Cadherin Conformations Associated with Dimerization and Adhesion

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To investigate conformations of C-cadherin associated with functional activity and physiological regulation, we generated monoclonal antibodies (mAbs) that bind differentially to monomeric or dimeric forms. These mAbs recognize conformational epitopes at multiple sites along the C-cadherin ectodomain aside from the well known Trp-2-mediated dimer interface in the N-terminal EC1 domain. Group 1 mAbs, which bind monomer better than dimer and the Trp-2-mutated protein (W2A) better than wild type, recognize epitopes in EC4 or EC5. Dimerization of the W2A mutant protein via a C-terminal immunoglobulin Fc domain restored the dimeric mAb-binding properties to EC4–5 and partial homophilic binding activity but did not restore full cell adhesion activity. Group 2 and Group 3 mAbs, which bind dimer better than monomer and wild type better than W2A, recognize epitopes in EC1 and the interface between EC1 and EC2, respectively. None of the mAbs could distinguish between different physiological states of C-cadherin at the cell surface of either Xenopus embryonic cells or Colo 205 cultured cells, demonstrating that changes in dimerization do not underlie regulation of adhesion activity. On the cell surface the EC3–EC5 domains are much less accessible to mAb binding than EC1–EC2, suggesting that they are masked by the state of cadherin organization or by other molecules. Thus, the EC2–EC5 domains either reflect, or are involved in, cadherin dimerization and organization at the cell surface.

Cadherins comprise a family of adhesion molecules that are important both for maintaining stable cell-cell adhesion and for regulating tissue morphogenesis during development (1). Cadherins mediate cell sorting (2, 3), and the pattern of expression of different cadherins often contributes to tissue morphogenesis (2, 4). Similarly, the level of expression of individual cadherins is regulated during developmental process as well as tumorigenesis (e.g. the epithelial and mesenchymal transition) (5). Furthermore, the adhesive activities of cadherin are also rapidly regulated during morphogenesis (6–8), although the mechanism of dynamic regulation is not well understood.

Classic cadherins are homophilic adhesion molecules and comprise five repeats of extracellular cadherin domains (EC), a transmembrane domain and a highly conserved cytoplasmic tail. Classic cadherins are regulated by proteins associated with its cytoplasmic tail, including β-catenin and p120, which are directly associated with cytoplasmic tail of cadherins, and α-catenin which is associated with β-catenin and thought to associate in some way with the actin cytoskeleton. Association with the cytoskeleton and clustering of cadherins is important to form strong adhesive interactions.

To understand how the adhesion activity of cadherins is regulated dynamically, it is necessary to understand the molecular nature of the homophilic adhesive bond. NMR and x-ray crystallographic and biophysical studies have provided evidence for the structure of the cadherin adhesive bond (9–15), but many different models have been proposed (1, 16). A key feature in all models is the role of the Trp-2 residue, located in the N-terminal EC1 domain, in the formation of a cadherin homodimer. Mutation of the Trp-2 has been shown to disrupt dimer formation and to abrogate adhesion (17, 18). In some models Trp-2 has been proposed to mediate cis dimerization of two cadherin subunits on the cell surface, and there is good cell biological and biochemical evidence for the presence of a cis dimer on cells (17–20). The Trp-2 has also been proposed to be the basis of the trans homophilic bond between cadherins on two different cells (9), and in one model there is thought to be an exchange between cis and trans dimer forms (20).

It has been proposed that control of cis dimerization mediated, at least in part, by the Trp-2 could underlie the regulation of cadherin-adhesive function (17, 21, 22), but so far changes in cis dimerization of the surface of cells has not been observed. However, no detailed analysis of cadherin structure at the cell surface of cells undergoing rapid, dynamic, physiological regulation of adhesive activity has yet been undertaken.

In some conditions, cadherin-mediated adhesion is regulated rapidly, sometimes in response to the action of growth factors (1). Also, there is some evidence for the inside-out signaling and outside-in signaling of cadherins in vitro and in vivo (1, 23, 24). We hypothesize that cadherins undergo conformational changes in response to regulation of cadherin accompanied with inside-out signaling. The phenomenon of inside-out regulation activated by signaling events is very well established for integrins, and integrins undergo well described conforma-

2 The abbreviations used are: EC, extracellular cadherin domain; mAb, monoclonal antibody; EGS, ethylene glycolbis(succinimidylsuccinate); ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; TBS, Tris-buffered saline; C-CHO, CHO/C-cadherin cells.
tion concentrations indicated, were bound via Ni²⁺ from a conditioned medium in the same way as wild type. After confirming that the sequence did not contain any other mutation, the plasmids were stably introduced in Chinese hamster cells. To cross-link proteins at the cell surface, Colo 205 cells, which were treated with or without 5 mM staurosporine overnight, were washed and then incubated with 2 mM Sulfo-EGS in Hepes buffer for 30 min. To obtain purified mAbs, hybridomas were cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 15% Ultra-Low IgG fetal bovine serum (Invitrogen) and 60 mM 2-mercaptoethanol, and mAbs were purified using Protein G column (HiTrapG, Amersham Biosciences) from the culture supernatant. Isotypes of all mAbs were determined using the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Applied Science). CCD1, CCD3, CCD5, and CCD23 are IgG1(k); CCD11, CCD19, and CCD21 are IgG1(l); and CCD25, CCD28, and CCD30 are IgG2b(k).

Chemical Cross-linking and Western Blotting—Purified CEC1–5 in Hepes buffer was incubated in 1 mM ethyleneglycol-bis(succinimidylsuccinate) (EGS, Pierce) for 30 min at room temperature to cross-link proteins. After being quenched by incubation with glycine to a final concentration of 50 mM for 30 min, proteins were separated on SDS-PAGE gel, transferred to nitrocellulose membranes, and detected with 6B6 anti-C-cadherin antibody and with horseradish peroxidase-conjugated goat anti-mouse Ig antibody (Bio-Rad) by a standard immunoblotting method.

To cross-link proteins at the cell surface, Colo 205 cells, which were treated with or without 5 mM staurosporine overnight, were washed and then incubated with 2 mM Sulfo-EGS in Hepes buffer for 30 min. Then samples were quenched with Tris to a final concentration of 50 mM for 30 min before lysing the cells with 1% Nonidet P-40 and 0.1 SDS in TBS containing protease inhibitor mixture (Complete #11897100, Roche Applied Science) and phenylmethylsulfonyl fluoride. The lysate was spun at 14,000 rpm for 20 min, and the proteins in the supernatant were analyzed by Western blot analysis as described above.

In the case of blastomeres, animal caps were dissected from embryos at stage 8, and one half were incubated with 5 ng/ml activin A for 2.5 h. Proteins were analyzed in the same way as Colo 205 cells, except that a lysis buffer without SDS was used.

Bead Aggregation Assay—The bead aggregation assay for Fc-tagged ectodomains of C-cadherin was conducted as described above (30). Briefly, Protein A-coated microspheres with 1.04-mm diameter (Bangs Laboratories Inc., Fishers, IN) were incubated with 1 mg/ml proteins for 90 min at room temperature. Beads were then washed and sonicated to obtain single beads determined by microscopy, divided, and supplemented with 1 mM CaCl₂ or 1 mM EDTA. The number of super-threshold-sized particles (set for size of large aggregates) as a function of time was determined using a Coulter counter, and the amount of protein coupled to beads was determined as described above.

Cell Aggregation Assay and Laminar Flow Cell Adhesion Assay—To obtain CHO-W2A cells, a W2A mutation was introduced to C-cadherin in pEE14 using a QuikChange mutagenesis kit. After the sequence was confirmed to be correct, the plasmid was stably transfected in CHO cells. The stable clones...
were subcloned three times, and the CHO-W2A cells were confirmed to express a similar amount of C-cadherin as the wild type. Cell aggregation assays were done with a standard method (31). Cells were washed, trypsinized in the presence of 1 mM CaCl₂, washed again, resuspended in Hanks' buffer (Invitrogen) with 2 mM CaCl₂ or 1 mM EDTA at 1 × 10⁵ cells/ml, and rotated at 100 rpm for the indicated time. The number of cells was counted, and the aggregation index was calculated as follows:

\[
\text{adhesion index} = \frac{N_t - N_0}{N_0}
\]

where \(N_t\) is number at certain time and \(N_0\) is the number at 0 min.

The laminar flow cell adhesion assay was conducted as described in a previous report (30). In brief, the cells were trypsinized in the presence of 1 mM CaCl₂, allowed to attach to glass tubes coated with a protein for 10 min, and washed away for 30 s at an indicated flow rate. The number of cells remaining after the wash was counted, and the adhesion percentage was calculated.

**Cellular ELISA**—Colo 205 cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium containing 10% fetal bovine serum. To induce cadherin-mediated cell adhesion, cells were incubated 20 nM staurosporine for 2 h, or washed once and incubated with 0.002% Trypsin in serum-free medium for 15 min (29). For cellular ELISA, cells were grown in 96-well plates pre-coated with 4 mg/ml collagen type IV, and incubated with purified mAbs for 2 h at 4°C. After washing three times with a medium containing 10% fetal bovine serum, cells were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) at 1:200 dilution for 1 h at 4°C, then washed three times with the medium and two times with Hanks’ buffer. The signals were detected with 3,3′,5,5′-tetramethylbenzidine as described above.

**Blastomere ELISA**—Animal cap explants from stage-8 embryo of *Xenopus laevis* were dissected and dissociated in calcium magnesium-free medium (7.5 mM Tris, pH 7.6, 2.4 mM NaHCO₃, 88 mM NaCl, 1 mM KCl). Dissociated blastomere cells were incubated with or without 5 nM activin for 90 min when necessary, 50 μl of cell suspension containing blastomeres from 10 animal caps was transferred to a 96-well ELISA plate pre-coated with poly-L-lysine, and cells were allowed to attach during the last 30 min of activin treatment. 150 μl of fetal bovine
serum in Marc’s modified Ringer’s medium, pH 7.4, was added to final 10% to block nonspecific binding sites and supply calcium, following by incubation for 15 min. Cells were then incubated with primary antibodies for 15 min at room temperature. A special method was developed to wash the blastomeres, which are much larger than tissue culture cells and lyse easily if exposed to air when medium is removed. The plate was submerged into 1 liter of wash buffer (Marc’s modified Ringer’s medium, pH 7.4, containing 1% glucose), and excess antibodies were washed away by very gentle removal of the buffer with a multiple tip pipette for 12 times. Then the plate was transferred to a bucket containing 1.7 liters of wash buffer, and then flipped and rotated several times. A set of washes in a tray and a bucket was repeated for a total of three times. After the last wash, most buffer was removed using a multiple tip pipette leaving 50 μl, and another 50 μl of horseradish peroxidase-conjugated secondary antibody, which was diluted at 1:100 in 20% fetal bovine serum/Marc’s modified Ringer’s medium, pH 7.4, was added to a well, and the mixture was bound for 30 min at room temperature. Excess secondary antibodies were washed away in the

![FIGURE 2](image)

**TABLE 1**

| Group | Name   | Binding activity | Epitope in | Western blot |
|-------|--------|-----------------|------------|--------------|
| Group 1 | CCD1   | M → D, W2A → Wt | Wt-Fc = W2A-Fc | EC1–5        |
|       | CCD3   | M → D, W2A → Wt | Wt-Fc = W2A-Fc | EC1–5        |
|       | CCD11  | M → D, W2A → Wt | Wt-Fc = W2A-Fc | EC1–5        |
|       | CCD19  | M → D, W2A → Wt | Wt-Fc = W2A-Fc | EC1–5        |
|       | CCD21  | M → D, W2A → Wt | Wt-Fc = W2A-Fc | EC1–5        |
| Group 2 | CCD5   | D → M, Wt → W2A | Wt-Fc > W2A-Fc | EC1–5        |
|       | CCD23  | D → M, Wt → W2A | Wt-Fc > W2A-Fc | EC1–5        |
| Group 3 | CCD25  | D → M, Wt | Wt-Fc | EC1–2        |
|       | CCD28  | D → M, Wt | Wt-Fc | EC1–2        |
|       | CCD30  | D → M, Wt | Wt-Fc | EC1–2        |

*D = dimer; M = monomer.*

**FIGURE 2.** Binding of monoclonal antibodies to W2A mutant C-cadherin. A, Trp-2 of CEC1–5 was replaced with Ala (CEC1–5,W2A). CEC1–5,W2A was analyzed for the dimerization by cross-linking with EGS, followed by Western blot analysis with anti-C-cadherin mAb. B, CEC1–5,W2A was run on a Super 12 gel filtration column to confirm that it did not form dimer. C, binding of monoclonal antibodies to CEC1–5 or CEC1–5,W2A determined by ELISA. Group 1 mAbs showed preference to W2A versus wild type. Group 2 mAbs showed a strong preference for wild type versus W2A, and Group 3 mAbs only bound to wild type.
Absorbance at 405 nm was measured. The supernatant without cells was transferred to a fresh well, and the absorbance at 405 nm was measured.

**RESULTS**

**Generation of Monomer- and Dimer-specific Monoclonal Antibodies**—To explore the potential conformational change in C-cadherin that accompanies its regulation during Xenopus gastrulation, we wanted to generate mAbs that distinguish states of C-cadherin. We previously reported that the ectodomain of C-cadherin (CEC1–5) forms monomeric or dimeric forms, and only the dimeric form had adhesive activity (21). Therefore, we used these protein species to screen mAbs. The purified histidine-tagged ectodomain of C-cadherin, CEC1–5, was separated by gel filtration to obtain monomeric and dimeric forms of CEC1–5 (Fig. 1A). CEC1–5 eluted from a Superose 12 column in earlier fractions was confirmed to form dimer by chemical cross-linking with EGS (Fig. 1B). The later fractions contained mainly monomeric forms. Fractions 10–13 or fractions 18–23 were combined and used as monomer or dimer forms, respectively.

Monomeric or dimeric species can be trapped by binding to nickel-coated substrates (21). Therefore, monomer or dimer fractions were bound to wells of nickel-coated 96-well plates, and mAbs were screened for preferential binding to either form using standard ELISA procedures (Fig. 1C). ~2,250 hybridoma supernatants from 2 fusions were screened, and we obtained mAbs CCD1, CCD3, CCD11, CCD19, and CCD21 that bound to monomer better than dimer (Fig. 1D). Also, mAbs CCD5 and CCD23 were found to bind dimer better than monomer (Fig. 1D). As shown in Table 1, we classify five mAbs that bind monomer better than dimer as Group 1, and the other 2 mAbs as Group 2 (see below for Group 3).

Because it has been shown that the tryptophan residue at the second position in EC1 has a critical role in E-, N-, and R-cadherin dimerization and cell adhesion (17, 18, 32), we analyzed whether the Trp-2 in C-cadherin is involved in cis dimerization. Wild-type and CEC1–5,W2A, the ectodomains of C-cadherin with Ala at the second residue instead of Trp, were chemically cross-linked with 2 mM EGS and analyzed by SDS-PAGE and immunoblotting. About one-fourth of wild-type CEC1–5 was cross-linked into dimers in these conditions, and cross-linking decreased significantly for CEC1–5,W2A (Fig. 2A). Loss of dimerization was further confirmed by gel filtration. Dimeric forms eluting in fractions 12–14 were not observed with CEC1–5,W2A (Fig. 2B).

The binding of the state-specific mAbs described above to CEC1–5,W2A were analyzed. All mAbs in Group 1, those that recognized monomer better than dimer, bound to CEC1–5,W2A better than wild-type CEC1–5 (Fig. 2C). Group 2 mAbs, which bound dimer better than monomer, bound to wild type better than the W2A mutant (Fig. 2C). The binding differences between W2A and wild type were more striking than the difference between monomer and dimer.

mAbs were also screened for their abilities to distinguish between wild-type CEC1–5 and CEC1–5,W2A. Three mAbs, CCD25, CCD28, and CCD30, bound to wild type but not to CEC1–5,W2A (Fig. 2C). Moreover, for the wild-type protein, these mAbs bound dimer better than monomer (Fig. 1D), consistent with the finding that CEC1–5,W2A does not dimerized significantly. We classified these three mAbs as Group 3, and all results are summarized in Table 1. Because monomer and dimer have the same amino acid sequences, all of these mAbs that differentially bind to dimer and monomer must recognize different conformations of CEC1–5.

We wished to map the epitopes on C-cadherin recognized by these monomer- or dimer-specific mAbs. Because the Group 2 and 3 mAbs showed striking reductions in their binding to the W2A mutant protein, we first tested whether the Trp-2 itself is a part of the epitope. A polypeptide of 15 amino acids from the C-cadherin N terminus and the one with W2A mutation were analyzed. All Group 2 mAbs exhibited a similar binding profile, and all Group 3 mAbs exhibited a similar binding profile.

**FIGURE 3.** Epitope mapping of mAbs. Binding of mAbs to series of C-cadherin ectodomains. 5.6 μg of Fc-tagged proteins was coated in each well of 96-well plates and detected by mAbs. A, binding of Group 1 mAbs. All exhibited a similar binding profile. B, binding of Group 2 and Group 3 mAbs. All Group 2 mAbs exhibited a similar binding profile, and all Group 3 mAbs exhibited a similar binding profile.

Monomer- or dimer-specific mAbs were obtained from the University of Virginia Biomolecular core facility. These mAbs were used to generate polyclonal sera against wild-type and W2A mutant CEC1–5, and analyzed by immunoblotting. About one-fourth of wild-type monomer CEC1–5 was cross-linked into dimers in these conditions, whereas CEC1–5,W2A did not exhibit similar binding profile, and all Group 3 mAbs exhibited a similar binding profile.

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facility and coated on a 96-well ELISA plate to test the binding of mAbs. None of the mAbs exhibited any binding to the peptides (data not shown). The peptides also failed to inhibit the binding of mAbs to full-length Wt or W2A mutant CEC1–5 (data not shown), indicating that the epitopes recognized by these mAbs probably do not include the Trp-2 or N-terminal 15 amino acids of C-cadherin.

We also mapped the epitopes recognized by the mAbs to specific EC domains in the C-cadherin protein. An ELISA binding assay using a series of different cadherin ectodomains constructs containing various combinations of EC domains fused at their C termini to the IgG Fc domain was performed for each of these mAbs (Fig. 3). Importantly, all of the mAbs within a group exhibited virtually the same EC domain binding profile,
suggesting that each group recognizes a similar or related set of epitopes.

The Group 2 mAbs, which prefer the dimer form and bind poorly to the W2A mutant protein, clearly recognize the EC1 domain (Fig. 3B). They all bind the minimal EC1-Fc construct and all EC1-containing constructs, but they fail to bind any construct lacking the EC1 domain, including EC2–5-Fc. Because these mAbs do not recognize the N-terminal Trp-2-containing peptide, they likely recognize a conformational epitope in EC1 that depends strongly on the formation of the Trp-2-dependent dimer.

The Group 3 mAbs, which prefer the dimer form and do not bind to the W2A mutant protein, recognize a more complex epitope requiring both EC1 and EC2 domains (Fig. 3B). They fail to bind EC1-Fc alone, but do bind well to EC1–2–Fc, suggesting that they might recognize EC2. However, they do not bind to EC2–5–Fc, indicating that they require EC1 for binding. Three explanations are possible: the Group 3 mAbs could bind a combined epitope at the interface of EC1 and EC2; they could recognize a conformational epitope in EC1 that requires the presence of adjacent EC2 domain to maintain the normal conformation of EC1; or they could recognize a conformational epitope in EC2 that requires the presence of adjacent EC1 domain to maintain the normal conformation of EC2. Because these mAbs prefer the dimer form and fail to bind the W2A mutant, these data suggest that Trp-2-dependent dimerization induces a conformation that is manifested in the interface between EC1 and EC2.

The Group 1 mAbs, which prefer the monomer form and prefer the W2A mutant protein over the wild-type protein, exhibit a complex binding profile, but the one or more epitopes they recognize clearly map toward the C terminus of the protein and involve domains EC4 and EC5 (Fig. 3A). They only bind to proteins containing EC5, suggesting initially that the epitopes reside in EC5. However, they bind poorly or not all to EC4–5 compared with either EC3–5 or EC1, -2, -4, and -5, indicating that the epitope requires an adjacent EC domain at N terminus of EC4–5 (although the specificity of this adjacent domain is not important). The two most likely explanations are: the mAbs recognize a conformational epitope in EC5 or at the interface of EC4 and EC5 and require another adjacent N-terminal domain to stabilize this conformation, or they recognize a conformational epitope in EC4 that requires adjacent EC domains at both the N terminus and the C terminus. Because Group 1 mAbs prefer monomer and the W2A mutant protein, these data suggest that Trp-2-dependent dimerization induces a conformation that is manifested in the interface between EC1 and EC2.

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Because we found that the mutation in Trp-2 makes CEC1–5 monomeric, we analyzed whether the mutation itself or dimer-
The adhesive activity of C-cadherin is down-regulated to undergo morphogenetic cell movements during gastrulation. The ectodomains CEC1–5 and CEC1–5, W2A were fused with Fc portion of human immunoglobulin (CEC1–5–Fc and CEC1–5, W2A–Fc) at the C terminus to force dimerization. The binding preferences of all five Group 1 mAbs disappeared, and they bound CEC1–5, W2A–Fc and CEC1–5–Fc almost equally (Fig. 4). Forced dimerization at the C terminus did not affect the binding preference of Group 2 and 3 mAbs; Group 2 mAbs still bound wild type better than CEC1–5, W2A–Fc, and all Group 3 mAbs only bound to wild-type CEC1–5–Fc (Fig. 4). These results indicate that the binding affinity for Group 1 mAbs, which bind to EC4–5, is dependent on dimerization but not the W2A mutation, and those of Group 2 and 3 are dependent on either the W2A mutation or require dimerization of the N terminus. Also, these results indicate that the forced dimerization makes the conformation or accessibility of epitopes of Group 1 mAbs on W2A mutant similar to wild type. We propose a model in Fig. 6 to understand these conformational changes (see “Discussion”).

Role of Trp-2 and Dimerization in Adhesion and Binding Activity of C-cadherin—Forced dimerization via the C-terminal Fc domain made the epitope recognized by Group 1 mAbs in EC4–5 behave like wild type. Therefore, we determined whether forced dimerization of the W2A mutant protein by the Fc domain influenced functional activity. We confirm that the Trp-2 is required for C-cadherin-mediated adhesion when expressed in cells (Fig. 5A), as has been shown for other classical cadherins (17, 32). We then tested the adhesion activity of purified W2A mutant proteins compared with normal. Interestingly, CEC1–5, W2A–Fc, which is force-dimerized at the C terminus and has dimeric conformation at EC4–5 recognized by Group 1 mAbs, did exhibit basic homophilic binding activity in the bead-aggregation assay (Fig. 5B). In contrast to the bead aggregation assay, CEC1–5, W2A–Fc did not exhibit any cell adhesion activity as measured by a cell-attachment flow assay (Fig. 5C). Therefore, forced dimerization via C terminus generated only partial homophilic binding activity, whereas it restored the wild-type conformation at EC4–5, as shown by binding of Group 1 mAbs (Fig. 6). But C-terminal dimerization was insufficient to restore full cell adhesion activity.

Assays for Conformational Change during Physiological Regulation of C-cadherin Adhesion Activity—It has been hypothesized that a monomer to dimer transition may underlie the physiological and dynamic regulation of cadherin adhesion (17, 21, 22, 33). Therefore, we asked whether our conformation sensitive mAbs could detect a conformational change in C-cadherin on the cell surface during cadherin regulation. We first examined C-cadherin regulation in Xenopus blastomeres stimulated to undergo morphogenetic cell movements during gastrulation. The adhesive activity of C-cadherin is down-regulated in response to the transforming growth factor-β type growth factor activin. Regulation occurs with no change in its amount at the cell surface or in the amount of catenin binding, suggestive of inside-out signaling. A special cell surface mAb binding assay had to be developed, because blastomeres are unusually large and fragile (see “Experimental Procedures”). Group 2 and 3 mAbs bound well to the blastomere surface at a low concentration of 0.5 μg/ml, but Group 1 mAbs did not (data not shown); therefore, we used a higher concentration of 20 μg/ml for Group 1 mAbs. mAb binding to blastomeres was reproducible, but there was no significant difference in binding to the blastomere surface with or without activin treatment (Fig. 7A). CCD3, CCD11, and CCD21 did not bind very well to the cells at high concentration, so it could not be ascertained whether any conformational changes occurred in epitopes recognized by these mAbs.

Another good model for cadherin physiological regulation is the human colorectal carcinoma cell line Colo 205. It has been reported that endogenous E-cadherin expressed in Colo 205 cells is completely inactive in regular culture, but can be strongly activated by incubation with a very low amount of trypsin or by treatment with the general protein kinase inhibitor, staurosporine (29). The mechanism of staurosporine-induced adhesion is not yet known, but it correlates with altered phosphorylation of p120 catenin. Nonetheless, E-cadherin activation occurred with no change in its amount at the cell surface or in the amount of catenin binding, suggesting an inside-out signaling mechanism. We expressed C-cadherin stably in Colo 205 cells, and the binding of mAbs to the cell with or without staurosporine-induced activation was tested by cellular ELISA (Fig. 7B). Group 2 and Group 3 mAbs bound to cells well, and their bindings were the same at all mAb concentrations with and without treatment with staurosporine (Fig. 7B). Because CCD5 showed cross-reactivity to purified human E-cadherin-Fc (data not shown), CCD5 was used to test whether binding to E-cadherin changed during activation of parental Colo 205 cells. Binding to E-cadherin with or without activation by staurosporine was also same (Fig. 7C). Even though all Group 1 mAbs bind well to purified C-cadherin proteins (Fig. 2), none of them bound to C-cadherin on the cell surface very well (see below). Therefore, it was not possible to ascertain whether the epitopes recognized by Group 1 mAbs undergo changes during cadherin activation.

We also used chemical cross-linking as an alternative approach to examine whether cadherin dimerization at the cell surface is affected during physiological regulation. C-cadherin at the cell surface was cross-linked with Sulfo-EGS, which is a membrane-impermeable cross-linker, and formation of the dimer was detected by Western blot analysis. E-cadherin of Colo 205 cells was also cross-linked with Sulfo-EGS, and no change in the amount of dimer was apparent as a result of stau- rosporine-induced activation (Fig. 8A). Cross-linking was less efficient for blastomeres than for Colo 205 cells, probably due to technical limitation of the cross-linking method. Blas-
### Cadherin Conformational Antibodies

**A**

|          | Staurosporine | Sulfo EGS |
|----------|---------------|-----------|
| C-cadherin | - | - | + | + |
| α-tubulin  | - | + | - | - |

**B**

|          | Activin | Sulfo EGS |
|----------|---------|-----------|
| E-cadherin | - | - | + | + |
| α-tubulin  | - | + | - | - |

**FIGURE 8.** Chemical cross-linking of cadherins at the cell surface during physiological regulation. A, E-cadherin of Colo 205 cells with or without staurosporine treatment was cross-linked with membrane-impermeable cross-linker Sulfo-EGS to cross-link membrane proteins. Proteins were extracted with 1% Nonidet P-40 and 0.1% SDS, and lysates were analyzed by Western blotting. B, blastomeres with or without activin treatment were incubated with Sulfo-EGS, proteins were extracted with 1% Nonidet P-40, and lysates were analyzed by Western blotting.

Accessibility of EC Domains at the Cell Surface—In the cell surface binding assays described above, we realized that some mAbs do not bind to the cell surface well. Therefore, we did a careful comparison of the binding of various C-cadherin mAbs to the purified protein and to the cell surface of Colo 205/C-cadherin cells and CHO/C-cadherin cells (C-CHO). All mAbs against C-cadherin, including those described in this report and several that were produced in previous studies (6, 8), showed similar binding to Fc-CEC1–5 (Fig. 9A). Strikingly, only Group 2 and 3 mAbs, which recognized EC1 or EC2 (blue), bound C-cadherin at the cell surface very strongly (Fig. 9B). Group 1 mAbs, which bind EC4–5, did not bind to the cell surface very well (Fig. 9B). Low binding of Group 1 mAbs was due to their preference for monomer, which may not even exist on the live cell surface, because they did not bind any better to the W2A mutant protein expressed in CHO cells (Fig. 9C). Moreover, other mAbs that bind to EC3–5 domains, but do not distinguish monomer from dimer, also bound poorly to the cell surface. mAb CCD6, which binds EC5, and two mAbs that recognize EC3, 6B6 and 5G5 (6), bound to C-cadherin on C-CHO and Colo 205/C-cadherin cells very poorly (Fig. 9B). mAb AA5, which binds EC5, also bound to cells poorly, although a little better to C-CHO cells, but this mAb may be different from the others, because it activates C-cadherin at the cell surface (8). Thus, the epitopes that project farthest from the cell surface in EC1–EC2 domains are much more accessible to antibodies than epitopes closer to the cell surface in EC3–5.

The properties of Group 2 and 3 mAbs binding to W2A mutant proteins expressed at the cell surface was the same as to purified soluble proteins (Fig. 9C); Group 2 mAbs bound wild-type C-cadherin better than W2A mutant C-cadherin, and Group 3 mAbs didn’t bind W2A mutant C-cadherin at all. This confirms that these antibodies are able to detect some of the conformational changes described in this report in the setting of the live cell surface.

**DISCUSSION**

In this study we were able to generate mAbs that recognize conformation-sensitive epitopes of C-cadherin associated with dimerization. Given the central role of the Trp-2-dependent dimer in the function of classic cadherins (1, 9, 14, 16, 20), the binding properties of these mAbs provide important insights into the structural conformations of cadherins involved in the control of adhesion. These mAbs also allowed us to test the hypothesis that a change in the formation of cis dimers at the cell surface is responsible for the dynamic physiological regulation of cadherin adhesion; i.e. the basis of inside-out signaling. Finally, experiments on the binding of many EC domain-specific mAbs to C-cadherin on living cells revealed novel information about the structural organization of the different EC domains at the cell surface.

The Trp-2 residue has been shown to mediate both cis and trans dimers between cadherin subunits (9, 14, 17–20). It is likely that the soluble C-cadherin proteins described in the present study are predominantly the Trp-2-mediated cis dimer forms. We know that that the CEC1–5–Fc protein used is a cis dimer, because the Fc domain at the C terminus forces parallel covalent (disulfide-mediated) dimerization between subunits. The His6-tagged version of CEC1–5 was shown in a previous study to exist in a simple monomer-dimer equilibrium (21), and in the present study we found that this dimerization depends on the Trp-2 residue. Because only the dimeric form of CEC1–5 exhibits homophilic aggregation activity, we argued that it represents the cis dimer form that is capable of engaging in the formation of the homophilic bond (21). Therefore, although we cannot rule out the presence of some trans Trp-2 dimers in the His6-C-cadherin protein samples, the experimental evidence indicates that we are analyzing primarily the cis dimer form.

A key finding in this report is that conformation-sensitive epitopes associated with Trp-2-mediated dimerization are located at many regions along the cadherin molecule, not just in the Trp-2-containing peptide. Group 2 mAbs, which prefer the dimer and bind poorly to the W2A mutant proteins, recognize an epitope in the EC1 domain. Because the epitope does not seem to reside in the N-terminal peptide containing the Trp-2, it is probably located at another site in EC1. Thus, dimerization is associated with changes in the conformation of epitopes at other regions of EC1, which is similar to the finding previously reported by Harrison et al. (34).

More interesting are the Group 3 mAbs, which implicate EC2 in the dimer conformation. Group 3 mAbs, which prefer the dimer form and do not bind to the W2A mutant protein,
recognize an epitope that requires both EC1 and EC2, suggesting that the epitope resides at the EC1–EC2 interface or perhaps in EC2. If so, this indicates that conformational changes associated with dimerization via Trp-2 are propagated beyond EC1 to EC2. Alternatively, the epitope resides in EC1, but absolutely requires adjacent EC2 to form its normal conformation. In this case EC2 and its interface with EC1 are coupled in some way to the formation of a dimer specific conformation.

The Group 1 mAbs, which prefer monomer forms, clearly demonstrate that conformational changes associated with dimerization are propagated all the way to the EC4 or EC5 domains. During dimer formation the epitopes in EC4/EC5 are either altered or become masked. Our experiments suggest that this change is due to dimerization per se, rather than Trp-2 binding to its pocket in EC1, because the Group 1 binding preference is reconstituted by forced dimerization via the C-terminal Fc, even in the W2A mutant protein. Therefore, the Group 1 epitopes in EC4/5 are potential candidates for the regulation of the cadherin subunits; they could either provide a novel dimerization interface in addition to the Trp-2 or become partially masked, because they reside near a dimerization interface; alternatively, a change in the conformation of the epitope in EC4/EC5 results from a dimerization at another nearby site independent of EC1. This is the first time that EC4 or EC5 have been associated with dimerization, suggesting that they have a role in cadherin function beyond serving as simple spacers, as some of the structural models have implied.

A role for the EC4/5 domains in adhesive function has come from many studies. In the present report, we find that forced dimerization of the W2A mutant protein via the C-terminal Fc domain can generate a protein with partial homophilic binding activity, as assessed by bead aggregation. Although it is not sufficient for full cell adhesive function without the Trp-2, this suggests that it may contribute weakly to homophilic binding. Similarly, biophysical studies on the adhesive forces produced by cadherins implicate C domains besides EC1 in homophilic bond formation (15). Genetic studies also provide strong evidence for a function of EC4 and EC5 domains in N-cadherin; missense mutations, resulting in single amino acid substitutions, in EC4 or EC5 of zebrafish N-cadherin cause phenotypic defects similar to the one caused by a mutation that truncates the protein from the membrane (35, 36). Moreover, EC5 has been implicated in the physiological regulation of C-cadherin, because it contains the epitope recognized by an activating mAb (8). It will be important to learn more about the structural conformations and functions of these domains to better understand the mechanisms of cadherin function.

Alterations in cadherin cis dimerization at the cell surface have been hypothesized to serve as a mechanism for the physiological regulation of adhesive function (17, 21, 22). Although it is well established that the Trp-2 can mediate cis dimerization (14, 17–20), the Trp-2 can also occur in other states, including formation of a trans dimer (9, 20), formation of an intramolecular bond (13), or unbound in a free monomeric form (17, 21). Dynamic changes in Trp-2-mediated interactions could therefore regulate adhesion. With our mAbs capable of detecting conformational epitopes associated with cis dimerization, we...
have been able to ask whether changes in dimerization occur on the surface of the living cell during physiological regulation. We used two powerful cellular models for cadherin regulation, both of which have implicated inside-out regulation of cadherin conformation at the cell surface. C-cadherin is regulated in Xenopus embryonic cells (blastomeres) during morphogenesis or treatment with the growth factor activin without any changes in cell surface levels or binding to catenins (6, 8). In nonadhesive Colo 205 cells, certain treatments cause a dramatic induction of cadherin-mediated adhesion without any changes in cell surface levels or binding to catenins (29). In neither model system could we detect any change in the binding of dimer or monomer preferring mAbs to the cell surface during changes in cadherin-mediated adhesion. We also found no evidence for changes in E-cadherin in the Colo 205 cells during adhesion induction. These findings were confirmed by chemical cross-linking experiments, which also failed to reveal any changes in the amount of cadherin dimer at the cell surface. Thus, changes in cadherin cis dimerization at the cell surface do not seem to underlie physiological regulation, and inside-out regulation must occur by other kinds of changes in cadherin conformation or oligomerization.

In the course of performing assays for mAb binding to the cell surface, we discovered a novel and interesting feature of cadherin structure and/or organization at the cell surface that may have important implications for function. The EC1 and EC2 domains are much more accessible to antibody binding than are EC3, EC4, and EC5, even though all domains are similarly accessible in the purified soluble C-cadherin protein. This suggests either of two likely general explanations: 1) The EC3–EC5 domains could be masked by the presence of other cell surface material, such as the glycocalyx. 2) Alternatively, inaccessibility could be due to the cadherin molecules interacting with other proteins or becoming organized in an oligomeric state that involves binding interactions with the EC3–EC5 domains. Such interactions could have implications for either the mechanism of adhesion and adhesion regulation or for outside-in signaling functions mediated by cadherins.

Very much has been learned about classic cadherin function from structural studies of the EC1 domain, especially the role of the Trp-2 in dimer formation (cis or trans). Yet there have been numerous indications of a role for the other EC domains, including genetic evidence for mutations that disrupt function, the presence of activation epitopes, and biophysical measurements of adhesive forces. Our mapping of conformational changes associated with dimerization and function to EC2–EC5 begins to provide insights into structural roles of these domains in the adhesive process.

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