RhoA and Rac1 are both required for efficient wound closure of airway epithelial cells

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RhoA and Rac1 are both required for efficient wound closure of airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 287: L1134–L1144, 2004. First published August 6, 2004; doi:10.1152/ajplung.00022.2004.—Repair of the airway epithelium after injury is critical for restoring normal lung. The reepithelialization process involves spreading and migration followed later by cell proliferation. Rho-GTPases are key components of the wound healing process in many different types of tissues, but the specific roles for RhoA and Rac1 vary and have not been identified in lung epithelial cells. We investigated whether RhoA and Rac1 regulate wound closure of bronchial epithelial cells. RhoA and Rac1 proteins were efficiently expressed in a cell line of human bronchial epithelial cells (16HBE) by adenovirus-based gene transfer. We found that both constitutively active RhoA and dominant negative RhoA inhibited wound healing, suggesting that both activation and inhibition of RhoA interfere with normal wound healing. Overexpression of wild-type Rac1 induced upregulation of RhoA, disrupted intercellular junctions, and inhibited wound closure. Dominant negative Rac1 also inhibited wound closure. Inhibition of the downstream effector of RhoA, Rho-kinase, with Y-27632 suppressed actin stress fibers and focal adhesion formation, increased Rac1 activity, and stimulated wound closure. The activity of both RhoA and Rac1 are influenced by the polymerization state of microtubules, and cell migration involves coordinated action of actin and microtubules. Microtubule depolymerization upon nocodazole treatment led to an increase in focal adhesions and decreased wound closure. We conclude that coordination of both RhoA and Rac1 activity contributes to bronchial epithelial wound repair mechanisms in vitro, that inhibition of Rho-kinase accelerates wound closure, and that efficient repair involves intact microtubules.

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ology, motility, and cytoskeleton. RhoA induces the formation of focal adhesions and stress fibers. This action of RhoA is mediated by downstream effectors, including Rho-kinase (ROCK), which inactivates myosin phosphatase by phosphorylation and increases myosin-based contractility (1, 21). Rho-GTPases are important in cell migration through effects on cell contractility that must be precisely regulated for efficient cell migration. RhoA is required for the formation and maintenance of focal adhesions (15, 34), whereas Rac1 and Cdc42 regulate formation of smaller “focal complexes,” structures associated with lamellipodia and filopodia (27). It has recently been proposed that turnover of adhesion sites in migrating cells may be regulated by microtubules growing toward these adhesion sites, and it has been speculated that microtubules destabilize adhesions (18, 20). Furthermore, it has been observed that depolymerization of microtubules leads to activation of RhoA (3, 9), whereas repolymerization of microtubules following disruption occurs with activation of Rac1 (47).

In this study, we examined whether RhoA and Rac1 regulate wound healing of bronchial epithelial cells. We demonstrate that both RhoA and Rac1 are required for the reorganization of the cytoskeleton for efficient wound closure. We show that inhibition of ROCK leads to activation of Rac1, formation of lamellipodia, and enhanced wound closure. Our results also demonstrate that intact microtubules are required for efficient wound healing of bronchial epithelial cells.

MATERIALS AND METHODS

Reagents. DMEM, penicillin-streptomycin, trypsin-EDTA solution, and PBS were purchased from Gibco Life Technologies (Grand Island, NY). FBS was obtained from Cellgro (Mediatech, Herndon, VA). HEPES, deoxycholic acid, Triton X-100, SDS, sodium vanadate, and nocodazole were purchased from Sigma (St. Louis, MO). Tween 20 was from Bio-Rad (Hercules, CA). Y-27632 dihydrochloride, and nocodazole were purchased from Sigma (St. Louis, MO). HEPES, deoxycholic acid, Triton X-100, SDS, sodium vanadate were obtained from Molecular Probes (Eugene, OR). Tocris Cookson (Ellisville, MO). Rhodamine-phalloidin and SlowFade were obtained from Molecular Probes (Eugene, OR).

Cell culture. 16HBE14o− (16HBE) cells, a simian virus 40 large T antigen-transformed human bronchial epithelial cell line that maintains tight junctions (6), was generously provided by Dr. D. Gruenert (Rochester, NY). 16HBE cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution buffered in 20 mM HEPES and 2.2 g/l NaHCO3, pH 7.2, at 37°C and 5% CO2.

Measurement of wound closure. Cells for wound healing experiments were cultured in 12-well plates, and the medium was exchanged every other day with fresh DMEM until cells were 80% confluent. Adenoviral infections were done in serum-deficient medium for 24 h, after which fresh medium without serum was added and incubated for another 24 h for maximum transfection efficiency. At this stage, monolayers were 100% confluent. Wounds of ~700 μm were produced by scraping the cell monolayers with a pipette tip across the diameter of the well. Images were collected with a Cool Snap-cooled charge-coupled device (CCD) camera (Roper Scientific, Trenton, NJ). Images were mounted on an Eclipse TE300 inverted microscope with a ×10 phase contrast objective (Nikon, Melville, NY) using Metamorph imaging software (version 4.6; Universal Imaging, West Chester, PA). Image dimensions were converted from pixels to micrometers using a calibration image. An outline of the wound in the field was obtained, and the mean wound width was calculated using Metamorph based on the perimeter and area of the wound as previously described (37). Data are expressed as a percentage of the time 0 wound width to normalize variability in wounding from well to well and experiment to experiment. To account for variations in wound closure due to size, wound widths were analyzed only when the size was between 650 and 700 μm. The mean initial wound width was 678 ± 14 μm. Wound width measurements were collected from two different locations in the same well and averaged as one measurement. All results reported are from six independent wells from two separate experiments (n = 6). Cells were washed with Dulbecco’s phosphate-buffered saline (DPBS), and medium containing 10% serum wash medium and without Y-27632 was added and maintained for the duration of the experiment. Images were obtained at the initial time of wounding and then at various times 15 h postwounding. Cell proliferation was minimal during this time (39).

Preparation and expression of adenoviral vectors. To investigate the effects of Rho-GTPases in airway epithelial cells, we used recombinant adenovirus-based expression as an efficient means to express the Rho-GTPases. The infection efficiency of 16HBE was initially evaluated using enhanced green fluorescent protein (EGFP) adenovirus. After 48 h of adenoviral infection, >80% of cells expressed fluorescence, and adenoviral infections did not affect the rate of wound healing. Previous studies have demonstrated that point mutations can result in expression of Rho-GTPase proteins that are either constitutively active (GTP-bound) or dominant negative (GDP-bound) conformational state (45). We utilized these mutations to investigate the mechanism of wound healing of airway epithelial cells. Adenoviral vectors expressing constitutively active RhoA (G14V-RhoA or CA-RhoA), dominant negative RhoA (T19N-RhoA or DN-RhoA), wild-type Rac1 (wt-Rac1), and dominant negative Rac1 (T17N-Rac1 or DN-Rac1), were used in this study. cDNA for G14V-RhoA, T19N-RhoA, and T17N-Rac1 were purchased from the Guthrie Institute. cDNA for RhoA and Rac1 alleles were flanked at the NH2 terminus by three copies of the hemagglutinin epitope. Recombinant adenoviral vectors expressing RhoA and Rac1 were prepared according to a published procedure with a kit provided by Beverly Davidson (Univ. of Iowa, Iowa City, IA) (2). Adenoviral vectors were purified with the use of a kit from BD Biosciences (Palo Alto, CA) and titrated according to the manufacturer’s specifications via a conventional assay (tissue culture infection dose) in HEK-293 cells. The titer of the adenoviral vectors used for infection ranged from 105 to 107 viral particles per milliliter for the different vectors. Cells were infected for 48 h before the experiments. Transfection efficiency was >80% as visualized by EGFP-expressing adenovirus. We also varied the expression of Rho-GTPases by increasing the levels of infection. Titration of the CA- and DN-RhoA mutants was performed, and we observed no cytopathic effect at viral dilutions of 1:10 or higher. In the case of EGFP-expressing adenovirus, the titer was higher (1011), and cytopathic effects were observed at a dilution of 1:104 so that this adenovirus was only used at a dilution of 1:105. At these levels of infection, cell viability was >87% as assessed by trypan blue exclusion, and this viability was comparable with that of uninfected cells.

Immunofluorescent staining. Confluent cells grown on glass coverslips and transfected with the RhoA and Rac1 adenoviral constructs for 48 h were wounded with a pipette tip and serum stimulated or stimulated with the ROCK inhibitor Y-27632 for 2 h. After 2 h, cells were fixed with 3.7% formaldehyde for 10 min at room temperature (RT), washed for 10 min three times with DPBS, and permeabilized with 1% Triton for 3 min at RT. Primary antibodies were diluted in PBS containing 5% BSA and included the antibodies against tubulin (1:250; clone B-5-1-2, Sigma) and vinculin (1:150; clone hVIN-1, Sigma). For the visualization of F-actin, cells were fixed, permeabilized, and incubated with rhodamine-phalloidin (Molecular Probes) for 30 min. Focal adhesion staining for vinculin was done using FITC-conjugated anti-mouse IgG (1:250) as secondary antibody. In double staining for vinculin and tubulin, tetramethylrhodamine B isothiocyanate-conjugated goat anti-mouse IgG (1:150; Zymed) and FITC-conjugated goat anti-mouse IgG (1:250) diluted in goat serum were used as secondary antibodies. Coverslips were washed three times with DPBS and mounted on slides with SlowFade antifade kit (Molecular Probes). Images were collected with a Cool Snap-cooled CCD camera (Roper Scientific) mounted on an Eclipse TE300 inverted
mM EDTA, 2% glycerol, 10% FBS). After 15 h, wounds in control uninfected cells had closed to 15% of the original wound width, and wound healing was unaffected by infection with EGFP-expressing adenovirus. By comparison, wounds in cells infected with CA-RhoA had closed to only 56% of the original wound width, and cells infected with DN-RhoA had closed to 33%. Under these experimental conditions, both CA-RhoA and DN-RhoA inhibited wound closure. To determine whether the inhibition of wound closure was dependent on the level of expression of CA-RhoA and DN-RhoA, we measured wound healing in cells infected with varying levels of adenovirus. Figure 1B shows that as the level of expression of CA-RhoA or DN-RhoA increased, the inhibition of wound healing was also increased. Cell viability was not affected at these levels of adenoviral infection.

The functionality of adenoviral expression of Rho-GTPases in 16HBE was confirmed by staining with rhodamine-phalloidin and examining changes in stress fibers by microscopic examination. As expected, infection with CA-RhoA increased stress fibers in cells (Fig. 2B) compared with uninfected cells.

**RESULTS**

Both activation and inhibition of RhoA inhibit wound healing. To test the hypotheses that RhoA is required for wound healing of airway epithelial cells but that increased activation of RhoA impairs wound healing, we infected 16HBE cells with adenoviral vectors expressing DN-RhoA or CA-RhoA. Wound closure studies of confluent monolayers of 16HBE cells transfected with RhoA mutants showed that expression of CA-RhoA and DN-RhoA both significantly decreased the rate of wound healing compared with control uninfected cells (Fig. 1A). After 15 h, wounds in control uninfected cells had closed to 15% of the original wound width, and wound healing was unaffected by infection with EGFP-expressing adenovirus. By comparison, wounds in cells infected with CA-RhoA had closed to only 56% of the original wound width, and cells infected with DN-RhoA had closed to 33%. Under these circumstances, both CA-RhoA and DN-RhoA inhibited wound closure. To determine whether the inhibition of wound closure is dependent on the level of expression of CA-RhoA and DN-RhoA, we measured wound healing in cells infected with varying levels of adenovirus. Figure 1B shows that as the level of expression of CA-RhoA or DN-RhoA increased, the inhibition of wound healing was also increased. Cell viability was not affected at these levels of adenoviral infection.

The functionality of adenoviral expression of Rho-GTPases in 16HBE was confirmed by staining with rhodamine-phalloidin and examining changes in stress fibers by microscopic examination. As expected, infection with CA-RhoA increased stress fibers in cells (Fig. 2B) compared with uninfected cells.
Uninfected cells did exhibit prominent actin bands at the wound edge, as indicated in the figure, but stress fibers spanning the cells away from the wound edge were much more prominent in CA-RhoA-infected cells. Cells infected with adenovirus expressing EGFP demonstrated actin structures at the wound edge, similar to that of uninfected cells (not shown). Expression of DN-RhoA (Fig. 2C) reduced stress fibers substantially, although cortical actin remained intact. In addition, uninfected control cells and cells expressing CA-RhoA and DN-RhoA also have lamellipodial extensions at the leading edge of the wound (Fig. 2, A–C), suggesting that expression of mutant forms of RhoA had little effect on lamellipodial extensions.

RhoA regulates wound healing via Rac1. Membrane ruffling and lamellipodial extensions occur at the leading edge of a wound as a result of activation of Rac1 (46), but this has not been examined in bronchial epithelial cells. We hypothesized that overexpression of Rac1 would enhance wound healing and that decreased Rac1 activity would inhibit wound healing. To test these hypotheses, we overexpressed wt-Rac1 and DN-Rac1 in 16HBE cells, wounded the monolayers, and measured wound closure. Both overexpression of wt-Rac1 and expression of DN-Rac1 significantly decreased wound healing (Fig. 1A). At 15 h, wounds in cells expressing wt-Rac1 had closed to 64% of the original wound width, whereas cells expressing DN-Rac1 had closed to 44%, compared with 15% for control uninfected cells (Fig. 1). Expression of wt-Rac1 induced intercellular gap formation and loss of cell-cell adhesion (as indicated by arrows in Fig. 2D), whereas DN-Rac1 did not have this effect (Fig. 2E). Visualization of the actin cytoskeleton showed that wt-Rac1 induced a phenotype with intercellular gaps and formation of lamellipodia along the cell periphery at the wound edge (Fig. 2D). We counted a total of 16 intercellular gaps from 5 fields of cells overexpressing wt-Rac1, but no gaps were observed in any of the other fields that we observed for the other conditions shown in Fig. 2. In addition, increased expression of wt-Rac1 induced an increase in cell size at the wound edge, indicating increased cell spreading at the wound edge. Lamellipodial extensions were significantly inhibited by expression of DN-Rac1 (Fig. 2E). Because we hypothesized that wt-Rac1 expression would enhance wound healing and this did not occur, we investigated whether wt-Rac1 induced upregulation of RhoA in 16HBE cells leading to a decrease in the rate of wound closure. We measured the levels of activated RhoA using the pulldown activity assay. In cells infected with wt-Rac1, serum stimulation increased RhoA activity more rapidly than uninfected cells and sustained activity longer (Fig. 3). These results suggest that the inhibition of wound closure in cells expressing wt-Rac1 was due to upregulation of RhoA and disruption of intercellular junctions as indicated by gaps between cells.

To overcome the upregulation of RhoA activity observed with wt-Rac1, we coexpressed wt-Rac1 with DN-RhoA. As shown in Fig. 2F, coexpression of DN-RhoA with wt-Rac1 induced formation of lamellipodia and blocked the formation of intercellular gaps. When we examined wound closure, cells coexpressing DN-RhoA with wt-Rac1 migrated at a rate comparable with that of control uninfected cells (Fig. 4). We observed that coexpression of DN-RhoA with wt-Rac1 resulted...
in a migratory phenotype comparable with uninfected controls but did not enhance the rate of wound closure.

ROCK inhibition stimulates activation of Rac1 and enhances wound healing. Our findings with the coexpressed DN-RhoA and wt-Rac1 mutant suggested that Rac1 activation in the lamellipodia accompanied by decreased RhoA activity resulted in efficient migration of airway epithelial cells. Because downregulation of RhoA could lead to decreased activity of downstream effectors of RhoA, we next examined the signaling mechanisms downstream of RhoA. Several Rho effectors have been identified. Among these effectors, protein kinases of the ROCK family have been reported to mediate effects of RhoA on cell spreading and motility, we determined whether formation of focal adhesions was altered in cells expressing Rac1 (Fig. 6C) exhibited decreased expression of vinculin in the cell body compared with control cells. The density of focal adhesions in cells expressing CA-RhoA (Fig. 6D) was decreased due to the loss of stress fibers compared with cells expressing CA-RhoA (Fig. 6E) with increased focal adhesions and stress fiber formation. The phenotype induced by wt-Rac1 (Fig. 6F) showed increased focal adhesion staining in membrane ruffles and at cell-cell adhesions compared with DN-Rac1 (Fig. 6G), which inhibited membrane ruffle formation and showed decreased vinculin staining at the wound edge. Coexpression of DN-RhoA and wt-Rac1 induced a phenotype with a reduced number of focal adhesions (Fig. 6H) compared with control cells (Fig. 6B), probably due to downregulation of RhoA-induced stress fibers.

Role of microtubules in wound healing. Microtubules have previously been shown to be involved in development of polarity needed for directional cell migration. Polarization and migration require an intact microtubule cytoskeleton (50). This has been attributed to an influence of microtubule signals on the actin cytoskeleton through the generation of active Rac1 to promote formation of lamellipodia or through the targeting of focal adhesions (20, 47). Changes in the polymerization state of microtubules influence RhoA and Rac1 activity, and microtubules interact directly with focal adhesions and promote their turnover (19). These interactions have not previously been investigated in wound closure of bronchial epithelial cell sheets.

To test the hypothesis that microtubules were required for efficient migration of 16HBE epithelial cell sheets, we examined the effect of microtubule disruption by nocodazole on wound healing and focal adhesion assembly. Confluent monolayers of 16HBE cells were incubated in the presence or absence of nocodazole, and wound healing was assayed. As a marker of focal adhesion assembly (7). As can be seen in Fig. 6A, immunofluorescence of cells stained with anti-vinculin antibody showed both cytoplasmic staining and focal adhesion staining at cell borders in control cells immediately following wounding. Two hours after wounding, the localization of vinculin appeared to be more concentrated at the cell borders and particularly at the wound edge (Fig. 6B). Cells treated with Y-27632 (Fig. 6C) exhibited decreased expression of vinculin in the cell body compared with control cells. The density of focal adhesions in cells expressing DN-RhoA (Fig. 6D) was decreased due to the loss of stress fibers compared with cells expressing CA-RhoA (Fig. 6E) with increased focal adhesions and stress fiber formation. The phenotype induced by wt-Rac1 (Fig. 6F) showed increased focal adhesion staining in membrane ruffles and at cell-cell adhesions compared with DN-Rac1 (Fig. 6G), which inhibited membrane ruffle formation and showed decreased vinculin staining at the wound edge. Coexpression of DN-RhoA and wt-Rac1 induced a phenotype with a reduced number of focal adhesions (Fig. 6H) compared with control cells (Fig. 6B), probably due to downregulation of RhoA-induced stress fibers.

Rho-GTPases regulate focal adhesion morphology. Because Rho-GTPases regulate the organization of cytoskeletal structures involved in cell spreading and motility, we determined whether formation of focal adhesions was altered in cells expressing Rho mutants. We examined localization of vinculin as a marker of focal adhesion assembly (7). As can be seen in Fig. 6A, immunofluorescence of cells stained with anti-vinculin antibody showed both cytoplasmic staining and focal adhesion staining at cell borders in control cells immediately following wounding. Two hours after wounding, the localization of vinculin appeared to be more concentrated at the cell borders and particularly at the wound edge (Fig. 6B). Cells treated with Y-27632 (Fig. 6C) exhibited decreased expression of vinculin in the cell body compared with control cells. The density of focal adhesions in cells expressing DN-RhoA (Fig. 6D) was decreased due to the loss of stress fibers compared with cells expressing CA-RhoA (Fig. 6E) with increased focal adhesions and stress fiber formation. The phenotype induced by wt-Rac1 (Fig. 6F) showed increased focal adhesion staining in membrane ruffles and at cell-cell adhesions compared with DN-Rac1 (Fig. 6G), which inhibited membrane ruffle formation and showed decreased vinculin staining at the wound edge. Coexpression of DN-RhoA and wt-Rac1 induced a phenotype with a reduced number of focal adhesions (Fig. 6H) compared with control cells (Fig. 6B), probably due to downregulation of RhoA-induced stress fibers.

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absence of nocodazole, and wound closure was measured for 15 h (Fig. 7). Because pretreatment with nocodazole followed by washout has been previously shown to stimulate Rac1 activity, we also determined whether nocodazole washout would alter wound closure. After 15 h, wounds in cells treated continuously with nocodazole had closed to 68% of the original wound width, whereas cells pretreated with nocodazole for 30 min followed by washout of the drug had an accelerated rate of wound closure. **Fig. 5.** Rho-kinase (ROCK) inhibition accelerates wound closure. 16HBE cells were serum-starved for 24 h before addition of the ROCK inhibitor Y-27632 in media containing serum. Data are means ± SE of 6 independent wells from 2 separate experiments (n = 6). *Significantly different from control, P < 0.05. A: time course of wound closure in cells treated with 0.5 μM Y-27632. B: dose-dependent stimulation of wound closure by Y-27632. Wound widths were measured after 15 h. C: effect of Y-27632 on actin cytoskeleton. Y-27632 inhibited central stress fibers compared with untreated control cells. Images are representative examples of fields examined from multiple samples 2 h after wounding. Bars, 20 μm. D: Y-27632 induced Rac1 activation as determined by glutathione-S-transferase-p21-activated kinase pulldown assay and Western blotting. The time indicated is the time after addition of serum and Y-27632. Shown is a representative blot of 3 individual experiments.

**Fig. 6.** Rho-GTPases alter focal adhesion assembly. 16HBE cells expressing Rho mutants were fixed after the initial wound (A) or 2 h after wound healing (B–H). Focal adhesions were identified using a monoclonal antibody against vinculin. Images at the wound edge were acquired using a ×60 oil-immersion objective and were digitally sharpened using the imaging tools in the Metamorph software. Representative images from at least 5 fields for each condition are shown. A and B: control uninfected cells; C: Y-27632-treated cells; D: DN-RhoA; E: CA-RhoA; F: wt-Rac1; G: DN-Rac1; H: DN-RhoA coexpressed with wt-Rac1. B–H: 2 h after wound healing. Bars, 20 μm.
expression of either CA-RhoA or DN-RhoA inhibited wound closure, and decreased RhoA activity, cell size, and intercellular gap formation, and decreased wound healing. Expression of DN-Rac1 decreased lamellipodial extensions and decreased wound healing. When we coexpressed wt-Rac1 with DN-RhoA, wound healing returned to control levels (Fig. 4).

Previous studies have demonstrated that both inhibition and constitutive activation of Rho-GTPases interfere with biological functions. Low-level expression of both constitutively active and dominant negative forms of RhoA disrupted the gate and fence functions of tight junctions in MDCK cells (17). Both inhibition and activation of Rac1 interfered with hepatocyte growth factor-induced disruption of adherens junctions in MDCK cells (14, 30). Defects in embryonic dorsal closure in Drosophila were observed when either dominant negative or constitutively active forms of Rho GTPases (DRac1, DCdc42, or Rhok) were expressed (13). Our studies show for the first time that both activation and inhibition of RhoA and Rac1 disrupt wound closure of bronchial epithelial cell sheets. There are several potential explanations for these findings. Cycling between GTP- and GDP-bound states may be required for the biological function (42), and shifting the level of activity predominantly to either side may block signaling pathways that emanate from the turnover. Related to this hypothesis, the role of Rho-GTPase signaling in the particular function may be tightly controlled both spatially and temporally. In the case of wound closure, it is likely that the coordinated movement of epithelial cell sheets requires levels of activity of RhoA, for example, that vary from the leading edge to the rear of the cells. Increased RhoA activity may be needed for cell contraction in the central region of the cells, whereas decreased RhoA activity may be necessary at the leading edge to allow relaxation of focal contacts. Alternatively, the biological function may depend on a critical level of activity necessary throughout the cells. We demonstrated that increasing levels of expression of RhoA mutants inhibited wound healing in an expression-dependent manner (Fig. 1B), but this does not rule out RhoA cycling or spatial activity as important components of the mechanism. Finally, expression of dominant negative or constitutively active forms may promote processes that damage the cells. The latter explanation is unlikely in our studies since we noted little difference in cell necrosis in these cells compared with controls as assessed by trypan blue staining. In addition, coexpression of DN-RhoA and wt-Rac1 restored wound healing to control levels (Fig. 4). These results also indicate that inhibition of wound closure in Fig. 1 was not due to cytotoxic effects of the adenovirus since higher levels of virus were present in the coexpression treatment (Fig. 4).

In a previous study by Nobes and Hall (28), microinjection of C3 transferase (a RhoA inhibitor) at low concentrations (70 μg/ml) in rat embryo fibroblasts resulted in the loss of stress fibers and focal adhesions. At fourfold higher concentrations (300 μg/ml), there was loss of substrate adhesions with cell retraction, and wound closure was significantly inhibited (80%). Microinjection of CA-RhoA severely inhibited wound closure but did not increase the number of actin stress fibers in wound edge cells, which were already abundant. Cells injected with DN-Rac1 revealed inhibition of lamellipodial activity and severe effects on cell migration (wound closure was blocked by 80%), whereas microinjection of CA-Rac1 had no inhibitory effect on cell migration, and cells migrated at rates comparable with controls.
The activities of Rho, Rac1, and Cdc42 are tightly coordinated as shown by the regulatory cascade identified in Swiss 3T3 cells, establishing that activation of Cdc42 leads to subsequent activation of Rac1, which in turn activates RhoA (26). Activated Rac1 or platelet-derived growth factor-mediated Rac1 activation was shown to induce the accumulation of Rac1 in membrane ruffles followed by the delayed Rho-dependent formation of stress fibers, suggesting activation of both RhoA and Rac1 (35). Expression of wt-Rac1 in airway epithelial cells caused the formation of intercellular gaps (Fig. 2D), whereas DN-Rac1 did not have this effect (Fig. 2E). Under our experimental conditions, expression of wt-Rac1 induced the upregulation of active RhoA. Wound healing of bronchial epithelial cells involves coordinated action of the cell sheet over time, and early differences in stimulation or inhibition can have a significant effect on wound closure, as we have demonstrated in previous studies involving keratinocyte growth factor (48) and inhibition of cyclooxygenase (37). Although the results shown in Fig. 3 do not offer direct proof that RhoA levels are continuously altered by wt-Rac1 expression, they do demonstrate that activation of RhoA occurs more rapidly and is sustained longer when wt-Rac1 is overexpressed.

To address the potential cross talk between RhoA and Rac1, we analyzed changes in cell morphology and rate of wound healing using coexpressed mutant. Coexpression of DN-RhoA and wt-Rac1 induced a migratory phenotype with enhanced...
Rac1 induces focal complex formation (26), whereas constitutive activation of RhoA. Furthermore, it has been reported that activation of RhoA activities are mutually antagonistic by monitoring the formation of RhoA- and Rac1-dependent adhesion structures (33). Several RhoA effectors are known to be targets of RhoA, such as ROCK, myosin binding of myosin phosphatase, and mammalian homolog of Drosophila diaphanosus (mDia). Chrzansowska-Wodnicka and Burridge (5) have shown that activated Rho stimulates contractility, thereby inducing formation of stress fibers and focal adhesions. Conversely, inhibition of contractility by several different mechanisms led to inhibition of Rho-induced stress fibers and focal adhesions. These findings indicate that stimulation of cell contractility could be an important step in Rho-dependent signaling pathways. Thus our finding that DN-RhoA failed to stimulate wound healing may be due to the need for some contractile activity associated with RhoA during cell migration or the involvement of downstream RhoA effectors other than ROCK. Furthermore, coexpression of DN-RhoA and wt-Rac1 resulted in a rate of wound healing comparable with controls.

Rottner et al. (36) showed that the ROCK inhibitor Y-27632 caused the dissolution of Rho-dependent focal adhesions but not Rac1-dependent peripheral focal complexes (34) that are associated with lamellipodium protrusion and membrane ruffling. The authors suggested that inhibition downstream of RhoA leads to activation of Rac1. Our results support this hypothesis (Figs. 5 and 6). By direct measurement of GTP-Rac, our results demonstrated that inhibition of ROCK with Y-27632 stimulated an increase in Rac1 activity. Concomitant with the loss of central stress fibers, focal adhesions were reduced. We observed that Y-27632 treatment induced a migratory phenotype compared with untreated cells (Fig. 5). Magdalena et al. (24) previously showed that Y-27632 could overcome the inhibition of cell migration caused by interfering with the mDia pathway in NIH/3T3 cells, but Rao et al. (32) showed that Y-27632 decreased cell migration in intestinal epithelial cells. Our results show for the first time that Y-27632 increased Rac1 activity and accelerated wound closure in epithelial cells.

Uehata et al. (44) showed that expression of DN-ROCK blocked the formation of stress fibers and focal contacts but did not suppress Rac1-mediated membrane ruffles. In a recent study on Swiss 3T3 cells, the authors concluded that Rac1 and RhoA activities are mutually antagonistic by monitoring the formation of RhoA- and Rac1-dependent adhesion structures (36). It was shown that focal complexes formed in response to Rac1 differentiated into focal contacts on upregulation of RhoA. Furthermore, it has been reported that activation of Rac1 induces focal complex formation (26), whereas constitutively activated RhoA leads to formation of large focal adhesions (34). We observed that expression of CA-RhoA (Fig. 2B) induced a substantial increase in stress fibers and focal adhesions compared with DN-RhoA (Fig. 2C). Adhesion sites in the lamellipodia of 16HBE cells coexpressing DN-RhoA and wt-Rac1 (Fig. 6) were similar in morphology to the adhesions induced by injecting CA-Rac1 into Swiss 3T3 cells while simultaneously suppressing Rho activity with the ribosylating enzyme C3 transferase (27).

Recent reports have demonstrated that localized Rho-GTPase activity may in part be regulated by microtubules and that microtubules play a role in directed cell migration. Krendel et al. (23) have reported that disassembly of microtubules in HeLa cells can activate RhoA by increasing the amount of free active GEF-H1 (Rho effector), whereas microtubule assembly downregulates RhoA by inactivating GEF-H1. Thus depolymerization of microtubules activates a signaling pathway that involves GEF-H1 and RhoA. This regulatory mechanism may be important in cell migration involving coordination of actin and microtubule networks. Ishizaki et al. (16) reported that in Hela cells, expression of an active mDia1 aligns microtubules in parallel to F-actin bundles along the long axis of the cell, terminating in a focal adhesion. In another study, Tsuji et al. (43) demonstrated that RhoA signaling in Swiss 3T3 fibroblasts has a built-in switch for Rac1 activation. This switch is mediated by mDia and inhibited by ROCK. Together, these results indicate that RhoA-dependent Rac1 activation may be switched on in a positive-feedback mechanism by mDia1. We demonstrated for the first time that disruption of microtubules with nucodazole significantly inhibited wound repair of bronchial epithelial cells. We observed that microtubule disassembly caused extensions of membrane protrusions at the wound edge. Our studies indicate that microtubules may be required for destabilizing focal adhesions at the wound edge of migrating cells. In contrast, nucodazole pretreatment for 30 min followed by washout induced a phenotype with microtubules growing into the lamellipodium and a reduced number of focal adhesions. We speculate that reassembly of microtubules following nucodazole washout leads to downregulation of RhoA (23) and activation of Rac1 (47), but this was not measured directly in our system. The similarity we observed in wound closure of 16HBE cells in the presence of Y-27632 and cells pretreated with nucodazole can be explained by the possibility that both treatments cause cell contraction accompanied by activation of Rac1 and targeting of focal adhesions by microtubules.

We conclude that wound closure of airway epithelial cells involves several coordinated events that are dependent on regulated levels of Rho-GTPase activity. We demonstrated that wound closure was impaired by constitutive activation of RhoA, by dominant negative-induced inhibition of RhoA or Rac1, or by overexpression of wt-Rac1. We showed that inhibition of ROCK with Y-27632 increased Rac1 activity and accelerated wound closure. Our studies are the first to demonstrate that microtubule disruption inhibits wound closure of epithelial sheets. Also, we demonstrated that microtubule disruption followed by washout resulted in an enhanced migratory phenotype. Further studies might investigate the role of Rho effectors and microtubule dynamics in inducing activation of Rho-GTPases and targeting focal adhesions to stimulate migration of airway epithelial cells.
REFERENCES


