Integrins stimulate E-cadherin-mediated intercellular adhesion by regulating Src-kinase activation and actomyosin contractility

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Summary

Cadherins and integrins are major adhesion molecules regulating cell-cell and cell-matrix interactions. In vitro and in vivo studies have demonstrated the existence of crosstalk between integrins and cadherins in cell adhesion and motility. We used a dual pipette assay to measure the force required to separate E-cadherin-producing cell doublets and to investigate the role of integrin in regulating the strength of intercellular adhesion. A greater force was required to separate cell doublets bound to fibronectin or vitronectin-coated beads than for doublets bound to polylysine-coated beads. This effect depended on cell spreading and the duration of stimulation. Cells expressing type II cadherin-7 also responded to fibronectin stimulation to produce a higher intercellular adhesion. Establishment of cadherin-mediated adhesion needed ROCK, MLCK and myosin ATPase II activity. The regulation of intercellular adhesion strength by integrin stimulation required activation of Src family kinases, ROCK and actomyosin contractility. These findings highlight the importance and mechanisms of molecular crosstalk between cadherins and integrins in the control of cell plasticity during histogenesis and morphogenesis.

Key words: Cadherin, Integrin, Force, Adhesion strength, Crosstalk, Contractility

Introduction

Cadherins and integrins constitute major classes of cell surface transmembrane receptors mediating cell-cell and cell-matrix adhesion, respectively (Hynes, 2002; Yagi and Takeichi, 2000). These adhesion molecules are involved in important biological processes, such as cell migration, cell proliferation, cell differentiation, cell survival and the regulation of gene expression. They are strictly regulated spatiotemporally during embryogenesis, and the coordination of their functions is essential for morphogenesis and wound healing (De Arcangelis and Georges-Labouesse, 2000; Gumbiner, 2005; Halbleib and Nelson, 2006), a process often deregulated in pathological states. Changes in the cadherin and/or integrin repertoire are often associated with epithelio-mesenchymal transition during embryogenesis and with the acquisition of tumorigenic and metastatic properties later on (Hazan et al., 2004; Maschler et al., 2005; Nakagawa and Takeichi, 1995; Wheelock and Johnson, 2003). Integrins and cadherins also interact with cytoplasmic partners, connecting them to the actin cytoskeleton and activating signaling cascades (Geiger et al., 2001; Mege et al., 2006). In this capacity they can act as mechanotransduction receptors, coupling the external surface and cytoskeleton of the cell, enabling cells to detect and respond to environmental constraints, as in durotaxis, chemotaxis and haptotaxis, key mechanisms in the control of biological processes (Ingber, 2006; Lecuit and Lenne, 2007).

There is a large body of evidence supporting the existence of crosstalk between integrins and cadherins in cell adhesion, motility and contraction (de Rooij et al., 2005; Genda et al., 2000; Marsden and DeSimone, 2003; Yano et al., 2004). Using in vivo and in vitro approaches, we have shown that changes in the repertoire of cadherins produced by cells modify the cellular response to β1-integrin-mediated interaction with the extracellular matrix (ECM) and prevent the β1-integrin-dependent phosphorylation of FAK (Dufour et al., 1999). Furthermore, the disruption of β1-integrin-mediated cell-ECM adhesion affects intercellular adhesion and impairs neural crest cell migration during embryogenesis (Breau et al., 2006; Pietri et al., 2004). However, it remains unclear how these adhesion systems are coupled to ensure adaptation of the adhesive status and mechanical properties of normal and pathological cells.

To address these issues, we used a dual pipette assay that permits us to measure the force required to separate newly formed cell doublets. When measured for doublets formed under a number a different conditions this separation force (SF) served as an index of intercellular adhesion to elucidate the mechanisms regulating adhesion strength. Specifically, we investigated the role of integrin-cadherin crosstalk in the regulation of intercellular adhesion, and we found that stimulating single cells by interaction with fibronectin-coated beads (FNbeads) increased E-cadherin-dependent adhesion strength (E-cadherin also known as cadherin-1). The stimulation of intercellular adhesion by integrins depends on the area of cell-bead contact and time of stimulation. The inhibition of Src family kinases (SFK) by PP1 did not affect the strength of E-cadherin-mediated adhesion, but impeded the integrin-dependent enhancement of cadherin-mediated adhesion strength. The inhibition of ROCK, MLCK or myosin II ATPase activity decreased the strength of cadherin-mediated adhesion and the integrin-dependent enhancement of E-cadherin homotypic adhesion. Our findings show that cellular tension, controlled by the actomyosin cytoskeleton,

regulates cell-cell adhesion and its modulation by cell-substrate adhesions.

Results

We have previously shown that the strength of E-cadherin-producing cell (Ecad cell) doublet adhesion is measurable within seconds of contact and increases rapidly with time (Chu et al., 2004), stabilizing after 30 minutes. In this study, we investigated the role of integrin stimulation in the development of cadherin-mediated adhesion by stimulating cells with FN-coated beads (FNbead) or polylysine-coated beads (PLbead) before bringing them into contact and subsequently measuring their SF (Fig. 1). We restricted our analysis to the impact of integrin ligation at an early stage of cadherin-mediated adhesion. The approach must optimize multiple parameters simultaneously. We must, (1) use cell doublets that have been in contact just long enough to have developed a significant adhesion but not long enough for that adhesion to have matured, (2) manipulate many doublets at each set of conditions in the same set of experiments, and (3) take into account the respective dynamics of cell-bead adhesion versus cell-cell adhesion, avoiding their putative reciprocal crosstalk during force measurement. A cell-cell contact time of 4 minutes meets all the criteria. In particular it allows measurements of SF in the linear growth phase of intercellular adhesion (Chu et al., 2004).

Integrin stimulation affects the strength of cadherin-mediated adhesion and requires cell spreading

Parental S180 cells and Ecad cells expressed similar levels of integrins at their cell surface as revealed by FACScan analysis (supplementary material Fig. S1). The incubation of FNbeads with parental S180 (not shown) and Ecad cells induced the recruitment of α5β1 integrin to small patches beneath the area of bead-cell contact (Fig. 2). Talin, which is known to interact with and to activate integrins, was colocalized with these patches, demonstrating the activation of integrins after stimulation with FNbeads. This finding is consistent with previous studies showing that the interaction of cells with integrin ligand-coated beads can promote integrin clustering and induce integrin-dependent signaling cascades (Miyamoto et al., 1995; Geiger et al., 2001). No integrin patches were detected when Ecad cells were incubated with PLbeads.

We investigated the conditions under which integrin stimulation affects the strength of E-cadherin-mediated adhesion by varying the number and size of the beads incubated with cells as well as the length of time for which cells and beads were allowed to interact before the cells were brought into contact. The SF required to separate doublets of Ecad cells bound for 15 minutes to a 10 μm FNbead was 39.9±8.5 nN, and that required to separate Ecad cell doublets not bound to FNbeads was 45.9±11.1 nN. A similar result was obtained for cells bound for 15 minutes to 6 μm beads (data are not shown). Thus, the stimulation of cells by incubation for 15 minutes with FNbeads had no effect on the intercellular adhesion strength of Ecad cell doublets. The SF did not differ significantly between unbound Ecad doublets and doublets made of Ecad cells bound for 60 minutes to one or two 10 μm FNbeads (Fig. 3A). By contrast, the SF for doublets of Ecad cells bound to three 10 μm FNbeads or one 15 μm FNbead increased by a factor of 1.6 in response to integrin-dependent stimulation (Fig. 3A,C and supplementary material Movie 1).

Thus, our observations suggest that the increase in E-cadherin-dependent adhesion strength observed following stimulation with integrins depends on the area of cell-bead contact and, consequently, on the number of integrins engaged in the interaction. All subsequent experiments were therefore performed with a single 15 μm bead, as it was difficult to manipulate cells adhering to three beads.

We next analyzed the effect of the duration of cell stimulation with an FNbead on E-cadherin-dependent adhesion. We compared

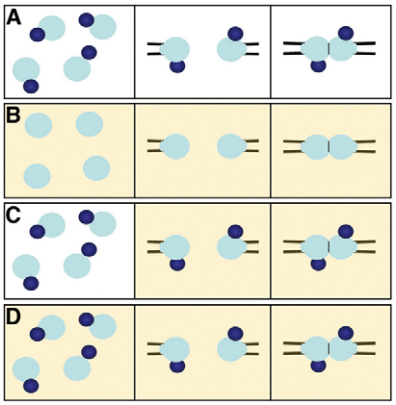

Fig. 1. Dual pipette assay and drug treatments during cell manipulation. The cells and ligand-coated beads are represented by light blue and dark blue circles, respectively. The presence of the drugs is indicated by panels colored in yellow. (**A**) Cells in suspension were incubated with ligand-coated beads for a specified length of time to produce cell-bead pairs (left panel; bound cells). Bound cells were selected and manipulated with micropipettes (middle panel) and were placed in contact for 4 minutes (right panel) before SF measurement, as indicated in the Materials and Methods section (see supplementary material Movie 1). (**B**) Isolated cells in suspension were incubated with drugs (left panel) for the specified period of time and then placed in contact for 4 minutes (right panel) before SF measurement. (**C**) Cells in suspension were incubated with ligand-coated beads. Bound cells were then incubated with the drug for 10 minutes (middle panel) and placed in contact for 4 minutes (right panel) before SF measurement. (**D**) Cells in suspension were incubated with ligand-coated beads in the presence of the drug and then manipulated (middle panel) and placed in contact for 4 minutes (right panel) before SF measurement.

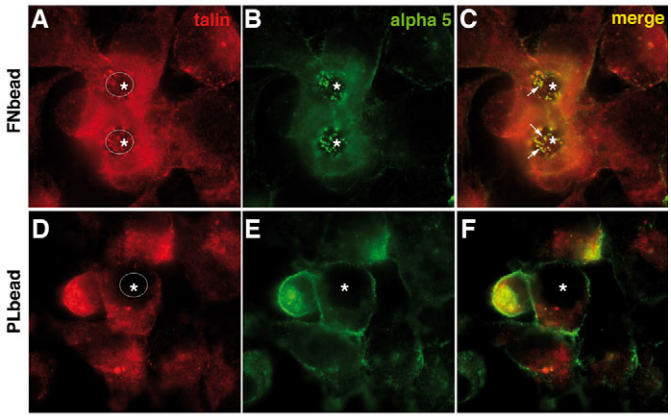

Fig. 2. Recruitment of α5β1 integrin and talin to adhesion complexes at the site of cell-bead contact. Cells bound to FNbeads (**A-C**) or PLbeads (**D-F**) were cytospun onto a glass coverslip, fixed and stained for talin (in red; A,D) or α5β1 integrin (in green; B,E). Merged images are presented in C and F. The asterisks and white circle indicate the bottom part of beads in contact with cells. The arrows show the adhesion complexes in which talin and α5β1 integrins are colocalized in the contact zone between an FNbead and a cell (C). These proteins are not detected at the site of contact between a PLbead and a cell (F).

the SF of doublets of Ecad cells allowed to bind to beads for 20 and 40 minutes before intercellular contact. The measured SF was not significantly higher than that measured for unbound Ecad cell doublets (Fig. 3B). Furthermore, the SF of doublets of Ecad cells bound to a 15 μm PLbead for 60 minutes before cell-cell contact was similar to that of unbound cells and significantly lower than that of cells bound to a 15 μm FNbead (Fig. 3B). We assessed whether the integrin-dependent increase in E-cadherin-mediated adhesion strength depended on the nature of the protein coating the bead. The SF for doublets of Ecad cells allowed to adhere to a FNbead or a vitronectin (VN)-coated bead (VNbead) was 1.6 times that for unbound cells and 1.5 times that for cells bound to PLbeads (Fig. 3C), indicating that the observed stimulation is dependent upon the ECM molecules used to coat the bead and involves the α5β1 or αV-integrins, respectively.

Taken together, these results demonstrate that stimulation with integrins increases E-cadherin-dependent adhesion strength. This effect depends on the number of integrins engaged and the duration of stimulation, but only above a relatively high threshold for surface area (at least 2.25 times the area of a 10 μm bead) and time (greater than 40 minutes).

We then explored the effect of surface area on the increase of intercellular adhesion and analyzed the kinetics of cell spreading on the bead (Fig. 4A,B). Isolated cells were put into contact with FNbead or PLbeads for 1, 4, 30 or 60 minutes. The cells adhered to PLbeads, but no spreading was observed over time (data not shown). By contrast, the area of cell-bead contact increased significantly over time for cells bound to FNbeads, with most of this expansion occurring after 30 minutes of interaction. An interesting side note is that it takes longer for S180 cells to spread out fully over a bead in our experimental conditions than reported in assays of cell adhesion to a two-dimensional substrate (Dufour et al., 1999).

The enhancement of cadherin-mediated adhesion strength involves several integrins and is not cell-type dependent

We performed stimulation experiments with other cell types expressing E-cadherins, such as L fibroblast cells, stably transfected with a cDNA encoding E-cadherin (EL cells), and squamous carcinoma SCC13 cells, which naturally produce cadherins (supplementary material Fig. S2). We obtained a SF of 26.4 nN for EL doublets and a SF of 43.6 nN for SCC13 doublets. Stimulation of EL and SCC13 cells by FNbeads yielded increases in SF by factors of 1.6 and 1.35, respectively. Thus, integrin-dependent changes in cadherin-based adhesion are not specific to S180 clones.

We also examined the response to integrin stimulation of cells producing another type of cadherin. Unlike Ecad cells, which responded only after 1 hour of integrin stimulation (Fig. 3B), cad7 cells, which produce type-II cadherin-7, responded to integrin stimulation after 15 minutes of contact between cells and beads. The SF measured for cad7 doublets bound to beads was twice that for cad7 doublets not bound to beads (supplementary material Fig. S3). Thus, although the basic response of Ecad and cad7 doublets

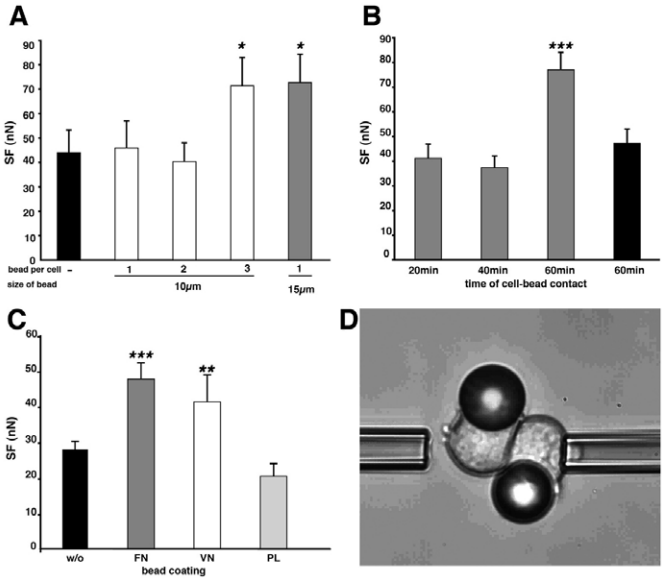

Fig. 3. Parameters of integrin stimulation before E-cadherin-mediated adhesion. (**A**) Mean SF measured for doublets of unbound Ecad cells (black bar) and bound Ecad cells allowed to adhere for 60 minutes, to one, two or three 10 μm FNbeads (white bars), and one 15 μm FNbead (gray bar) before the formation of cell-cell contact. (**B**) Mean SF measured for doublets of Ecad cells bound to one 15 μm FNbead for 20, 40 or 60 minutes (gray bars), and one 15 μm PLbead for 60 minutes (black bar) before the establishment of cell-cell contact. (**C**) Mean SF for doublets of unbound Ecad cells (black bar) or of cells bound for 60 minutes to one 15 μm FNbead (dark gray bar), one 15 μm VNbead (white bar) or one 15 μm PLbead (light gray bar). (**D**) Typical morphology of a doublet of Ecad cells bound to a 15 μm FNbead for 60 minutes before SF measurement. Error bars indicate s.e.m.; ***P<0.0001, **P<0.01, *P<0.05 (Student's t-test).

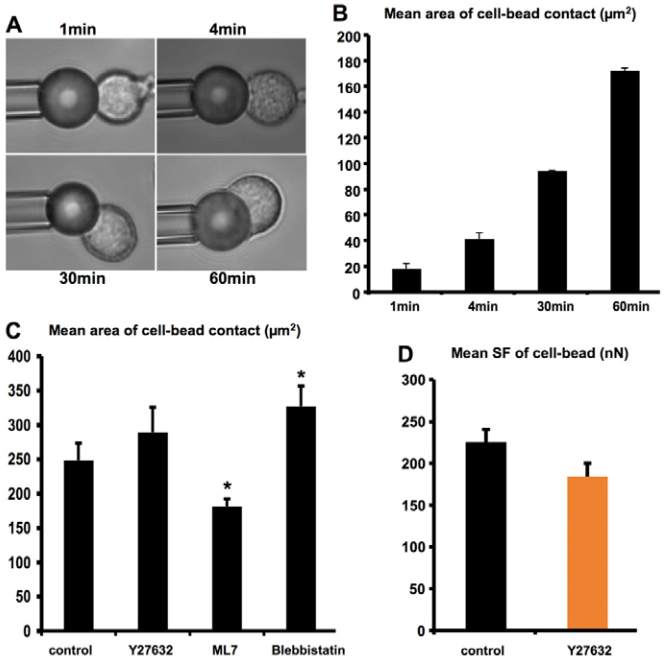

Fig. 4. Rate of spreading of Ecad cells on a 15 μm FNbead. (**A**) Spreading of Ecad cells bound for 1, 4, 30 or 60 minutes. (**B**) Changes in cell-bead contact area over time of interaction. (**C**) Mean contact area measured between Ecad cells and a 15 μm FNbead in the control condition or in the presence of Y27632, ML-7 or blebbistatin for 60 minutes. (**D**) Mean SF measured for cell-FNbead contact (integrin-based adhesion strength) of Ecad cells in control conditions (black bars) or in the presence of 10 μM Y27632 for 60 minutes (orange bars). Error bars indicate s.e.m.; *P<0.02 (Student's t-test).

to integrin stimulation is similar, their respective time courses are quite different. Unless otherwise specified, in the rest of this results section, 'bound cells' indicate Ecad cells incubated with beads for 60 minutes.

We next examined the relationship between the extent of cell spreading over the bead and cell-bead adhesion strength. We observed that the increase in Ecad-cell-bead contact area (Fig. 4A,B) was correlated with an increase in cell-bead adhesion strength over time (supplementary material Fig. S4). Moreover, in each case examined, for Ecad as well as for cad7 cells, the time course of integrin-dependent stimulation of cadherin-based adhesion correlated with the time course of cell-FN adhesion strength (supplementary material Fig. S4).

Src family kinases play a role in the integrin-dependent stimulation of intercellular adhesion

Integrins and cadherins interact with cytoplasmic partners, connecting them to the actin cytoskeleton and activating signaling cascades. Among the signaling events, SFK-dependent tyrosine phosphorylations are considered to play an important role in controlling integrin-dependent cell signaling (Mitra and Schlaepfer, 2006; Playford and Schaller, 2004). Moreover, an association between the receptor-like tyrosine phosphatase α (RPTP-α) and integrins is required for the activation of SFK, the assembly of focal complexes, and the strengthening of the integrin-actin cytoskeleton linkage (von Wichert et al., 2003; Pallen, 2003).

We conducted studies to elucidate the role of SFK, in both cadherin-mediated adhesion and the integrin-dependent regulation of intercellular adhesion strength. The SF of doublets of Ecad cells and of Ecad cells exposed to FNbeads for 60 minutes were used as controls for cadherin-mediated adhesion and integrin-dependent regulation of intercellular adhesion strength, respectively. Then, we compared these values with those measured in the presence of PP1, a selective inhibitor of Src kinases, or PP3, an inactive analogue (Fig. 5). The treatment with PP1 or PP3 did not modify the cadherin-based adhesion strength measured after 4 minutes of contact. By contrast, the incubation with PP1, but not PP3, abolished the integrin-dependent increase in SF measured for Ecad cells stimulated for 1 hour with FNbeads. This indicates that SFK are not involved in the development of nascent E-cadherin-based adhesion in cell doublets in suspension, but they play a role in mediating the integrin-dependent regulation of intercellular adhesion strength.

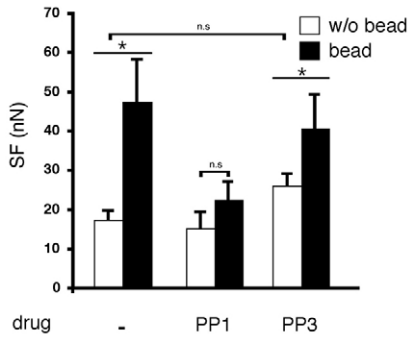

Fig. 5. Involvement of Src tyrosine family kinases on the regulation of E-cadherin-mediated adhesion strength by integrin stimulation. Mean SF for doublets of Ecad cells that were untreated (-) or treated with PP1 or PP3 were incubated simultaneously with (black bars) or without (white bars) FNbeads for 60 minutes before SF measurement (see also Materials and Methods section, Fig. 1A and D, respectively). Error bars indicate s.e.m.; *P<0.03 (Student's t-test); n.s, not statistically different.

ROCK and MLCK inhibition decrease MLC phosphorylation but have not effect on cadherin or integrin surface expression onto FNbeads

As described above, we observed that the kinetics of integrin-dependent increase of intercellular adhesion correlated with the extent of cell spreading over the FNbead. Cell adhesion to ECM is known to induce signaling cascades and to regulate the Rho family of small GTPases and actomyosin contractility leading to cell spreading and migration (Huveneers and Danen, 2009; Geiger et al., 2001; Cox et al., 2001; Hotchin et al., 1995). The activity of myosin II is mainly controlled by the phosphorylation of its light chain (MLC), which is controlled by the opposing activities of the enzymes MLC kinase (MLCK) and MLC phosphatase (MLCP) (Somlyo and Somlyo, 1994). MLCK and ROCK, which is a downstream effector for Rho GTPase (Matsui et al., 1996), are the two major kinases that phosphorylate MLC on its Ser19 (Amano et al., 1996; Kamm and Stull, 2001), in vitro as well as in vivo. It has been shown that the myosin binding subunit of myosin phosphatase (MYPT1 or MBS) can be phosphorylated on Thr696 and Thr850 by ROCK, resulting in a decrease in MLCP activity in vitro (Feng et al., 1999; Kimura et al., 1996).

Based on these reports, we investigated the possible role of the ROCK-MLCK pathway in Ecad cell adhesion to FN. We observed that cell surface expression levels of E-cadherin and α-5 integrin were not affected in Ecad cells treated for 60 minutes with Y27632, ML-7 (that inhibit ROCK and MLCK functions, respectively) or with blebbistatin (a specific myosin II ATPase inhibitor; Fig. 6A). We then checked the phosphorylation of MLC and of the ROCK substrate MYPT1 in the context of crosstalk between cadherins and integrins. Ecad cell monolayers were dissociated in trypsin-free medium to preserve cadherins and integrins at the cell surface. Then cells were seeded onto FN at 80% confluency for 1 hour. At that time, most of the cells adhering to FN formed intercellular adhesions with the closest neighboring cells, allowing the crosstalk between integrins and E-cadherin to occur. In lysates obtained from these adhering cells, MYPTI and MLC were phosphorylated. These phosphorylations were reduced when cells were treated with Y27632 or ML-7 (Fig. 6B,C). These results indicate that ROCK and MLCK are involved when Ecad cells interact together and with FN-coated surfaces.

Then we investigated the consequences of drug treatment both on Ecad cell spreading and adhesion strength onto the FNbead. The area of cell-bead contact was not significantly modified in Y27632-treated cells, but was slightly decreased and increased in cells treated with ML-7 or blebbistatin, respectively (Fig. 4C). Furthermore, the cell-bead adhesion strength was not affected in cells treated with Y27632 (Fig. 4D). These results indicate that inhibition of contractility by ROCK inhibitor does not perturb integrin-mediated adhesion strength, suggesting that it does not affect the integrin stability, activity, density or recruitment during our assay.

ROCK, MLCK and myosin functions regulate cadherin-mediated adhesion strength

We investigated the possible role of the ROCK-MLCK pathway in the regulation of the strength of intercellular adhesion. We first evaluated the effects of Y27632 on the development and maintenance of E-cadherin-dependent adhesion. An SF of 51.6 nN

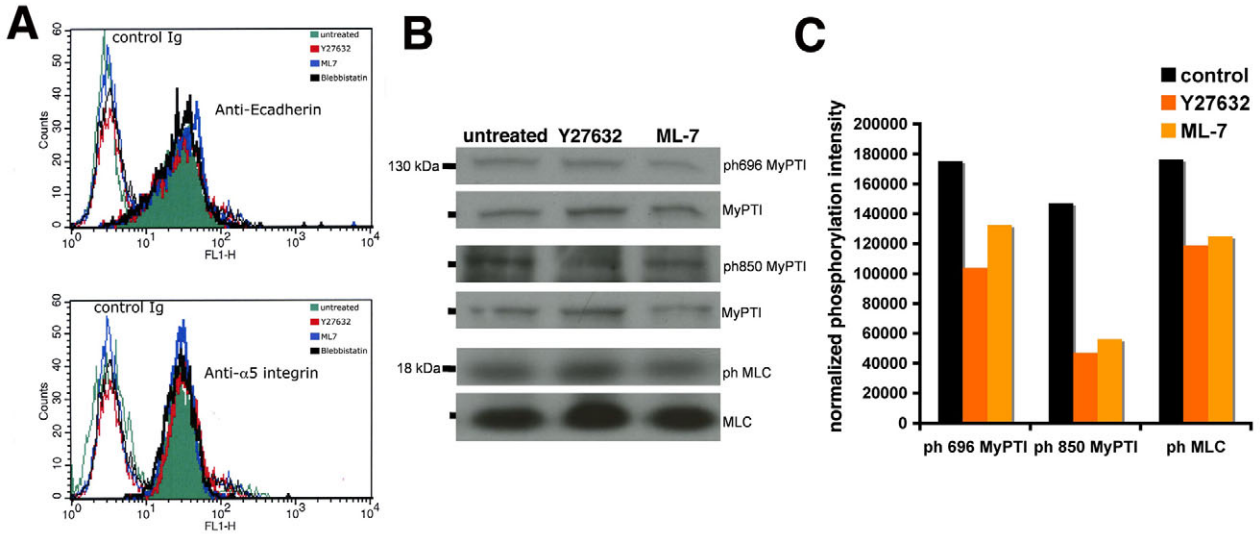

Fig. 6. Effect of drugs on cadherin and integrin surface expression levels, cell spreading and MLC phosphorylation. (**A**) FACScan profiles obtained with isotype control Ig and with antibodies directed against the extracellular domain of E-cadherin (top panel) and α5-integrin chain (lower panel) on untreated cells (green) or on cells treated for 60 minutes with Y27632 (red), ML-7 (blue) or blebbistatin (black). (**B**) Phospho-MLC, phospho-MYPT1 and total MLC and MYPT1 content on Ecad cells plated on FN in control condition or in the presence of Y27632 and ML-7. (**C**) Normalized graph for phosphorylated MLC and MYPT1 in drug-treated cells compared with the controls.

was measured for doublets of Ecad cells treated with Y27632 for 10 minutes. This value is similar to that recorded for untreated Ecad cell doublets (55.9 nN). By contrast, doublets of Ecad cells treated for 60 and 120 minutes had significantly lower SF values, at 21.4 nN and 11.4 nN, respectively (Fig. 7A). A previous study showed that the SF required to separate preformed doublets (never disrupted by the dissociation procedure of cell monolayers and thus highly cohesive) was 310 nN (Chu et al., 2004). Here, we observed a SF of 54 nN for preformed doublet treated with Y27632, which was much lower than that of untreated cell doublets (Fig. 7B). In this assay, Y27632 inhibited both the development and maintenance of cell adhesion in a time-dependent manner.

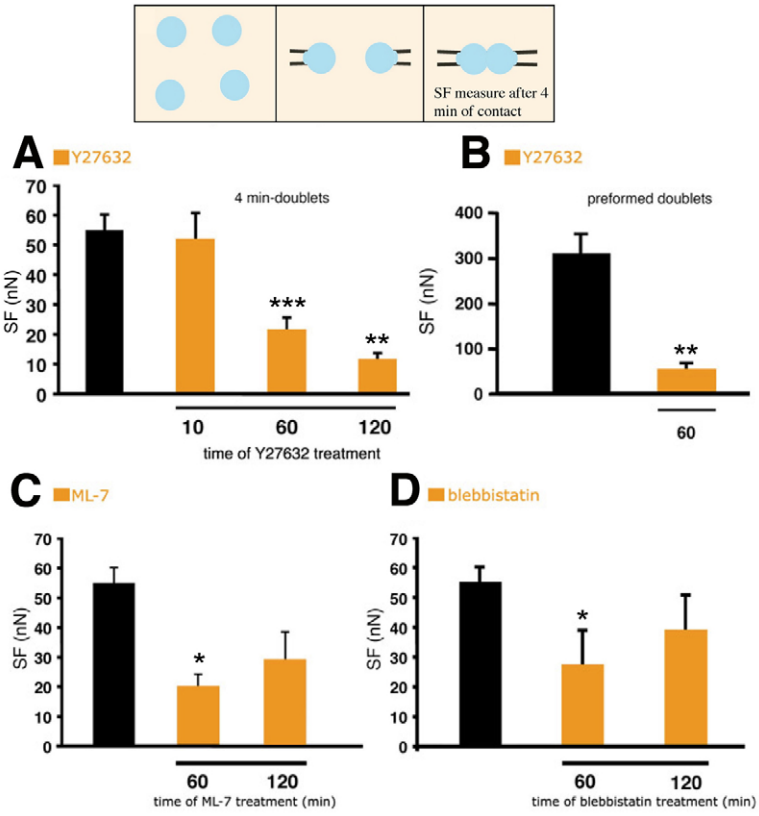

Fig. 7. Effect of ROCK, MLCK and myosin II ATPase inhibition on E-cadherin-mediated adhesion strength. (**A**) Mean SF measured for doublets of Ecad control cells (black bars) or cells treated for 10, 60 or 120 minutes with Y27632 (10 μM; orange bars). (**B**) Mean SF for pre-existing doublets treated with Y27632 for 60 minutes. (**C**,**D**) Mean SF for doublets of Ecad control cells or cells treated for 60 or 120 minutes with 10 μM ML-7 (**C**) or blebbistatin (**D**). The inset at the top shows the experimental procedure (see also Materials and Methods section and Fig. 1B). Error bars indicate s.e.m.; ***P<0.0001, **P<0.001, *P<0.01 (Student's t-test).

Doublets of Ecad cells treated with ML-7 had a lower SF than untreated doublets (Fig. 7C). The contribution of myosin II was assessed using the specific myosin II ATPase inhibitor, blebbistatin. The SF measured for doublets of Ecad cells treated with this drug was lower than that for the controls (Fig. 7D). These results indicate that ROCK, MLCK and myosin II ATPase are involved in the control of the development and regulation of cadherin-based adhesion strength.

ROCK, MLCK and myosin functions are required for the integrin-dependent stimulation of intercellular adhesion

We further explored the putative role of ROCK in the integrin-dependent regulation of intercellular adhesion strength. As described above, the stimulation of Ecad cells with FNbeads for 60 minutes resulted in a higher SF for the bound doublets than for unbound doublets (see Fig. 3A,B). In the experimental conditions that did not affect E-cadherin adhesion strength (see Fig. 7A), the treatment of bound cells for 10 minutes with Y27632 not only completely abolished this increase in SF, but it resulted in a large decrease in SF (Fig. 8A, compare the first and last black and orange bars). Comparable results were obtained with doublets of Ecad cells stimulated for short periods of time with FNbeads (20 minutes, a period for which no effect of integrin stimulation on cadherin-based adhesion was observed in control conditions; see Fig. 3B, Fig. 8A, compare first and second black and orange bars). Thus, ROCK inhibition greatly impairs the integrin-stimulated establishment of cadherin-based adhesion whether the drug was added during the stimulation of Ecad cells by FNbeads or after (Fig. 8B). Taken together, these results show that ROCK activity is involved in the positive regulation of cadherin-based adhesion by integrin-dependent signaling.

Finally, we investigated the role of MLCK or myosin II motor activity in the integrin-dependent regulation of intercellular adhesion. We found that ML-7 or blebbistatin abolished the increase in SF observed in FNbead-stimulated Ecad doublets (Fig. 8B). Thus, MLCK and myosin II, and, consequently, actomyosin contractility, are involved in the crosstalk between integrin and cadherin to the control of cell adhesion strength.

Discussion

Many studies have shown a coordinated regulation of cell-cell and cell-ECM adhesion. For example, during embryogenesis, integrins have been shown to regulate cadherin-dependent adhesion during convergent extension movements in amphibian embryos (Marsden and DeSimone, 2003). Zebrafish gastrulation requires fibronectin to control the movement of involuting mesodermal cells by upregulating and/or activating E-cadherin (Trinh and Stainier, 2004). TGFβ can promote intercellular adhesion through ECM

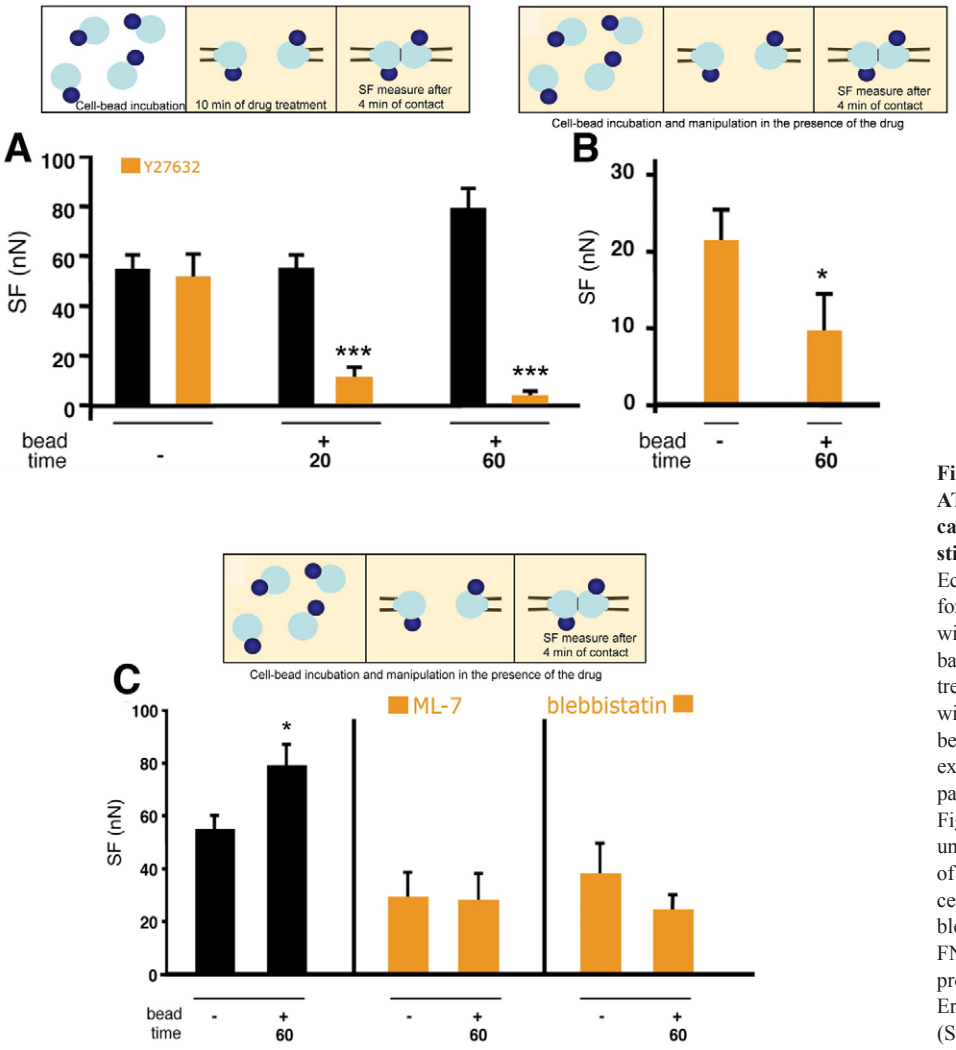

Fig. 8. Effect of ROCK, MLCK and myosin II ATPase inhibition on the regulation of E-cadherin-mediated adhesion strength by integrin stimulation. (**A**) Mean SF for doublets of unbound Ecad cells (–) or of cells adhering to FNbeads (+) for 20 or 60 minutes and then treated for 10 minutes with Y27632 (orange bars) or untreated (black bars). (**B**) Mean SF for doublets of Ecad cells treated with Y27632 and incubated simultaneously with (+) or without (–) FNbeads for 60 minutes before SF measurement. The insets show the experimental procedures followed for cell-bead pairs (see also Materials and Methods section and Fig. 1C,D). The same procedure was also applied to unbound cells (Fig. 1B). (**C**) Mean SF for doublets of unbound (–) Ecad control cells (black bars) or cells treated for 60 minutes with ML-7 or blebbistatin (orange bars) and adhering to one FNbead for 60 minutes (+). The experimental procedure was as shown in the inset of Fig. 1D. Error bars indicate s.e.m.; ***P<0.0001, *P<0.01 (Student's t-test).

remodeling and FAK activation (Wang et al., 2004) in vitro. In keratinocytes, integrins α6β4 and α3β1 stimulated E-cadherin homophilic interactions (Chartier et al., 2006; Hintermann and Quaranta, 2004; Hintermann et al., 2005). Both α2β1 integrin clustering and increased PKA activity are required to induce HT-1080 intercellular adhesion on collagen type IV (Whittard and Akiyama, 2001). By contrast, several studies have implicated signaling cascades in the coordinated destabilization of cell-cell contacts via integrin-ECM engagement and Src activation (Avizienyte et al., 2002; Genda et al., 2000; Koenig et al., 2006; Wang et al., 2006; de Rooij et al., 2005). The loss of FAK or paxillin prevents the efficient establishment of N-cadherin adhesion and affects the plasma membrane dynamics of migrating HeLa cells (Yano et al., 2004). The coordinated interplay between cell-cell and cell-substratum adhesion has also been reported in primary neural crest cell cultures where β1 and β3 integrins activated signaling cascades controlling N-cadherin cell surface distribution and activity (Monier-Gavelle and Duband, 1997). Cells can also use integrins as mechanosensors to sense the environmental stiffness and respond by transducing local tension through actomyosin contractility. The manipulation of cell adhesion and shape has an impact on cell spreading (Chen et al., 1997; Discher et al., 2005; McBeath et al., 2004), directional cell motility (Jiang et al., 2005; Parker et al., 2002), cell contractility (Polte et al., 2004; Tan et al., 2003; Bhadriraju et al., 2007) and malignancy (Guck et al., 2005). Recently, it was shown that rigidity could modulate the crosstalk between integrins and cadherins in MCF, but not in MDCK cells (Tsai and Kam, 2009). Thus, crosstalk clearly occurs between integrins and cadherins, and this crosstalk may enhance or suppress adhesion depending on the cellular and environmental context.

The present study analyzes the effect of the integrin-cadherin crosstalk on the mechanics of cadherin-based adhesion in term of separation forces. We used cells in suspension to eliminate ECM-mediated signaling and minimize the events induced by cells adhering to and spreading over a surface. We locally stimulated cells using FNbeads and showed that integrin-cadherin crosstalk positively regulates intercellular adhesion strength (Fig. 3). One possible explanation for this effect is an increase in cadherin expression in stimulated cells. However, when we used Ecad-GFP doublets for these experiments, we observed no significant changes in GFP fluorescence at the cell-cell contact site compared with unstimulated cells (data not shown). This suggests that the increase in adhesion strength by integrin stimulation is not produced by a modulation of cadherin levels, but more probably through a regulation of cadherin adhesive activity (see below).

It has been shown that integrin-ECM adhesion can positively regulate adhesive interactions between tumor cells (Whittard and Akiyama, 2001), but it may also physically disrupt cadherin-mediated adhesion without decreasing the intrinsic adhesive function of cadherins (de Rooij et al., 2005). In the present study, we showed that E-cadherin-mediated cell-cell adhesion strength was increased by integrin ligation in both tumorigenic and non-tumorigenic cells (supplementary material Fig. S2). During the formation, maintenance and remodeling of epithelial sheets, cells must simultaneously slow down their migration and increase their attachment to their neighbors. Collective cell migration occurs naturally during embryonic development and is aberrant during carcinoma invasion and metastasis, and cells use signals from cell-ECM interactions to maintain cell-cell contact as they migrate (Friedl and Wolf, 2003; Yano et al., 2004; Christofori, 2003). The leading cells interact with the ECM and generate a mechanical tension that is transmitted to the cells following them. Cadherins are good candidates for transducers of these forces at cell-cell contacts and for myosin II-driven contractility (Lecaudey and Gilmour, 2006). Our results support this hypothesis and provide new insight into the mechanisms at work.

Cells producing different types of cadherins differ in intercellular adhesiveness (Chu et al., 2006). Here, we show that integrin stimulation increased cadherin-mediated adhesion strength in cells expressing E-cadherin or cadherin-7 (cad7 cells). However, whereas these cells show a similar increase in intercellular adhesion strength following FNbead stimulation, they differ in their sensitivity to this stimulus (supplementary material Fig. S3). Cad7 cells responded after only 15 minutes of stimulation, whereas Ecad cells required 60 minutes of stimulation. This result supports and extends previous observations of differences in adhesion properties and spatiotemporal regulation during morphogenesis and pathological processes between type-I and type-II cadherins (Chu et al., 2006; Takeichi, 1995). Thus, the cadherins and integrins produced by cells, together with environmental cues, may strongly affect integrin-cadherin crosstalk, with potentially far-reaching implications for the regulation of tissue morphogenesis and, probably, metastasis. For example, forces of several hundred nN are required to detach epithelial and fibroblastic cells from an ECM-coated surface (Wu et al., 2005). However, forces of about 400 nN are required to separate cell doublets producing type-I cadherins, while even weaker forces (a few nN) suffice to separate cell doublets producing type-II cadherins (Chu et al., 2006). Furthermore, the strengthening of cell-ECM adhesion requires different kinetics depending on the type of cadherins expressed by cells (supplementary material Fig. S4). Thus, even when integrin-cadherin crosstalk increases intercellular adhesion, the repertoire of cadherins expressed and the range of the resulting forces developed at both the cell-ECM and cell-cell interfaces appear to be sufficient for the regulation of differential cell adhesion. This can have a dramatic effect on cell dispersal and migration, particularly given the time frame over which such forces are likely to act in vivo.

The mechanisms underlying these observations are numerous and complex. For example, integrin ligation changes the activity of several signaling molecules, including tyrosine kinases. An increase in tyrosine phosphorylation of cytoskeletal components and signaling by SFK plays an important role in controlling the actin cytoskeleton. In colon cancer cells, activated Src kinase induces rearrangement of actin and controls β-actin dynamics by tyrosine phosphorylation in the vicinity on integrin-dependent adhesion sites at the cell periphery (Avizienyte et al., 2007). SFK signaling plays a crucial role during the development and maintenance of cell-cell contacts (McLachlan et al., 2007) involving the tyrosine phosphorylation of cortactin (Ren et al., 2009). Our study revealed that PP1 treatment abolished the integrin-dependent stimulation of cadherin-mediated adhesion (Fig. 5). This result indicates that integrin-cadherin crosstalk positively regulates intercellular adhesion strength via a mechanism involving the SRK activation.

We observed that the strengthening of E-cadherin-based adhesion correlated with the cell spreading onto the FNbead and with an increase in FN-adhesion strength (Fig. 4B and supplementary material S4). The signaling pathways involving MLCK and ROCK act downstream of integrin activation and regulate the contractility of the actin cytoskeleton by modulating MLC phosphorylation and the formation of focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992; Kamm and Stull, 2001; Sorokina and Chernoff, 2005).

Recently, MRCK-containing complex was shown to regulate actomyosin retrograde flow and cell protrusions (Tan et al., 2008). Cadherin ligation also affects the activity of Rho family GTPases (Noren et al., 2001; Charasse et al., 2002; Braga and Yap, 2005), and several reports provide evidence that ROCK and acto-myosin contraction mediate opposite effects on intercellular adhesions. They can promote either the formation of cell-cell contacts (Conti et al., 2004; Shewan et al., 2005) or the disassembly of the cadherins at adhesion sites (Ivanov et al., 2009; Ivanov et al., 2004; Gavard and Gutkind, 2008; de Rooij et al., 2005; Avizienyte et al., 2004; Sahai and Marshall, 2002). Our results indicate that ROCK and myosin-based contraction of the actin cytoskeleton is required for cadherin-mediated adhesion and maintenance (Fig. 7). Such variations may be one of the reasons for cell type-specific responses to depletion of myosin II or to inhibitors of MLC phosphorylation in cell adhesion and migration.

Y27632, ML-7 and blebbistatin treatments did not change the surface levels of E-cadherin and α5-integrin (Fig. 6A). We observed that, for similar cell-FNbead area, the SF required to detach the bead from the cell is comparable for control and Y27632-treated cells, indicating that the drug does not change cell-FNbead adhesion properties (Fig. 4D). This result is in agreement with a previous study showing that collagen-bead interaction, collagen-induced Rap1 activity and the activation state of β1-integrin were not affected by blebbistatin or Y-27632 (Arora et al., 2008). In addition, a decrease in cadherin-mediated adhesion strength of Y27632-treated cells stimulated for 60 minutes with FNbeads was measured. By contrast, this drug did not affect the strength of cell adhesion to FNbead. These results indicate that the drugs do not broadly and non-specifically alter the intracellular signaling and trafficking in the treated cells. Furthermore, the increase of cadherin-based adhesion strength occurred when the cells had strengthened their adhesion to FN. Y27632, ML-7 and blebbistatin have either no effect, a slightly negative effect, or a slightly positive effect, respectively, on cell-bead spreading (Fig. 4C) and any significant differences in the mean SF displayed by cell-bead contact was observed between control Ecad cells and Y27632-treated cells (Fig. 4D). However, these drugs inhibited the integrin-cadherin crosstalk. Collectively, these findings show that this inhibitory effect is not correlated with a modulation of FN-adhesion strengthening, but rather with an alteration of the downstream signaling involving the actin cytoskeleton, contractility or cell tension operating at the intercellular adhesive sites. In agreement with these observations, we found that ROCK and MLCK inhibitors reduce the phosphorylation level of MLC (Fig. 6B) in adherent cells.

The integrin-dependent stimulation of cadherin-based adhesion required a threshold of cell adhesion onto the FNbead to occur and was inhibited by ROCK and myosin II ATPase inhibitors. This result is in agreement with a previous report showing that the activation of ROCK by Rho small GTPase during cell adhesion is dependent on cell shape and cytoskeletal tension (Bradhiraju et al., 2007). In this study, it was suggested that the cell spreading modulates the coupling between RhoA and ROCK downstream to integrin ligation. The decrease in myosin II ATPase activation and cytoskeletal tension by drugs in our FNbead-stimulated cells may alter RhoA-ROCK coupling. Taken together these results suggest a role for this coupling in integrin-mediated regulation of intercellular adhesion. However, we showed that inhibition of ROCK also had a dramatic effect on cadherin-based adhesion in cells stimulated for 20 minutes by one FNbead (where cells were not well spread onto the bead, see Fig. 8A). Another pathway independent of this coupling may act to regulate integrin-cadherin crosstalk to modulate intercellular adhesion strength.

Our experiments using pharmacological inhibitors of SFK, ROCK, MLCK and myosin II allow us to study their implication in both the development and the maintenance of cell adhesion in a time- and dose-dependent manner. We chose to use these inhibitors because other methods altering ROCK, MLCK or SFK function cannot be used in our study. For example, we did not use dominant-negative and constitutively active mutants of Rho because in cells expressing $RhoA^{N19}$ and $RhoA^{V14}$ mutants the binding to the coated-bead was strongly impaired (data not shown). Similarly, we did not use ROCK- or MLCK-siRNA-treated cells in our approach. Recent studies have shown that these siRNA strongly alter integrin- or cadherin-mediated adhesion (Barkan et al., 2008; Harb et al., 2008), thus no SF could be measured in such conditions to confirm the involvement of these proteins in the integrin- and cadherin-dependent strengthening of adhesion.

Our results indicate that ROCK and myosin-based contraction of the actin cytoskeleton is required for crosstalk between integrins and cadherins to regulate adhesion strength (Fig. 9). However, it remains unclear how the strength of interaction between cadherins of contacting cells can be modified by intracellular signaling. We can suggest several possibilities. Although we did not directly analyze the link between Src kinases and cell contractility in our cells, several studies have shown that Src can associate with, phosphorylate and activate MLCK (Garcia et al., 1999; Fincham et al., 2000; Shi et al., 2000). Another explanation could be that the integrin-cadherin crosstalk via ROCK and/or MLCK and the actomyosin pathway may inhibit Rac activation and consequently reduce cell protrusions. Supporting this possibility, previous studies have shown that Rho and ROCK suppress Rac activation at the protrusions (El-Sibai et al., 2008) and that activated Rac induces

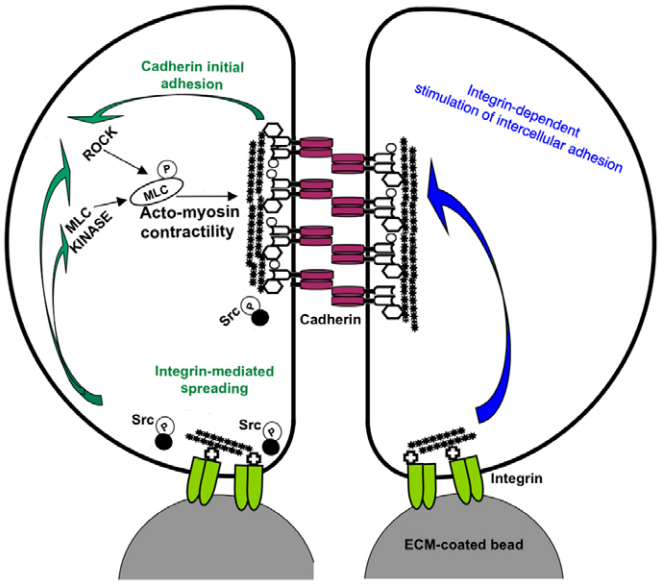

Fig. 9. Integrin-dependent regulation of cadherin-based adhesion strength. Model for the integrin-dependent regulation of cadherin-based adhesion strength (blue arrow) in a dual pipette assay via Src activation and ROCK-MLCK actomyosin contractility (green arrows). For clarity, the green and blue arrows are represented in only one cell.

protrusions in Ecad cells and is a potent suppressor of the cadherin-based adhesion strength (Chu et al., 2004). Although we showed that cadherin levels were not affected, integrin-dependent stimulation may change the cadherin organization at the surface or trafficking and speed up the formation of cadherin clusters (Adams and Nelson, 1998), leading to the strengthening of adhesion through the coupling of cadherins to actin cables (Mege et al., 2006; Lambert et al., 2007). In addition, we cannot exclude an effect on the stability of cadherin at the cell-cell contact upon integrin stimulation. However, mDia1 has been shown to promote cadherin-mediated adhesion strengthening in a myosin II-dependent manner (Carramusa et al., 2007) and is one of the downstream targets of Rho GTPases (Watanabe et al., 1997). A dynamic balance between these inputs may serve to regulate the effect of integrin ligation on the strength of intercellular adhesion.

Our findings provide new insight into the roles of SFK, cellular tension and the actomyosin cytoskeleton in the control of cadherin-based intercellular adhesion and its stimulation by cell-FN adhesion. They also highlight the important role played by ROCK and actomyosin contractility in both the development and maintenance of well-established cell-cell contacts and their positive regulation by short- and long-term integrin-dependent signaling. These findings highlight the importance of molecular crosstalk between cadherins and integrins in the control of cell plasticity and cell behavior with implications during histogenesis, morphogenesis and tumorigenesis.

Materials and Methods

Antibodies and reagents

The ECCD-2 monoclonal antibody (mAb) directed against mouse E-cadherin was obtained from CAMBREX Bio Sciences (Emerainville, France). The 7D9 mAb directed against chicken E-cadherin was obtained from the Developmental Studies Hybridoma Bank. MAbs against α5 integrin chain (cl 5H10-27), β1 integrin chain (cl 9EG7) and MYPT1 (the myosin binding subunit of myosin phosphatase) were obtained from BD Biosciences. Polyclonal antibodies directed against phosphorylated MYPT1 (Thr696) and phosphorylated MYPT1 (Thr850) were obtained from Euromedex (Souffelweyersheim, France)). Antibodies directed against myosin light chain (MLC2) and phosphorylated MLC2 (Ser19) were purchased from Cell Signaling Technology. MAbs against talin (cl 8D4) and bovine plasma FN were purchased from Sigma. Secondary antibodies coupled to Texas Red and FITC were obtained from Jackson ImmunoResearch Laboratories; those coupled to Alexa Fluor 488 and 594 were purchased from Promega. Secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG) were obtained from Jackson Laboratories. VN fragment 1-112 (VN) containing the cell adhesion site was kindly supplied by J. Parello (UMR5074, CNRS). PP1, PP3, Y27632, ML-7 and blebbistatin were obtained from Calbiochem. Stock solutions of PP1 and PP3 (5 mM) were made up in DMSO, and stored at –20°C. Stock solutions of blebbistatin (5 mM), ML-7 (10 mM) or Y27632 (20 mM) were made up in DMSO, 50% ethanol, and water, respectively, and stored at –20°C. Polystyrene microbeads (mean diameter 6, 10 and 15 μm) were obtained from Polysciences, Inc.

Coating of beads

Beads were coated by incubation with 50 μg/ml FN, VN or PL in phosphate-buffered saline (PBS), pH 7.4, for 1 hour at room temperature and were then centrifuged at 12,000 ***g*** for 5 minutes at 4°C. The FN- and VN-coated beads were saturated by incubation with 10 mg/ml BSA in PBS for 30 minutes at room temperature, and were then stored in 0.1% NaN_3-5% glycerol at 4°C. Binding of ligand was confirmed by immunodetection with a home-made Rabbit polyclonal anti-FN (Duband et al., 1986) and anti-VN (AbCam).

Cell lines and stable transfections

Ecad cells and cad7 cells are stably transfected S180 clones producing chicken E-cadherin and cadherin-7, respectively, as previously described (Dufour et al., 1999). EL cells, a stably transfected fibroblast L cell line producing E-cadherin, was described by Nagafuchi et al. (Nagafuchi et al., 1987). The SCC13 squamous cell carcinoma was provided by Faraldo-Martin (UMR 144 CNRS-Institut Curie). All cell lines were cultured at 37°C, under an atmosphere containing 5% CO_2, in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Confluent cultures were routinely passaged, following treatment with 0.05% trypsin + 0.02% EDTA in PBS.

Cell dissociation and adhesion assays

For SF measurements, cell monolayers were treated with non-enzymatic cell dissociation buffer (Sigma) and completely dissociated into single cells by pipetting. The cells were rinsed several times in Ca^{2+}, Mg^{2+}-free Hepes-buffered saline medium and resuspended in Eagle's minimal essential medium (Invitrogen) to a final concentration of 10^6 cells/ml. For SF measurement, cells were resuspended in a working medium consisting of CO_2-independent medium (Invitrogen) supplemented with 1% FCS. Control measurements were performed with cells from the cell suspension. For integrin stimulation experiments, single cells were incubated for 20, 40 or 60 minutes at 37°C in working medium, in the presence of ligand-coated beads at a ratio of 1 bead per cell (bound cells; Fig. 1A) for SF measurement. Alternatively, three different assays were carried out in which ROCK, MLCK or myosin II ATPase activity was disrupted. (1) For the analysis of the effects of drugs on cadherin-based adhesion, single cells or preformed cell doublets were incubated in the presence of 10 μM Y27632, ML-7 or blebbistatin for different periods of time (Fig. 1B). (2) For investigations of the effect of Y27632 on the adhesive cell response to integrin stimulation, cells were incubated with 15 μm coated beads for 20 or 60 minutes, and the drug was added for the last 10 minutes of the incubation period before the cells were brought into contact for SF measurement (Fig. 1C). (3) Finally, cells were incubated with 15 μm coated beads in the presence of the drugs (10 μM) for 1 hour (Fig. 1D). Control experiments were performed for each set of experiments in the presence of similar amounts of solvent to ensure that the solvent did not have an effect on the force measured.

Immunofluorescence microscopy

Immunodetection was carried out as previously described (Chu et al., 2004). Cells were viewed under a Leica DMRBE6000 epifluorescent microscope with a highly sensitive cooled interlined CCD camera (CoolSnap HQ, Roper). Cadherin and integrin expression were analyzed by flow cytometry as previously described (Chu et al., 2004; Ramprasad et al., 2008). Deconvolution microscopy was carried out as described by Angenieux et al. (Angenieux et al., 2005). Cells were examined under a classical upright motorized microscope (Leica DMRA2). Images were acquired using an oil-immersion objective (100 PL APO HCX, 1.4 NA) and a CoolSnap HQ camera. Rapid and precise Z-positioning was achieved with a piezoelectric device (LVDT, Physik Instrument) mounted beneath the objective lens. The system was controlled by Metamorph 5.0.7 Software (Universal Imaging Corp). Deconvolutions were performed on stacks of images taken with a 0.2-μm plane-to-plane distance, using the three-dimensional deconvolution module of Metamorph and the fast iterative constrained PSF-based algorithm.

Western blotting

Ecad cell monolayers were detached using cell dissociation buffer and seeded onto FN-coated culture dishes at 80% confluency and then treated for 1 hour with Y27632 or ML-7, or left untreated. Cell were briefly rinsed in PBS and lysed by the direct addition of SDS-PAGE sample buffer to the cultures and scraping. The lysates were sonicated for 10 seconds and boiled for 5 minutes as described by Garton et al. (Garton et al., 2008), The samples were subjected to 7.5% and 12% SDS-PAGE for MYPT1, phospho-MYPT1, MLC and phospho-MLC, respectively. Western blot analysis was performed with antibodies directed against MYPT1, pMYPT1(696), pMYPT1(850), MLC and pMLC, and the proteins were detected using SuperSignal West pico chemoluminescent substrate (Pierce). Quantitative analysis was done using the ImageJ64 software on two independent experiments and the phMYPT1/MYPTI and phMLC/MLC ratio calculated for untreated or drug treated cells.

Measurement of SF and area of the cell-bead contact

The micromanipulation technique used has been previously described (Chu et al., 2004). Cells were manipulated at 37°C with two micropipettes, each held by a micromanipulator connected to a combined hydraulic-pneumatic system and a pressure sensor. Two cells were brought into contact for 4 minutes. The pipettes were then moved apart to detach the adherent cells. If a doublet was pulled intact from the left pipette it was moved back to the orifice of that pipette, and the degree of aspiration was then increased. The cycle was repeated until the *n*th cycle, at which the cells separated. SF for each doublet was calculated using the equation: $SF=\pi(d/2)^2(P_{n-1}+P_n)/2$ where d is the inner diameter of the left pipette, P_{n-1} and P_n are the values of aspiration at the last two cycles. The results for 30-50 measurements obtained were used to obtain the mean SF. The same procedure was applied to the quantification of cell-bead adhesion, with the bead held at the tip of the right pipette and the cell at the tip of the left pipette. For the calculation of the area of the cell spreading onto the bead we assume that the cell-bead pair system can be represented as two spheres in contact and has a cylindrical symmetry. Considering r as the radius of the bead and θ as the angle between the long axis of symmetry and the edge of the cell-bead contact zone, the adhesion area of the cell onto the bead is then given by $2\pi(1-\cos\theta)r^2$.

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