

Conformational epitopes at cadherin calcium-binding sites and p120-catenin phosphorylation regulate cell adhesion

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ABSTRACT We investigated changes in cadherin structure at the cell surface that regulate its adhesive activity. Colo 205 cells are nonadhesive cells with a full but inactive complement of E-cadherin–catenin complexes at the cell surface, but they can be triggered to adhere and form monolayers. We were able to distinguish the inactive and active states of E-cadherin at the cell surface by using a special set of monoclonal antibodies (mAbs). Another set of mAbs binds E-cadherin and strongly activates adhesion. In other epithelial cell types these activating mAbs inhibit growth factor–induced down-regulation of adhesion and epithelial morphogenesis, indicating that these phenomena are also controlled by E-cadherin activity at the cell surface. Both types of mAbs recognize conformational epitopes at different interfaces between extracellular cadherin repeat domains (ECs), especially near calcium-binding sites. Activation also induces p120-catenin dephosphorylation, as well as changes in the cadherin cytoplasmic domain. Moreover, phospho-site mutations indicate that dephosphorylation of specific Ser/Thr residues in the N-terminal domain of p120-catenin mediate adhesion activation. Thus physiological regulation of the adhesive state of E-cadherin involves physical and/or conformational changes in the EC interface regions of the ectodomain at the cell surface that are mediated by catenin-associated changes across the membrane.

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INTRODUCTION

The dynamic regulation of cadherin-mediated adhesion is important for many morphogenetic and physiological processes, including cell rearrangements during embryonic development, junctional remodeling for epithelial morphogenesis, cell invasion during cancer metastasis, wound healing, and regulation of endothelial barrier function during inflammatory responses (Gumbiner, 2005; Dejana *et al.*, 2008; Vestweber *et al.*, 2010; Baum and Georgiou, 2011; Takeichi, 2011). Although transcriptional regulation often underlies cadherin loss or switching, there is abundant evidence that cadherins are

regulated rapidly and posttranslationally during many of these processes. Several different mechanisms have been proposed to mediate dynamic regulation of cadherin adhesion, including internalization and trafficking via endocytosis (Lu *et al.*, 2003; Ulrich *et al.*, 2005; Ogata *et al.*, 2007; Baum and Georgiou, 2011), tyrosine phosphorylation and dissociation of catenins, and cytoskeletal interactions (Brembeck *et al.*, 2004; Lilien and Balsamo, 2005; Daugherty and Gottardi, 2007). There is also evidence that cadherin adhesion activity on the cell surface is directly subject to regulation, independent of internalization or catenin dissociation (Brieher and Gumbiner, 1994; Aono *et al.*, 1999; Zhong *et al.*, 1999; Chen *et al.*, 2009).

Regulation of cell adhesion requires the breaking and remaking of homophilic adhesive bonds, and even regulation by internalization and trafficking requires control of cadherin disengagement from the homophilic bond between neighboring cells. Although much has been learned about the signaling pathways, catenins, and cytoskeletal proteins that affect adhesion, the mechanisms by which they regulate cadherin homophilic binding on the cell surface is unknown. This contrasts greatly with our understanding of the regulation of integrin-mediated adhesion, for which it is known that conformational changes in the integrin extracellular domain and associated changes

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Abbreviations used: EC, extracellular cadherin repeat domain; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; Fab, monovalent antigen-binding fragment of antibody; HGF, hepatocyte growth factor; mAb, monoclonal antibody; MDCK, Madin–Darby canine kidney (cell line).

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in clustering and ligand binding affinity are controlled by associated cytoplasmic proteins such as talin and kindlins in response to signaling events (Hynes, 2002; Ginsberg *et al.*, 2005; Shattil *et al.*, 2010). This is probably due to the lack of experimental systems that lend themselves to such studies of changes in cadherin binding activity, in contrast to the integrins, which are rapidly activated during triggering of platelet aggregation or leukocyte adhesion.

A great deal of progress has been made understanding the high-resolution structure of the classical cadherin ectodomain and the structural basis of the cadherin homophilic bond. The x-ray crystal structure of the entire C-cadherin ectodomain provides a model for understanding cadherin structure and binding properties (Boggon *et al.*, 2002). A critical Trp-2 residue in the N-terminal extracellular cadherin repeat domain 1 (EC1; or Trp-2 and Trp-4 for type II cadherins; Patel *et al.*, 2006) mediates strand exchange between cadherins to form a dimer, and this key dimer interaction is believed to represent the adhesive homophilic bond between cadherins on neighboring cells. This bond is generally believed to represent a strong state of adhesion.

Several biophysical and structural findings suggest that additional interactions between cadherin ectodomains might occur. Surface force and atomic force measurements have provided evidence for three different interactions (Zhu *et al.*, 2003; Shi *et al.*, 2010). An electron microscopic study of a fragment of VE-cadherin suggests that it forms a stable hexamer (Hewat *et al.*, 2007), although such structures have not been observed in other studies (Brasch *et al.*, 2011). An alternative dimer (X-dimer) involving the calcium-binding interface between EC1 and EC2 domains has also been observed in x-ray crystal structures of cadherins, and this X-dimer may form as an intermediate in the formation of the final Trp-2–dependent adhesive bond (Harrison *et al.*, 2010; Hong *et al.*, 2011). However, little is known about how the structure of the cadherin ectodomain may change during the physiological regulation of adhesive states on cells.

We provided evidence that physiological regulation of C-cadherin in response to growth factors during embryonic morphogenesis involves changes in the adhesive state of cadherins at the cell surface, without changes in either expression levels at the cell surface or amounts of associated catenins (Brieher and Gumbiner, 1994; Chen and Gumbiner, 2006; Chen *et al.*, 2009; Zhong *et al.*, 1999). We hypothesized that the adhesive functions of cadherins, like those of the integrins, can be regulated by changes in conformation and/or physical state at the cell surface. To investigate potential changes in the molecular structure, conformation, or physical state of the cadherin homophilic-binding ectodomain, we used an approach that was successful for integrins (Schwartz *et al.*, 1995; Stuiver and O' Toole, 1995; Humphries, 1996; Kiosses *et al.*, 2001) to screen for monoclonal antibodies (mAbs) that either differentially recognize cadherins on the cells in the inactive or active adhesive states or bind to and stimulate the adhesive activity of cadherins, and we used them to ascertain structural changes and associated signaling events involved in regulation. Such activity-associated mAbs also provide powerful tools for evaluating the roles of adhesion regulation in a range of biological processes. Indeed, we previously identified one mAb that activates C-cadherin at the surface of embryonic cells and used it to demonstrate the role of adhesion regulation in morphogenetic movements of gastrulation (Zhong *et al.*, 1999). In the present study, we chose a much more accessible cellular model to identify changes with an immunochemical approach—the Colo 205 human colorectal tumor cell line. Colo 205 cells grow in culture as rounded, nonadhesive cells despite having high E-cadherin and associated catenins at the cell surface, and they

can be triggered to adhere, compact, and form epithelial monolayers by a variety of stimuli (Aono *et al.*, 1999). Because of these properties, they provide a model analogous to the rapid triggering of integrin activation on platelets and leukocytes to investigate the mechanisms of E-cadherin activation.

RESULTS

Distinguishing E-cadherin activity states with monoclonal antibodies

Nonadhesive Colo 205 cells can be triggered to adhere, compact, and form epithelial monolayers by a variety of stimuli of unknown mechanism, including treatment with very low levels of trypsin or with the broad-spectrum protein kinase inhibitor staurosporine (Aono *et al.*, 1999; also see Figure 2A later in the paper). We hypothesized that the adhesive functions of cadherins, like those of the integrins, could be regulated by changes in conformation and/or physical state at the cell surface. To try to detect such changes, we raised mAbs to purified human E-cadherin ectodomain and screened for ones that can differentially recognize E-cadherin on the cells in the inactive or active adhesive states. The vast majority of mAbs out of more than 100 tested did not distinguish between the two states, exhibiting virtually identical binding curves in enzyme-linked immunosorbent assays (ELISA; Figure 1, left). This finding confirms that the amount of E-cadherin on the cell surface does not change during activation of adhesion and demonstrates that accessibility of the antibodies to E-cadherin is not limited during adhesion and subsequent cell compaction and epithelialization.

Five different mAbs did exhibit differential binding between untreated and activated Colo 205 cells when determined with a quantitative ELISA (Figure 1, right). In all five cases the binding was reduced in the activated state. Because general antibody accessibility is not limiting during activation, this finding suggests that certain E-cadherin epitopes selectively undergo some change in either conformation or local physical masking during adhesion activation. It is not clear why the reduction in binding is only partial; it could be due either to a large change in mAb binding to a small fraction of the total pool of E-cadherin molecules at the cell surface or to a small change in mAb binding to all or most of the E-cadherin molecules. We do not know how many E-cadherin molecules must be activated to induce changes in Colo 205 adhesion and compaction. Although E-cadherin did accumulate at cell–cell contact regions of activated Colo 205 cells, a large fraction did not redistribute out of the free surface at the edge of cell colonies (Figure 2A), suggesting that it may not all be activated or engaged in cell adhesion. Nonetheless, these and other findings (see *Discussion*) indicate that E-cadherin undergoes some sort of change in physical state and/or conformation during adhesion activation.

Direct activation of E-cadherin adhesion at the cell surface through mAb binding

We also sought mAbs that could bind to E-cadherin at the cell surface and induce a change in state and activate its adhesive function. To do so, we screened for mAbs raised to purified human E-cadherin ectodomain that could trigger adhesion and compaction of Colo 205 cells, similar to treatment with low levels of trypsin or staurosporine. Although the vast majority of anti-E-cadherin mAbs were either neutral or in fact inhibitory for adhesion (unpublished data; Table 1), we did obtain several mAbs that could trigger the formation of epithelial morphology (Figure 2A). Four such mAbs were strong activating mAbs—they effectively triggered the morphological change within 4 h; two others were considered weak activating mAbs, since they triggered a less extensive change (Table 1).

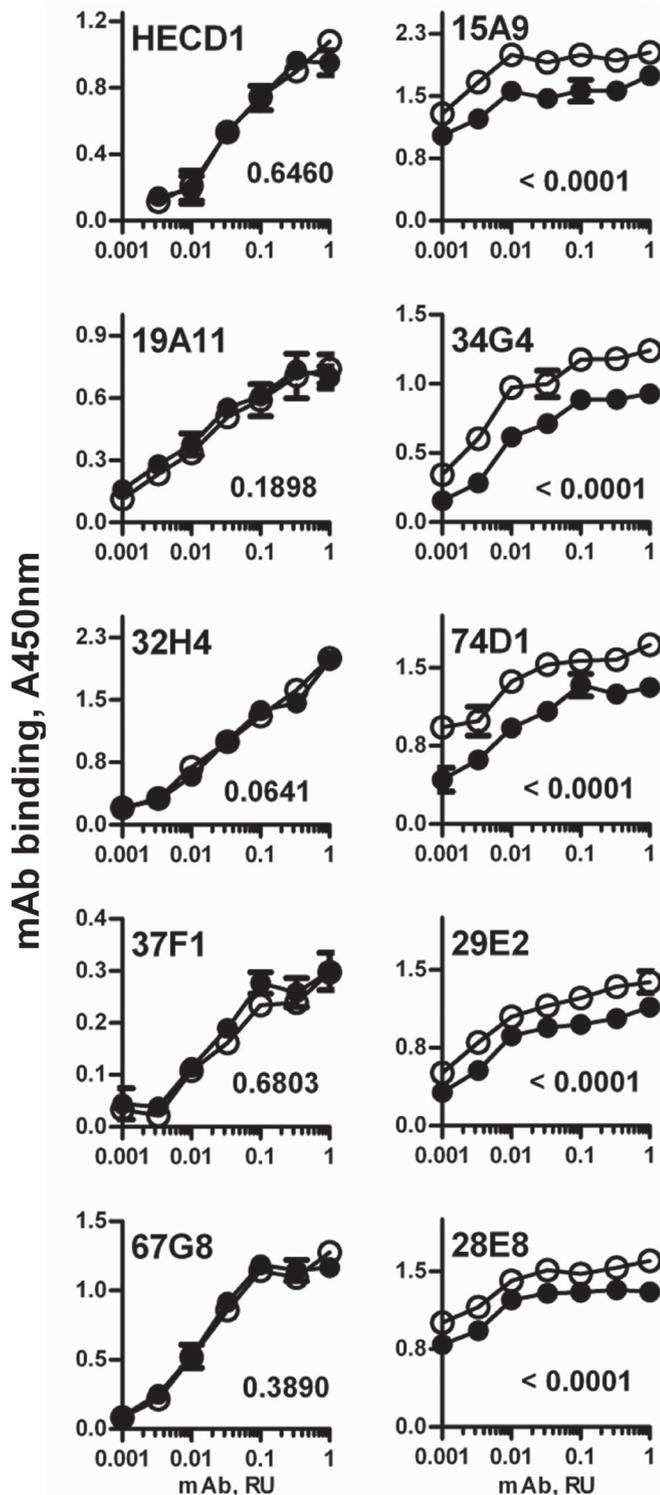


FIGURE 1: Five different anti-E-cadherin mAbs exhibit differential binding between untreated and activated Colo 205 cells. Colo 205 cells were untreated or treated overnight with 7 nM staurosporine to activate adhesion. mAbs were applied to cells at serial dilutions starting from nondiluted hybridoma cell supernatant or 3 μ g/ml purified mAbs. Antibodies were allowed to bind for 2 h at 37°C, and the binding was detected with peroxidase-labeled anti-mouse secondary antibodies and colorimetric measurements of peroxidase activity. Left, five examples of the binding properties of most mAbs; right, distinguishing mAbs. Open symbols, untreated cells; closed symbols, staurosporine-activated cells. Error bars, SD of

Monovalent antigen-binding fragment of antibody (Fab) prepared from the mAbs retained activating activity (Figure 2A), which rules out the possibility that they facilitate adhesion simply by cross-linking E-cadherin molecules between two neighboring cells. Thus these mAbs appear to induce a change in E-cadherin that leads to activation of adhesion.

To determine whether the activating mAbs act through specifically enhancing E-cadherin-mediated adhesion, we performed E-cadherin-specific adhesion assays. A flow adhesion assay was used in which strength of cell attachment to a substrate coated with purified E-cadherin protein is measured by the shear flow required to detach cells (Chappuis-Flament *et al.*, 2001). Under control untreated conditions, Colo 205 cells failed to attach significantly to the E-cadherin substrate (Figure 2B). Treatment of Colo 205 cells with low levels of trypsin stimulated adhesion to the E-cadherin substrate, as evidenced by the increase shear flow required for cell detachment. Activating mAb 19A11 stimulated adhesion to the E-cadherin substrate even more, indicating that the mAbs work by directly and specifically triggering E-cadherin adhesive activity. Furthermore, activating mAbs were able to cause a change in cell surface E-cadherin that is detected by the distinguishing antibody (Figure 2C), suggesting that the activating mAbs trigger similar physical changes in E-cadherin to those due to staurosporine and low levels of trypsin.

Roles of cell surface E-cadherin activity in epithelial morphogenesis

Although dynamic regulation of cadherin adhesive function in response to morphogens is an important mediator of tissue morphogenesis (Meiners *et al.*, 1998; Gumbiner, 2005; Ulrich *et al.*, 2005), activation of E-cadherin from a completely inactive state probably occurs more rarely; compaction of the 8- to 16-cell-stage mouse embryo is the one obvious example (Vestweber *et al.*, 1987; Fleming and Johnson, 1988). Therefore we wanted to determine whether the activity states controlled by our activating mAbs have roles in the more common phenomenon of partial adhesion down-regulation by growth factor signaling. Such down-regulation occurs during processes involving cell rearrangements, such as convergent-extension movements in gastrulating embryos (Zhong *et al.*, 1999; Ulrich *et al.*, 2005; Chen and Gumbiner, 2006; Ogata *et al.*, 2007), wound closure and healing (Gorfinkiel and Arias, 2007), and junctional remodeling during epithelial morphogenesis/tubulogenesis (Meiners *et al.*, 1998; Pollack *et al.*, 1998; Baum and Georgiou, 2011).

We first tested whether activating mAbs had any effect on the regulation of adhesion by growth factors using A431 cells, an epidermal growth factor (EGF)-responsive human epithelial cell line. Using the flow adhesion assay, we observed that A431 cells exhibited reduced adhesion strength in response to EGF treatment (Figure 3A). Treatment of the cells with activating mAb along with EGF significantly strengthened adhesion. E-cadherin is known to inhibit cell motility and scattering in epithelial monolayers, and scattering can be enhanced by growth factors, in part through down-regulation of E-cadherin adhesion (Birchmeier, 1995; Meiners *et al.*, 1998; Lu *et al.*, 2003; Gumbiner, 2005). We therefore asked whether

three replicate cell samples. The significance of the difference between curves was determined using two-way ANOVA (see *Materials and Methods* for details). Corresponding column factor p values are shown on each graph. $p < 0.05$ represents a significant difference.

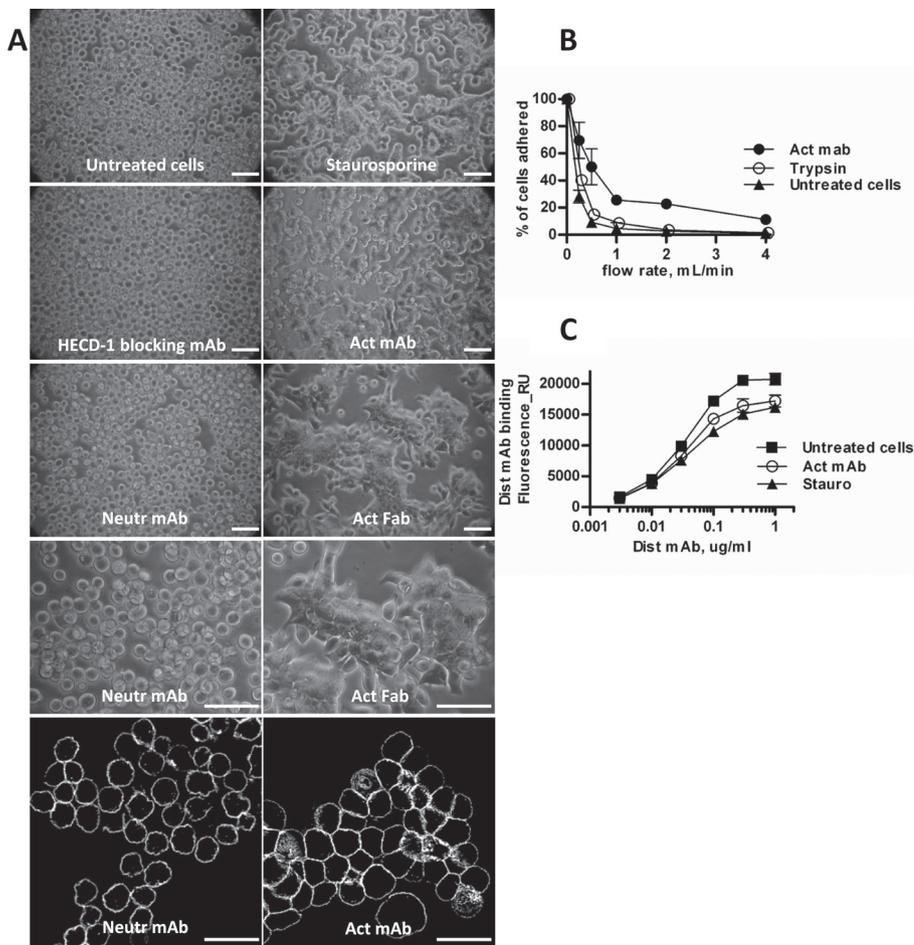


FIGURE 2: Activating mAbs stimulate cadherin-mediated adhesion and acquisition of epithelial morphology. (A) Activating mAbs and Fab fragments triggered compact epithelial morphology. Colo 205 cells, either untreated or treated overnight with 3 $\mu\text{g}/\text{ml}$ of neutral, adhesion-blocking or activating mAb 19A11. Scale bar, 50 μm . Bottom, immunofluorescence staining for E-cadherin. Scale bar, 25 μm . (B) Activating mAb 19A11 stimulated adhesion of cells to pure E-cadherin substrate. Colo 205 cells were untreated, pretreated with 3 $\mu\text{g}/\text{ml}$ 19A11 activating mAb for 2 h, or treated with 0.001% trypsin for 30 min, and cell adhesion strength to E-cadherin-coated capillary tubes was evaluated using increasing laminar flow to determine the force required to detach cells. Error bars, SD of two independent measurements. (C) A distinguishing mAb detected a change in E-cadherin caused by the activating antibody. Colo 205 cells were pretreated overnight with 3 $\mu\text{g}/\text{ml}$ 32H4 (IgG2a) activating mAb or 7 nM staurosporine. The 34G4 (IgG1) distinguishing mAb was allowed to bind for 2 h at 37°C, and the binding was revealed by goat anti-mouse IgG1–Alexa 488 antibodies. Error bars, SD of three replicate cell samples.

the rate of scratch wound closure in A431 monolayer cultures is affected by activating mAbs (Figure 3, B and C). A431 cells gradually filled in scratch wounds, and the rate of closure was enhanced by treatment with EGF. Activating mAbs slowed the rate of wound closure in both normal culture medium and EGF-treated cultures. Neutral mAbs that bind E-cadherin without activating it fail to slow wound closure, demonstrating that the effect was specific to activating mAbs. Therefore the activating mAbs enhance adhesion in a constitutively adhesive epithelial cell line and can counteract the down-regulation of E-cadherin-mediated adhesion caused by growth factors, thereby affecting epithelial cell migration.

Experiments on the physiological regulation of adhesion were performed with Madin–Darby canine kidney (MDCK) cells because they are normal, well-behaved epithelial cells and undergo epithelial morphogenesis in culture (Gumbiner and Simons, 1986; Gumbiner et al., 1988; Qin et al., 2005). MDCK cells can be induced

to undergo tubulogenesis by treatment with hepatocyte growth factor (HGF)/scatter factor when they are cultured in three-dimensional collagen gels (Pollack et al., 1998). Activating mAb 19A11, which cross-reacts with canine E-cadherin (unpublished data), inhibited the formation of epithelial tubules, causing the cells to remain as round epithelial cysts (Figure 4A). Neutral mAbs that bind E-cadherin without activating adhesion had no effect on tubulogenesis. Activating mAb 19A11 also enhanced the adhesion of HGF-treated MDCK cells to E-cadherin when analyzed with the flow adhesion assay (Figure 4B), suggesting that its effect on tubulogenesis is due to enhanced adhesion. HGF is also known to cause scattering of MDCK cells in two-dimensional culture by decreasing cell–cell adhesion and stimulating motility (Pollack et al., 1998). We therefore used the scratch wound assay with MDCK cells (Figure 4C). Activating mAb 19A11 slowed the rate of wound closure in both normal culture medium and HGF-treated cultures compared with neutral mAb controls. Therefore, activating mAbs are able to specifically stimulate adhesion in a normal epithelial cell line and inhibit morphogenetic changes that depend on down-regulation of cell adhesion.

Epitope mapping reveals antibody recognition of interface regions between cadherin domains

To begin to determine the molecular and structural basis for the regulation of E-cadherin activity states, we mapped the epitopes recognized by distinguishing and activating mAbs. Initially a rough mapping to the cadherin EC domain was performed by immunoblotting using a set of expressed human E-cadherin deletion constructs (Supplemental Figure S1). The findings are summarized in Table 1. The EC domains recognized by the different independent mAbs tended to cluster, with four strongly activating mAbs recognizing EC1, two weakly activating mAbs recognizing EC3, and four distinguishing mAbs recognizing EC4 (one recognizes EC1-2). Moreover, binding of the distinguishing mAbs and the weak activating mAbs, but not that of the strong activating mAbs, depends on calcium (Table 1), suggesting that distinguishing and weak activating mAb epitopes are associated with conformations of the cadherin controlled by calcium-binding sites.

We then carried out a fine mapping of the epitope(s) recognized by the strongly activating mAbs. In vitro-translated deletion constructs of the EC1 domain mapped the C-terminus required for binding of the four mAbs to the very C-terminal end of EC1, to either T97 or V98 (Figure 5A). It was surprising that the four mAbs failed to bind a construct lacking the N-terminal residues of EC1 (unpublished data), suggesting that they recognize conformational epitopes comprising discontinuous segments. We therefore decided to examine residues over the entire length of EC1. Of

Antibody	Isotype	Epitope location	Amino acids involved in recognition	Calcium dependence	Comment
Activating mAbs					
19A11	IgG1	EC1	V98, R70, P16	No	Recognizes denatured ECad
59D2	IgG1	EC1	V98, R70, E64	No	Recognizes denatured ECad
66E8	IgG1	EC1	V98, R70, E64	No	Recognizes denatured ECad
74A2	IgG1	EC1	V98, R70, E64, P16	No	Recognizes denatured ECad
32H4	IgG2a	EC3	V323_A329	Full	Weak activator, recognizes denatured ECad
70B4	IgG3	EC3	V323_A329	Partial	Weak activator, recognizes only native protein
Distinguishing mAbs					
15A9	IgG2a	EC4	S342, S432_V434	Partial	Recognizes denatured ECad
28E8	IgG1	EC4	S342, S432_V434	Partial	Recognizes denatured ECad
29E2	IgG1	EC4	S342, V434	Full	Recognizes denatured ECad
34G4	IgG1	EC1/2	ND	Partial	Recognizes only secreted Fc-ECad in native gel
74D1	IgG1	EC4	S432_V434	Full	Recognizes denatured ECad
Neutral mAbs					
27D2	IgG2b	EC3	ND	Full	Recognizes denatured ECad
37F1	IgG1	EC5	ND	ND	Recognizes only native protein
46H7	IgG2a	EC3	ND	No	Recognizes denatured ECad
76D5	IgG1	EC4	ND	Full	Recognizes denatured ECad
Adhesion-blocking mAbs					
52F9	IgG1	EC5	ND	ND	Recognizes only native protein
67G8	IgG1	EC5	ND	ND	Recognizes only native protein

mAbs used in this study or identified in the screen were characterized by binding to human E-cadherin (ECad) using Western blotting or ELISA. ND, not determined.

TABLE 1: Antibody summary.

importance, none of the activating mAbs required the Trp2 residue involved in homophilic bond formation, since they all strongly recognize the W2A mutant protein (Figure 5A'). None of the mAbs recognize mouse E-cadherin (not shown), presumably because they were generated in mouse and the protein is strongly expressed in many tissues. Because human and mouse E-cadherin are highly conserved (Figure 5A''), we were able to map the epitopes recognized by independently mutating the small number of nonconserved residues in EC1 to the mouse versions. This revealed that the mAbs do recognize a discontinuous epitope, which includes residues P16, E64, and R70 (Figure 5A') in addition to T97/V98. Of note, these residues all lie near residues that contribute to the calcium-binding site between EC1 and EC2 (Figure 5A''). Mapping these residues onto the published x-ray crystal structure of E-cadherin EC1-EC2 reveals that the epitope comprises three different loops in EC1 that come together to form the EC1 side of the calcium-binding site at the opposite end of the EC1 domain from the Trp2 homophilic adhesion binding site (Figure 6A, A'). Of interest, binding of this group of mAbs does not require calcium (Table 1), indicating that the structural arrangement of these loops in EC1 must be at least partially independent of calcium binding. That all four strongly activating mAbs recognize very similar conformational epitopes implicates this structure of the E-cadherin molecule in the control of adhesion activity.

The epitope in EC3 recognized by the weak activating mAbs was also partially refined by using secreted protein constructs containing the pre-pro and three N-terminal domains expressed in mammalian

cells. C-terminal deletions of the EC3 domain were examined for mAb binding by using immunoblotting (Supplemental Figure S2). Both weak activating mAbs required residues 329–332 at the boundary between EC3 and EC4 for binding, which overlaps with the calcium-binding site between EC3 and EC4 (Figure 6, B and B'), consistent with partial or full dependence of the binding of these two mAbs on calcium (Table 1). Although we have not yet determined whether other loops from EC3 that contribute to the calcium-binding site are part of the epitope, these findings suggest that similar to the strongly activating mAbs, these mAbs activate E-cadherin adhesive function by binding to a calcium binding site, albeit a distinct one.

Fine mapping of the epitope recognized by the four EC4-recognizing distinguishing mAbs described in Figure 1 was done similarly using mutations of secreted mammalian expression constructs containing the pre-pro and four N-terminal domains (Figure 5, B, B', and B''). First, the C-terminal region required for binding was mapped using a series of C-terminal deletion constructs. All four distinguishing mAbs required residues 429–434 at the very C-terminus of EC4 for binding (Figure 5B). Because this region again overlaps with part of the calcium-binding site between EC4 and EC5, we asked whether nonconserved residues between mouse and human E-cadherin near the calcium-binding site contribute to the epitope. It is not surprising that a point mutation of S432L residue near the C-terminus of EC4 reduced binding of three of the four distinguishing mAbs (Figure 5B'). More interesting is the observation that mutation at a distant region of EC4 that also contributes to

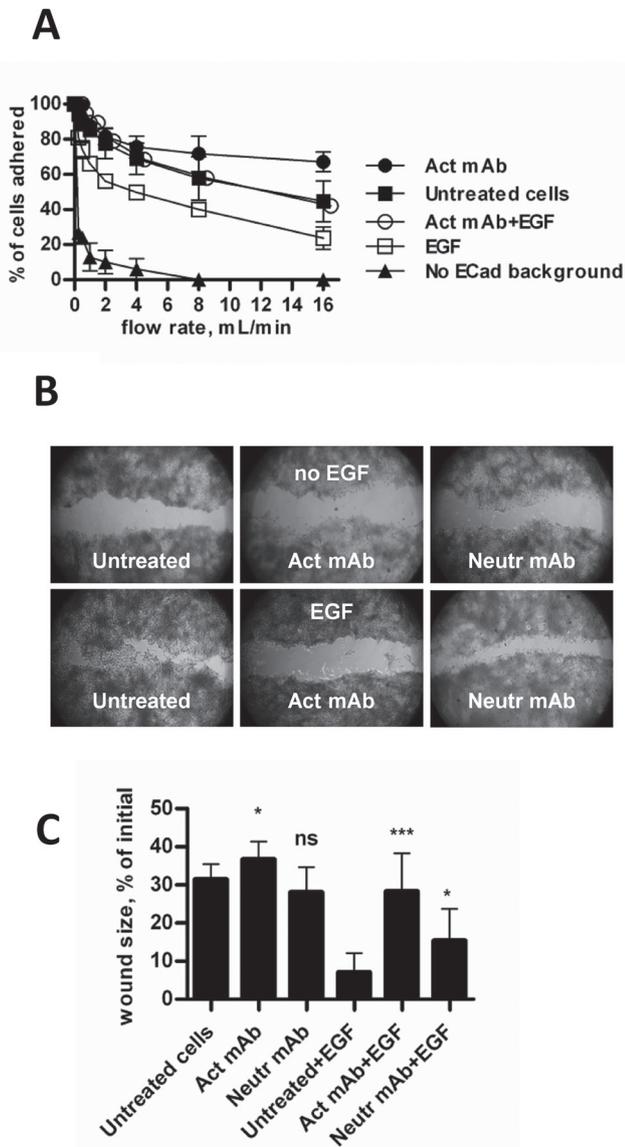


FIGURE 3: Activating mAbs counteract adhesion down-regulation caused by growth factor treatment. (A) Activating mAb strengthened the adhesion of A431 cells treated with EGF. Cells were either untreated or treated overnight with 3 $\mu\text{g/ml}$ of 19A11 activating mAb, followed by 2 h of incubation with either medium containing 1% NBS or the same medium containing 10 ng/ml EGF. Cell adhesion strength was evaluated using increasing laminar flow to determine the force required to detach cells. Error bars, SD of two independent experiments (N = 4). (B) Activating mAb decreased the rate of wound closure in A431 monolayer cultures. Wounded cultures were treated with 3 $\mu\text{g/ml}$ of either neutral or activating mAbs, and wounds were allowed to close in medium with or without EGF for 24 h. Shown is one example out of many samples and experiments. (C) A431 wound closure quantification. Error bars, SD of three independent experiments (N = 12). Significance of difference was determined by Student's t test; ns, no significant difference with $p > 0.05$; * $p < 0.05$; *** $p < 0.005$.

this calcium-binding site, S342P, greatly reduces binding of a different subset of three of the four distinguishing mAbs (Figure 5, B' and B''). Mapping these residues onto the published x-ray crystal structure of mouse E-cadherin reveals that the epitope comprises different loops from discontinuous parts of EC4 that form the EC4 side of the calcium-binding site (Figure 6, C and C'). The binding of this

group of mAbs does depend either partially or fully on the presence of calcium (Table 1). Therefore the distinguishing mAbs appear to recognize a conformational epitope of EC4 near the calcium-binding site between EC4 and EC5.

An activating mAb to *Xenopus* C-cadherin, AA5, reverses the down-regulation of C-cadherin adhesive function in response to morphogens and blocks morphogenesis of embryonic tissues (Zhong *et al.*, 1999). The AA5 epitope had only been mapped to the EC5 domain of C-cadherin (Zhong *et al.*, 1999), and we therefore decided to refine it. C-terminal deletion mutagenesis of the in vitro-translated C-cadherin EC5 domain shows that AA5 binding requires residues 533–540 at the very C-terminus of EC5 (Figure 7A). Moreover, the epitope appears to be conformational, because binding is completely abolished by reducing and alkylating the two intramolecular disulfide bonds near the C-terminus of this domain (Figure 7B), suggesting that it may comprise loops from discontinuous segments. Site-directed mutation of cysteine residues involved in the formation of these disulfide bonds greatly reduced AA5 mAb binding (Figure 7C). These disulfide bonds are unique to the EC5 domain and are conserved features of classic cadherins (Boggon *et al.*, 2002). Although there is no calcium-binding site at the C-terminus of the EC5 domain, the epitope recognized by the AA5-activating mAb is located in an analogous position at the base of the domain (Figure 6, D and D', diagram). Thus the C-cadherin-activating mAb may also recognize a potential interface region between domains, in this case the interface between EC5 and the transmembrane domain, although the structure of the latter domain is not known.

E-cadherin activation triggers biochemical changes across the plasma membrane in the catenins and the cadherin cytoplasmic tail

Previous work with Colo 205 activation by treatment with low-level trypsin or staurosporine showed that activation was associated with a decrease in the apparent molecular weight of p120-catenin on SDS-PAGE and implicated p120 in activation (Aono *et al.*, 1999). Activating mAb treatment caused a shift in p120-catenin very similar to the one caused by low trypsin treatment (Figure 8A). The effect was specific to mAb activation, because neutral and blocking mAbs that bind well to E-cadherin did not cause the shift. The shifted p120-catenin band was enriched in digitonin lysates of the Colo 205 cells (Figure 8A) but was detectable in lysates made with a variety of other detergents (Figure 8B). A shift to a lower apparent molecular weight is consistent with dephosphorylation of p120-catenin during activation. Indeed, using phospho-specific mAbs to p120-catenin (Xia *et al.*, 2004), we find that activation is associated with dephosphorylation of S268, S288, and T310 (Figure 8C). Thus, activating mAbs specifically affect p120 dephosphorylation via direct binding to the cadherin ectodomain and therefore act like agonists for this adhesion-associated event.

The observed dephosphorylation of p120-catenin resulting from mAb-induced activation could either be a direct effect of mAb treatment that may play a role in adhesion activation or an indirect consequence of cell adhesion resulting from activation. The time course of the mAb-induced shift in p120-catenin was similar to the time course of the development of strong adhesions detected by morphological observation, beginning in 1–2 h and fairly complete by 5 h (Figure 8D); therefore it is not possible to discern which happens first. However, we were able to separate activation from the development of overt adhesion by using a blocking mAb to E-cadherin. HECD1 completely blocked the ability of activating mAb to trigger adhesion (Supplemental Figure S3) but did not prevent the

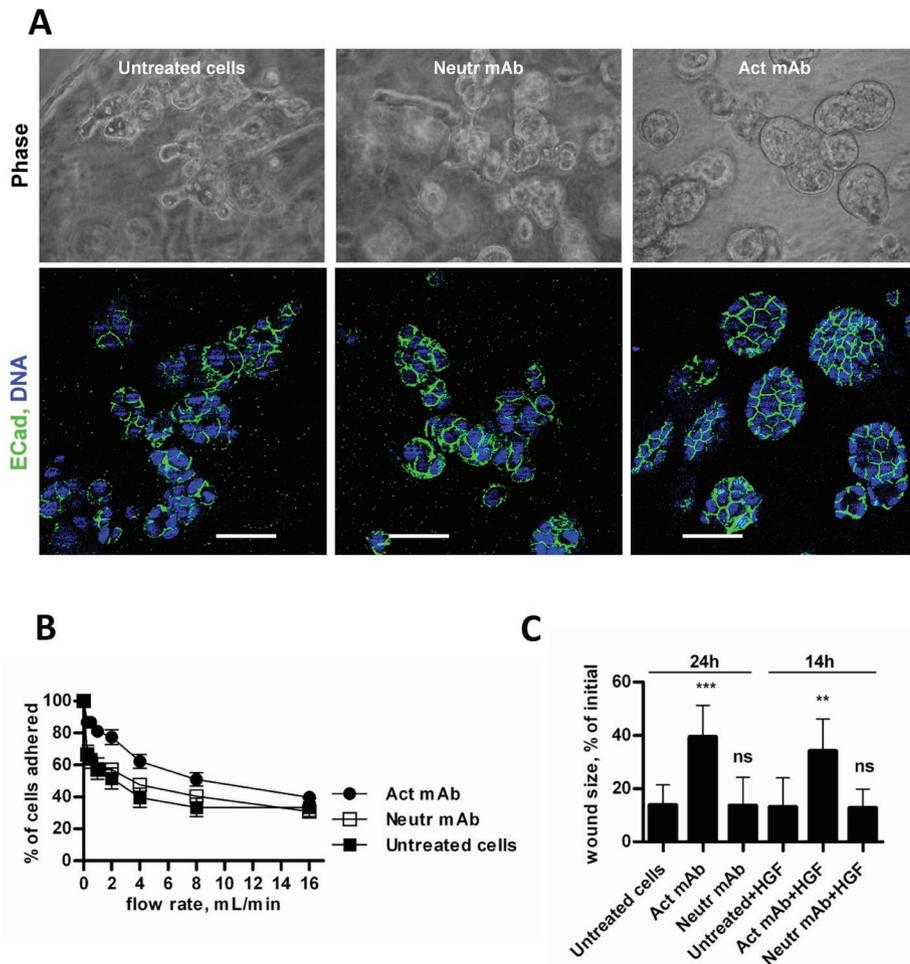


FIGURE 4: Activating mAb enhances adhesion in MDCK cells and inhibits growth factor-induced epithelial tubulogenesis. (A) Tubulogenesis assay. MDCKII cells were cultured in type I collagen gels containing HGF and treated with neutral mAb, activating mAb, or no mAb. Cells were grown for 7 d, and phase contrast pictures were taken to assess morphology. Immunofluorescence staining for E-cadherin shows epithelial organization. (B) Activating mAb increased E-cadherin-mediated adhesion in MDCKII cells treated with HGF. Cells were either untreated or treated overnight with 3 µg/ml 19A11 activating mAb in a medium containing 20 ng/ml HGF. Cell adhesion strength was evaluated using increasing laminar flow to determine the force required to detach cells. (C) Activating mAb decreased rate of scratch wound closure in MDCKII monolayers. Wounded cultures were treated with 3 µg/ml neutral or activating mAbs, and wounds were allowed to close in medium with or without HGF for 14 or 24 h, respectively. Error bars, SD of two independent experiments (N = 8). Significance of difference was determined by Student's *t* test; ns, no significant difference with $p > 0.05$; ** $p < 0.01$; *** $p < 0.005$.

molecular weight shift in p120-catenin (Figure 8E). Therefore the activating mAb is able to trigger activation-associated biochemical changes across the membrane independent of the formation of the adhesive bond between cells. This suggests that activating mAb binding induces a transmembrane change in the E-cadherin cytoplasmic domain that leads to the dephosphorylation of p120-catenin, which in turn may be involved in eliciting changes in E-cadherin to activate adhesion.

Indeed a transmembrane change in E-cadherin caused by activating mAb was also revealed by the unmasking of an epitope in the E-cadherin cytoplasmic domain (Figure 9). Indirect immunofluorescence staining of E-cadherin with a commercial mAb to the cytoplasmic domain (BD clone 36) in nonactivated Colo 205 cells (neutral mAb treated) was very poor, exhibiting virtually no staining of the E-cadherin in the plasma membrane relative to immunostaining by

ectodomain mAbs (the low levels of cytoplasmic staining may be background). Activation of adhesion, by activating mAb, staurosporine, or low trypsin, stimulated the appearance of staining by the BD cytoplasmic domain mAb at the plasma membrane, as revealed by colocalization with anti-ectodomain immunostaining. Epitope mapping of the region recognized by the BD clone 36 mAb (Supplemental Figure S4) indicates that the epitope is at or near portions of the p120-catenin-binding region, as determined previously by mutagenesis experiments (Thoreson *et al.*, 2000), as well as the crystal structure of the p120-cadherin complex (Ishiyama *et al.*, 2010). Thus adhesion-activating mAbs trigger the unmasking of an epitope at the p120-catenin-binding region of the E-cadherin cytoplasmic domain.

Previous work with Colo 205 activation by treatment with low trypsin or staurosporine implicated p120-catenin functionally in adhesion activation (Aono *et al.*, 1999). Because our activating mAb induced similar changes in p120-catenin phosphorylation, as well as changes in or near the p120-binding domain of E-cadherin, we asked whether mAb activation depends on p120-catenin interactions with the cadherin cytoplasmic domain. To do so, we used E-cadherin-deficient cells expressing a variety of E-cadherin mutant constructs (Figure 10). E-cadherin-deficient MDA-MB-231 cells were previously transfected to stably express wild-type E-cadherin and several different cytoplasmic domain mutant constructs (Wong and Gumbiner, 2003; Kim *et al.*, 2011). The parental MDA-MB-231 cells do not adhere to purified E-cadherin in the flow adhesion assay (Figure 10, top, curve with triangles), nor do they exhibit morphologically discernible cell-cell adhesion in culture (unpublished data). Activating mAb increases the adhesion of cells expressing full-length E-cadherin to E-cadherin, as evidence by increased resistance to detachment by shear flow (Figure 10, top). The activating mAb also increased the adhesion of cells expressing a construct lacking the β -catenin-binding domain and one with α -catenin sequences directly fused to the cytoplasmic domain (which lacks the β -catenin binding region but still contains the p120-binding region). In contrast, activating mAb had no effect on the adhesion of cells expressing a construct with targeted mutations that abrogate p120-binding (Figure 10, bottom).

Similar results were observed for the cadherin construct expressing MDA-MB-231 cells using the wound closure assay (Supplemental Figure S5). As in Figure 3, activating mAb slows the scratch wound closure by cells expressing full length E-cadherin. The activating mAb also slowed the rate of wound closure by cells expressing a construct lacking the β -catenin-binding domain and one with α -catenin sequences directly fused to the

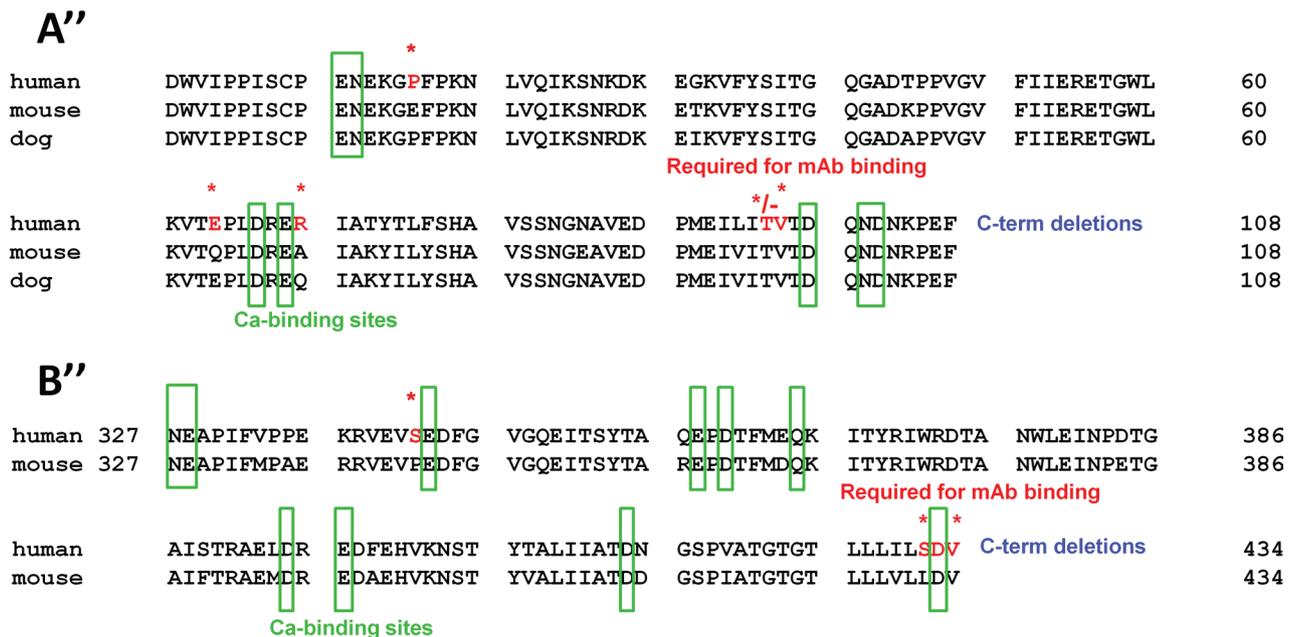
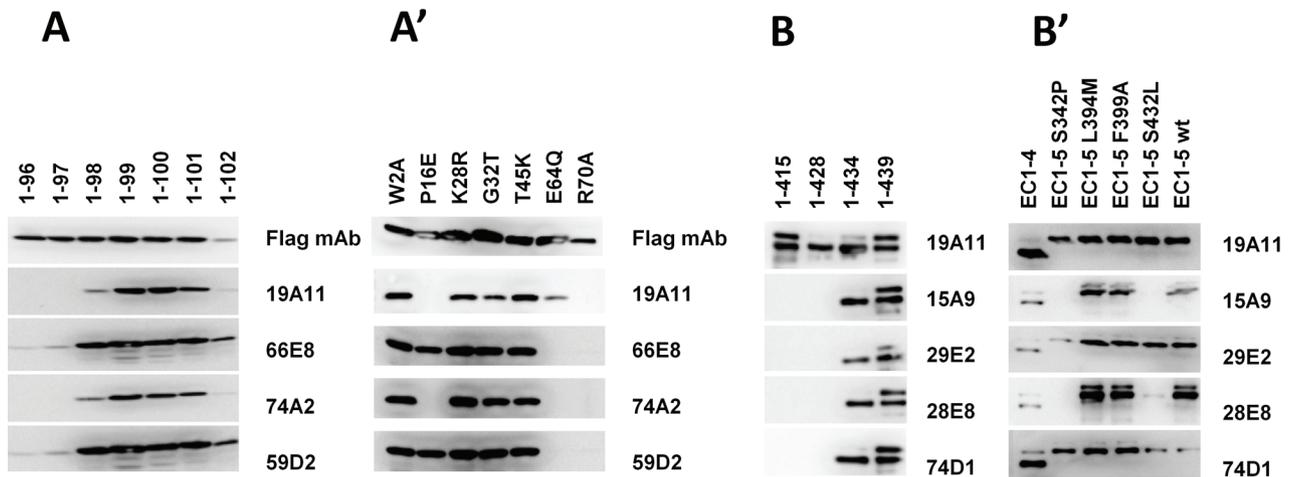


FIGURE 5: Strongly activating mAbs recognize a discontinuous epitope close to the calcium-binding site between EC1 and EC2, whereas distinguishing mAbs recognize a discontinuous epitope near the calcium-binding site between EC4 and EC5. (A, A', A'') strongly activating mAbs; (B, B', B'') distinguishing mAbs. (A) In vitro–translated deletion constructs show that mAb binding requires the very C-terminal end of EC1. (A') Site-directed mutations revealed the requirements for residues P16, E64, and R70 for binding of different mAbs. (A'') Location of residues that contribute to formation of the activating epitope. Amino acids important for antibody binding are shown in red; green rectangles show amino acids involved in Ca binding. (B) In vitro–translated deletion constructs mapped the binding of four out of five distinguishing mAbs to the very C-terminal end of the EC4 domain; 34G4 distinguishing mAb recognizes EC1/EC2 and was not included this analysis. (B') Site-directed mutations revealed the requirement for residues S342 and S432 in mAb binding. (B'') Location of residues that contribute to the formation of the distinguishing epitope. Amino acids important for antibody binding are shown in red; green rectangles show amino acids involved in Ca binding.

cytoplasmic domain. In contrast, activating mAb had no effect on the rate of wound closure by cells expressing a construct with targeted mutations that abrogate p120-binding. The MDA-MB-231 cells do express very low levels of cadherin 11, similar to the other “cadherin-deficient” fibroblastic cell lines, such as L-cells and Chinese hamster ovary cells, which potentially could have secondary effects on cell migration phenomenon. However, taken together with the results using direct E-cadherin adhesion assays in Figure 10, these findings indicate that p120-catenin binding to the E-cadherin cytoplasmic domain appears to be required for the adhesion-enhancing activity of the activating mAbs.

Regulation of adhesion activation by p120-catenin phosphorylation

The findings in Figures 8–10 suggest that p120-catenin and, in particular, p120-catenin dephosphorylation are involved in the regulation of E-cadherin activation. We therefore tested whether dephosphorylated p120-catenin can activate E-cadherin and whether blocking p120-catenin dephosphorylation inhibits mAb induced E-cadherin activation. To do so, we expressed specific mutant forms of p120-catenin harboring mutations in the Ser/Thr sites that have been shown to be phosphorylated (Xia *et al.*, 2003) and for which we observe dephosphorylation during activation (Figure 8). Either proteins harboring mutations in several Ser/Thr

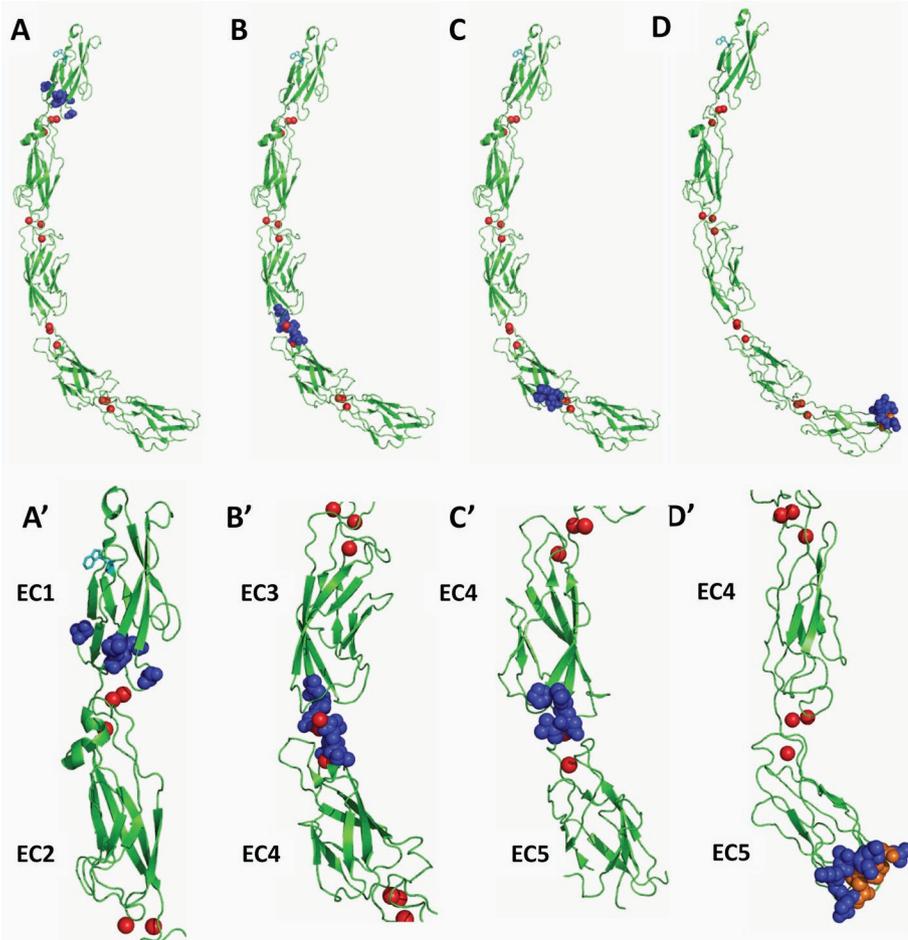


FIGURE 6: Structural modeling of activity state-associated epitopes located at the interfaces between domains. (A, A') Epitope for strongly activating mAbs located between EC1 and EC2, close to the calcium-binding site. (B, B') Epitope for weakly activating mAbs located at the calcium-binding site between EC3 and EC4. (C, C') Epitope for distinguishing mAbs located at the calcium-binding site between EC4 and EC5. (D, D') Epitope for C-cadherin-activating mAbs AA5 located at the base of EC5 near the transmembrane domain. Amino acid residues involved in epitopes are shown in blue, calcium ions are shown in red, cysteine residues that form disulfide bonds are shown in orange, and Trp-2 structure is shown in cyan.

sites (Figures 11 and 12) or those with single-site mutations (Supplemental Figures S6 and S7) were analyzed. The effects of the multiple-site mutants were more penetrant. Mutant mouse p120-catenin proteins were expressed in Colo 205 cells using retroviral vectors (Figure 11) either in the presence of endogenous human p120-catenin (control small interfering RNA [siRNA]) or when endogenous human p120-catenin was substantially knocked down using siRNA; expressed mouse p120-catenin was detected with an antibody specific for the mouse version. The siRNA treatment effectively depleted endogenous human p120-catenin (Figure 11), and similar to observations in the literature, the levels of E-cadherin were significantly reduced due to the role of p120-catenin in stabilizing cadherin expression (Ireton *et al.*, 2002; Davis *et al.*, 2003; Xiao *et al.*, 2005). Of importance, expression of mouse p120-catenin completely rescued the levels of E-cadherin expression, irrespective of whether it contained any of the Ser/Thr mutations, either the S → A phosphorylation-deficient mutants or the S → E phosphomimetic mutations (Figure 11). Thus the state of Ser/Thr phosphorylation had no detectable role in the well-known function of p120-catenin in the regulation of E-cadherin expres-

sion levels, similar to the results reported by Reynold's group (Xia *et al.*, 2006).

We then tested whether the Ser/Thr mutations had any effects on activation of E-cadherin-mediated adhesion in Colo 205 cells (Figure 12). Images are shown in part I of Figure 12 (A–H and A'–H'), and quantitation of this and two additional similar experiments is shown in part II of Figure 12 (with corresponding a–h and a'–h'). It is not surprising that siRNA depletion of p120-catenin blocked the activation of adhesion by activating mAb, as well as reducing the levels of both p120-catenin and E-cadherin (Figure 12, A' and B') compared with control cells, in which activating mAb caused cells to aggregate and compact compared with neutral mAb-treated cells (Figure 12, A and B). Cells expressing wild-type mouse p120-catenin behaved the same as normal cells, with activating mAb inducing aggregation and compaction compared with neutral mAb treatment (Figure 12, C, D, C', and D'). It is striking that expression of a phosphorylation-defective mutant of p120-catenin with six Ser/Thr residues mutated to Ala (6S,T→A) caused constitutive aggregation and compaction of the cells, even without treatment with activating mAb (Figure 12, E and E'); activating mAb had no additional effect (Figure 12, F and F'). Constitutive activation occurred even without depletion of endogenous human p120-catenin (Figure 12E), suggesting that the effect of the mutant is dominant. The constitutive activation of adhesion by the 6S,T→A mutant is also evident after quantitation of the data to measure the number of cells present in different-size aggregates (Figure 12, part II). Wild-type p120-catenin-expressing cells aggregated in response to treatment with activating mAb (Figure 12, c, d, c', and d'),

but 6S,T→A-expressing cells aggregated constitutively (Figure 12, e, f, e', and f'). These data suggest that the dephosphorylated forms of p120-catenin induced during E-cadherin activation (Figure 8) are sufficient to mediate adhesion activation.

To determine whether dephosphorylation is required for mAb-induced activation of adhesion, we similarly expressed phosphomimetic forms of mouse p120-catenin harboring S,T→E mutations. Our findings again indicate that the combination of four N-terminal S/T mutations (Figure 12) was more penetrant than individual-site mutations (Supplemental Figure S7). Of importance, unlike the S,T→A mutations, the S,T→E mutations did not constitutively activate adhesion, showing that they did not act as phosphorylation-deficient mutations but instead are likely to be phosphomimetics. Expression of 4S,T→E p120-catenin in the absence of endogenous human p120-catenin significantly inhibited the ability of the activating mAb to induce cell aggregation and compaction (Figure 12, G' and H'). In the presence of endogenous human p120-catenin it partially blocked aggregation and compaction (Figure 12, G and H). These findings are confirmed by quantitation of the data, which indicates that the 4S,T→E mutants significantly blocked induction of

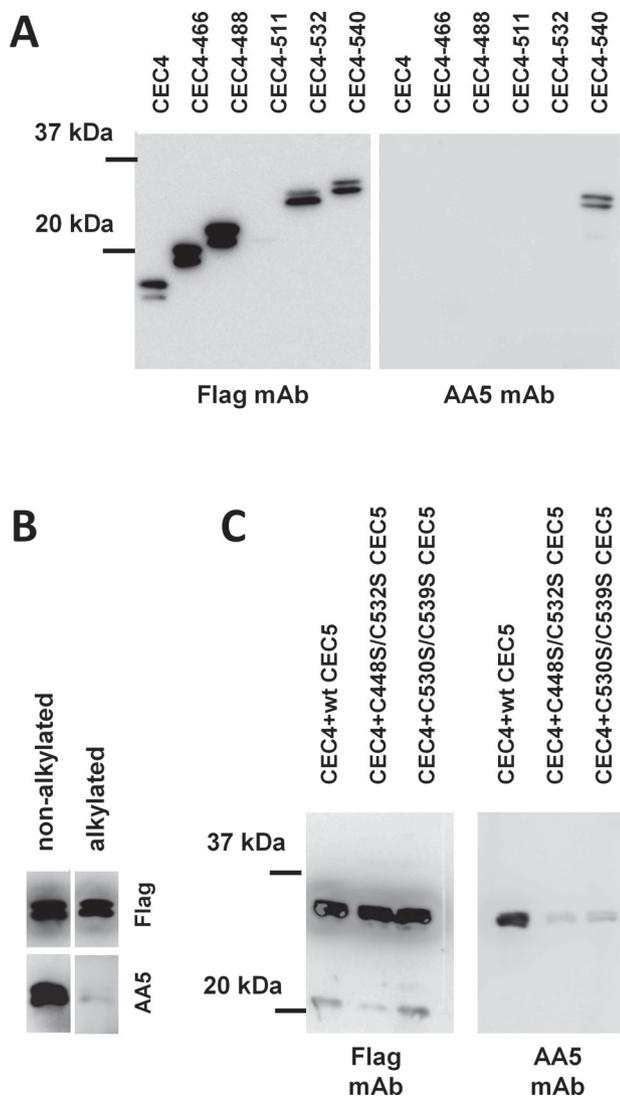


FIGURE 7: The AA5 C-cadherin-activating mAb recognizes a conformational epitope at the interface between EC5 and the transmembrane domain. (A) In vitro-translated deletion constructs mapped AA5 binding to the very C-terminal end of the EC5 domain. (B) AA5 binding is abolished by reducing and alkylating the two intramolecular disulfide bonds in the EC5 domain. (C) Site-directed mutations revealed that the AA5 binding depends on disulfide bonds formed by C448/C532 and C530/C539.

cell aggregation by activating mAb treatment (Figure 12, part II, g, h, g', and h'), especially when endogenous human p120-catenin is depleted. Therefore expression of phosphomimetic mutants of p120-catenin shows that p120-catenin dephosphorylation is required for mAb-induced activation of adhesion.

We also examined whether phosphorylation of individual Ser/Thr sites in the N-terminus have especially important roles in adhesion activation of Colo 205 cells, and the results are summarized in Table 2. Both phosphorylation-deficient S→A (Supplemental Figure S6) and phosphomimetic S→E mutants (Supplemental Figure S7) of the best candidate sites, as determined by their dephosphorylation during activation (Figure 8) or by their presence in the multisite mutants (Figure 12), were examined using the same approach as for the multisite mutants.

The effects of the single-site mutations were weaker than those of the multisite mutations, but several sites showed statistically sig-

nificant partial effects that could be discerned through the quantitative measure of cell aggregation. In particular, S268, S288, and to a lesser extent S312 were found to have roles in constitutive activation of adhesion (Supplemental Figure S6, c, c', e, e', and g') or in the inhibition of antibody-induced adhesion (Supplemental Figure 7, d', f', and h') compared with other Ser-site mutations. The fact that the multisite mutants had stronger effects in the assays suggests that phosphorylation of these sites works in combination to regulate E-cadherin-mediated adhesion.

To determine whether phosphorylation of these sites has a more general role in regulating E-cadherin-mediated adhesion, we expressed the multisite 6S,T→A and 4S,T→E p120-catenin mutations in A431 cells in the presence of endogenous human p120-catenin (Figure 13). Both mutant proteins were expressed at similar levels as wild-type mouse p120-catenin (Supplemental Figure S8). As expected, expression of wild-type p120-catenin had no effect on the strength of adhesion to purified E-cadherin protein as assessed with the flow adhesion assay compared with mock-infected controls (Figure 13). Of interest, the 6S,T→A mutant significantly increased the strength of E-cadherin-mediated adhesion, whereas the 4S,T→E mutant significantly decreased the strength of adhesion (Figure 13). These findings suggest that the phosphorylation status of these Ser/Thr residues of p120-catenin have a general role in regulating the strength of E-cadherin adhesion, with phosphorylation decreasing overall adhesive strength.

DISCUSSION

Previous work on both *Xenopus* C-cadherin (Brieher and Gumbiner, 1994; Zhong *et al.*, 1999; Chen *et al.*, 2009) and human E-cadherin (Aono *et al.*, 1999) provided evidence for the regulation of cadherin adhesion activity independent of cell surface expression levels. This led us in the present study to test the hypothesis that conformational changes or other physical changes (oligomerization/clustering) in the cadherin homophilic binding ectodomain are involved in the regulation of adhesion. We obtained evidence to support this hypothesis by two criteria—mAbs that distinguish activity states of E-cadherin, and mAbs that bind to E-cadherin and induce its activation at the cell surface.

The distinguishing mAbs identified in this study exhibited relatively small changes in binding to cell surface E-cadherin as a result of activation by staurosporine or activating mAbs. Nonetheless, the change in binding was very selective and observed only for a rare group of mAbs and therefore not simply due to changes in accessibility to the cell surface in general. Of importance, most of these mAbs recognized a very similar epitope, indicating that they target a very specific feature of the protein.

Adhesion-activating mAbs could work in either of two general ways, as exemplified by antibody regulation of integrin adhesive function (Schwartz *et al.*, 1995; Stuver and O' Toole, 1995; Humphries, 1996). One simple mechanism observed for integrin-activating mAbs is the stabilization of the high-affinity binding conformation for ligands, such as von Willebrand's factor. The other mechanism observed for integrins is more complex, with activating mAbs acting like signaling ligands that trigger changes in cytoplasmic factors that regulate integrin affinity state. The stimulation of p120-catenin dephosphorylation and the unmasking of the epitope near the p120-binding site suggest that cytoplasmic changes are similarly involved in antibody-induced E-cadherin activation. Of importance, these changes appear to be direct consequences of mAb binding rather than secondary effects subsequent to cadherin-mediated adhesion. Indeed, our functional analyses using mutations in the E-cadherin cytoplasmic domain and in

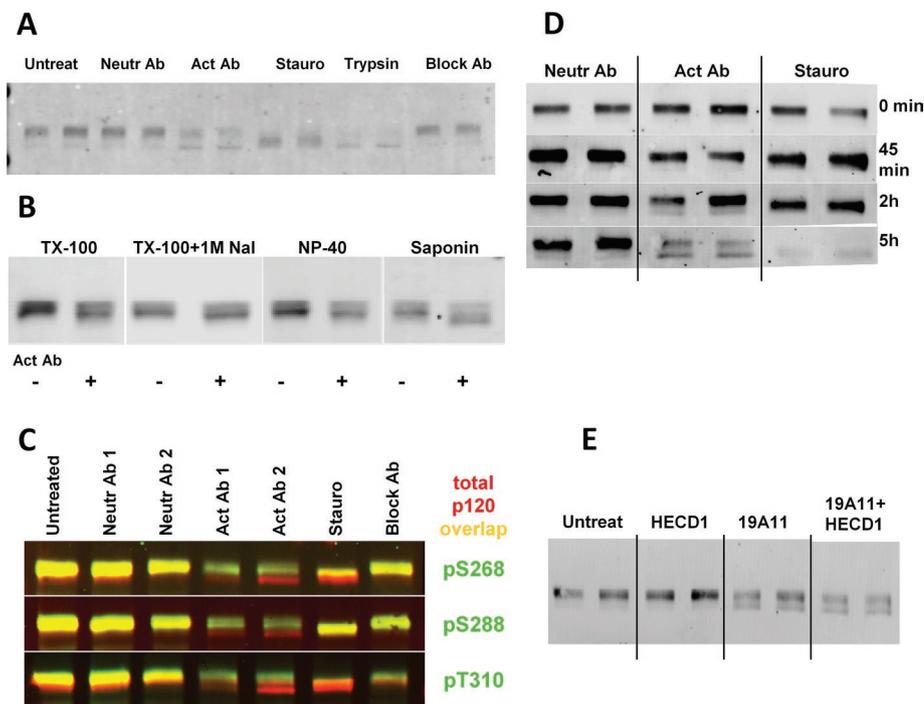


FIGURE 8: p120-catenin dephosphorylation in response to mAb-induced E-cadherin activation. (A) Activating mAb treatment caused a molecular weight shift in p120 on SDS gels. Colo 205 cells were untreated, treated with 3 $\mu\text{g}/\text{ml}$ 19A11 activating mAb, 76D5 neutral mAb, SHE78-7 blocking mAb, or 7 nM staurosporine for 5 h, or treated with 0.001% trypsin for 1 h. Cells were lysed in 1% digitonin, and p120 was detected by Western blotting. (B) Activating mAb-mediated p120 molecular weight shift detected using lysis with different detergents. Colo 205 cells were either untreated or treated with 3 $\mu\text{g}/\text{ml}$ 19A11 activating mAb for 5 h, lysed in 0.5% Triton X-100, 0.5% Triton X-100 plus 1 M NaI, 0.5% NP-40, or 1% saponin, and p120 was detected by Western blotting. (C) The p120 molecular weight shift is associated with dephosphorylation in response to mAb activation. Colo 205 cells were either untreated or treated with 0.3 $\mu\text{g}/\text{ml}$ activating mAb 66E8 (act Ab2), 3 $\mu\text{g}/\text{ml}$ 19A11 activating mAb (Act Ab1), 76D5 and 46H7 neutral mAbs (Neutr Ab1 and 2), HECD-1 blocking mAb, or 7 nM staurosporine for 5 h. Cells were lysed in 1% digitonin and analyzed by Western blotting with rabbit p120 mAb (total p120, shown in red) in combination with phosphospecific p120 mAbs that recognize pS268, pS288, or pT310 (shown in green). Yellow represents signal overlapping. (D) Time course of p120 molecular weight shift in response to activating mAb. Colo 205 cells were treated with 3 $\mu\text{g}/\text{ml}$ 19A11 activating mAb, 76D5 neutral mAb, or 7 nM staurosporine for 10 min, 45 min, 2 h, and 5 h, lysed in 1% digitonin, and analyzed by Western blotting. (E) Activating mAb-induced p120 molecular weight shift is independent of cell adhesion. Colo 205 cells were pretreated with 19A11 mAb for 1 h and then incubated with or without blocking mAb for 5 h. Cells were lysed in 1% digitonin, and p120 was analyzed by Western blotting.

p120-catenin phosphorylation sites provide evidence that p120-catenin function is required for the activating mAb to enhance adhesion, consistent with studies using other activators (Aono *et al.*, 1999). In this way, the activating mAbs can be considered to be agonists that trigger proximal events in the process of cadherin activation.

Our findings suggest that the regulation of cadherin adhesion activity involves the transduction of molecular changes throughout the entire cadherin molecule, entailing changes in homophilic binding via EC1, interfaces between several of the EC domains, and cytoplasmic signaling events via the catenins. We previously observed that the binding to a cadherin substrate causes cadherin clustering at the cell surface (Yap *et al.*, 1998), which in turn seems to regulate the state of adhesion (Yap *et al.*, 1997). Thus cadherins may undergo multiple physical or conformational changes back and forth across the plasma membrane during the overall process of cell adhesion, similar to those well documented for the EGF receptor (Macdonald-Obermann and Pike, 2009; Yang *et al.*, 2010), and the

activating mAbs may be able to induce some of these changes.

The epitopes recognized by the distinguishing and activating mAbs all map to interfacial regions between adjacent cadherin EC domains, especially at or near the calcium-binding regions between domains. The C-cadherin-activating mAb recognizes a region at a potential interface between the membrane-proximal EC5 domain and the transmembrane domain, even though this region does not contain a calcium-binding site. Moreover, most of the mAbs seem to recognize conformational epitopes, raising the possibility that conformational changes at interface regions and/or calcium-binding sites may be involved in the regulation of the adhesive-binding state. How potential changes at several different EC interfaces recognized by all these mAbs (EC1–EC2, EC3–EC4, EC4–EC5, EC5–transmembrane domain) regulate the state of the homophilic adhesive bond is not yet clear. One attractive hypothesis is that changes transmitted across multiple EC domain interfaces propagate an overall conformational change through the entire ectodomain to affect the distal homophilic binding site in EC1 and across the membrane to affect the cytoplasmic domain. There is evidence that changes in one EC domain can influence the properties of distal EC domains (Tsuiji *et al.*, 2007; Shi *et al.*, 2010). Similarly, we find that cytoplasmic processes are involved in mAb activation. It is not clear how cytoplasmic factors in the Colo 205 cells could control the state of Trp-2-mediated homophilic binding, preventing its formation in the absence of activation, without invoking some more complicated mechanism involving the whole ectodomain.

Our findings also provide direct evidence that phosphorylation of p120-catenin at specific Ser/Thr residues regulates adhesion activation in Colo 205 cells.

Several specific N-terminal sites are dephosphorylated during mAb-induced adhesion activation. This appears to be directly associated with the transmembrane activation process, since dephosphorylation did not depend on the ultimate formation of adhesive contacts *per se*. Furthermore, the phosphorylation status of these same residues was found to control the state of activation. Blocking phosphorylation of these specific Ser/Thr residues in the N-terminus by mutations caused constitutive activation of adhesion, even in the presence of endogenous p120-catenin. This finding is similar to that observed by Aono *et al.* (1999) using an N-terminally truncated p120-catenin mutant, and it is possible that their results were due to the loss of these phosphorylation sites. Indeed we find that dephosphorylation of these N-terminal Ser/Thr sites is required for mAb-induced adhesion activation, since phosphomimetic mutations prevent activation. How the phosphorylation status of these N-terminal Ser/Thr residues of p120-catenin can so strikingly regulate the activity state of E-cadherin at the cell surface is not yet clear. Although we observe an

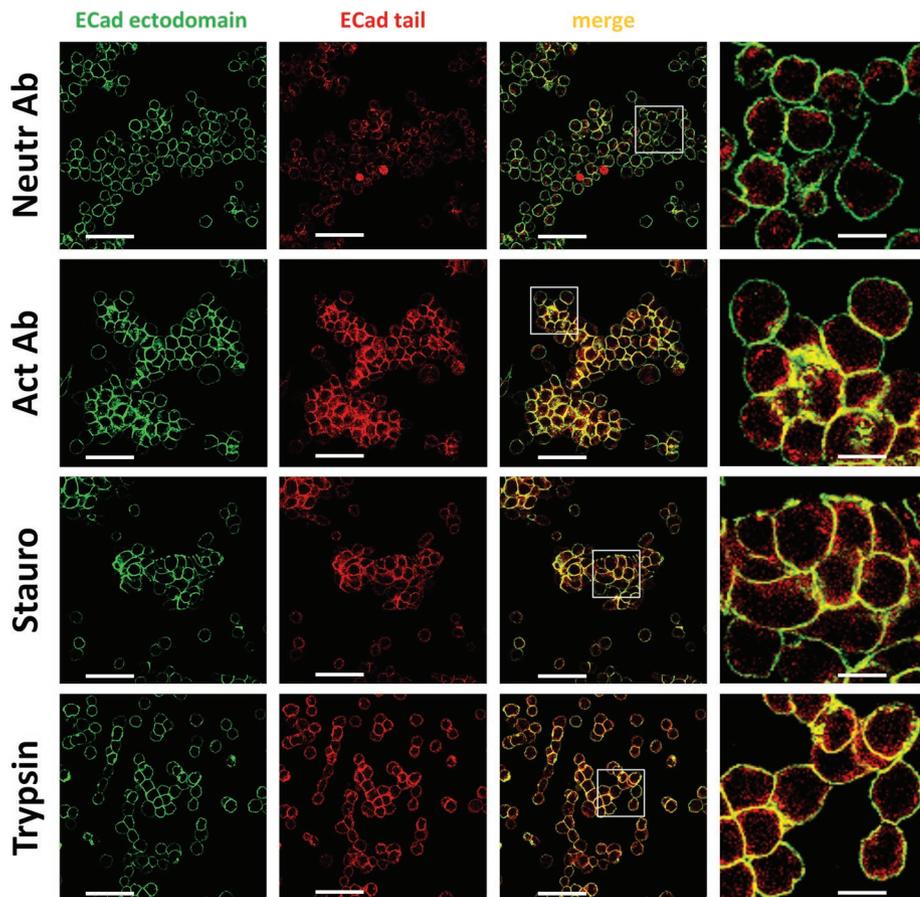


FIGURE 9: Adhesion-activating mAb stimulates the unmasking of an epitope in the p120-binding site of the E-cadherin cytoplasmic tail. Unmasking of cytoplasmic epitope was detected by cell surface colocalization with constitutively accessible extracellular E-cadherin epitope. Immunofluorescence confocal microscopy of nonadhesive and adhesion-activated Colo 205 cells. Cells were treated with 3 $\mu\text{g}/\text{ml}$ 76D5 neutral mAb, 19A11 activating mAb, or 7 nM staurosporine for 5 h or treated with 0.001% trypsin for 1 h and then fixed and stained with E-cadherin cytoplasmic tail-specific IgG2a mAb (clone 36; BD) and extracellular domain (ectodomain)-specific IgG2b mAb 27D2 to avoid staining conflict with antibodies used for treatment (both IgG1). Images represent a single focal plane; last column, cropped and magnified merged image. Scale bar, 50 μm (left) and 10 μm (last column).

associated change in the accessibility of a site in the cadherin cytoplasmic tail at or near the p120-catenin-binding site, activation is not associated with changes in the amount of p120-catenin or other catenins associated with the cadherin cytoplasmic domain (Aono *et al.*, 1999; our present findings). Presumably, more subtle alterations in the structure of the cadherin cytoplasmic domain are involved.

Although the Colo 205 cell line used to identify the activating mAbs is atypical in being nonadhesive in the untreated state, the activating mAbs also affect growth factor-regulated adhesion in A431 and MDCK cells. In addition, their inhibition of cell migration, wound closure, and tubulogenesis suggests that regulation of cadherin activity state at the cell surface is important for tissue morphogenesis, similar to previously observed inhibition of the elongation of embryonic tissue via convergent extension by C-cadherin-activating mAbs (Zhong *et al.*, 1999). Moreover, we observe a similar role for p120-catenin N-terminal Ser/Thr phosphorylation in the control of adhesive strength in the constitutively adhesive A431 cell line. Thus regulation of adhesion at the cell surface via physical changes in the cadherin protein may be a common feature of adhesive function in general.

Our findings have important implications for understanding the roles of cadherins in tissue morphogenesis and disease. For example, in evaluating the roles of cadherins in cancer it may be important to assess the activity state of the protein on the cell surface in addition to its level of expression. Furthermore, the ability to stimulate or maintain high cadherin activity at the cell surface may provide a novel way to enhance cadherin function to affect disease processes.

MATERIALS AND METHODS

Cell culture and antibodies

All cell lines used were from the American Type Culture Collection (Manassas, VA). Cells were cultured in standard CO_2 /temperature conditions in medium containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA). EGF and HGF were purchased from PeproTech (Rocky Hill, NJ). E-Cadherin cytoplasmic tail-specific mAb (clone 36; 610182) was from BD Biosciences (San Diego, CA). The p120 C-terminus-specific rabbit mAb (2806-1) was from Epitomics (Burlingame, CA), and p120 phosphospecific mAbs (Xia *et al.*, 2004) were kind gifts from Albert Reynolds (Vanderbilt University, Nashville, TN).

Screening of hybridomas for activating and distinguishing mAbs

Mice were immunized with purified ECad1-5 protein described later, and hybridoma fusions were plated at low density in eighty 96-well plates to ensure that distinguishing mAb activity was not covered up by contaminating hybridomas secreting nondistinguishing mAbs. Supernatants of hybridomas were first screened for binding to the ectodomain of human E-cadherin using an ELISA described later. E-Cadherin-positive

hybridoma supernatants were tested for binding to live Colo 205 cells with an ELISA for differential binding to nonactivated versus staurosporine-activated cells. For activating mAbs, hybridoma supernatants were screened for their ability to trigger adhesion and compact morphology, similar to what is observed as a result of activation with low-level trypsin or staurosporine (Aono *et al.*, 1999). Clones of interest were checked for specificity using ELISA on pure E-cadherin protein, ELISA on live Chinese hamster ovary cells stably expressing either human E- or *Xenopus* C-cadherin, in a competitive cell ELISA with pure E-cadherin 1-5EC domain, by immunofluorescence staining, and by Western blot analysis.

E-cadherin protein, expression, and epitope mapping

The E-cadherin ectodomain fused with the Fc region of human immunoglobulin G1 (IgG1) at the C-terminus (Fc-ECad1-5) was purified as described before (Chappuis-Flament *et al.*, 2001). The Fc fragment was cleaved off by Factor Xa through an enzyme-specific site introduced into the fusion protein between ECad1-5 and Fc fragment. The latter was removed by protein A affinity chromatography. Mutations in the E-cadherin ectodomain were introduced using a QuikChange Site-Directed Mutagenesis kit

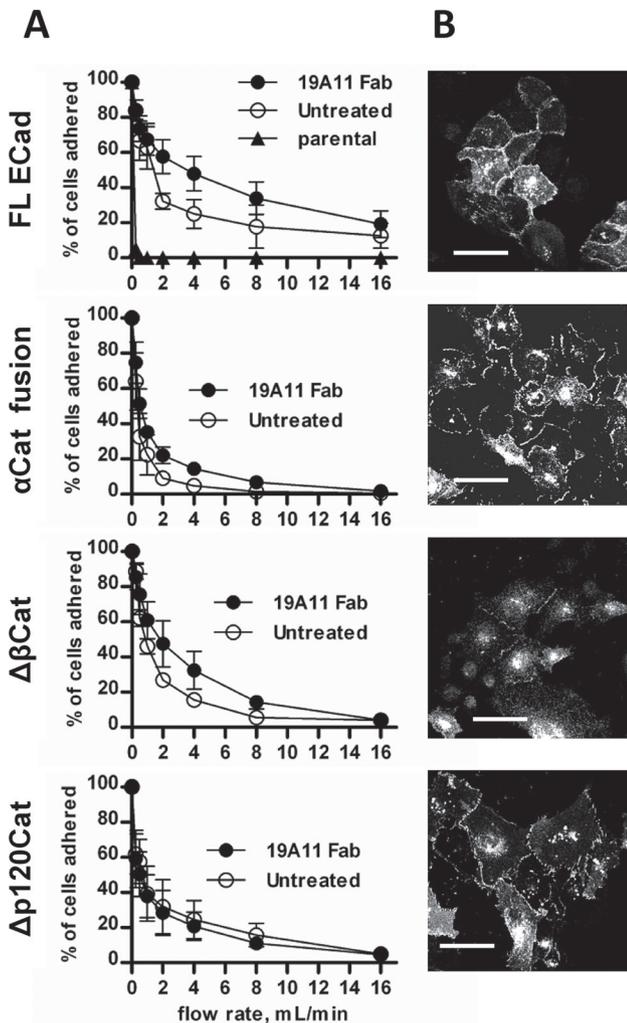


FIGURE 10: Requirement for p120 binding site on E-cadherin for mAb-mediated adhesion activation. (A) Effect of activating mAb on E-cadherin-mediated adhesion of MDA-MB-231 cells expressing various E-cadherin mutant constructs. Cells expressing full-length E-cadherin (FL ECad), E-cadherin- α -catenin fusion protein (α Cat fusion), C-terminus-truncated E-cadherin lacking β -catenin-binding site ($\Delta\beta$ Cat), and Δ p120Cat cell line lacking p120-binding site were tested. Cells were either untreated or treated for 5 h with 1 μ g/ml 19A11 activating mAb Fab fragment before harvesting. Cell adhesion strength was evaluated using increasing laminar flow to determine the force required to detach cells. Error bars, SD of two independent experiments, two independent runs for each condition (N = 4). (B) Localization of different E-cadherin constructs at cell-cell contacts when stably expressed in MDA-MB-231 cells. Volume-rendered confocal images of fixed cells stained with 19A11 mAb. Scale bar, 50 μ m.

(Agilent Technologies, Santa Clara, CA). The cDNAs for epitope mapping were cloned either into the pCS2 vector containing a C-terminal FLAG tag (Kim *et al.*, 2009) for expression in the wheat

FIGURE 12: Effects of nonphosphorylatable (S,T \rightarrow A) and phosphomimetic (S,T \rightarrow E) p120-catenin mutations on activation of Colo 205 cell adhesion. Cells from the experiment described in Figure 11 were examined for adhesion activation. At 72 h postelectroporation, cells were fixed with PFA and stained with human E-cadherin extracellular domain-specific IgG2b mAb 27D2 (all cell lines) in combination with p120 catenin rabbit mAb (Epitomics) in the case of mock-transfected cells or with mouse p120 catenin-specific IgG2a mAb 8D11 in case of mouse p120-transfected cells. Images show representative merged epifluorescence of three independent experiments for each cell line. Scale bar, 50 μ m. Bar graphs represent quantification of cell adhesion activation by measuring the distribution of cells in three categories: single cells, small aggregates of up to five cells, and large aggregates of six and more cells. Each bar graph shows quantification of three images from three independent experiments (N = 3) with a SD.

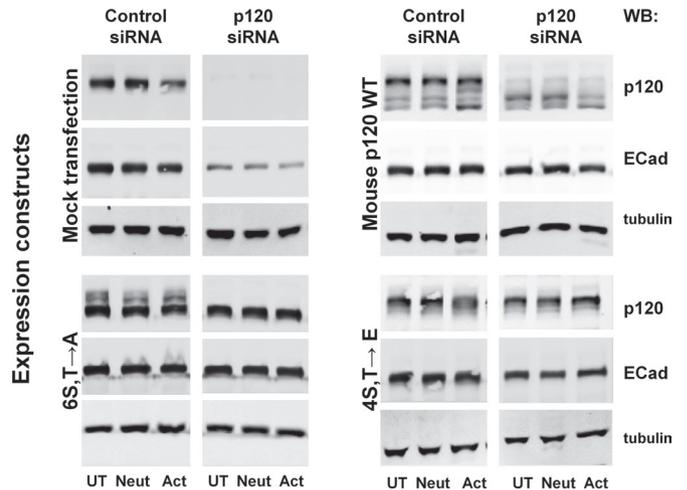


FIGURE 11: Wild-type and phosphorylation mutants of mouse p120-catenin all-rescue E-cadherin expression levels in Colo 205 cells. Colo 205 cells were infected with retroviruses containing various mutant mouse p120-catenin constructs and selected with neomycin before siRNA-mediated knockdown of human p120-catenin. The mouse p120 catenin 6S,T \rightarrow A mutant harbors S252A, S268A, S288A, T310A, S312A, and T916A mutations; the 4S,T \rightarrow E mutant harbors S268E, S288E, T310E, and S312E mutations. Mock-treated cells were infected with retrovirus containing empty vector and subjected to selection like other lines. Human p120 catenin knockdown was achieved by electroporation of infected Colo 205 cells with human p120-specific siRNA. At 60 h postelectroporation, cells were subjected to overnight treatment with 1 μ g/ml indicated mAb Fab or remained untreated. At 72 h postelectroporation, cells were lysed with electrophoresis sample buffer and analyzed by Western blotting. Representative data are shown from three independent experiments for each cell line. Act, activating mAb 19A11 Fab-treated cells; Neut, neutral mAb 76D5-treated cells; UT, untreated cells.

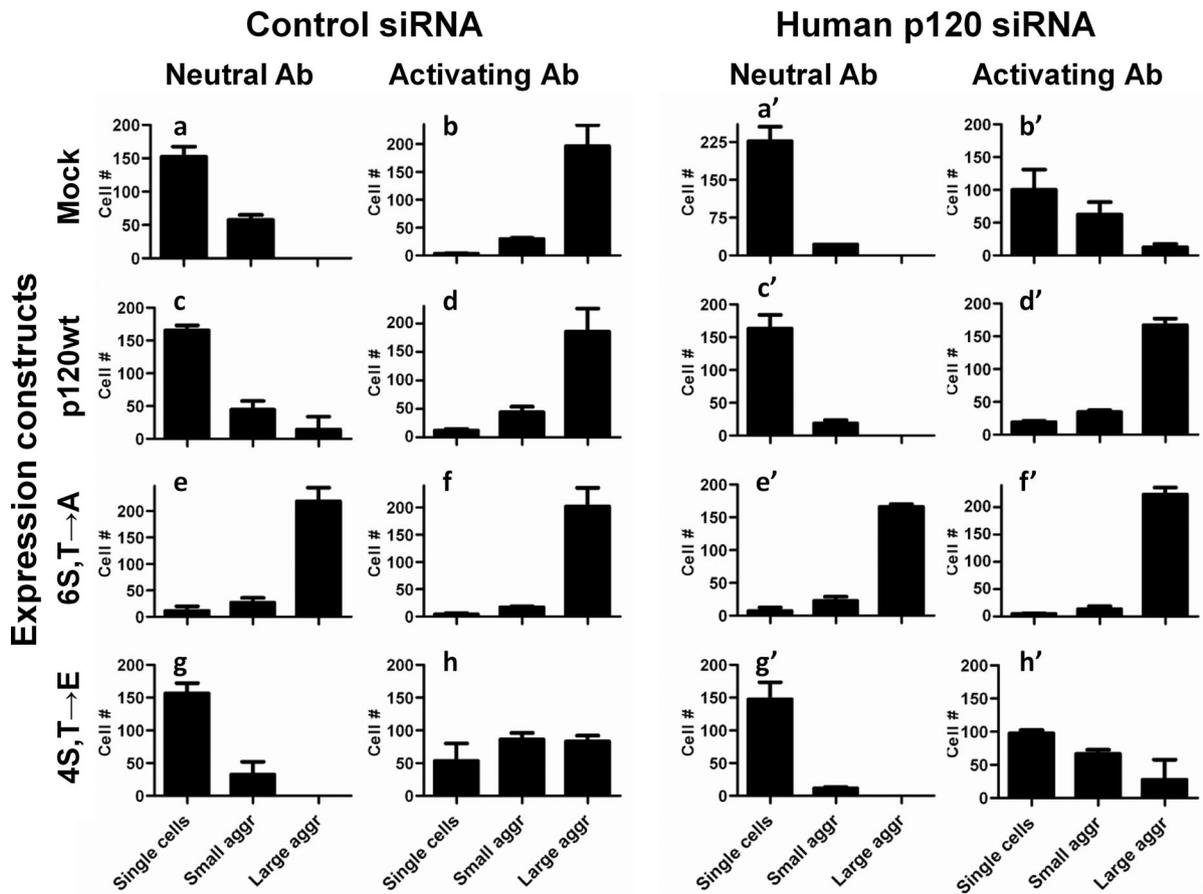
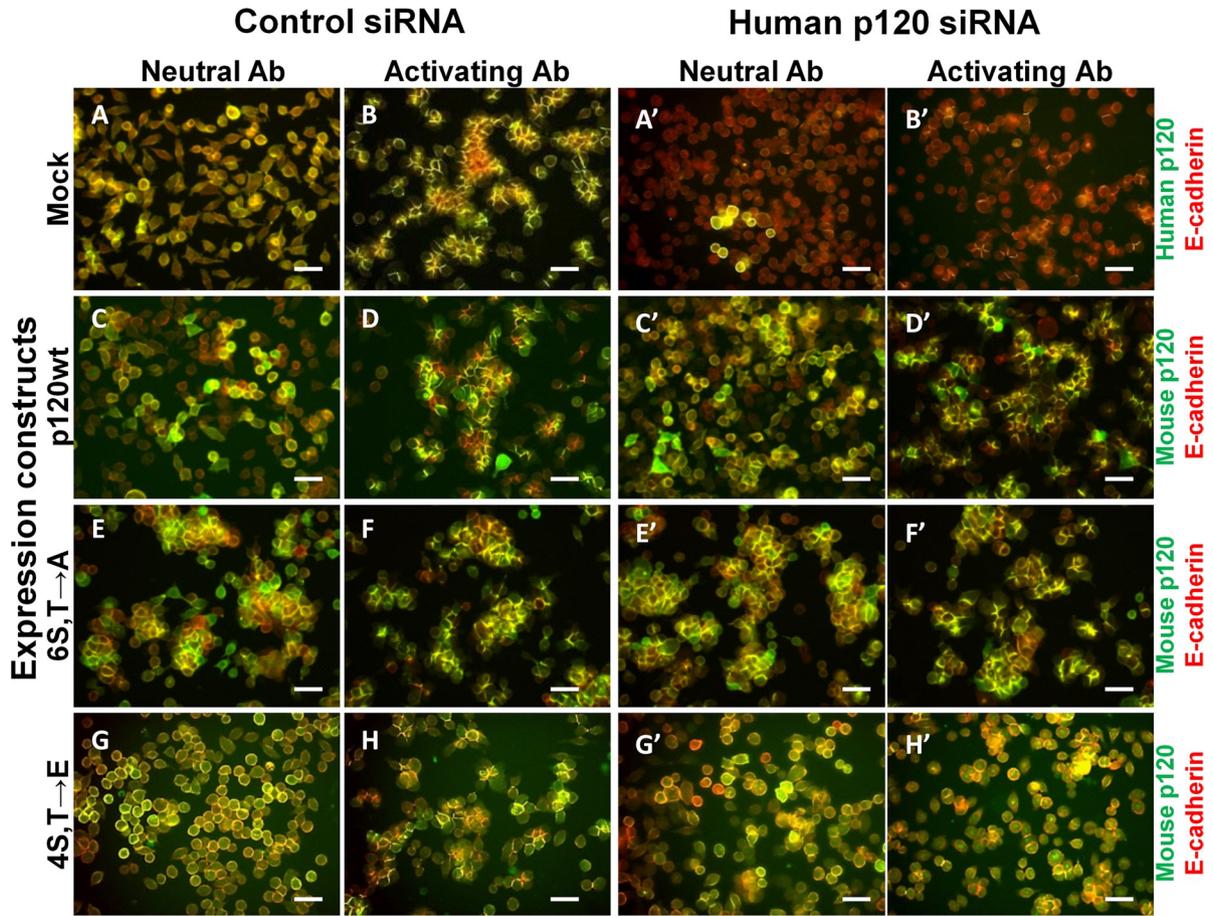
germ *in vitro* system or into the pEE14 vector with a C-terminal Fc tag for transient transfection in Chinese hamster ovary cells. In the case of Chinese hamster ovary expression the expressed proteins were purified from the cell culture media using protein A agarose.

Colo 205 cell activation assay

For the activation assay Colo 205 cells were seeded on 96-well plates precoated with rat-tail collagen type I (Sigma-Aldrich, St. Louis, MO) at 2×10^5 cells per well and allowed to recover overnight after harvesting. The activities of hybridoma supernatants or purified mAbs were determined by application to cells at serial dilutions. Colo 205 cell adhesion activation was determined by the extent of morphological change to compact epithelial appearance, and the degree of activation was estimated by eye.

Live-cell ELISA

Tissue culture 96-well plates were precoated with 5 μ g/ml of rat-tail collagen type I for 2 h at 37°C for seeding of Colo 205 cells or used



Residue	Dephosphorylation during mAb-induced activation	S,T→A activation	S,T→E blocks mAb-induced activation
S252	ND	-	-
S268	+	+	+
S288	+	+	+
T310	+	ND	ND
S312	ND	+/-	+/-
T916	-	ND	ND

Sites involved in adhesion regulation determined by single mutations (see Supplemental Figures S6 and S7) and sites shown to be dephosphorylated during adhesion activation determined using phosphospecific antibody staining (Figure 8) are shown. +, observed effect; -, no effect; ND, not determined.

TABLE 2: Summary of roles of individual p120-catenin phospho-Ser/Thr sites in adhesion activation of Colo 205 cells.

without precoating for other cell types. Cells were seeded at densities close to confluence, kept overnight to allow cadherin cell surface levels to recover, and incubated in duplicates (or greater multiples) with serial dilutions of hybridoma supernatants or purified mAbs in culture medium for 1 h at 37°C. After washing three times with culture medium, cells were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA) for 1 h at 37°C and then washed three times. Amount of binding was detected with 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich). In some cases secondary goat anti-mouse IgG1 isotype antibodies labeled with Alexa 488 fluorescent dye were used. The significance of the difference between curves was determined using two-way A (two-factor analysis of variance [ANOVA]), where two factors considered were "-/+ activation" (column factor) and "concentration" (row factor). For all curves analyzed, an interaction between these two parameters was not significant (cell activation and antibody concentration were independent), whereas the row factor was significant (good dose dependence). Column factor p values that ask whether the curves are different are presented on each graph. $p > 0.05$ indicates no significant difference, and $p < 0.05$ represents a significant difference. Analysis was performed using Prism software, version 5 (GraphPad Software, La Jolla, CA).

Laminar flow cell adhesion assay

The laminar flow cell adhesion assay was conducted as described in a previous report (Chappuis-Flament *et al.*, 2001). In brief, the cells were trypsinized in the presence of 2 mM calcium and allowed to attach to glass capillary tubes coated with a protein for 10, 15, or 20 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.

Wound-healing and MDCK branching tubulogenesis assays

For wound-healing assay, monolayers of cells were wounded by scraping with a plastic pipette tip and placed back in 2% NBS medium with or without growth factor (10 ng/ml EGF or 20 ng/ml HGF) and antibody supplements. Cells (four parallel wells per condition) were cultured for 14–30 h, stained by 0.1% crystal violet in 25% methanol, and photographed. Relative wound size was measured at three different points per each photo. Significance of difference was counted according to Student's *t* test; ns, nonsignificant difference with $p > 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. For tubulogenesis MDCKII dog epithelial cells were maintained and cultured

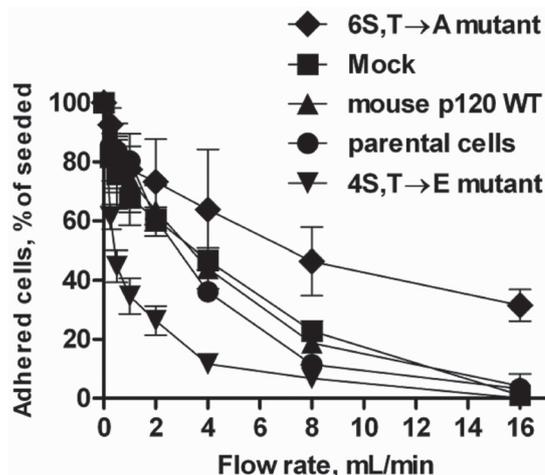


FIGURE 13: p120-catenin phosphorylation mutants regulate E-cadherin-mediated adhesion in A431 epithelial cells. A431 cells were infected with retroviruses containing different constructs and selected with neomycin. Mouse p120 catenin 6S,T→A mutant harbored S252A, S268A, S288A, T310A, S312A, and T916A mutations; 4S,T→E mutant harbored S268E, S288E, T310E, and S312E mutations. Mock-treated cells were infected with retrovirus containing empty vector and subjected to selection as other lines. No knockdown of endogenous p120 catenin was performed. Cell adhesion strength was evaluated using increasing laminar flow to determine the force required to detach cells. Error bars, SD of two independent runs in two independent experiments (N = 4).

in type I rat-tail collagen gels as described elsewhere (Wozniak and Keely, 2005). Briefly, cells were cultured in floating collagen gels containing 20 ng/ml HGF either untreated or treated with 2 µg/ml neutral mAb or activating mAb. Cells were grown for 7 d and phase contrast pictures taken to assess morphology using a Nikon 35-mm camera (Nikon, Melville, NY) attached to an Axiovert inverted microscope (Carl Zeiss, Jena, Germany). For immunofluorescence, gels were placed on glass coverslips, fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature, and permeabilized with 0.025% Triton X-100. Then general staining protocol was applied. Images were acquired using an Eclipse TE2000 confocal microscope (Nikon).

Structural modeling

Structural modeling was done using PyMOL 1.3 software (DeLano Scientific, Palo Alto, CA). For E-cadherin modeling, the mouse E-cadherin 3Q2V.pdb file was used (Harrison *et al.*, 2010); for C-cadherin, 1L3W.pdb was used (Boggon *et al.*, 2002).

Electrophoresis and Western blotting

SDS-PAGE was reformed using standard procedures. For basic-native gels a protocol from Mario Lebediker (Wolfson Center for Applied Structural Biology, Hebrew University of Jerusalem, Jerusalem, Israel) was used. Briefly, SDS and reducing agents were excluded from all solutions; otherwise general Laemmli electrophoresis protocol was applied. Before loading, the gels were prerun for 15–20 min. Western blot analysis was performed using standard procedures. Blots were developed with HRP-labeled secondary antibody and further processed with a Fujifilm LAS-3000 instrument (Fujifilm, Tokyo, Japan). Alternatively, blots were developed using IRDye 800CW goat anti-mouse (926-32210; LI-COR Biosciences, Lincoln, NE) and IRDye 680 goat-anti-rabbit (926-32221; LI-COR Biosciences) secondary antibodies and processed in a LI-COR Odyssey infrared imaging system.

Immunofluorescence, confocal, and bright-field imaging

For immunofluorescence, cells were grown on glass coverslips coated with 5 µg/ml of either fibronectin (for MDA-MB-231) or rat-tail collagen (Colo 205), fixed with 4% PFA for 10 min at room temperature, and permeabilized with 0.25% Triton X-100. For secondary antibodies, goat anti-mouse IgG (H+L)-Alexa 488 (A11001), goat anti-mouse IgG2b-Alexa 488 (A21141), IgG2b-Alexa 546 (A21143), IgG2a-Alexa 488 (A21131), and IgG2a-Alexa 546 (A21133), and goat anti-rabbit IgG-Alexa 546 (A11010) (all Invitrogen, Carlsbad, CA) were used in different combinations. Nuclei were counterstained with To-Pro-3 iodide (T3605; Invitrogen). All images were acquired at room temperature. For confocal imaging, coverslips were mounted in ProLong Gold anti-fade reagent (P36930; Invitrogen). Images were acquired using an Eclipse TE2000 confocal microscope (Nikon), PlanApo 60×/1.40 objective lens with immersion oil, high-quality charge-coupled device camera, and EZ-C1 software (Nikon). For general immunofluorescence, images were acquired using an IX-71 fluorescence microscope (Olympus, Center Valley, PA), UPlanFL N 10×/0.30 objective lens, a digital charge-controlled device camera C10600-10B (Hamamatsu, Hamamatsu, Japan), and SlideBook 5.0 Software (Intelligent Imaging Innovations, Denver, CO). Phase contrast and bright-field images were acquired using an Axiovert inverted microscope (Zeiss), LD-APlan 20×/0.3 or AChroPlan 4×/0.1 objective lenses, and PowerShot G6 camera (Canon, Lake Success, NY).

Mouse p120-catenin retroviral constructs, infections, and human p120 knockdown

Retroviral constructs in the pLZRS Neo vector, including mouse wild-type p120 catenin isoform 3A and several mutants thereof (S252A, S252E, S268A, S268E, S288A, S288E, S312A, S312E, 6S,T→A) were kind gifts from Albert Reynolds (Ireton *et al.*, 2002; Xia *et al.*, 2006). The 6S,T→A mutant harbors S252A, S268A, S288A, T310A, S312A, and T916A mutations. Additional mutations in mouse p120 catenin isoform 3A were introduced using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) and cloned into the pLZRS Neo vector. The 4S,T→E mutant harbors S268E, S288E, T310E, and S312E mutations. All retroviral vector and viruses were produced using the Phoenix retrovirus producer cell line (Garry P. Nolan, Stanford University) according to the Nolan lab protocol. Cells were infected with respective retroviruses by spinoculation in six-well tissue culture plates at 1800 × g for 2 h at 33°C and selected with 1 mg/ml neomycin for 10 d. In the case of Colo 205 cells, multiple infections with the same virus were performed to achieve the desired expression level. Mock-treated cells were infected with retrovirus containing empty vector and subjected to selection as for the other lines. Mouse p120 catenin expression levels were estimated by Western blot analysis using mouse p120-specific mAb 8D11 (Wu *et al.*, 1998), a kind gift from Albert Reynolds. Human p120 catenin knockdown was achieved by electroporation of infected cells with human p120-specific siRNA (Davis *et al.*, 2003) using Amaxa Nucleofector (Lonza, Basel, Switzerland) according to the manufacturer's instructions.

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