Cadherin-dependent mechanotransduction depends on ligand identity but not affinity

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Summary
This study investigates the relationship between classical cadherin binding affinities and mechanotransduction through cadherin-mediated adhesions. The mechanical properties of cadherin-dependent intercellular junctions are generally attributed to differences in the binding affinities of classical cadherin subtypes that contribute to cohesive energies between cells. However, cell mechanics and mechanotransduction may also regulate intercellular contacts. We used micropipette measurements to quantify the two-dimensional affinities of cadherins at the cell surface, and two complementary mechanical measurements to assess ligand-dependent mechanotransduction through cadherin adhesions. At the cell surface, the classical cadherins investigated in this study form both homophilic and heterophilic bonds with two-dimensional affinities that differ by less than threefold. In contrast, mechanotransduction through cadherin adhesions is strongly ligand dependent such that homophilic, but not heterophilic ligation mediates mechanotransduction, independent of the cadherin binding affinity. These findings suggest that ligand-selective mechanotransduction may supersede differences in cadherin binding affinities in regulating intercellular contacts.

Key words: Cadherin, Mechanotransduction, Binding selectivity, Magnetic twisting cytometry, Micropipette manipulation, MTC

Introduction
Cadherins are essential adhesion proteins at intercellular junctions in all cohesive tissues. In mature tissues, they maintain the mechanical integrity of cell–cell junctions, and regulate the barrier properties of tissues such as the vascular endothelium and intestinal epithelia. In development, cadherins are essential for morphogenesis (Gumbiner, 2005). Following the first demonstrations that dissociated embryonic cells re-aggregated with cells from the same germ layer in vitro (Steinberg and Gilbert, 2004; Townes and Holtfreter, 1955), in vitro assays demonstrated that different cadherins induce cells to segregate away from each other (Friedlander et al., 1989; Nose et al., 1988). In vitro assays, which visualized cell aggregate compositions either in hanging droplets or in agitated cell suspensions, suggested that cell-sorting might be driven by differential adhesion (Friedlander et al., 1989; Steinberg, 1962; Steinberg, 1963; Townes and Holtfreter, 1955) and the minimization of surface free energies, attributed to differences in cadherin binding affinity or surface expression (Foty et al., 1996; Foty and Steinberg, 2005; Steinberg, 1962; Steinberg, 1963; Steinberg, 2007).

Observations support the hypothesis that surface free energy minimization determines cell organization in vitro and in vivo. Cells in the ommatidium of the Drosophila retina adopt patterns that are similar to surface-tension-dependent shapes of soap bubbles (Hayashi and Carthew, 2004; Hilgenfeldt et al., 2008; Käfer et al., 2007). In Drosophila, oocyte positioning in the egg chamber requires DE-cadherin expression and appears to correlate with the local DE-cadherin expression levels (Godt and Tepass, 1998). Genetic switches control the programmed down regulation and expression of different cadherins during neural crest cell emergence and migration out of the neurepithelium (Niessen et al., 2011; Takeichi et al., 1990).

Cadherins were initially thought to mainly form homophilic bonds, based on the tendency of cells expressing the same cadherin to co-aggregate in vitro (Nose et al., 1988). However, semi-quantitative estimates of interfacial tension and cell adhesion (Duguay et al., 2003; Niessen and Gumbiner, 2002), as well as quantitative measurements of cadherin adhesion energies and solution binding affinities (Chien et al., 2008; Harrison et al., 2010; Katsamba et al., 2009; Prakasam et al., 2006; Shi et al., 2008; Vendome et al., 2011), contributed to a more nuanced view that cadherin subtypes cross-react, but that their relative adhesion energies determine cell segregation patterns. Comparisons of cadherin-dependent, in vitro cell sorting with solution binding affinities suggest that affinities differing by more than fivefold support cell segregation (Katsamba et al., 2009; Tabdili et al., 2012). However, in other cases, smaller adhesive differences did not predict cell-sorting outcomes (Niessen and Gumbiner, 2002; Prakasam et al., 2006; Shi et al., 2008).

Despite the appeal of the surface-free-energy-minimization arguments, cell surface adhesion energies do not account for the effect of cortical tension on intercellular interactions. Increasing evidence suggests that myosin II-dependent contractile forces are central to determining cell shape, intercellular extension, and the maintenance of tissue boundaries (Bertet et al., 2004; Lecuit, 2005; Lecuit and Le Goff, 2007; Lecuit and Lenne, 2007; Lecuit et al., 2011). Studies increasingly also show that mechanical forces exerted during development alter signaling and actomyosin
dynamics (Kasza and Zallen, 2011). Cell elongation and intercalation are driven by anisotropic tension in cells that originates from asymmetric intracellular myosin II and actin distributions (Bertet et al., 2004; Cavey et al., 2008; Rauzi et al., 2010; Tepass et al., 2002). During cell division, the strict maintenance of some cell compartments appears to be regulated largely by cortical actomyosin bundles adjacent to the membrane (Monier et al., 2010). The elastic-like properties of tissues also appear to influence cell organization in Xenopus embryos (Luu et al., 2011). In a comparison of the relative influence of adhesion versus cortical tension, single cell force spectroscopy measurements demonstrated that cohesive forces between zebrafish germine progenitor cells did not specify cell localization in the embryo (Krieg et al., 2008). Instead, cortical tension appeared to determine cell positioning. Theoretical analysis predicts that cortical tension and adhesion energies coordinate influence sorting (Manning et al., 2010).

The seemingly overriding role of cell mechanics in directing some cell movements in vivo was puzzling in light of the cadherin requirement for morphogenesis and cell segregation in vitro. The connection between cadherin-dependent sorting and cortical tension was not obvious. However, the recent discovery that cadherin complexes are intercellular force sensors suggests a cadherin-dependent mechanism that could bridge both the cadherin requirement for cell sorting and cadherin-mediated changes in cortical tension (Ladoux et al., 2010; le Duc et al., 2010; Liu et al., 2010; Yonemura et al., 2010). Cadherins are both adhesion proteins and cytoskeletal regulatory proteins (Niessen et al., 2011). Although cadherin ligation alone activates changes in cytoskeletal organization through Src and GTPases, cadherin complexes actively respond to applied force to alter cell mechanics (Ladoux et al., 2010; le Duc et al., 2010; Yonemura et al., 2010). An unanswered question has been whether cadherin-binding specificity could also modulate cell mechanics.

This study demonstrates that mechanotransduction at cadherin complexes is ligand dependent, but that ligand-selective force sensation is not determined by the affinities of the cadherin bonds. Magnetic twisting cytometry and traction force microscopy assessed mechanotransduction in response to acute, bond shear and to endogenous contractile forces on cadherin bonds, respectively. Micropipette measurements of cadherin-mediated intercellular binding kinetics determined the two-dimensional (2D) binding affinities and dissociation rates of the identical cadherin pairs as probed in mechanical measurements. Comparisons of cadherin binding affinities with mechanotransduction responses show that homophilic, but not heterophilic cadherin ligands trigger junction reinforcement, independent of the cadherin affinities. Qualitatively similar results were obtained with five different cell lines and three different classical cadherins. They suggest that, although classical cadherin binding affinities differ, the ligand-dependent modulation of cell mechanics may play a greater role in regulating intercellular boundaries.

Results

EC1-dependent cadherin binding affinities

Micropipette manipulation measurements (Fig. 1A) (Chesla et al., 1998; Chien et al., 2008; Huang et al., 2007; Huang et al., 2010; Long et al., 2001; Zhang et al., 2005) were used to determine the two-dimensional EC1-dependent binding affinities between cell surface cadherins (Fig. 1B) and recombinant human immunoglobulin Fc-tagged cadherin extracellular domains immobilized on an apposing red blood cell (Fig. 1C) (Chien et al., 2008). This experiment quantifies the intercellular binding probability, which is the number of intercellular binding events $n_b$ divided by the number of cell–cell contacts $N_c$, as a function of the intercellular contact time. The binding probability is related to the number of inter-surface bonds (Chen et al., 2008). Fig. 2 shows the time dependence of the binding probability measured between an MDCK cell (E-cad) and a red blood cell (RBC) modified with oriented, Fc-tagged extracellular domains of canine E-cadherin (k9E-cad.Fc). As observed previously (Chien et al., 2008), the time course exhibits two kinetic stages. There is a fast, initial rise to a plateau at a probability $P_1 \sim 0.4$—that is, four out of ten cell–cell contacts results in binding. After a 2–5-second delay, the binding probability again rises to a final, limiting plateau at $P_2 \sim 0.53$.

A prior study with Xenopus C-cadherin mediated cell–cell binding first reported the two-stage binding kinetics (Chien et al., 2008). The use of domain deletion mutants localized the different features in kinetic time course to structural regions of the extracellular domain. The latter approach demonstrated that the fast, first step requires the EC1 domain, whereas the second rise to the second plateau $P_2$ requires the full ectodomain, e.g. EC1-5 (Chien et al., 2008). Because EC1 embeds the specificity-determining region (Nose et al., 1990), the analyses of the kinetic
Table 1. Best fit parameters from nonlinear least squares fits of EC1-dependent cell–cell binding kinetics to Eqn 1

<table>
<thead>
<tr>
<th>Test cell</th>
<th>Cadherin</th>
<th>Density (#/μm²)</th>
<th>Red cell</th>
<th>Density (#/μm²)</th>
<th>2D affinity (×10⁻⁴ μm²)</th>
<th>Off rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDCK</td>
<td>k9 E-cadherin</td>
<td>17</td>
<td>k9 E-cad.Fc</td>
<td>7</td>
<td>3.6±0.2</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>MCF7</td>
<td>k9 E-cadherin</td>
<td>17</td>
<td>k9 E-cad.Fc</td>
<td>7</td>
<td>2.5±0.2</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>hN-cadherin</td>
<td>93</td>
<td>ck N-cad.Fc</td>
<td>32</td>
<td>0.70±0.06</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>hN-cadherin</td>
<td>93</td>
<td>k9 E-cad.Fc</td>
<td>7</td>
<td>1.2±0.2</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>MCF7</td>
<td>hE-cadherin</td>
<td>7</td>
<td>k9 E-cad.Fc</td>
<td>18</td>
<td>4.2±0.2</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>MCF7</td>
<td>hE-cadherin</td>
<td>7</td>
<td>ck N-cad.Fc</td>
<td>37</td>
<td>2.7±0.1</td>
<td>2.2±0.4</td>
</tr>
</tbody>
</table>

Fig. 2. Kinetics of cadherin-mediated binding between an MDCK cell and a red cell modified with oriented k9-E–cad.Fc extracellular domains.

The E-cadherin densities on the MDCK cell and the red blood cells were 17 cadherin/μm² and 7 cadherin/μm², respectively. The contact area A_c was 7 μm². The solid line is the best, nonlinear least squares, fit of Eqn 1 to the data for the first, EC1-dependent binding step, and the dashed lines are the 95% confidence intervals. Best fit parameters are summarized in Table 1. The data described in this study focused on the first EC1-dependent binding step.

The trans dimerization of EC1 domains occurs by the mutual exchange of tryptophan at position 2 (W2), in which the side chain of W2 docks in the binding pocket of the EC1 domain of an apposing cadherin (Boggon et al., 2002). This mechanism is described by the reaction scheme:

\[ R + L \xrightarrow{k_r} B \]

The following equation (Eqn 1) gives the analytical expression for the time-dependent binding probability P(t) for this reaction (Chesla et al., 1998):

\[ P = 1 - \exp \left\{ -[m_R]_{2} m_R A_c K_s (1 - \exp(-k_c t)) \right\} \tag{1} \]

where \( m_R \) and \( m_R \) are the receptor and ligand surface densities (no./μm²) on the two cells, \( A_c \) is the contact area (μm²), \( K_s \) is the two-dimensional binding affinity (μm²), and \( k_r \) is the dissociation rate (s⁻¹). Therefore, \( K_s \) and \( k_r \) can be determined from nonlinear, least-squares fits of the first EC1-dependent binding step to Eqn 1. Details of the data analyses are described in Materials and Methods.

The solid line in Fig. 2 is the weighted, nonlinear least squares fit of the first binding step to Eqn. 1, and the best-fit two-dimensional affinity for homophilic N-cadherin binding was 1.9±0.3×10⁴ μm², and the heterophilic binding affinity is intermediate between the homophilic affinities at 2.8±0.3×10³ μm². The best-fit parameters are summarized in Table 1. The results of measurements with MCF7 cells probed with canine E-cad.Fc or with ck N-cad.Fc are also in Table 1. In these measurements, the homophilic E-cadherin affinity exceeds that of N-cadherin, although the absolute differences are not large.

**Cadherin-dependent mechanotransduction is ligand dependent**

To determine how cadherin affinity differences affect force transduction through cadherin adhesions, magnetic twisting cytometry measurements (MTC; Fig. 1D) were carried out with different cells and different cadherin ligands. These measurements quantify changes in cadherin junction mechanics in response to shear forces applied to cadherins on the cell surface by ligand-modified beads. These studies used ferromagnetic beads that were modified with recombinant, extracellular domains of either the same cadherin subtype as expressed on the cell (homophilic ligand) or a different subtype (heterophilic ligand). In these measurements, the bead is magnetized parallel to plane of the cell, and an orthogonal oscillatory magnetic field induces a torque on the bead, causing it to twist (Fig. 1D). The resultant bead displacement reflects the viscoelasticity of the bead–receptor–cytoskeletal junction, such that changes in the bead displacement reflect junction remodeling and cell contractility.

Fig. 3 compares bond shear measurements conducted with four different cell lines: MDCK, C2C12, MCF7 and MDA-MB-435 cells. The probe beads were modified with Fc-tagged ectodomains of chicken N-cadherin, canine E-cadherin, or *Xenopus* C-cadherin, as in the micropipette measurements. Fig. 3A shows the percent change in the stiffness of the bead-cell junction, relative to unperturbed bonds. Here, the adhesive junction was between N-cad.Fc coated beads and N-cadherin on C2C12 cells. As reported previously with F9 cells (le Duc et al., 2010), the cadherin junction stiffens in response to acute, applied bond shear. This stiffening response is ablated by treatment with EGTA (Fig. 3A), which chelates Ca²⁺ ions required for cadherin function. It is also abolished following F-actin depolymerization by treatment with latrunculin B (Fig. 3B). The mechanotransduction is therefore cadherin and F-actin dependent, in agreement with previous findings (le Duc et al., 2010). By contrast, when the beads were modified with a different cadherin subtype, e.g. C-cad.Fc or E-cad.Fc, there was no change in junction stiffness relative to controls. Bead pulls with an anti-N-cadherin antibody, which recognizes the N-terminal EC1 domain, also failed to induce junction remodeling (Fig. 3B). The results of measurements with
Fig. 3. Cadherin-dependent mechanotransduction is ligand selective. (A) MTC measurements of the force-induced stiffening response of C2C12 cells probed with beads coated with N-cad.Fc (black squares), E-cad.Fc (black circles) or C-cad.Fc (gray circles). Controls were with N-cad.Fc beads and 4 mM EGTA (white diamonds). (B) Control measurements with C2C12 cells probed with beads coated with anti-N-cadherin antibody (white diamonds), or with N-cad.Fc in the absence (black squares) and presence (black circles) of latrunculin B. (C) MTC measurements of the force-induced stiffening response of MDA-MB-435 cells probed with beads coated with N-cad.Fc (black squares), E-cad.Fc (black circles) or C-cad.Fc (gray circles). Controls were with N-cad.Fc in the presence of 4 mM EGTA (white diamonds). (D) MTC measurements of MCF7 cells probed with beads coated with E-cad.Fc (black squares), N-cad.Fc (black circles) or C-cad.Fc (gray circles). Controls were with E-cad.Fc, in the presence of EGTA (white diamonds). (E) MCF7 controls with E-cad.Fc-coated beads in the presence of Lat B (black circles) or with beads coated with anti-E-cadherin antibody. (F) The stiffening response of MDCK cells probed with E-cad.Fc (black squares) or N-cad.Fc (black circles). Control measurements were with EGTA (white diamonds). In A–F, each point represents measurements of >200 beads.

CHO cells stably transfected with N-cadherin (N-CHO) are in supplementary material Fig. S1.

In all cases investigated (Fig. 3A–F), only homophilic ligand-induced junction stiffening during the first 120 s of shear modulation, and heterophilic ligands consistently failed to induce any response (Fig. 3). C2C12 and MDA-MB-435 cells both express endogenous N-cadherin (Fig. 3A–C), and shear applied only to beads coated with N-cad.Fc, but not E-cad.Fc or antibody, triggered force-activated junction remodeling. Similarly, MCF7 and MDCK cells (Fig. 3D–F), which express endogenous E-cadherin, required E-cad.Fc-coated beads to induce junction stiffening.

The finding that only homophilic cadherin ligation induces the mechanoresponse was unexpected, in light of the binding affinities quantified with the identical cells and cadherin ligands (Table 1). Both protein adhesion measurements and solution binding affinities show that cadherin subtypes cross-react, often with heterophilic affinities that are intermediate between those of the homophilic bonds (Katsamba et al., 2009; Prakasam et al., 2006; Steinberg, 2007). Here, although the heterophilic ligands bind cell-surface cadherins, as demonstrated by micropipette manipulation measurements, they do not trigger force transduction.

The ligand requirement for mechanotransduction is further demonstrated by measurements with beads modified with anti-N-cadherin or with anti-E-cadherin antibodies. Both antibodies recognize the N-terminal domains of the respective target cadherins. Although the antibody-modified beads firmly bound to cadherins on C2C12 and MCF7 cells, neither triggered an active response to applied bond shear (Fig. 3B,E). This agrees with a similar result obtained with beads modified with DECMA-1 (E-cadherin blocking antibody) and F9 cells (le Duc et al., 2010).

Fig. 4. Cadherin-based traction forces are rigidity and ligand dependent. (A) Root-mean-square (RMS) traction forces (Pa) exerted by MDCK cells on polyacrylamide gels with elastic moduli of 34 kPa and 0.6 kPa, when coated with E-cad.Fc or with N-cad.Fc ligand. At ~160 ng/μm², the immobilized protein densities were similar for both cadherin ectodomains on both hydrogels. MDCK cells generated

Cadherin complexes are rigidity sensors

To address the possibility that bead pulls may not reflect physiologically relevant stress, complementary traction force measurements tested the ability of cadherin complexes to sense substrate rigidity, and to proportionally alter endogenous contractile stress. Prior studies of force sensation at focal adhesions demonstrated that mechanoresponses to exogenous force parallel substrate rigidity sensing (Geiger et al., 2009). In these studies, endogenous contractile forces exert physiological forces at cadherin adhesions to elastomeric substrata coated with cadherin ectodomains (Fig. 1E). The cadherin pairs mediating these cell-substratum adhesions are identical to those probed by micropipette manipulation and by MTC.

Fig. 4A compares traction forces generated by MDCK cells on polyacrylamide gels with elastic moduli of 34 kPa and 0.6 kPa, when coated with E-cad.Fc or with N-cad.Fc ligand. At ~160 ng/μm², the immobilized protein densities were similar for both cadherin ectodomains on both hydrogels. MDCK cells generated
greater traction on rigid gels coated with E-cad.Fc than on soft gels (Fig. 4A), confirming that E-cadherin complexes also sense substrate rigidity. MDA-MB-435 cells similarly exhibited rigidity-dependent traction forces on N-cad.Fc-coated hydrogels (Fig. 4C). This rigidity sensing via homophilic cadherin ligation agrees with a prior report of myoblast traction forces on elastomeric pillars coated with N-cadherin (Ladoux et al., 2010). Blebbistatin (50 μM) and cytochalasin D (4 μM) substantially reduced the traction forces generated on rigid substrata (Fig. 4B,D), in agreement with prior findings (Ladoux et al., 2010).

By contrast, on the stiffer gels (34 kPa) coated with heterophilic cadherin ligands, both MDCK and MDA-MB-435 cells generated lower traction forces (Fig. 4A,C). The lower traction forces exerted by MDCK cells on N-cad.Fc coated gels might be explained by the lower heterophilic bond affinity relative to the homophilic E-cad.Fc affinity (Table 1). However, this would not explain the behavior of MDA-MB-435 cells because the measured homophilic N-cadherin affinity is lower than the heterophilic affinity (Table 1). Yet the cells exert greater traction forces on N-cad.Fc coated gels. On soft hydrogels (0.6 kPa), both cell types were more rounded, and traction forces were low and ligand independent, within experimental error.

To further test the role of cell contractility in traction force generation MDA-MB-435 cells treated with nocodazole (20 μM) on gels with elastic moduli of 0.6 kPa and 34 kPa. Microtubule depolymerization increases cell contractility via a Rho-GTPase-dependent pathway (Danowski, 1989). As expected, nocodazole treatment increased the traction forces. As shown in supplementary material Fig. S3 the magnitudes of the increase in traction forces were almost the same in cells adhering to homophilic versus heterophilic cadherin ligands, on the gels with the same elastic modulus.

Different from the MTC measurements, heterophilic ligation reduced the traction forces by only ~50% relative to controls with blebbistatin or cytochalasin D treated cells. This could be due to compensatory mechanisms regulating cell contractility on these substrata. For example, cells also generated rigidity-dependent traction forces on poly-L-lysine-coated gels ~3 hr after seeding in serum-free medium (not shown), suggesting that parallel mechanisms may regulate global cell contractility. This behavior is not due to integrin interference because immunofluorescence did not detect focal adhesions at the basal surface.

Consistent with a functional role of tension in stabilizing cadherin adhesions, more cells attached and spread on the more rigid substrata coated with homophilic cadherin ligands (Fig. 5A,B). Again, the greater MDCK cell densities on E-cad.Fc than on N-cad.Fc-coated substrata might initially be attributed to the relative affinities of the homophilic versus heterophilic E-cadherin bonds. However, greater numbers of MDA-MB-435 cells adhered to rigid substrata coated with N-cad.Fc relative to E-cad.Fc, despite the lower affinity of the homophilic N-cadherin bond (Table 1). On the softer gels, there was no statistically significant difference in cell attachment densities to either homophilic or heterophilic ligands.

In Fig. 6A–C, well defined actin stress fibers and punctate Paxillin staining are apparent in MDA-MB-435 cells spread on collagen-coated semi-rigid polyacrylamide (34 kPa), in the presence of serum (10 v/v% FBS). The absence of Paxillin staining at the basal cell surface, on both soft and rigid gels (Fig. 6D,E), ruled out integrin interference in either the traction force or cell attachment measurements.

**Discussion**

These findings demonstrate that mechanotransduction at cadherin adhesions requires homophilic ligation, and that force-activated junction remodeling appears to be insensitive to differences in the intrinsic two-dimensional cadherin binding affinities. The binding affinity and cell surface adhesion energy do not determine the magnitude of force-dependent, cadherin-mediated changes in cell mechanics. This is somewhat analogous to findings that focal adhesion-mediated rigidity sensing and adhesion are independent (Engler et al., 2004). However, the mechanisms of cadherin adhesion, binding selectivity and mechanotransduction are distinct from integrins.

The results suggest that differences in mechanical changes at stressed cadherin junctions could supersede more subtle differences in cadherin affinities. This could explain, in part, the finding that cortical tension better predicted cell positioning in zebrafish embryos than the cohesiveness of the germinal progenitor cells (Krieg et al., 2008). It was unclear how to reconcile the latter result with the extensive literature suggesting that cadherin-dependent differences in adhesion energies could direct cell sorting (Duguay et al., 2003; Foty and Steinberg, 2004; Foty and Steinberg, 2005; Katsamba et al., 2009; Niessen et al., 2011; Nose et al., 1990; Steinberg, 1963; Steinberg and Takeichi 1994; Steinberg, 2007). Our findings suggest a cadherin-dependent mechanism that could both determine cohesive energies and regulate junctional or possibly global (Chopra et al., 2011) cell mechanics.
The two-dimensional affinities determined from micropipette studies are equilibrium, time-independent properties of EC1-EC1 bonds, but the MTC and traction force measurements are mechanical approaches that may reflect different properties of cadherin bonds. For example, single bond rupture forces depend on dissociation rates rather than affinities (Dudko et al., 2007; Evans and Ritchie, 1997). However, none of the bond parameters determined thus far generally favor homophilic over heterophilic bonds. The dissociation rates determined from micropipette measurements (Table 1), single bond rupture studies (Shi et al., 2008), or SPR studies (Katsamba et al., 2009) do not correlate with the mechanoselectivity. Neither do the single bond rupture forces (Shi et al., 2008). Some studies suggest that the heterophilic binding frequency, which is related to the association rate, might be lower than for homophilic bonds (Berx and van Roy, 2009; Panorchan et al., 2006), but this is not the case for the protein pairs considered here. Thus, the cadherin binding properties alone do not appear to confer mechanical selectivity.

The second step in the kinetic profile was not considered in this analysis for two main reasons. First, studies of cadherin-dependent cell segregation have focused on EC1 (Katsamba et al., 2009; Nose et al., 1990), and one aim of these studies was to determine how EC1-dependent binding properties influence mechanotransduction. Second, the second binding stage in the binding time course does not involve the EC1 domain. Prior studies with C-cadherin showed that the second step requires EC3 (Chien et al., 2008). Because of the conserved binding behavior of different classical cadherins (Perret et al., 2004; Prakasam et al., 2006; Shapiro and Weis, 2009; Shi et al., 2008), we assume the C-cadherin findings apply to classical cadherins. Consistent with this view, N-glycans on EC2-EC3 of N-cadherin modulate the second kinetic step, but not the EC1 affinity (Langer et al., 2012). This indicates that the two kinetic steps involve different structural regions. EC1 mutants also altered both the affinity for the first step and cell sorting behavior, which is attributed to EC1-dependent cell cohesion (Tabdili et al., 2012).

The ligand-dependent mechanical differences are manifest at stressed cadherin junctions, and are expected to exert greater influence at stressed intercellular adhesions. On soft hydrogels, for example, where cells exert low contractile stress, there was no distinguishable ligand-dependence of the traction forces (Fig. 4A,C). Thus, interfacial energies might dominate cell-cell interactions in some cases, such as in soft tissue environments or in vitro cell sorting assays, where intercellular forces may be insufficient to effect significant differences in cortical tension. In this context, it is noteworthy that CHO cells, which are commonly used for in vitro sorting assays do not exert large contractile forces (Leader et al., 1983) (see supplementary material Fig. S1). Conversely, large intercellular forces generated during convergence extension or cell intercalation movements may be sufficient to activate cadherin-dependent changes in intercellular mechanics (Kasza and Zallen, 2011).

These results are also intriguing, in light of the postulated mechanism of cadherin-dependent mechanotransduction. Cadherin bond stress is thought to induce a conformational change in β-catenin bound to the cadherin/β-catenin complex that exposes a cryptic site in β-catenin (Yonemura et al., 2010). The latter recruits actin-binding proteins such as vinculin to junctions. The finding that mechanical stimulation with anti-cadherin antibodies (le Duc et al., 2010) or heterotypic ligands fails to activate mechanotransduction indicates that tension on cadherin ectodomains alone is insufficient to trigger the requisite change in β-catenin.

The molecular basis for cadherin mechanoselectivity remains to be determined. The independence cadherin bond properties and
mechanotransduction selectivity suggests that additional molecular factors may contribute to force transduction at intercellular junctions. There is precedence for this. Anti-VE-cadherin antibody-coated beads failed to activate mechanotransduction via VE-cadherin (Tzima et al., 2005). However, the VE-cadherin complex comprises the vascular endothelial growth factor receptor and PECAM, which appears to be the flow-sensitive mechanotransducer between endothelial cells subject to fluid shear stress (Hahn and Schwartz, 2009). Whether other membrane proteins contribute to selective force transduction by classical cadherins remains to be determined. Possible candidates are protocadherins (Chen and Gumbiner, 2006; Deplazes et al., 2009; Taveau et al., 2008), receptor tyrosine kinases (Brady-Kalnay et al., 1995; Hellberg et al., 2002; McLachlan and Yap, 2011) or growth factor receptors (Perrais et al., 2007; Shibamoto et al., 1994; Tzima et al., 2005; Williams et al., 2001). Recent findings demonstrated a role for protocadherin-19 in the regulation of N-cadherin-dependent cell–cell adhesion and migration (Papushueva and Heisenberg, 2010; Taveau et al., 2008), and PAPC regulates the adhesive activity of C-cadherin during Xenopus morphogenesis (Chen and Gumbiner, 2006). Nevertheless, the mechanoselectivity demonstrated here with five cell lines and three classical cadherin subtypes demonstrates that cadherin selectivity can modulate both intersurface adhesion energies and cell mechanics, both of which instruct morphogenesis and maintain compartment barriers in mature tissues.

Materials and Methods

Materials

Blebbistatin, cytocchalasin D (Cyt D), latrunculin B (Lat B), monoclonal anti-N-cadherin antibody (clone GC-4), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), poly-L-lysine, 3-aminopropyl-triethoxysilane (APS), and glutaraldehyde were purchased from Sigma-Aldrich (St Louis, MO). Nocodazole was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-E-cadherin and anti-paxillin antibodies were purchased from BD Biosciences (San Jose, CA). Acrylamide, N,N′-methylene-bis-acrylamide (Bis), TEMED and ammonium persulfate (AP) were obtained from Bio-Rad ( Hercules, CA). N-hydroxysuccinimide ( NHS) and N-succinimidyl-6-[4-(azido-2'-nitrophenyl) amino] hexanoate (Sulfo-SNAPH) were from Pierce Biotech (Rockford, IL).

Plasmids and cell lines

The cDNA encoding the full-length chicken N-cadherin in the pEGFP-N1 plasmid was provided by Andre Sobel (Institut de Fer a Moulin; Gif-sur-Yvette, France). Canine E-cadherin cDNA in pEGFP-N1 plasmid was a gift from James Nelson (Stanford University, Palo Alto, CA). These plasmids were transfected into Chinese hamster ovary (CHO-K1) cells, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Clones expressing wild-type N-cadherin (N-CHO) were selected in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 v/v% FBS and 400 μg/ml G418. The cadherin expression levels of all cells were determined by quantitative flow cytometry. The antibody-modified RBCs were washed twice with 0.9 w/v% NaCl, and suspended in 12 ml 0.3% NaN3 in 0.9 w/v% saline at 1.5 × 1010/ml. The thus isolated RBCs can be used for up to 3 weeks, after which they are disposed of as biohazardous waste.

Polyclonal, anti-FC-domain; goat polyclonal anti-human immunoglobulin G (IgG); or polyclonal anti-Fc-domain, goat anti-mouse IgG (both from Sigma-Aldrich, St Louis, MO) were covalently immobilized on RBC surfaces to capture cadherin-Fc constructs. These antibodies were covalently bound to glycoproteins on red blood cells, following CrCl3 activation (Gold and Fadenburg, 1967; Koller and Wick, 1977). Approximately 107 RBCs were rinsed five times with 0.85 w/v% NaCl, and then resuspended in 250 μl 0.3% w/v% NaN3 with 4 μg/ml of the desired antibody. The CrCl3 solution used to chemically activate carbohydrates on the RBC surface was diluted to concentrations below 0.01 w/v% with 0.02 mM sodium acetate in 0.85 w/v% NaCl (pH 5.5). To control the protein immobilization densities, serial dilutions of the CrCl3 solution were used. To bind antibodies to the cell surface, 250 μl of the diluted CrCl3 solution was added to 250 μl of RBC antibody mixture, and mixed for 5 min. The reaction was halted by adding 500 μl of ‘Stop Solution’ (PBS with 5 mM EDTA and 1 w/v% BSA). The cells were washed twice with Stop Solution. The immobilized antibody density was determined by quantitative flow cytometry. The antibody-modified RBCs were suspended in 10 μl EAS45 and stored at 4°C until use.

Micropipette measurements of cell binding dynamics

The intercellular binding probability versus contact time was quantified with the micropipette manipulation technique, described previously (Chesla et al., 1998; Chien et al., 2008; Evans et al., 2004; Huang et al., 2010; Zhang et al., 2005). The binding probability P is the number of detected binding events nB divided by the total number of cell–cell contacts, or nN/N. In these measurements, a cadherin-expressing cell and a cadherin-Fc modified RBC were partially immobilized on micropipettes and held in a chamber containing L15 medium supplemented with 1 w/v% BSA (Fig. 1). The cells were visualized with a 100 x oil immersion objective on a Zeiss Axiosvert 200 microscope, and the image output was recorded with a DAGE-MTI (Michigan City, MI) CCD100 camera interfaced to an LCD monitor. The cells were positioned with piezo-electric controllers, and cyclically brought in and out of contact. Adhesion events are identified from deformation of the RBC and recoil after bond rupture, as visualized with the microscope. The contact time was controlled with the piezo-electric actuator, which manipulated the position of one of the pipettes. The contact area was ~7 μm2 (~1.5 μm diameter), and was quantified with Zeiss Axiovision software. Binding probabilities were measured for 50 cell–cell contacts, for each cell pair tested. Each contact time represents measurements with at least three different pairs of cells, such that N>150. The probabilities P are presented as the mean ± standard deviation from the mean.

Model fits to the EC1-mediated binding step

When determining binding parameters from data fits to Eqn 1, we first determined the spread in the data at each time point. In this case, the best, unbiased estimated parameter is determined by weighted least squares analysis, with the weighting factor for each time point being the inverse of the variance at that time point. The kinetic parameters that best fit the data were then determined using weighted, non-linear least squares regression (OriginLab, Northampton, MA).
where \( n_i \) is the number of trials at each time point, \( N \) is the number of separate time points measured, \( P_{\text{mean}} \) is the mean probability at each time point, \( P_{\text{pred}} \) is the predicted probability of the model (i.e., Eqn 1), \( P_{\text{pred}} \) is each probability observed in the full data set, and \( N \) is the total number of observations. When the test statistic is larger than the critical value on an \( F \) distribution with \((N-n, n-2)\) degrees of freedom, the model is rejected. The best-fit parameters summarized in Table 1 were obtained from fits of Eqn 1 to the maximum number of data points that did not fail the lack-of-fit test.

**Magnetic twisting cytometry**

In magnetic twisting cytometry (MTC) experiments (Fig. 1D), polystyrene-coated, 4.9 μm diameter, ferromagnetic beads with carboxyl surface groups (Spherotech, Lake Forest, IL) were covalently coated with specific Fc-Tagged protein, poly-L-lysine (PLL), or blocking antibody, and then allowed to bind to the cell surface. The beads were first activated with EDC/NHS, by incubation with EDC (10 mg/ml) and NHS (10 mg/ml), in MES buffer (50 mM, 100 mM NaCl, pH 5.0) for 15 min at room temperature, on a shaker. The beads were centrifuged at 12,000 g for 15 min, and then incubated with 75 μg of the ligand of interest (Fc-tagged cadherin ectodomains, PLL, or blocking antibody) per mg beads for 2 hr at room temperature, in coupling buffer (20 mM HEPES, 100 mM NaCl, 5 mM CaCl₂, pH 8.0). In order to prevent bead aggregation, beads were sonicated for 3 seconds before they were added to the cells. The protein-coated beads were then allowed to settle on a confluent cell monolayer for 20 min, before applying torque. To disrupt F-actin, cells were treated with 4 μM latrunculin B or 4 μM cytochalasin D for 10 min before twisting measurements.

All MTC studies were carried out with cells cultured in glass-bottomed dishes on a heated microscope stage at 37°C. Cells and beads were visualized with an inverted microscope (Leica) with a 20x, 0.6 numerical aperture objective and a cooled charge-coupled device camera (Orca2; Hamamatsu Photonics). First, beads were magnetized horizontally by applying a short, 1 Tesla field for 1 ms. Next, an oscillating magnetic field of 60 Gauss at a frequency of 0.3 Hz was applied orthogonal to the magnetic moment of the beads, for up to 120 seconds. This generates a modulated shear stress on the bead-cell junctions. The bead displacements were recorded with a CCD camera, and displacements were converted to the complex modulus of the junction, using custom software. The specific modulus is the ratio of the applied torque \( T \) to the bead displacement \( D \), \( G = T/D \), which gives the complex modulus of the bead-cell junction. The thus determined specific modulus follows a log-normal distribution, from which the geometric mean and standard deviation are obtained.

**Traction force microscopy**

Fourier transform traction force microscopy was carried out with compliant polyacrylamide gels surface-modified with cadherin ectodomains. Polyacrylamide gels were prepared as described previously (Beningo et al., 2002). Red fluorescent microspheres (0.2 μm, Molecular Probes, Eugene, OR) were embedded in gels. The Young’s modulus of the gels used in this study were 0.6 kPa and 34 kPa. The gels were covalently modified with 0.2 mg/ml of human or chicken anti-Fc antibody was 7 cpm/ng. Polyacrylamide hydrogels (0.6 kPa and 34 kPa) were prepared as described previously (10 mm diameter, 1 mm thickness). 125I-labeled immunoglobulin Fc was covalently bound to the gels that were chemically activated with Sulfo-SANPAH. Gels of defined size were then rinsed with buffer to remove unbound protein, and placed in scintillation vials containing 5 ml of scintillation cocktail. The radioactivity in each sample was recorded with an LS 6500 scintillation counter (Beckman Instruments) with specified settings for 125I detection. Control measurements used hydrogels incubated with the labeled protein, but not the Sulfo-SANPAH cross-linker.

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**References**


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Fig. S1. N-cadherin mechanotransduction is ligand selective. MTC measurements of the force-induced stiffening response of NCHO cells probed with beads coated with N-cad.Fc (black squares), E-cad.Fc (black circles) or C-cad.Fc (gray circles).
Fig. S.2. Traction force ‘heat maps’ of cells on rigid (34 kPa) or soft (0.6 kPa) hydrogels coated with E-Cad.Fc or with N-Cad.Fc ligand. (A-C) Traction force heat maps of single MDA-MB-435 cells on soft (A: 0.6 kPa) and rigid (B: 34 kPa) gels coated with N-Cad.Fc (homophilic ligand), and an MDA-MB-435 cell on a rigid (C: 34 kPa) gel coated with N-Cad.Fc (heterophilic ligand). (D-F) Traction force heat maps of single MDCK cells on soft (D: 0.6 kPa) and rigid (E: 34 kPa) gels coated with E-Cad.Fc (homophilic ligand), and an MDCK cell on a rigid (F: 34 kPa) gel coated with N-Cad.Fc (heterophilic ligand).
Fig. S3. Nocodazole treatment increased traction stresses exerted by the cells on cadherin substrates. RMS (root-mean-square) traction forces (Pa) generated by MDA-MB-435 cells on cadherin-coated gels. Cells were cultured in the absence of serum on soft (0.6 kPa) and semi-rigid (34 kPa) polyacrylamide gels coated with either N-cad.Fc or E-cad.Fc. The filled bars indicate traction stresses in the absence of nocodazole, and the white bars show the increase in traction forces after treating the cells with nocodazole.