Cell–Cell Adhesion and RhoA-Mediated Actin Polymerization are Independent Phenomena in Microtubule Disrupted Keratinocytes

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E-cadherin-mediated adherens junction formation and maintenance are thought to involve actin filament rearrangements through the action of small GTPases. Recently, we demonstrated that microtubule disruption in normal human epidermal keratinocytes grown in low calcium media conditions induces cell–cell adhesion by redistribution of endogenous E-cadherin, and it promotes stress fiber formation. This actin rearrangement was apparently mediated by RhoA activation. This model system therefore provides a tool with which to dissect relationships between cell–cell adhesion and Rho-mediated stress fiber formation. In this study, we have demonstrated in normal human epidermal keratinocytes that disruption of actin structures including stress fibers does not interfere with E-cadherin redistribution during microtubule-induced cell–cell adhesion. Moreover, this cell–cell adhesion could not be blocked by RhoA inactivation at the level for inhibition of stress fiber formation. Additionally, in the immortalized HaCaT keratinocyte cell line, which does not undergo cell–cell adhesion after microtubule disruption in low calcium conditions, expression of dominant-active RhoA could induce stress fiber formation without inducing adhesion. On the other hand, a variant of the HaCaT cell line, HC-R1, showed microtubule-disruption-induced cell–cell adhesion without stress fiber formation. Together, our results suggest that, in keratinocytes, the process of cell adhesion can occur independently of RhoA-mediated stress fiber formation.


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adherins are calcium-dependent transmembrane adhesion molecules that mediate cell–cell interactions through adherens junctions (AJ) and desmosomes between epidermal keratinocytes (Takeichi, 1995). Mammalian cell–cell adhesion is often initiated by E-cadherin-mediated AJ formation, which results in desmosome assembly at flanking sites (Vasioukhin et al, 2000). Cadherin family proteins including E-cadherin have a large extracellular domain, a short transmembrane domain, and a cytoplasmic tail. The extracellular domains interact homophilically with cadherins on adjacent cells, and their cytoplasmic tails provide attachment to the actin cytoskeleton via catenins and other cytoskeletal proteins. This association between cadherins and the actin cytoskeleton does not appear to affect cadherin homophilic binding per se (Yap et al, 1997), but it is an important mechanism to strengthen adhesion forces (Tsukita et al, 1992; Imamura et al, 1999). Recently, actin polymerization and filopodial penetration into adjacent cells has been described as an integral mechanism of cell–cell adhesion (Vasioukhin et al, 2000). In addition various lines of evidence indicate that Rho family GTPases are key regulators of actin rearrangements in the formation and maintenance of AJ (Braga et al, 1997; Takaishi et al, 1997). Together, these observations have provided new insights into the role of actin rearrangements in cell–cell adhesion processes in epidermal keratinocytes.

The Rho family GTPases regulate cytoskeletal dynamics and participate in many different cellular processes. Stress fiber formation was the first reported cytoskeletal phenotype produced by activated Rho. In contrast, Rac and Cdc42 activation result in lamellipodial and filopodial protrusions, respectively (Bishop and Hall, 2000). Recently, numerous data on the roles of Rho family GTPases as crucial regulators of cadherin-dependent adhesion have emerged (reviewed in Braga, 2000). For example, in epithelial cells including keratinocytes, inactivation of Rac1 and Rho were found to perturb the organization of actin filaments and dislocate E-cadherin complexes from AJ (Braga et al, 1997; 1999; Takaishi et al, 1997; Jou and Nelson, 1998). In contrast, it was reported that sustained Rac activation per se is sufficient to disassemble cadherin-mediated cell–cell adhesion in simple epithelial cells and keratinocytes (Quinlan, 1999; Braga et al, 2000). Also, transforming growth factor (TGF-β-1) rapidly activates RhoA, resulting in loss of junctional E-cadherin localization and acquisition of a fibroblastoid morphology (epithelial–mesenchymal transition, EMT) in epithelial cells (Bhowmick et al, 2001). Furthermore, dominant-active forms of Rho that are able to induce stress fiber formation could not promote cell–cell adhesion, suggesting that Rho activation alone is not sufficient for cell–cell adhesion (Braga et al, 1999).

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Abbreviations: AJ, adherens junction; EMT, epithelial–mesenchymal transition; FAK, focal adhesion kinase; MT, microtubule; NHEK, normal human epidermal keratinocytes.
Thus, the mode of action of Rho remains obscure, and it appears that its role(s) in cell–cell adhesion may vary according to different cellular requirements (Braga et al., 1999).

Recently, two apparently different mechanisms of E-cadherin-mediated cell–cell adhesion have been proposed (reviewed in Vasioukhin and Fuchs, 2001). In Madin-Darby canine kidney cells, cell–cell adhesion occurs concurrently with the formation of circumferential actin structures. These circumferential structures appeared to be a prerequisite for cell–cell adhesion in certain epithelial cells (Quinlan and Hyatt, 1999). In contrast, primary epidermal keratinocytes could adhere to each other without redistribution to membranes upon switching to high calcium conditions (below 0.08 mM) does not allow AJ formation (Vasioukhin et al., 2000). Thus, it appears that actin organization and structure can play variable roles in cell–cell adhesion depending upon the cell type. The detailed mechanisms of these events remain to be resolved, however.

It is generally believed that culture of epidermal cells under low calcium conditions (below 0.08 mM) does not allow AJ formation (Vasioukhin and Fuchs, 2001). We (Kee and Steinitz, 2001) have described, however, how cell–cell adhesion can be induced by microtubule (MT) disruption in normal human epidermal keratinocytes (NHEK) cultured in low calcium media. In addition to cell–cell adhesion in this system, MT disruption also induced stress fiber formation. These phenomena in this model system led us to suspect a role for actin organization in cell–cell adhesion.

Furthermore, we showed that HaCaT cells, which are derived from normal epidermis, show neither cell–cell adhesion nor stress fiber formation after MT disruption under low calcium conditions. Rho activation has been implicated in both cell–cell adhesion and stress fiber formation in many other experimental systems (Bershadsky et al., 1996; Braga et al., 1997; 1999). Accordingly, in this study, we have explored whether RhoA activation is essential for the observed increases in cell–cell adhesion and stress fiber formation by MT disruption in NHEK. Our results document that nocodazole-induced cell–cell adhesion can occur without stress fiber formation and RhoA activation.

**Materials and Methods**

**Cells, chemicals, and antibodies** NHEK (Clonetics, San Diego, CA) were derived from neonatal foreskin. They were propagated on collagen-coated dishes (0.1 mg per ml, collagen type I, Sigma, St. Louis, MO) using serum-free keratinocyte growth medium supplemented with 0.05 mM CaCl₂, bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, transferrin, epinephrine, and gentamycin (KGM, Clonetics, San Diego, CA). HC-R1 cells, a clonal derivative of NHEK (Clonetics), were grown in a 5% CO₂ atmosphere in a humidified incubator. The HaCaT cell line (Boukamp et al., 1988) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. We also used the HC-R1 cell line. This line was obtained from HaCaT cells following transfection of the neomycin resistant gene (pCDNA3.1) and selection with G418 (500 μg per ml) for 3 wk. Among the neomycin-resistant HaCaT cells, HC-R1 cells, which showed the strongest nocodazole-induced cell–cell adhesion, were chosen. All cells were grown in a 5% CO₂ atmosphere.

The MT disrupting agent, nocodazole, was used at a final concentration of 33 μM. For actin microfilament disruption, cytochalasin B (25 μM) was used. Monoclonal antibodies against E-cadherin, focal adhesion kinase (FAK), phospho-paxillin, phosphotyrosine (PY), and β-catenin were all purchased from Transduction Laboratories (Lexington, KY). A monoclonal antibody against β-tubulin was from Roche (Indianapolis, IN). Rabbit polyclonal antibodies against RhoA and Rhob and a goat polyclonal antibody against α-catenin were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antibody against the Xpress epitope was from Invitrogen (Carlsbad, CA). Species-specific fluorescein isothiocyanate (FITC) or Texas Red conjugated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). A peroxidase-conjugated secondary antibody was from Bio-Rad Laboratories ( Hercules, CA). For the immunofluorescence staining of F-actin, FITC-conjugated phallolidin (Sigma) was used.

**Induction of cell–cell adhesion** NHEK were grown on coverslips or tissue culture dishes in low calcium KGM until they reached 60%–70% confluence, and were then treated as indicated for 1 h. Because confluent growth of NHEK induces some cell–cell adhesion even in low calcium medium, we did not use cells that were more than 70% confluent. To induce cell–cell adhesion, the treated cells were analyzed for E-cadherin immunoprecipitation between cell–cell boundaries by an immunofluorescence (IF) assay (Kee and Steinitz, 2001). HaCaT and HC-R1 cells were grown at 50% confluence, transferred from maintenance media to low calcium KGM, and cultured for 24 h to dissociate adherent cells. Because HaCaT have a higher growth rate than NHEK, we performed experiments using less-confluent HaCaT cells than NHEK. Cell–cell adhesion was induced as above.

**IF assay** For IF staining, treated cells were rinsed with ice-cold phosphate-buffered saline (PBS; pH 7.4) and fixed with 4% paraformaldehyde in PBS. In some experiments, for enhancing visualization of cytoskeletal proteins by removal of soluble cytosolic proteins, pretreatment with 0.4% Triton X-100, 0.4% paraformaldehyde in PBS for 3 min was performed prior to 4% paraformaldehyde fixation. After fixation, cells were permeabilized by incubation with 0.4% Triton X-100 in PBS. Permeabilized cells were incubated with 5% bovine serum albumin in PBS containing 0.05% Tween-20 (PBST) for 30 min, and then reacted with primary antibodies diluted in blocking solution. After washing three times with PBST, bound primary antibodies were detected by species-specific fluorochrome-conjugated antibodies (Texas Red or FITC). The stained cells were mounted onto a slide glass with mounting medium (Vectorshield, Vector) and then observed in a fluorescence microscope (Microphot-FXA, Nikon, Japan). For F-actin staining, phallolidin conjugated with FITC was used.

**Construction and expression of Rho mutants** Full-length RhoA cDNA was amplified from NHEK total RNA after reverse transcription using a pair of primers (forward, 5′-CCGGG ATCCA TGGCT GCCAT CCGG-3′; reverse, CTACT CGAGG TTTCA CAAGA CAAG-3′). The nucleotide sequence of the amplified RhoA gene was confirmed by DNA sequencing. Also cDNAs for RhoB (gift of Dr. H. L. Moss) and RhoC (gift of Dr. Sofia D. Merajver) were used. These Rho cDNAs were subjected to site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) to generate Rho dominant-active (DA) (mutation of nucleotide 41G to T), RhoA dominant-negative (DN) (56-C to A, 57-A to C), RhoB DN (56-C to A, 57-A to C), RhoB DN (56-C to A, 57-G to C), and RhoC DN (56-C to A). The resulting Rho mutant were thus Gly14Val in DA and Thr19Asn in DN. The final mutant Rho cDNAs were inserted into the pcDNA3.1/His mammalian expression vector (Invitrogen). For monitoring expression of these mutants, the Xpress antibody epitope tag was placed at the N-terminus of the end of the Rho open reading frame.

**Microinjection and transfection** Microinjections were performed using the micromanipulator and an intracell (Eppendorf, Hamburg, Germany). Cells were injected with 80% coenzyme (100 μg per ml in PBS, Cytokeleton, Denver, CO), together with Oregon Green dextran (Molecular Probes, Eugene, OR) for visualization. Groups of four to ten cells or single cells were injected at 150 pA pressure for 0.2 ms. The microinjection conditions used did not significantly alter cell morphology. Multiple groups (n > 3) of cells were analyzed for each condition.

For transient expression of Rho proteins, the above-mentioned Rho constructs were transfected into NHEK or HaCaT cells grown on coverslips using Fugene 6 (Roche) according to the manufacturer’s protocol. The transfected cells were grown for 24 and 36 h in low calcium medium, then treated with nocodazole or dimethylsulfoxide (DMSO, vehicle control) for 1 h, and examined by IF staining. Because results were the same at 24 or 36 h, we show only the data from 36 h cultures.

**Rho activity assay** Rho activities were measured by an affinity precipitation method using Rhotekin Rho-binding domain (Upstate Biotechnology, Lake Placid, NY). Briefly, cells were homogenized using lysis buffer containing 50 mM Tris–HCl (pH 7.2), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 500 mM NaCl, 10 mM MgCl₂, and a protease inhibitor cocktail (Roche). These lysates were mixed with Rhotekin Rho-binding domain proteins, which were then immunoprecipitated with anti-RhoA antibody, and then incubated for 45 min at 4°C. After extensive washing with lysate buffer, the resulting precipitates were subjected to Western blot analysis.

**Immunoprecipitation and Western blot analyses** For an analysis of the phosphorylation states of FAK and paxillin, tyrosylphosphorylated proteins were detected by Western blot analysis as described (Braga et al., 1999). Briefly, lysates were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% nonfat dry milk in PBS and incubated with antibodies. After washing three times, bound primary antibodies were detected with peroxidase-conjugated secondary antibodies and chemiluminescence reagents (Amersham, Sunnyvale, CA).
proteins were immunoprecipitated from cell extracts using PY20 (Transduction). Briefly, cells cultured on 100 mm culture dishes were homogenized in 1 ml of lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM MgCl₂, 1 mM sodium orthovanadate, and a protease inhibitor cocktail (Roche). After insoluble material was removed by centrifugation at 10,000 g for 15 min, the remaining supernatants were precleared by incubation with 5 µl of normal mouse serum and protein A agarose beads (Santa Cruz) for 2 h at 4°C. Then 1 µg of PY20 antibody was mixed with these lysates for 1 h on ice, and antigen–antibody complexes were precipitated with protein A agarose beads. After extensive washing of precipitates with lysis buffer, bound proteins were eluted with sample buffer (Invitrogen) and subjected to Western blot analysis using anti-FAK and antipaxillin monoclonal antibodies. Western blot analysis was carried out as described previously (Kee and Steinert, 2001). Proteins were detected by enhanced chemiluminescence (Renaissance, NEN). Antibody dilutions followed the manufacturer’s recommendation.

RESULTS

MT disruption induces E-cadherin-mediated cell–cell adhesion and stress fiber formation in NHEK but not in HaCaT cells

Previously, we demonstrated that MT disruption induces cell–cell adhesion in NHEK cells (Kee and Steinert, 2001). This adhesion did not occur in HaCaT cells, however (compare Fig 1A, B). In addition, NHEK cells showed strong stress fiber formation upon nocodazole treatment (Fig 1A) but HaCaT cells did not (Fig 1B), even though MTs were almost completely disrupted in both cell types (Fig 1Af, Bf).

It is generally believed that Rho is involved in tension/contraction of cells by regulation of actin bundle formation and also cell–cell adhesion (Sander and Collard, 1999; Braga, 2000). Thus, we asked whether stress fiber formation induced by MT disruption is required for cell–cell adhesion, because our data indicated that stress fiber formation and cell–cell adhesion are concomitantly regulated in NHEK cells. Further, it has been reported that MT disruption in fibroblasts induces stress fiber formation, which is accompanied by an increase of cell-substrate focal adhesions with the concomitant phosphorylation of FAK and paxillin (Bershadsky et al, 1996; Liu et al, 1998). To explore whether increased stress fiber formation in NHEK cells is accompanied by focal adhesion signaling, the phosphorylation status of FAK and paxillin were analyzed. In NHEK cells cultured in low calcium medium, we found that tyrosine phosphorylation of both proteins was similarly increased after nocodazole treatment, reaching a maximum by around 30 min of treatment (Fig 2A, C). Also, the longer persistence of paxillin phosphorylation than FAK is similar to previous observations in fibroblasts (Bershadsky et al, 1996). These results for stress fiber and focal adhesion formation after MT disruption imply that the same molecular events occur in NHEK cells and fibroblasts. In contrast, the phosphorylation status of FAK and paxillin did not change in HaCaT cells regardless of...
staining, were significantly reduced upon cytochalasin B treatment, whereas MT remained intact (Fig 3Bb, c). Together, our data suggest that long bundles of polymerized actin to the level of visible stress fiber formation are not essential for E-cadherin redistribution to membrane and minimal cell–cell adhesion.

**Transient expression of Rho mutants does not interfere with nocodazole-induced cell–cell adhesion**

Although nocodazole-induced cell–cell adhesion can occur without formation of actin filament bundles, the possibility of Rho involvement in cell–cell adhesion nevertheless still remained. Indeed, there are cellular processes in which Rho plays a role that cannot be easily explained by regulatory roles for actin, e.g. growth control (Braga, 2000). To explore these issues in more detail, we performed experiments to alter Rho activity. First, various Rho mutant proteins were transiently expressed in NHEK. Double-labeling using Xpress (transfected epitope) and RhoA antibodies showed proper cytoplasmic expression of RhoA proteins (Fig 4Aa–d). The dominant-active form (RhoA-V14) induced stress fiber formation even in control NHEK (Fig 4e, f). The dominant-negative form (RhoA-N19) suppressed nocodazole-induced stress fiber formation, whereas surrounding cells continued to show strong stress fibers (Fig 4g, h). The dominant-negative forms of RhoB and RhoC could not induce suppressor activity (Fig 4Ba, b, Ca, c), however, suggesting that stress fiber formation in NHEK was indeed mediated by RhoA activation. On the other hand, all three dominant-negative Rho mutants completely failed to inhibit nocodazole-induced cell–cell adhesion (Fig 4A1–l, Bc, d, Cc, d). Furthermore, cell–cell adhesion could not be induced by the dominant-active forms of RhoA, RhoB, or RhoC in untreated NHEK (our unpublished results), as expected from previous reports (Braga et al, 1999). In fact, Rho protein action is closely related to cell–matrix adhesion (Weisberg et al, 1997; Keely et al, 1998) in addition to cell–cell adhesion. Thus, it could be speculated that long-term inactivation of Rho proteins could abolish both cell–cell and cell–matrix adhesions, which in turn results in cell detachment. This possibility led us to suspect that our assay system, based on attached cells, reflects only incomplete inactivation of Rho, even though the level of inactivation observed was sufficient for inhibition of stress fiber formation. To explore this issue further, the C3 transferase, which is known to specifically inhibit activation of Rho proteins, was microinjected into NHEK cells, and calcium- and nocodazole-induced cell–cell adhesion was analyzed. Consistent with previous reports (Braga et al, 1997; 1999), we found that C3 microinjection inhibited cell–cell adhesion induced by high calcium (Fig 5b, b′) and even by MT disruption (Fig 5c–d′), whereas control microinjection did not (Fig 5a, a′). Such inhibition by C3 could be observed when single cells were injected (Fig 5c, c′), as well as in cells injected in clusters (Fig 5d, d′).

The results of C3 microinjection and the transient expression of dominant-negative Rho initially appear contradictory. Because C3 is a particularly potent inhibitor of Rho, however, this suggests stepwise or distinct actions of Rho on stress fiber and focal adhesion versus cell–cell adhesion. Nevertheless, it is important to emphasize that our results show that RhoA inactivation at the level for inhibition of stress fiber formation could not inhibit cell–cell adhesion. Moreover, based on the failure of induction of cell–cell adhesion by Rho activation, it could be suggested that MT disruption generates at least two different signals, one for cell–cell adhesion and another for stress fiber formation, although the mechanism of Rho involvement in cell–cell adhesion remains to be further clarified.

Neomycin-resistant HaCaT cells acquire nocodazole-induced cell–cell adhesion, but not stress fiber formation and increased focal adhesions. Next, we examined whether failure of RhoA activation in MT-disrupted HaCaT cells is responsible for the absence of cell–cell adhesion. Expression of dominant-active RhoA, however, induced stress fiber formation (Fig 6a, b) without any noticeable increase in cell–cell adhesion.
Thus, it is likely that the absence of cell–cell adhesion in MT-disrupted HaCaT cells is not due to absence of RhoA activation. To verify this conclusion, we generated HaCaT cells that gained the ability to form cell–cell adhesions upon nocodazole treatment. To do so, we produced a neomycin-resistant HaCaT (HC-R1) cell line through neomycin gene transfection and selection with G418. Interestingly, these cells acquired nocodazole-induced cell–cell adhesion in low calcium medium (KGM). In notable contrast to NHEK cells, however, HC-R1 cells showed intense accumulation of actin in addition to E-cadherin at sites of cell–cell adhesion after nocodazole treatment (Fig 7Ac, b). Importantly, this cell–cell adhesion was not accompanied by either stress fiber formation (Fig 7Ab) or an increase of the tyrosine phosphorylation state of either FAK or paxillin (Fig 7B). These results showed that cell-cell adhesion by MT disruption could occur without stress fiber formation, and provides support for our results in NHEK cells.

Figure 5. Microinjection of C3 coenzyme inhibits nocodazole-induced cell–cell adhesion. Injected cells were monitored by fluorescence emission from Oregon Green–dextran (Dex, a, b, e, f), and cell–cell adhesion was assessed by E-cadherin IF (E-cad, c, d, f, d’). Control injection (dextran only, a) did not inhibit nocodazole-induced cell–cell adhesion (a’). In contrast, C3 injection (with dextran) markedly inhibited cell–cell adhesion induced by high calcium for 3 h (b’) or nocodazole (c, d’). These inhibition patterns were consistent regardless of whether single (c’) or multiple (d’) cell injections were performed. Arrowheads indicate dislocation of E-cadherin complex from former cell–cell adhesion sites. Scale bar: 30 μm.

Figure 6. Actin stress fiber formation can be induced without cell–cell adhesion in HaCaT cells cultured in low calcium medium. (a) Transient expression of dominant-active RhoA in HaCaT was alone sufficient to induce stress fiber formation. AC indicates actin staining with phalloidin. (b) Transfected cell monitored by Xpress IF staining. (c) RhoA activation failed to induce cell–cell adhesion. Cell–cell adhesion was assessed by α-catenin (α-cat) IF staining. (d) Transfected cell monitored by Xpress IF staining in same field as (c). Bar: 20 μm. Identical experiments were done using DN forms of RhoB (B) and RhoC (C). DN RhoB and RhoC could not block either nocodazole-induced stress fiber formation (Ba, b, Ca, b) or cell–cell adhesion. Arrows indicate transfected cells.

(Fig 6c, d). Thus, it is likely that the absence of cell–cell adhesion in MT-disrupted HaCaT cells is not due to absence of RhoA activation.

To verify this conclusion, we generated HaCaT cells that gained the ability to form cell–cell adhesions upon nocodazole treatment. To do so, we produced a neomycin-resistant HaCaT (HC-R1) cell line through neomycin gene transfection and selection with G418. Interestingly, these cells acquired nocodazole-induced cell–cell adhesion in low calcium medium (KGM). In notable contrast to NHEK cells, however, HC-R1 cells showed intense accumulation of actin in addition to E-cadherin at sites of cell–cell adhesion after nocodazole treatment (Fig 7Ac, b). Importantly, this cell–cell adhesion was not accompanied by either stress fiber formation (Fig 7Ab) or an increase of the tyrosine phosphorylation state of either FAK or paxillin (Fig 7B). These results showed that cell-cell adhesion by MT disruption could occur without stress fiber formation, and provides support for our results in NHEK cells.

This nocodazole-induced cell–cell adhesion also could be observed in hygromycin-resistant HaCaT cells (our unpublished results) but not in zeocin-resistant HaCaT (Fig 7Ac, d). Therefore
After cotreatment with nocodazole and cytochalasin B (Fig. 8), for NHEK, cell-cell adhesion in HC-R1 cells was still induced nocodazole-induced cell-cell adhesion in HC-R1 cells. As seen to be more similar to MDCK than NHEK in high calcium. Thus, accumulation at cell-cell adhesion sites (Fig. 8Aa) to be more similar to MDCK than NHEK in high calcium. Thus, accumulation at cell-cell adhesion sites (Fig. 8Aa). In contrast to NHEK cells, however, E-cadherin staining in HC-R1 cells treated with nocodazole and cytochalasin B showed a linear pattern (Fig. 8Ab), even though actin microfilaments were disrupted and present only as aggregated deposits (Fig. 8Ad).

Furthermore, transient expression of dominant-negative RhoA under low calcium conditions could not block nocodazole-induced actin accumulation in HC-R1 cells (Fig. 8Bb, b'), whereas the dominant-active RhoA readily induced stress fiber formation (Fig. 8Ba, d'). These data suggest that actin accumulation at cell-cell adhesions is induced by a different mechanism than for stress fiber formation. Moreover, both suppressing and activating RhoA mutants failed to interfere with nocodazole-induced cell-cell adhesion in HC-R1 cells (Fig. 8Bc, e', d, d'). Although the intensity of E-cadherin staining of cells expressing the dominant-negative RhoA appeared weaker than that of surrounding nontransfected cells (compare with Fig. 5d, d'), we could not find cells in which E-cadherin staining was completely abolished. Taken together, our results show that the behavior of actin in HC-R1 cells is quite different from that in NHEK cells. In both cell types, however, RhoA inactivation at the level for inhibition of stress fiber formation is not sufficient to block the nocodazole-induced cell-cell adhesion.

**DISCUSSION**

There are several ways to induce cell-cell adhesion in epithelial cells in vitro. One long-established method involves calcium-induced cell-cell adhesion, which is achieved simply by raising the calcium concentration in the medium (Hennings et al., 1980). This cell-cell adhesion is rarely accompanied by stress fiber formation, and recent evidence has revealed that the switch to high calcium reduces RhoA activity in MDCK cells (Noren et al., 2001). Another experimental method to induce cell-cell adhesion is activation of protein kinase C by phorbol ester (PMA) treatment of epithelial cells (Strasheim et al., 1999). In this model, PMA treatment also induces myosin-containing stress fiber formation. In addition, we have demonstrated that disruption of MT in NHEK by nocodazole promotes cell-cell adhesion through AJ assembly, and it also induces stress fiber formation (Kee and Steinert, 2001). These findings suggest a role for Rho isoforms, as stress fiber formation is a well-known cytoskeletal phenotype of Rho activation. Interestingly, although there are several lines of evidence documenting a requirement for Rho in AJ formation in keratinocytes (Braga et al., 1997, 1999), to date, the precise role(s) of Rho activity in cell-cell adhesion remain unclear. Accordingly, we have explored these processes using our nocodazole-induced cell-cell adhesion model in NHEK cells.

**Stress fibers are not required for nocodazole-induced cell-cell adhesion** In AJ of epithelial cells, the E-cadherin-catenin complex can associate directly or indirectly with actin through α-catenin (Tsukita et al., 1992). Indeed, a recent report documented a pronounced defect in AJ formation in α-catenin-deficient mouse skin (Vasioukhin et al., 2001), which strengthens the concept that actin plays an important role in AJ formation.

In the calcium switch model, transferring keratinocytes to high calcium medium containing cytochalasin caused E-cadherin to redistribute to cell borders in a punctated rather than a continuous pattern, and with incomplete sealing of AJ (Vasioukhin et al., 2000). In our study in low calcium conditions, however, it was difficult to see such gaps in NHEK cells cotreated with cytochalasin and nocodazole (Fig. 3Aa). An explanation of these data may be that small pieces of actin filaments that were products of incomplete disruption might still play a partial role in cell-cell adhesion resulting in heterogeneous patterns mixing complete and incomplete cell-cell adhesion. Nevertheless, our data clearly document that well-developed actin filament bundles or stress fibers are not required for nocodazole-induced cell-cell adhesion.

![Figure 7](image-url) In an HaCaT variant cell line, HC-R1, the cells could acquire nocodazole-induced cell-cell adhesion in the absence of stress fiber formation and increased focal adhesions. (A) HC-R1 cells in low calcium medium showed cell-cell adhesion when MT were disrupted (a, E-cadherin staining). This cell-cell adhesion was not accompanied by stress fiber formation, and instead a dense circumferential actin structure was developed (b, actin staining). In the case of the zeocin-resistant HaCaT cell line (Zeo-R), neither cell-cell adhesion nor actin cytoskeletal changes were found after MT disruption (c, d). Scale bar: 30 μm. (B) In HC-R1 cells, MT disruption could not induce increased tyrosine phosphorylation of FAK and paxillin

it appeared that acquisition of sensitivity to nocodazole-induced cell-cell adhesion occurred only in aminoglycoside antibiotic-resistant cells. Accordingly, this phenomenon may be related instead to other pharmacologic actions of aminoglycosides, such as inhibition of phospholipase activity (Lipsky and Lietman, 1982; Liscovitch et al., 1991).

**Neither disruption of actin filaments nor RhoA inactivation can interfere with nocodazole-induced cell-cell adhesion in HC-R1 cells** Recently, two types of calcium-induced cell-cell adhesion models were proposed (Vasioukhin and Fuchs, 2001). In contrast to the NHEK cell system, MDCK cells form a circumferential actin cable, which surrounds cells, and it was thought that this process might be a prerequisite for cell-cell adhesion formation. Our data for the HC-R1 cells treated with nocodazole in low calcium medium showed an intense actin accumulation at cell-cell adhesion sites (Fig. 7Ab), which appeared to be more similar to MDCK than NHEK in high calcium. Thus, we tested whether this actin accumulation was required for nocodazole-induced cell-cell adhesion in HC-R1 cells. As seen for NHEK, cell-cell adhesion in HC-R1 cells was still induced after cotreatment with nocodazole and cytochalasin B (Fig. 8Aa), although the intensity of E-cadherin staining was much lower than in cells treated only with nocodazole (see Fig. 7Aa).
In the case of HC-R1 cells, nocodazole treatment induced actin accumulation at cell–cell adhesion sites instead of stress fiber formation. In some cells such as MDCK, circumferential actin organization appears to play an important role in cell–cell adhesion in the high calcium model (Vasioukhin and Fuchs, 2001). Thus, nocodazole-induced cell–cell adhesion in HC-R1 cells seems to be more similar to that of MDCK cells than NHEK cells, although it was not clear whether this actin accumulation preceded cell–cell adhesion. In fact, the underlying mechanism for the generation of circumferential actin seems to be quite different from stress fiber formation. For example, in adenoviral E1A-expressing primary baby rat kidney (BRK) cells, the addition of activated Ras results in the loss of stress fibers but not circumferential actin (Fischer and Quinlan, 1998). Also, activated Rac resulted in disruption of circumferential actin organization in the same cells (Quinlan and Hyatt, 1999). Further, we show here that nocodazole-induced circumferential actin organization in HC-R1 cells was not affected by RhoA inhibition (Fig 8Bb, b’). Nocodazole-induced cell–cell adhesion in HC-R1 cells, however, was not inhibited by disruption of actin filaments in low calcium conditions (Fig 8A), although the intensity of E-cadherin staining was significantly reduced. These results are quite different from those in the adenoviral E1A-expressing BRK model, and they support our speculation that short pieces of actin filaments may still be sufficient for cell–cell adhesion formation, albeit less efficiently.

RhoA activation to levels involving actin reorganization is not required for nocodazole-induced cell–cell adhesion

Rho involvement in calcium-induced cell–cell adhesion has been reported by several groups. Inhibition of Rho by C3 microinjection is known to disrupt E-cadherin cytoskeletal links in A549, and to block the assembly of new A549 cell junctions (Braga et al., 1997; 1999; see Fig 5). Because Rho proteins are thought to be involved in matrix adhesions as well (Weisberg et al., 1997; Keely et al., 1998), complete inactivation of Rho proteins should detach the anchored cells, which precludes assay. Thus, short-term inactivation (1 h) through C3 microinjection would be more appropriate for studying complete blockage of Rho activation than long-term inactivation by transient transfection (24–36 h). In fact, our results showed that C3 microinjection blocked the nocodazole-induced cell–cell adhesion but the transient expression of dominant-negative Rho could not. One interpretation of this finding is that a minimal level of activity of Rho, which is lower than that required for stress fiber formation, is sufficient for promoting cell–cell adhesion. Another possibility is that Rho plays a role in cell–cell adhesion that is totally different than for stress fiber formation. In fact, there are some phenomena that are hard to explain based on Rho-mediated actin organization (Braga, 2000). For example, Rho inactivation can lead to altered cell proliferation (Yamamoto et al., 1993; Olson et al., 1995; Liu et al., 1998). Thus, it could be speculated that incomplete inactivation of Rho in attached cells could still play some roles other than in stress fiber formation.

Furthermore, the fact that inactivation of RhoA, RhoB, or RhoC could not interfere with nocodazole-induced cell–cell adhesion raises the possibility of redundant functions of Rho isoforms. There are several isotypes of Rho proteins. RhoA, RhoB, and RhoC have similar biologic functions, such as positive roles in stress fiber formation. RhoD activation induces disassembly of stress fibers in fibroblasts, however (Khosravi-Far et al., 1998; Tsubakimoto et al., 1999). Because C3-transferase has the potential to inhibit activation of all of the Rho isoforms, we cannot exclude a role for RhoD activation in nocodazole-induced cell–cell adhesion. The strong stress fiber formation we see in MT-disrupted NHEK, however, tends to argue against this possibility.

There is also evidence for an inhibitory role of Rho in cell–cell adhesion, however. Some exogenous factors such as transforming growth factor β1 (TGF-β1) are known to induce EMT with a characteristic loss of E-cadherin at cell junctions (Bhowmick et al., 2001). In epithelial cells including mouse primary epidermal keratinocytes, TGF-β1 treatment rapidly induced RhoA activation, and blocking of this activation inhibited TGF-β1-mediated EMT (Bhowmick et al., 2001). In fibroblasts, transient platelet-derived-growth-factor-induced as well as sustained Rac activation by

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**Figure 8. Neither disruption of actin filaments nor RhoA inactivation could interfere with nocodazole-induced cell–cell adhesion in HC-R1 cells.** (A) HC-R1 cells cultured in KGM-2 were cotreated with cytochalasin B and nocodazole and then IF stained with the E-cadherin antibody (a) and phalloidin (b). Accumulation of E-cadherin at sites of cell–cell adhesion was clearly visualized (a), although the intensity was weaker than that with nocodazole alone (see Fig 4Aa). (b) HC-R1 cells were transfected with RhoA mutant constructs and assayed for nocodazole effects under low calcium conditions. (a) RhoA-DA could induce stress fiber formation in HC-R1 cells; (b) but RhoA-DN could not block the accumulation of actin at cell–cell adhesion sites in nocodazole-treated HC-R1 cells. (c), (d) Neither DA nor DN forms of RhoA could block nocodazole-induced cell–cell adhesion.
Acquisition of nocardazole-induced cell–cell adhesion in HaCaT cells. We found that aminoglycoside antibiotic-resistant HaCaT cells such as HC-R1 acquired sensitivity to nocodazole-induced cell–cell adhesion (Fig 7). In addition to their antibiotic effects, aminoglycoside antibiotics have inhibitory effects on phosphotyrosine turnover through inhibition of phospholipase C and D (Lipsky and Lietman, 1982; Schwartz et al, 1984; Lisovitch et al, 1991). Further, this effect eventually blocks IP3-mediated calcium release from intracellular pools (Vassbotn et al, 1990; Harris and Hanrahan, 1993). For these reasons, G418 and neomycin sulfate have been used for phosphotyrosyl phospholipase C and phospholipase D (Maio and Brown, 1991; Chen et al, 2000). In addition, neomycin can induce cytoplasmic protrusions (lamellipodia) and characteristic actin cytoskeletal changes (actin ring structures) in fibroblasts (Hedberg et al, 1993; Hedberg and Bell, 1995). These lamellipodial protrusions are replaced temporarily with stress fibers at later stages, suggestive of late RhoA activation (Safiejko-Mroczka and Bell, 1995). Recently, data have accumulated on the regulation of phospholipase D activity by RhoA, and a direct interaction between these proteins has been reported (Abousalham et al, 1997; Hess et al, 1997; Bae et al, 1998), in addition to a modulation of cytoskeletal and actin cytoskeletal dynamics by phospholipases C and D (Frohman et al., 1988; Janmey, 1994). These studies have suggested that phospholipids and small GTPases might regulate each other very tightly in a reciprocal manner. Nevertheless, in view of our data, which showed complete inhibition of nocardazole-induced stress fiber formation by RhoA inactivation, we can conclude that RhoA activation to levels needed to regulate actin cytoskeletal organization is not essential for nocardazole-induced cell–cell adhesion in keratinocytes.

REFERENCES


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