of total aggregates counted. Aggregates in which green and red cells were clearly separated were scored as a separate group and for final analysis, evenly divided as being either red or green aggregates. For each condition a minimum of 50 aggregates were counted. All the mixing experiments were done independently at least three times.

Immunoprecipitation, Western blotting, and immunofluorescence

To check for cell surface expression of cadherins, each cell line was cell surface biotinylated as described in Gottardi et al. (1995), after which cells were lysed in 1% NP-40/150 mM NaCl/20 mM Tris, pH 7.6/1 mM EDTA. Lysates were cleared by spinning for 10 min at 14,000 rpm and the concentration of the lysate was determined by Bradford assays (Bio-Rad Laboratories). For each cell lysate, equal concentrations of protein were incubated with a β -catenin polyclonal antiserum for 2 h at 4°C, after which 40 μ l of protein A was added to pull down the antigen-antibody complex (2 h, 4°C). Beads were washed three times with lysis buffer, two times with PBS, and were subsequently resuspended in SDS sample buffer. For the determination of total levels of expression in the stable lines, cells were directly lysed in 1% NP-40/150 mM NaCl/20 mM Tris, pH 7.6/1 mM EDTA and equal amounts of protein were analyzed. Proteins were separated by SDS-PAGE and transferred to nitrocellulose using standard methods (Laemmli, 1970; Towbin et al., 1979). After incubation with the appropriate primary and secondary antibodies, labeled proteins were visualized with ECL (Amersham Pharmacia Biotech). Biotinylated proteins were visualized by ECL after incubation of the membrane with streptavidin-conjugated to HRP. To detect membrane-associated cadherins and catenins,

cells were fixed with methanol and processed according to standard immunofluorescence protocols (Yap et al., 1998).

We thank Urs Rutishauser, Filippo Giancotti, and all members of the Gumbiner laboratory for valuable discussions during this project. We especially thank Cara Gottardi for critically reading the manuscript.

This work was supported by a National Institutes of Health grant (GM52717) awarded to Barry M. Gumbiner, by the Dewitt Wallace Fund for Memorial Sloan-Kettering Cancer Center, and by a Cancer Support grant NCI-P30-CA-08784. C.M. Niessen was a Charles A. Dana Fellow and supported by the Charles H. Revson Foundation and The Dutch Cancer Society.

Submitted: 8 August 2001

Revised: 4 December 2001

Accepted: 5 December 2001

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