Microtubule dynamics and Rac-1 signaling independently regulate barrier function in lung epithelial cells

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Lorenowiec MJ, Fernandez-Borja M, van Stalborth AD, van Sterkenburg MA, Hiemstra PS, Hordijk PL. Microtubule dynamics and Rac-1 signaling independently regulate barrier function in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 293: L1321–L1331, 2007. First published September 7, 2007; doi:10.1152/ajplung.00443.2006.—Cadherin-mediated cell-cell adhesion controls the morphology and function of epithelial cells and is a critical component of the pathology of chronic inflammatory disorders. Dynamic interactions between cadherins and the actin cytoskeleton are required for stable cell-cell contact. Besides actin, microtubules also target intercellular, cadherin-based junctions and contribute to their formation and stability. Here, we studied the role of microtubules in conjunction with Rho-like GTPases in the regulation of lung epithelial barrier function using real-time monitoring of transepithelial electrical resistance. Unexpectedly, we found that disruption of microtubules promotes epithelial cell-cell adhesion. This increase in epithelial barrier function is accompanied by the accumulation of β-catenin at cell-cell junctions, as detected by immunofluorescence. Moreover, we found that the increase in cell-cell contact, induced by microtubule depolymerization, requires signaling through a RhoA/Rho kinase pathway. The Rac-1 GTPase counteracts this pathway, because inhibition of Rac-1 signaling rapidly promotes epithelial barrier function, in a microtubule- and RhoA-independent fashion. Together, our data suggest that microtubule-RhoA-mediated signaling and Rac-1 control lung epithelial integrity through counteracting independent pathways.

RhoA; cell-cell contact; transepithelial resistance

Epithelia form tightly regulated barriers that protect the body from the external environment. Loss of epithelial integrity greatly increases the risk of microbial infection and is often a hallmark of chronic (lung) inflammatory disorders, such as asthma or chronic obstructive pulmonary disease (13, 36, 45). In polarized epithelium, adhesion between adjacent cells is mediated by various types of intercellular junctions, such as tight junctions (TJs), adherens junctions (AJs), and desmosomes (34). AJs are characterized by the presence of E-cadherin, a transmembrane adhesion molecule that mediates Ca2+-dependent homophilic binding through its extracellular domain. The intracellular domain of E-cadherin interacts with βγ- and α-catenins, which link E-cadherin with the actin cytoskeleton (47, 50).

The members of the Rho family of small GTPases are key regulators of the actin cytoskeleton and are involved in the formation, maintenance, and breakdown of AJs. Activation of Rac-1 is associated with increased cell-cell adhesion, and a guanine nucleotide-exchange factor for Rac-1, Tiam-1, promotes cadherin-based cell-cell adhesion (3, 12, 14, 33). In contrast, activation of RhoA is also associated with disassembly of cell-cell contacts (4, 40, 49). Similar to Rac-1, RhoA promotes and inhibits cell-cell contact. Inhibition of RhoA with C3 toxin dislocalizes E-cadherin from cell-cell junctions (6), and several effectors of activated RhoA, e.g., Dia1 and Dia2, induce epithelial cell-cell adhesion (39). In contrast, overexpression of constitutively active RhoA or Rho kinase (a RhoA effector kinase) stimulates stress fiber formation and contractility, resulting in loss of cell-cell contact (5, 16). Thus Rho-like GTPases play divergent roles in the control of cadherin-mediated intercellular contacts.

Microtubule (MT) dynamics regulates intercellular, cadherin-mediated adhesion in endothelial and epithelial cells (15, 20, 59). MTs are polarized polymers of α- and β-tubulin dimers, which show fast growth at their plus ends and slow growth at their minus ends (30). In polarized epithelial cells, MT minus ends orient toward the apical part of the cell, and the plus ends to the basal part of the cell (28). MT disassembly disrupts TJs in thyroid epithelial cells and decreases endothelial barrier function (2, 52, 60). Conversely, MT stabilization counteracts thrombin-induced endothelial permeability (1), whereas formation of AJ by expression of E- and N-cadherin in fibroblasts results in the stabilization of MT minus ends (7). Thus the role of MTs in the control of cell-cell adhesion differs, possibly as a function of cell type.

Here, we studied the role of MT dynamics in conjunction with the Rho GTPases Rac-1 and RhoA in the regulation of lung epithelial barrier function. We show that MT depolymerization promotes transepithelial resistance (TER) in a RhoA-dependent fashion in lung epithelial cells. In addition, we found that inhibition of Rac-1 rapidly promotes TER, through a MT- and RhoA-independent pathway. Our findings show that lung epithelial barrier function is controlled by MT dynamics, in conjunction with counteracting Rac-1 and RhoA-driven signaling.

MATERIALS AND METHODS

Reagents. Nocodazole, taxol, cytochalasin B, and C3 toxin were purchased from Sigma. Y-27632 and ML-7 were from Biomol (UK). Tat-ctrl, Tat-Rac-1, Tat-Rac-2, Tat-CDC42, and Tat-RhoA COOH-terminal peptides, as well as TAT-CRIB and Tat-17-32, have been described elsewhere (32, 35, 48, 51). Mouse monoclonal antibodies to

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tyrosinated (clone TUB-1A2) was from Sigma (1:1,000). Mouse monoclonal antibody to β-catenin was purchased from Transduction Laboratories (1:100). Rabbit polyclonal antibody to occludin was from Zymed Laboratories (1:50).

Cell culture. All cell lines were purchased from ATCC (Manassas, VA) and were cultured at 37°C and 5% CO2. H292 cells (bronchial epithelial cell line) and A549 cells (alveolar epithelial cell line) were maintained in RPMI-1640 medium (GIBCO) containing 10% heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Madin Darby canine kidney (MDCK) cells were cultured in Iscove’s modified Dulbecco’s medium (BioWhittaker, Brussels, Belgium) containing 10% heat-inactivated FCS (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Primary bronchial epithe-

Fig. 1. Depolymerization of microtubule (MT) promotes lung epithelial cell barrier function. Cells were plated on fibronectin-coated gold electrodes and grown to confluency. Transepithelial resistance (TER) was measured in real time, as described in MATERIALS AND METHODS. A: H292 cells were treated with 8 μM nocodazole, 10 μM taxol, or 25 μg/ml cytochalasin B (arrow indicates time of addition). X, control (ctrl); •, nocodazole; ▲, taxol; ■, cytochalasin B. Representative results from at least 5 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2-h time point when resistance values plateaued. Values are means (± SE) of 5 independent experiments performed in duplicate. *P < 0.05; **P < 0.005. B: A549 cells were treated with 8 μM nocodazole, 10 μM taxol, or 25 μg/ml cytochalasin B (arrow indicates time of addition). X, control; •, nocodazole; ▲, taxol; ■, cytochalasin B. Representative results from 2 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2-h time point when resistance values plateaued. Values are means (± SE) of 2 independent experiments performed in duplicate. C: primary human bronchial epithelial cells were cultured to confluence on golden electrodes (8W10E), differentiated, and treated or not with 8 μM nocodazole at the time indicated by arrow (left panel). TER was measured in real time as described in MATERIALS AND METHODS. •, Control; ▲, nocodazole. Representative results from 2 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2-h time point. Values are means (± SE) of 2 independent experiments performed in duplicate. D: Madin Darby canine kidney (MDCK) cells were treated with 8 μM nocodazole, 10 μM taxol, or 25 μg/ml cytochalasin B (arrow indicates time of addition). X, control; ▲, nocodazole; •, taxol; ■, cytochalasin B. Representative results from 3 independent experiments are shown. Bar graph (right panel) represents the relative resistance at the 2-h time point when resistance values plateaued. Values are mean (± SE) of 3 independent experiments performed in duplicate. *P < 0.05.
lial cells were isolated, cultured, passaged, and differentiated by culture in high calcium (1 mM CaCl₂) medium for 36 h before the experiment, as described (29). Cells were grown to confluence on 8W1E ECIS electrodes coated with a mixture of collagen (type I bovine collagen, PureCol, Inamed) and fibronectin (isolated from human plasma), differentiated, and treated as indicated in the figure legends.

Transcellular electrical resistance and permeability measurements. Cells were seeded at 3 × 10⁵ cells per well (0.8 cm²) on fibronectin-coated electrode arrays (8W1E, unless stated otherwise; Applied Biophysics) and grown to confluence. Measurements of transepithelial electrical resistance were performed in real time by means of an electrical cell-substrate impedance sensing system (ECIS, Applied Biophysics) at 37°C, 5% CO₂ (46). Briefly, the small measuring electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier, and a 1-V, 4,000-Hz signal was supplied by a 1-MΩ resistor to approximate a constant-current source of 1 µA. The in-phase and out-phase voltages between the electrodes were monitored in real time, followed by conversion to scalar measurements of transepithelial impedance. The indicated relative electrical resistance values were obtained by subtracting the resistance at time t = 0 from every resistance value in the successive time points. As measured with 8W1E electrodes, H292 baseline values read around 12,000 Ω; A549 baseline values read around 9,000 Ω; MDCK baseline values read around 9,000 Ω. As measured with the 8W10E electrodes, human primary bronchial epithelium baseline values read around 1,000 Ω. This value is ~10-fold lower compared with the cells measured on the 8W1E electrodes, as impedance is measured over a 10-fold larger area. Permeability of epithelial monolayers was determined using Transwell assays, as described (61).

Immunofluorescence. Cells were cultured on glass coverslips. After treatment, cells were fixed with 3.7% (wt/vol) formaldehyde for 10 min at room temperature and permeabilized with 0.1% (wt/vol) Triton X-100 for 5 min. Thereafter, cells were incubated with the indicated primary antibodies, followed by incubation with a goat-anti-mouse-Ig antibody or a goat-anti-rabbit-Ig antibody conjugated to Alexa 488 (1:500; Molecular Probes, Leiden, The Netherlands). F-actin was visualized with Texas Red-labeled phalloidin (1:200; Molecular Probes, Leiden, The Netherlands). Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope with Plan-Neofluar ×100/1.3 oil objective using appropriate filters. Instrument settings were kept constant between different experiments. Fluorescence distribution profiles were created with Image-Pro Plus 6.0 software (Media Cybernetics) to allow semiquantitative analysis of the distribution of cellular and junctional proteins.

RhoA activation assay. RhoA pull-down experiments were performed as previously described (41). In brief, after stimulation, cells were lysed in buffer containing 50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1% (wt/vol) Nonidet P-40, 10% (wt/vol) glycerol, 1 mM NaF, 2 mM NaVO₃, and protease inhibitor cocktail (Roche) for 10 min on ice. Lysates were cleared by centrifugation at 10,000 g for 10 min at 4°C. GST-Rhotekin fusion protein, coupled to glutathione-Sepharose beads (Amersham Biosciences), was added to the supernatants and incubated for 1 h at 4°C. Beads were washed three times in lysis buffer, and bound proteins were eluted with Laemmli sample buffer. Total cell lysates and precipitates were analyzed by Western blotting with a mouse monoclonal antibody anti-RhoA (Santa Cruz Biotechnology), anti-mouse IgG antibodies coupled to horseradish peroxidase, and enhanced chemiluminescence (Amersham Biosciences).

Statistical analysis. All results were expressed as the means ± SE or SD of the indicated number of independent experiments. Where applicable, values were compared in a paired two-tailed Student t-test. A P value <0.05 was considered significant.

RESULTS

Depolymerization of MTs increases lung epithelial barrier function. To establish the role of MTs in epithelial barrier function, we monitored TER of monolayers of H292 cells in real time. Cells were treated with nocodazole, a MT-disrupting agent, or with taxol, a MT-stabilizing agent. Depolymerization of MTs by nocodazole resulted in a fast and pronounced increase in TER, which leveled off after 30 min, and remained stable over the 3-h period of measurement. Taxol had a minor, lowering effect on bronchial epithelial TER (Fig. 1A). As expected, disruption of the epithelial actin cytoskeleton with cytochalasin B induced a dramatic decrease in TER (Fig. 1A).

Disruption of MTs by nocodazole also increased the TER of monolayers of the alveolar epithelial A549 cells (Fig. 1B), albeit to a lower extent, as observed with the bronchial H292 cells. As in H292 cells, treatment of A549 cells with taxol induced a small decrease in TER, while disruption of the actin cytoskeleton with cytochalasin B caused a pronounced decrease in the TER (Fig. 1B). To confirm these findings in primary cells, we used subcultures of primary human bronchial epithelial cells and treated these, after differentiation, with nocodazole. These experiments showed that the primary cells also responded with a clear increase in TER following breakdown of MTs (Fig. 1C). Together, these data show that MT disassembly promotes epithelial barrier function in lung epithelial cells. In contrast, depolymerization of MTs in MDCK cells (kidney epithelium) did not result in an increase in TER. Moreover, cytochalasin B induced a significant increase in TER in MDCK cells, underscoring the differential regulation of cell-cell contact in lung vs. kidney epithelial cells (Fig. 1D).

To further substantiate our findings, we also measured paracellular permeability of H292 monolayers following MT disruption. The data show that paracellular permeability is regulated by nocodazole in a qualitatively similar fashion as TER. Similarly, taxol had little effect, whereas cytochalasin B increased permeability (Fig. 2).

A common modification of MT is the enzymatic addition of a tyrosine residue at the COOH-terminal end of α-tubulin, resulting in increased dynamic behavior (high polymerization/depolymerization rates). Since nocodazole is known to act primarily on dynamic, tyrosinated MTs (22), we performed

Fig. 2. Depolymerization of MT reduced lung epithelial cell barrier permeability. H292 cells were grown to confluence on 0.45-µm Transwell filters, washed, treated with 8 µM nocodazole (noc), 10 µM taxol (tax), or 25 µg/ml cytochalasin B (cyt. B), and permeability to FITC dextran was measured after 24 h incubation in the presence of the drugs. Values represent means ± SD of a representative experiment performed three times in triplicate. *P < 0.05.
immunofluorescence labeling of tyrosinated MTs on nocodazole-treated H292 cells. Indeed, nocodazole induced an almost complete loss of tyrosinated MT in the lung epithelial cells (Fig. 3A).

Epithelial integrity depends on junctional E-cadherin-β/γ-catenin complexes and on their interaction with the actin cytoskeleton. Since MT dynamics regulate the actin cytoskeleton (26, 53), we investigated whether the increase in TER, induced by MT disassembly, was accompanied by changes in the organization of the actin cytoskeleton and/or of intercellular junctions. MT depolymerization caused an increase in immunostaining for β-catenin at cell-cell junctions and at the apical side of the cells (Fig. 3B). This suggests that MT depolymerization induces accumulation of E-cadherin-β-catenin complexes at the plasma membrane, possibly through an effect on protein trafficking. The F-actin levels at the cell-cell junctions did not change significantly in cells treated with nocodazole (Fig. 3C, left). However, MT disassembly induced distinct F-actin bundling at the bottom side of bronchial epithelial cells, generating long actin spikes (Fig. 3C, middle). Moreover, X-Z imaging (Fig. 3C, right) showed that actin was more regularly distributed in nocodazole-

![Fig. 3. Disruption of MTs results in actin bundling and increased levels of β-catenin at cell-cell junctions. A: H292 cells, grown on glass coverslips, were treated or not with 8 μM nocodazole for 1 h, fixed, and stained for tyrosinated MTs (tyr-MT). Bars, 20 μm. Images are representative of at least 3 independent experiments. Fluorescence intensity profiles along the indicated dashed line between points indicated as a and b are shown. B: H292 monolayers were treated or not with 8 μM nocodazole for 1 h, fixed, and stained for β-catenin. Images are representative of 4 independent experiments. Fluorescence intensity profiles along the indicated dashed line between points a and b are shown. X-Z images show β-catenin distribution along the junctions and the apical membrane. C: H292 cells, cultured and stimulated as in A and B, were fixed and stained for F-actin. Confocal sections of the middle section of the cells (left) and from the floor (bottom) of the cells are shown. Bars, 10 μm. Fluorescence intensity profiles along the indicated dashed line between points a and b are shown. X-Z images show F-actin distribution along the junctions and the apical membrane. D: H292 cells, grown on glass coverslips, were treated or not with 8 μM nocodazole for 1 h, fixed, and stained for occludin. Bars, 10 μm. Images are representative of at least 2 independent experiments. Fluorescence intensity profiles along the indicated dashed line between points indicated as a and b are shown.]

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treated cells compared with controls, in particular at the apical side of the cells, resulting in a more “rigid” appearance of the actin distribution following MT breakdown. Immunostaining for the TJ protein occludin did not reveal a major increase in recruitment of this protein to cell-cell contact sites (Fig. 3D).

MT disassembly activates RhoA. Rho-like GTPases are key regulators of cytoskeletal dynamics and act as signaling intermediates between MTs and F-actin (10, 11, 24, 38). Depolymerization of MTs resulted in a transient activation of RhoA, with a maximum at 5 min and return to basal levels within 30–60 min (Fig. 4A). To test whether RhoA was required for the MT disassembly-induced increase in TER, we blocked RhoA signaling by preincubation of epithelial monolayers with a cell-permeable peptide corresponding to the hypervariable COOH-terminus of RhoA (Tat-RhoA) (48). Subsequently,
cells were treated with nocodazole, followed by real-time recording of TER. Inhibition of RhoA attenuated partially, but significantly, the increase in TER induced by MT depolymerization (Fig. 4B). The peptide by itself did not affect basal TER (Fig. 4B). To analyze this in more detail, we tested whether the RhoA effector Rho kinase (ROCK), known to induce myosin phosphorylation and actin bundling, was required for this effect. The ROCK inhibitor Y-27632 significantly blocked the nocodazole-induced increase in TER (Fig. 4C). In contrast, inhibition of myosin light chain kinase, which directly phosphorylates myosin and induces actin bundling, did not affect the increase in TER induced by MT disassembly (Fig. 4D). These data show that the increase in lung epithelial barrier function induced by MT breakdown is, in part, mediated by a RhoA-ROCK pathway and does not require myosin light chain kinase activity.

**Inhibition of Rac-1 increases lung epithelial barrier function.** Rac-1 is another Rho-like GTPase that has previously been clearly implicated in the control of E- and VE-cadherin-based cell-cell contact (6, 12, 14, 42, 49, 56). Moreover, the balance between Rac-1 and RhoA signaling regulates cell-cell adhesion (5, 12, 41). Because RhoA activation is involved in the increase in TER of lung epithelial cells upon MT depolymerization (Fig. 4), we subsequently tested the relevance of Rac-1 for this effect. To block Rac-1 signaling, we preincubated H292 monolayers with a cell-permeable peptide corresponding to the hypervariable COOH-terminal domain of Rac-1 (Tat-Rac-1), previously shown to inhibit Rac function (32, 43, 48), and monitored changes in TER. This peptide blocks Rac-1 signaling within minutes by competing with Rac-1-targeting proteins. Unexpectedly, inhibition of Rac-1 induced an immediate and dramatic increase in basal TER (Fig. 5A). Inhibition of RhoA using the corresponding COOH-terminal peptide did not have any major effect on TER (Fig. 5A).

To further establish the role of Rac-1, we blocked Rac-1 signaling in H292 cells with a different Rac-1-inhibiting peptide, Tat-17–32, which encodes part of the effector domain of the PAK-CRIB domain (Tat-CRIB), which specifically binds GTP-loaded Rac-1 (35). This CRIB domain peptide also enhanced TER, albeit to a lesser extent (Fig. 5B). As with nocodazole, we confirmed that the effect of the Rac-1 COOH-terminal peptide in primary human bronchial epithelial was similar as in the lung cell lines (Fig. 5C). Thus inhibition of Rac-1 signaling strongly promotes TER of monolayers of lung epithelial cells.

The Rac-1 inhibiting COOH-terminal peptide did not induce a detectable increase in the immunostaining for β-catenin at cell-cell junctions (Fig. 5D), as was observed in nocodazole-treated cells (Fig. 3B). However, Rac-1 inhibition did induce the reorganization of the actin cytoskeleton, i.e., the induction of F-actin and formation of stress fibers, and markedly promoted cell spreading, particularly of cells at the periphery of small clusters of cells (Fig. 5E). Inhibition of RhoA had little effect and did not induce an increase in cell stress fibers, as expected (not shown). Immunostaining for occludin in cells treated with the Rac-1 COOH-terminal peptide showed a minor increase in occludin recruitment to junctional areas compared with controls (Fig. 5F).

The nocodazole-induced increase in lung epithelial barrier function is, at least in part, mediated by RhoA-ROCK signaling (Fig. 4). Since Rac-1 inhibition also promoted barrier function and F-actin formation (Fig. 5), we tested whether the effect of Rac-1 inhibition is mediated by RhoA-ROCK signaling. Inhibition of ROCK with Y-27632 did not change the effect of the Tat-Rac-1 peptide on TER (Fig. 6A). Similarly, addition of Y-27632 following incubation of cells with the Rac-1 COOH-terminal peptide did not abrogate the barrier-enhancing effect of Rac-1 inhibition (Fig. 6B). Moreover, direct inhibition of RhoA by preincubation of H292 monolayers with the RhoA inhibitory peptide or C3 toxin (31) also failed to attenuate the increase in TER induced by inhibition of Rac-1 (Fig. 6C). These data suggest that RhoA/ROCK signaling is not required for the increase in TER that follows the inhibition of Rac-1.

**MT disassembly promotes barrier function independently of Rac-1.** To further investigate the relation between Rac-1 and MT-mediated regulation of epithelial barrier function, we monitored the TER of H292 monolayers that were treated with nocodazole before the addition of the Rac-1-inhibiting peptide. Interestingly, inhibition of Rac-1 in nocodazole-treated cells further enhanced TER (Fig. 7A), suggesting that the pathways
that regulate barrier function downstream of Rac-1 inhibition and MT depolymerization are separate and not redundant. Stabilization of MTs with taxol, before inhibition of Rac-1, did not have any effect on the increase in TER (Fig. 7B). This further indicates that Rac-1 inhibition does not increase TER through the regulation of MT dynamics. These data, together with the differential requirement for RhoA in MT- and Rac-1-mediated signaling, suggest that MT dynamics and Rac-1 control lung epithelial cell-cell adhesion through independent pathways.

DISCUSSION

One of the main findings of this work is that the loss of dynamic MT promotes cell-cell adhesion in lung epithelial cells. This finding is in line with data from Ivanov et al. (15) and Kee et al. (20), who showed that MT dynamics negatively regulate cell-cell contacts in keratinocytes and in intestinal epithelium. In differentiated keratinocytes, however, MT disruption-induced increased cell-cell contact was suppressed (20). Cell differentiation may alter cytoskeletal organization or
signaling properties, mediating very different roles for MTs in cell-cell contact. Similarly, MT dynamics are required for proper barrier function of thyroid epithelial cells (60), and MT depolymerization induces loss of cell-cell adhesion in newt lung epithelial cells (54). Also, in kidney epithelial MDCK cells, MT disassembly reduces barrier function (Fig. 1D).

Together, these data suggest that the regulation of epithelial cell-cell contact by MT is cell-type specific and may also be controlled by the level of differentiation.

The observed increase in TER upon MT disassembly in lung epithelial cells was accompanied by increased immunostaining for β-catenin, but not occludin, at cell-cell junctions. This indicates that MTs regulate trafficking of E-cadherin-β-catenin complexes to and from cell-cell junctions, leading to an internalization defect in nocodazole-treated cells. Accordingly, Ivanov et al. (15) reported that inhibition of the MT motor kinesin attenuated junction disassembly in intestine epithelium. Similarly, N-cadherin-positive vesicles were found to move in a MT-dependent, kinesin-driven fashion in fibroblasts (27), and junctional complex proteins such as p120-catenin and β-catenin were shown to interact with MTs and with the MT-associated motors kinesin and dynein (8, 25, 44, 58).

Depolymerization of MT in keratinocytes not only promotes recruitment of E-cadherin to the sites of cell-cell contact, but also induces an increase in F-actin at cell-cell junctions (20). Although we did not find such an increase of F-actin at cell-cell junctions, MT disassembly induced pronounced actin rearrangements at the basal side of the cells. These comprised extensive formation of actin stress fibers, which were oriented from the center radiating outward to the cellular periphery. Interestingly, the actin rearrangements shown in Fig. 3 are very similar to those described for epithelial cells in which...
expression of Par3 is reduced (9). Moreover, these actin rearrangements are also reminiscent of the nocodazole-induced transition of epithelial cell-cell contact from tangential to radial (23). The nocodazole-induced actin rearrangements thus suggest increased cell-cell contact, in particular in the basal side of the cells. This may well influence epithelial polarity and the consequent organization of cell-cell contact in a way such that TER of lung epithelial cells is increased.

Formation of stress fibers induced by MT disassembly is a RhoA-dependent phenomenon in fibroblasts as well as in endothelial cells (26, 52). In line with this notion, we found that, also in lung epithelial cells, MT disassembly stimulates RhoA activation. More importantly, we show that the increase in lung epithelial barrier function, stimulated by MT depolymerization, is partially mediated through RhoA and ROCK signaling. This finding is in good agreement with data from others, implicating RhoA in the stimulation of cell-cell adhesion. For example, thrombin was found to increase TER of A549 cells in a Rho-dependent fashion (18). Conversely, inhibition of RhoA signaling by the C3 toxin or using a dominant-negative mutant of RhoA in epithelial cells was found to disrupt junctional localization of E-cadherin (6, 17, 42). However, constitutively active RhoA failed to increase epithelial cell-cell contacts (5), indicating that RhoA by itself is not sufficient to enhance epithelial cell-cell adhesion. Interestingly, in keratinocytes, MT depolymerization-induced cell-cell adhesion appears to be dissociated from MT disassembly-induced RhoA-driven cytoskeletal dynamics, further indicating that, in addition to RhoA, other factors are involved in the regulation of epithelial barrier function (19).

Our data demonstrate that inhibition of Rac-1 signaling, by means of cell-permeable peptides, rapidly and dramatically improves lung epithelial barrier function. Accordingly, activation of Rac-1 in epithelial cells and various carcinoma cell lines induces disassembly of cell-cell contact (4, 21, 37). Similarly, in the epithelial cells lacking the polarity protein Par3, Rac activity is upregulated, leading to loss of integrity. Also here, inhibiting Rac signaling promotes junction assembly (9). In contrast, several studies have shown that Rac-1 activity in epithelial as well as endothelial cells is also required for the formation of cadherin-based adhesion (6, 14, 42, 57). This discrepancy may relate to differences in experimental conditions, cell type, and state of maturation of the junction or the type of extracellular matrix used (40).

Activation of Rac-1 promotes MT growth into lamellipodia of migrating kidney epithelial cells, while a dominant-negative mutant of Rac-1 inhibits MT polarization toward the leading edge of migrating cells and blocks lamellipodia formation (54, 55). Conversely, MT growth activates Rac-1 in migrating fibroblasts, whereas MT depolymerization inhibits Rac-1 activation (55). We found that, although both MT disassembly and inhibition of Rac-1 induced actin polymerization, the consequent increase in lung epithelial barrier function occurs through independent pathways. In contrast to MT disassembly, the increase in TER induced by the inhibition of Rac-1 signaling did not induce increased immunostaining for E-cadherin/β-catenin complexes at cell-cell junctions and did not require RhoA/ROCK signaling. Also for occludin, we could not detect a major increase in junctional staining, indicating that the mechanism by which Rac inhibition promotes lung epithelial TER is not based on increased formation of tight junctions. MT depolymerization and inhibition of Rac-1 were additive in the enhancement of lung epithelial TER. Our data suggest that, in contrast to their functional coupling in cell migration, Rac-1 and MT act independently in the control of lung epithelial barrier function.

In conclusion, our current findings provide new and unexpected insights in the control of lung epithelial intercellular contacts by MT and Rho GTPases. It is important to stress that the regulation of epithelial integrity, as described in this paper, even in primary human cells, may deviate from the situation in vivo, for example as a result of altered cell differentiation.
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Future research will need to address the relative contribution of cytoskeletal changes and traffic of junctional components to the strength of cell-cell contact. In addition, additional in vivo studies will be necessary to further establish the mechanisms that control cell-cell contact in epithelial cells and to gain insight in its apparent, striking cell-type specificity.

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