Organized migration of epithelial cells requires control of adhesion and protrusion through Rho kinase effectors

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Hopkins AM, Pineda AA, Winfree LM, Brown GT, Laukoetter MG, Nusrat A. Organized migration of epithelial cells requires control of adhesion and protrusion through Rho kinase effectors. Am J Physiol Gastrointest Liver Physiol 292: G806–G817, 2007. First published November 30, 2006; doi:10.1152/ajpgi.00333.2006.—Migration of epithelial cell sheets, a process involving F-actin restructuring through Rho family GTPases, is both physiologically and pathophysiologically important. Our objective was to clarify the mechanisms whereby the downstream RhoA effector Rho-associated coil-coil-forming kinase (ROCK) influences coordinated epithelial cell motility. Although cells exposed to a pharmacological ROCK inhibitor (Y-27632) exhibited increased spreading in wound closure assays, they failed to migrate in a cohesive manner. Two main phenomena were implicated: the formation of aberrant protrusions at the migrating front and the basal accumulation of F-actin aggregates. Aggregates reflected increased membrane affiliation and detergent insolubility of the actin-binding protein ezrin and enhanced coassociation of ezrin with the membrane protein CD44. While F-actin aggregation following ROCK inhibition was recapitulated by inhibiting myosin light chain (MLC) phosphorylation with the MLC kinase inhibitor ML-7, the latter did not influence protrusiveness and, in fact, significantly decreased cell migration. Our results suggest that excessive protrusiveness downstream of ROCK inhibition reflects an influence of ROCK on F-actin stability via LIM kinase 1 (LIMK-1), which phosphorylates and inactivates cofilin. Y-27632 reduced the levels of both active LIMK-1 and inactive cofilin (phospho forms), and expression of a dominant negative LIMK-1 mutant stimulated leading edge protrusiveness. Furthermore, Y-27632-induced protrusions were partially reversed by overexpression of LIMK-1 to restore cofilin phosphorylation. In summary, our results provide new evidence suggesting that adhesive and protrusive events involved in organized epithelial motility downstream of ROCK are separately coordinated through the phosphorylation of (respectively) MLC and cofilin.

CELL MIGRATION is a dynamic and highly regulated process that shapes the organization of every tissue in the body. Whether it involves the extension of neuronal dendrites to transfer electrical signals or the mobilization of leukocytes at sites of infection, cell migration is intrinsic to several developmental and physiological processes. However, cell migration also plays an important role in several pathophysiological processes including cancer. As the majority of solid tumors are carcinomas derived from epithelial tissue, a better understanding of epithelial cell migration and the factors that drive it is important for our understanding of cancer. This firstly requires an appreciation of the fundamental differences between the migration of epithelial cells versus single cells such as leukocytes (for reviews, see Refs. 25 and 51). An important difference is that epithelia by nature must maintain their intercellular contacts during migration; thus, migrating epithelial cells must couple cohesive forward movement with coordinated dynamic retraction at the cell rear (12).

Since any form of migration necessitates extensive reorganization of the F-actin cytoskeleton, an examination of the signaling pathways governing the cytoskeletal structure is important. Rho family GTPases such as Rho, Rac, and Cdc42 orchestrate key F-actin rearrangements including the development of (respectively) stress fibers, lamellipodia, and filopodia (24, 33, 34, 36–38). Several RhoA-dependent cytoskeletal rearrangements are regulated by the effector Rho-associated coil-coil-forming kinase (Rho kinase/ROCK). However, the role of ROCK in orchestrating single cell versus coordinated epithelial migration is incompletely understood. For example, pharmacological inhibition of ROCK has been shown to enhance cell migration in single cell fibroblast models (28, 32), whereas the same pharmacological inhibitors have shown promise as antimotility agents in models of tumor epithelial cell migration (20, 40, 43).

In light of these ambiguities, the aim of our study was to define the role of ROCK in coordinated epithelial cell motility. ROCK has been shown to be important in the closure of small oligocellular epithelial wounds (39), so our model addressed the closure of larger wounds by sheet migration rather than purse string-based contraction. Many parallels have been drawn between cell migration in wound healing and tumor development (10, 16), so in our models of cell migration we performed wound closure experiments under conditions where the function of ROCK had been antagonized. We also reproduced the same conditions of ROCK inactivity in unwounded models of epithelial colony spreading to exclude any anomalies associated with models of a purely pathophysiological nature.

Our experiments revealed interesting results that help to reconcile previously published reports of both stimulatory and inhibitory outcomes of ROCK inhibition. Specifically, our morphological experiments showed that epithelial cell migration is artificially driven forward in the absence of ROCK activity but at the expense of epithelial sheet integrity, as evidenced by tearing of migrating sheets. This was associated with two distinct abnormalities at morphological and biochemical levels. First, ROCK inhibition stimulated the formation of...
abnormal F-actin aggregates, which coincided with an increased abundance of ezrin/CD44 focal cell-matrix contacts. Second, ROCK inhibition was also responsible for stimulating excessive protrusiveness at the migrating edge of both wounded epithelial sheets and unwounded epithelial colonies. Our investigations suggested that abnormal aggregates are linked to alterations in regulatory light chain of myosin II (MLC) phosphorylation downstream of ROCK, whereas abnormal protrusive events link to LIM kinase 1 (LIMK-1)-mediated phosphorylation of the actin-depolymerizing factor cofilin (also downstream of ROCK). Thus, despite an apparent enhancement of epithelial cell motility following ROCK inhibition, this actually represents a false effect in which the forward extension and rear retraction events necessary for organized epithelial migration are apparently uncoupled. Therefore, our results support the potential use of pharmacological ROCK inhibitors as net inhibitors of epithelial cell motility, which may have implications in preventing the spread of epithelial carcinomas.

**EXPERIMENTAL PROCEDURES**

**Cell culture, wounding, and pharmacological inhibition of ROCK.** T84 and Caco-2 intestinal epithelial cells (American Type Culture Collection, Rockville, MD) were passaged as described previously and seeded on 0.33- or 5-cm² collagen-coated permeable supports (Costar, Cambridge, MA) (18). Mycoplasma testing was routinely performed on all parental cultures to confirm the absence of contamination. Scratch wounds were made through a metal tip or wounding comb (for 0.33- and 5-cm² filters, respectively). Monolayers were allowed to migrate for 18 h in antibiotic-free, serum-free medium in the presence of the ROCK inhibitor Y-27632 (50 μM, Calbiochem, San Diego, CA) (17, 19, 48) or vehicle (sterile water). For wound closure measurements, monolayers on 24-well plates were scratch wounded and photographed at 0 and 18 h on an inverted phase-contrast microscope linked to a charge-coupled device camera (Olympus). A minimum of three replicates were used per condition. In addition to T84 and Caco-2 cells, the nontransformed rat intestinal epithelial cell line IEC-6 (35) was also used as an internal control to exclude artifactual observations in immortalized cell lines. Wound sizes were measured at each time point by taking the average length of six parallel lines from edge to edge of each wound using Scion Image freeware (Scion Image, Frederick, MD). Data from at least three independent experiments were pooled and statistically analyzed using two-tailed unpaired Student’s t-tests.

**Transient transfections.** Caco-2 cells were plated at low density on coverslips and transfected after 24 h with 0.4 μg myc-tagged wild-type (WT) ROCK or a kinase-defective, Rho-binding dominant negative (DN) mutant of p160ROCK (KDIA ROCK) in pCAG-myc plasmids (a kind gift of S. Narumiya, Kyoto, Japan). A 2:1 (v/v) ratio of FuGENE 6 (Roche Applied Science, Indianapolis, IN) to DNA was used throughout. Monolayers were harvested at 48 h, fixed in 3.7% paraformaldehyde (10 min at room temperature), permeabilized in 0.5% Triton X-100 (30 min at room temperature), and immunostained for myc and F-actin as described below. For LIMK-1 experiments, Caco-2 cells were transfected with LIMK-1 constructs in pcDNA3 plasmids as follows: myc-tagged full-length mouse LIMK-1 or a DN form of LIMK-1 lacking part of the catalytic domain (LIMK-1s) (1). LIM plasmids were a kind gift of P. Caroni (Basel, Switzerland). Transfected cells were treated with Y-27632 (50 μM) or vehicle 1 h after transfection, harvested at 48 h, and stained for myc and F-actin as described below.

**Immunofluorescence/confocal microscopy.** Cells were fixed in ice-cold ethanol (20 min at −20°C) or paraformaldehyde (3.7%), with the latter subsequently being permeabilized in 0.5% Triton X-100 for 30 min. After being blocked in 5% normal goat serum or 3% BSA (1 h at room temperature), monolayers were incubated (1 h at room temperature) with primary antibodies to CD44 (Santa Cruz Biotechnology, Santa Cruz, CA), ezrin (BD Biosciences Pharmingen/Transduction, San Diego, CA), or myc (BD Biosciences/Clontech, Palo Alto, CA) followed by fluorophore-conjugated secondary antibodies (Alexa dye series, Molecular Probes, Eugene, OR). Nuclei were stained using To-Pro-3-iodide (Molecular Probes, 10 min at room temperature). Phalloidin coupled to Alexa-488 or Alexa-568 dyes (Molecular Probes) was used (1 h at room temperature) to visualize F-actin in paraformaldehyde-fixed cells. To visualize Triton X-100-insoluble ezrin, monolayers were washed in HBSS¹⁺ and orbitally rotated (30 min at 4°C) in intracellular-type buffer [containing 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 10 mM HEPES (pH 7.4), 1% Triton X-100, and protease/phosphatase inhibitor cocktails (Sigma, St. Louis, MO)]. Triton-soluble proteins were washed away, and monolayers were stained as above for Triton X-100-insoluble ezrin. Monolayers were mounted in 1:1:0.01 (vol/vol/vol) PBS-glycerol-p-phenylenediamine or ProLong (Molecular Probes) and visualized on a Zeiss LSM510 metaconfocal microscope (Carl Zeiss Microimaging, Thornwood, NY). Composite images were built using Adobe Photoshop.

**Biochemistry.** To evaluate the effect of ROCK inhibition on levels of key proteins in Triton X-100-insoluble pools likely affiliated with the cytoskeleton (7, 49), wounded T84 monolayers on 5-cm² permeable supports were allowed to migrate for 18 h in the presence or absence of Y-27632 (50 μM). Monolayers were washed in HBSS¹⁺ and rotated for 30 min at 4°C in intracellular buffer (as above) containing 1% Triton X-100 and protease/phosphatase inhibitors. The Triton X-100-soluble fraction was removed, centrifuged to remove cell debris, and mixed with an equal volume of 2X Laemmli sample buffer. Cells remaining on the permeable supports, constituting the Triton X-100-insoluble protein pool, were scraped into Laemmli sample buffer. Equal volumes of both fractions were subjected to SDS-PAGE and Western blot analysis. Biochemical levels of key proteins involved in cell migration were also separately assessed in membrane versus cytosolic fractions of Y-27632-treated epithelia. Cells were washed in HBSS¹⁺, scraped into detergent-free intracellular buffer (as above) containing protease and phosphatase inhibitors, and boiled with anti-CD44 or isotype-matched control IgG. Bound CD44 was retrieved with protein G-Sepharose (3 h at 4°C), washed and boiled in sample buffer, and Western blotted with antibodies to CD44 (Santa Cruz Biotechnology, Santa Cruz, CA), ezrin (BD Biosciences Pharmingen/Transduction, San Diego, CA), or myc (BD Biosciences/Clontech, Palo Alto, CA) followed by fluorophore-conjugated secondary antibodies (Alexa dye series, Molecular Probes, Eugene, OR). Equivalent protein concentrations of membrane and cytosolic fractions (as determined by BCA assay, Pierce, Rockford, IL) were analyzed by SDS-PAGE/Western blot analysis. Biochemical levels of key proteins involved in cell migration were also separately assessed in membrane versus cytosolic fractions of Y-27632-treated epithelia. Cells were washed in HBSS¹⁺, scraped into detergent-free intracellular buffer (as above) containing protease and phosphatase inhibitors, and boiled with anti-CD44 or isotype-matched control IgG. Bound CD44 was retrieved with protein G-Sepharose (3 h at 4°C), washed and boiled in sample buffer, and Western blotted with antibodies to CD44 (Santa Cruz Biotechnology, Santa Cruz, CA), ezrin (BD Biosciences Pharmingen/Transduction, San Diego, CA), or myc (BD Biosciences/Clontech, Palo Alto, CA) followed by fluorophore-conjugated secondary antibodies (Alexa dye series, Molecular Probes, Eugene, OR). Equivalent protein concentrations (as determined by BCA assay) were electrophoresed and immunoblotted for ezrin.
RESULTS

Altered migration of ROCK-inhibited epithelial sheets is associated with F-actin aggregation and abnormal forward protrusiveness. Rho family GTPases and the major RhoA downstream effector ROCK exert significant control over F-actin restructuring (24, 33, 34, 36–38), such as that required during cell migration. Our aim was to dissect the role of ROCK in organized migration of epithelial sheets, a migratory process distinct from that of single (nonattached) cells. We first determined the migration of scratch-wounded epithelial monolayers treated with the specific ROCK inhibitor Y-27632. Our choice of inhibitor concentration (50 μM), although high (8), has been used in another important study (38) and also coincides with our previous observations of selective effects on F-actin without disruption of intercellular junctions or inhibition of protein kinase A/C activity (Ref. 49 and data not shown). Furthermore, although we did notice similar functional effects in both cell types at lower concentrations of the inhibitor, 50 μM was our preferred choice because of consistent effects in the two cell types, allowing for a meaningful cross-comparison between the two cell types.

ROCK inhibition significantly enhanced the migration of both T84 and Caco-2 epithelial monolayers (relative to vehicle controls) at 18 h postwounding (Fig. 1A). Overall, migratory characteristics were similar in both epithelial cell types. Cells at the leading edge of control monolayers consistently flattened out into the wound parallel to each other and perpendicular to the direction of migration. As shown for T84 cells in Fig. 1B, migrating edges of control cells were smooth and extended in an organized fashion into the wound space without the formation of any gaps between cells. In contrast, leading edges of cell sheets exposed to Y-27632 were highly irregular, with holes frequently observed to form between cells (Fig. 1B, arrowheads).

To examine whether irregularities at the migrating edge of epithelial cells exposed to Y-27632 reflected a pathological response to damage or a global effect on epithelial sheet spreading, we examined spreading in unwounded subconfluent

Fig. 1. Rho-associated coil-coil-forming kinase (ROCK) inhibition influences coordinated migration and F-actin restructuring in epithelial cells. A: T84 and Caco-2 epithelial monolayers were wounded and allowed to migrate for 18 h in the presence of the pharmacological ROCK inhibitor Y-27632 (50 μM) or a vehicle control (sterile water). Wound images were captured with a charge-coupled device camera, and wound diameters measured at times of 0 and 18 h to calculate the percent wound closure. Y-27632 treatment significantly enhanced wound closure to a similar degree in both cell types. Data represent means ± SE of 4 independent experiments with several replicates per experiment. B: wounded T84 monolayers treated with Y-27632 or vehicle were photographed by phase-contrast microscopy after 18 h of migration. Whereas control epithelial cells migrated as a cohesive sheet, prominent holes (arrowheads) were observed to form behind the leading edge of the wound in ROCK-inhibited monolayers. C: phase-contrast images of T84 epithelial colonies spreading in the presence or absence of Y-27632 (50 μM). Control colonies displayed smooth regular edges spreading uniformly into the wound, whereas the spreading edges of ROCK-inhibited colonies were jagged and cells at the leading edge extended excessive abnormal protrusions (arrows) into the wound. D: wounded T84 epithelial monolayers were allowed to migrate for 18 h in the absence or presence of Y-27632 (50 μM). F-actin was labeled with Alexa-568-phalloidin and analyzed by confocal microscopy. Abundant F-actin filaments were seen at the leading edge and base of control migrating cells (arrow). F-actin aggregates (arrowhead) were observed behind the leading edge of epithelial cells exposed to Y-27632. Note the gap (*) in the migrating epithelial sheet exposed to Y-27632. Reconstructed images in the xy plane highlight F-actin aggregates in epithelial cells migrating in the presence of Y-27632 (arrowhead). E: nontransformed rat intestinal epithelial IEC-6 cells were wounded and allowed to migrate for 18 h in the presence or absence of Y-27632 (50 μM). F-actin labeling in control cells revealed typical basal stress fiber networks close to the wound edge (arrows). Organized stress fiber networks were not visible in ROCK-inhibited cells, but F-actin aggregates were observed (arrowheads). Gaps were also seen close to the leading edge (•).
T84 epithelial monolayers exposed to Y-27632 (Fig. 1C). The spreading edges of control unwounded colonies extended smoothly and uniformly into the vacant space, analogous to the spreading behaviour of wounded epithelial sheets. In contrast, the edges of spreading colonies exposed to Y-27632 were not smooth and regular but rather extruded multiple aberrant protrusions (arrows in Fig. 1). As in the wounding assay, significant gaps were observed behind the spreading edges of ROCK-inhibited cells (Fig. 1C, asterisk).

Since the Rho-ROCK pathway regulates F-actin restructuring, we analyzed F-actin organization in wounded T84 epithelial cells by rhodamine phalloidin labeling and confocal microscopy. Under control conditions, F-actin was enriched in the tips of lamellipodia in migrating cells [Fig. 1D, en face (xy), arrow]. The same enrichment was not observed in lamellipodia from ROCK-inhibited cells, and, in addition, Y-27632 induced the formation of prominent F-actin aggregates behind the leading edge (Fig. 1D, arrowhead). As noted in the phase-contrast micrographs in Fig. 1B, holes were visible behind the leading edge of ROCK-inhibited monolayers (Fig. 1D, asterisk). In the reconstructed xz or vertical plane, F-actin was visualized in subcortical membrane networks, at the cell base, and at the leading edge of control migrating cells. Prominent intracellular F-actin aggregates were observed in the rear of T84 cells migrating in the presence of Y-27632 (Fig. 1D, open arrowhead).

Since both T84 and Caco-2 cell lines are immortal cell lines derived from colonic tumors, we also tried to exclude the possibility that the observed effects of ROCK inhibition were artifacts of malignant transformation. Thus, we examined F-actin restructuring in response to Y-27632 in IEC-6 cells, a nontransformed rat intestinal epithelial cell line. As shown in Fig. 1E, control IEC-6 cells displayed prominent F-actin stress fiber bundles toward the wound edge (arrows); in contrast, IEC-6 cells exposed to Y-27632 (50 µM for 18 h) showed fewer stress fibers along with evidence of monolayer discontinuities (asterisks) and F-actin aggregates (arrowheads).

To verify that such effects were due to ROCK inhibition rather than nonspecific effects of the inhibitor Y-27632 on other signaling systems, we transiently transfected Caco-2 epithelial cells with myc-tagged DN ROCK (KDIA ROCK) and analyzed the restructuring of F-actin (red) in small spreading colonies of 5–10 cells in size (Fig. 2). To focus our analysis on the contribution of ROCK to cell spreading, the only colonies photographed were those containing transfected cells at spreading edges (rather than in the nonspreading center). Due to the high concordance previously noted between unwounded and wounded epithelial models (Fig. 1), wounding experiments were not performed as quantitation would be meaningless unless the transfection efficiency was almost 100%. Control epithelial cells were transfected with myc-tagged empty vector (EV) or WT ROCK, and transgene expression in all cases was verified by immunofluorescence labeling of the myc epitope tag (green).

F-actin restructuring in spreading cells transfected with EV (Fig. 2, top, green) was visually indistinguishable from that in surrounding nontransfected cells. However, as ROCK is known to be expressed in both Caco-2 (39) and T84 (50) cells,
it can be assumed that some basal level of ROCK activity would exist in these models and contribute to the observed structural organization of F-actin. In contrast, cells overexpressing myc-tagged WT ROCK exhibited prominent basal F-actin fibers (Fig. 2, middle, arrow, green). Due to the detrimental effects of transfecting cells with myc-tagged KDIA ROCK (Fig. 2, bottom, green), we observed large distended cell “islands” containing prominent intracellular F-actin aggregates (arrowheads) rather than colonies of organized epithelial cells. This resembled the abnormal spreading events observed in monolayers exposed to Y-27632 (Fig. 1).

**ROCK inhibition promotes the formation of membrane-linked adhesion complexes in migrating epithelial sheets.** Having determined that the two main phenomena arising from ROCK inhibition in migrating epithelial cells were 1) abnormal basal F-actin aggregation behind the leading edge and 2) excessive cellular protrusiveness at the leading edge, we next set about establishing which pathways downstream of ROCK activity could be responsible for such effects.

Dealing first with the former, we speculated that basal aggregates of F-actin could reflect enhanced binding to basal actin-binding proteins that anchor to the extracellular matrix. Thus, we examined the impact of ROCK inhibition on the F-actin-binding protein ezrin, a member of the ezrin-radixin-moesin (ERM) family that binds CD44 in the cell membrane to form a bridge between the cytoskeleton and matrix. Our focus was on ezrin since it, unlike other ERM family members, is heavily expressed in intestinal epithelial cells (47). Confocal localization experiments revealed an enrichment of ezrin at the leading edge of wounded control migrating monolayers (Fig. 3A,a, en face, arrow). In the reconstructed xz or vertical plane (Fig. 3A,b), ezrin was observed to localize predominantly at the basal membrane of cells at the leading edge. Pools of ezrin were also observed in the apical and basolateral membranes of migrating control cells just behind the wound edge (Fig. 3A,b, xz plane). Nuclei localized at the basal membrane of these cells. In contrast, ezrin-enriched fringes were not typically observed at the leading edge of epithelial sheets migrating in

**Fig. 3.** Localization, polarization, and membrane affiliation of ezrin in migrating cells is altered by ROCK inhibition. **A:** the F-actin-binding ezrin-radixin-moesin protein ezrin (green) was immunolocalized in T84 cells migrating in the presence of vehicle (a and b) or Y-27632 (c and d; 50 μM) for 18 h. Nuclei were visualized with Tro-Pro3 (red). In control cells, ezrin was enriched at the leading edge (le), and it was distributed beneath the membranes. In contrast, ROCK inhibition prevented ezrin from concentrating at the leading edge of migrating cells (c), and monolayer discontinuities (*) were observed in both en face (c) and xz (d) planes. Prominent basal ezrin aggregates (arrowheads) were identified in these cells. **B:** membrane and cytosolic fractions were prepared from migrating T84 epithelial cells incubated with vehicle or Y-27632 for 18 h and immunoblotted for ezrin. The majority of ezrin was cytosolic in both treatment groups. ROCK inhibition induced cosedimentation of a pool of ezrin with cellular membranes. **C:** Triton-X-100-soluble (S) and -insoluble (I) extracts were prepared from wounded T84 epithelia migrating for 18 h in the presence or absence of Y-27632 and immunoblotted for ezrin. ROCK inhibition induced an increase in detergent-insoluble ezrin. **D:** wounded T84 epithelia migrating for 18 h in the presence or absence of Y-27632 (50 μM) were immunostained for total ezrin versus Triton X-100-insoluble ezrin. Triton X-100-insoluble ezrin was virtually undetectable in control monolayers, but prominent aggregates of detergent-insoluble ezrin (arrowheads) were observed behind the leading edge of ROCK-inhibited epithelial cells.
the presence of Y-27632 (Fig. 3A,c, en face). Discontinuities were frequently seen in migrating epithelial sheets (Fig. 3A, asterisks), as also observed in Figs. 1 and 2. In the xz plane, Y-27632-treated cells extruded lamellipodia similar to control cells, but ezrin failed to localize to the leading edge or the apical/basolateral membranes. Instead, ezrin was distributed in prominent aggregates close to the basal membrane of ROCK-inhibited cells (Fig. 3A,d, arrowheads). Furthermore, unlike under control conditions, nuclei of cells exposed to Y-27632 did not polarize along the cell base.

To biochemically understand the nature of ezrin aggregates at the basal membrane of ROCK-inhibited migrating cells, we investigated whether Y-27632 treatment increased ezrin affiliation with cell membranes (Fig. 3B). As ezrin is a cytosolic protein, increased membrane association should indicate its presence in a complex with a membrane-binding partner such as CD44. In extracts from control migrating epithelia, ezrin partitioned mainly into cytosolic fractions (Fig. 3B). In ROCK-inhibited migrating epithelia, the vast majority of ezrin also partitioned into cytosolic fractions; however, a significant band was also detected in membrane fractions.

In a complementary approach, we also probed the expression of ezrin in Triton X-100-insoluble cellular fractions, which enrich for membrane/cytoskeletal elements (Fig. 3C). The majority of ezrin in both control and ROCK-inhibited cells was Triton X-100 soluble. However, ROCK inhibition greatly increased the pool of ezrin recovered from Triton X-100-insoluble fractions. The Triton X-100 solubility profiles of occludin and E-cadherin, two proteins involved in cell-cell adhesion (unlike ezrin, which is associated with cell-matrix adhesion), were unaffected by ROCK inhibition (data not shown). In parallel, migrating T84 epithelial monolayers were immunostained for Triton X-100-insoluble ezrin (Fig. 3D). A large increase in immunoreactivity of Triton X-100-insoluble ezrin was observed in monolayers treated with the ROCK inhibitor Y-27632. This was distributed mainly as prominent aggregates behind the leading edge (Fig. 3D, arrowheads), consistent with our other observations. The validity of our detergent extraction immunostaining procedure was verified by the fact that F-actin was Triton X-100 insoluble in these models (data not shown).

Since the increased aggregation and membrane affiliation of ezrin likely reflected binding to the membrane-anchored cell adhesion molecule CD44, we next investigated if ROCK inhibition enhanced the coprecipitation of CD44 and ezrin. As shown in Fig. 4A, ROCK-inhibited T84 cells exhibited an approximately twofold increase in CD44-ezrin coprecipitation relative to that in control migrating cells. No ezrin was pulled down in migrating cells immunoprecipitated with a control IgG antibody instead of CD44. Increased levels of actin were also detected in CD44 immunoprecipitates of ROCK-inhibited cells (data not shown), suggesting that ROCK inhibition enhanced the recovery of a ternary complex containing CD44, ezrin, and F-actin. We did not observe alterations in total CD44 protein

A

IP CD44

Control

Y-27632

IgG

Ezrin ~81 kDa

IgG heavy chain

B

Control

Y-27632

Fig. 4. Y-27632 increases CD44/ezrin affiliation and alters CD44 localization. A: CD44 was immunoprecipitated from wounded T84 epithelial monolayers migrating for 18 h in the presence of vehicle or Y-27632 (50 μM), and immunoblotted for ezrin. Densitometric analysis of immunoblotted protein revealed that ROCK inhibition induced a 2-fold increase in the coprecipitation of CD44 and ezrin. Control samples were immunoprecipitated with isotype-matched IgG. Similar results were obtained in 3 independent experiments. B: wounded T84 epithelial monolayers migrating in the presence or absence of Y-27632 were immunostained for CD44 (green), F-actin (red), and nuclei (blue) and analyzed by confocal microscopy. CD44 was enriched at the tips of F-actin filaments (arrow) extending into control wounds and along the leading edge. ROCK inhibition influenced CD44 distribution at the leading edge, in favor of a more diffuse cytoplasmic localization of CD44 with holes frequently observed behind the leading edge (*).
levels, membrane affiliation, or detergent solubility profiles following ROCK inhibition (data not shown).

We also examined whether the morphological localization of CD44 protein could be altered following ROCK inhibition in migrating T84 cells (Fig. 4B). In control cells, CD44 (Fig. 4B, green) was enriched in a fringe-like pattern at the leading edge and localized at the tips of F-actin stress fiber bundles (Fig. 4B, red, arrow) protruding into the wound. Following incubation with Y-27632, basal F-actin fibers were disorganized, and CD44 was not at the leading edge but rather highlighted the perimeter of gaps (Fig. 4B, asterisk) anterior to the edge. Gap formation (previously commented upon in Figs. 1–3) and mislocalization of CD44 in response to Y-27632 was a specific outcome of ROCK inhibition, since disruption of F-actin with cytochalasin D induced the aggregation of CD44 at the leading edge without gap formation (data not shown).

F-actin aggregation but not abnormal protrusiveness can be recapitulated by inhibition of MLC phosphorylation. Having demonstrated that abnormal F-actin aggregates formed in ROCK-inhibited cells reflect increased membrane affiliation of the linker protein ezrin and its binding to the cell-matrix adhesion protein CD44, we were next interested to explore which pathway downstream of ROCK could be involved in this phenomenon. Since an important downstream target of ROCK is MLC, and since phosphorylation of MLC itself regulates cell-matrix adhesion (14, 22), we therefore examined whether aggregates could be recapitulated by direct inhibition of MLC phosphorylation. Wounded T84 monolayers allowed to migrate for 18 h were incubated with the specific MLC kinase (MLCK) inhibitor ML-7 (20 μM) during this time and examined by immunofluorescence/confocal microscopy for the presence of F-actin aggregates. Inhibition of MLC phosphorylation with ML-7 did indeed recapitulate F-actin aggregation behind the leading edge, as observed in both xy and reconstructed xz planes (Fig. 5, A and B, respectively, arrowheads), but protrusiveness was not different from that in control monolayers. Further evidence for a lack of involvement of MLC phosphorylation in stimulating protrusiveness in our models was the observation that ML-7 significantly inhibited epithelial sheet migration (Fig. 5B), in direct contrast to the proprotrusive, promigratory effects of Y-27632 described earlier.

Abnormal protrusiveness downstream of ROCK inhibition is sensitive to cofilin phosphorylation status in migrating epithelial cells. Having linked proaggregative but not proprotrusive activity downstream of ROCK inhibition to possible effects on MLC phosphorylation, we next considered other effector pathways potentially implicated in the abnormal protrusiveness observed following Y-27632 treatment. As protrusiveness critically relates to F-actin filament turnover, we considered the dependence of this phenomenon on the ROCK effectors LIMK-1 and cofilin. Phosphorylation of LIMK-1 by ROCK activates the kinase activity of LIMK-1, which, in turn, phosphorylates and inactivates the actin-depolymerizing factor cofilin. This terminates the activity of active (unphosphorylated) cofilin, which normally binds to F-actin filaments and induces monomer dissociation from the pointed end to facilitate actin polymerization at the barbed end by treadmilling (5). Thus, we considered alterations in cofilin phosphorylation as a potential mechanism for promoting abnormal protrusive activity in ROCK-inhibited migrating cells.

As shown in Fig. 6A, we first examined the effect of Y-27632 on levels of unphosphorylated and phosphorylated LIMK-1 and cofilin in wounded T84 epithelial sheets after 18 h of migration. ROCK inhibition was associated with a 34% increase in inactive (unphosphorylated) LIMK-1 and a 66% decrease in active (phosphorylated) LIMK-1. This was accompanied by a decrease of 41% in inactive (phosphorylated) cofilin levels following treatment with Y-27632. Overall, the expression levels of active (unphosphorylated) cofilin and actin were unchanged by ROCK inhibition.

To investigate whether reduced levels of inactive (phosphorylated) cofilin were responsible for abnormal protrusiveness in migrating epithelial cells, we transiently transfected Caco-2 cells with LIMK-1s, which exerts negative effects on cofilin phosphorylation (1). Spreading epithelial cells stained for F-actin (Fig. 6B, green) and expressing LIMK-1s (Fig. 6Ba, red fluorophore) extended broad irregular protrusions atypical of the rest of the spreading colonies. To further probe this, we next reasoned that restoring cofilin phosphorylation to ROCK-inhibited cells should correct the abnormal protrusive phenotype. We therefore transfected Caco-2 cells with full-length LIMK-1 and subsequently treated colonies for 18 h with Y-27632 or vehicle control. Characteristic basal F-actin networks (Fig. 6B, green) and wide F-actin “cables” were identified in the spreading edge of control cells (Fig. 6Bb, asterisk). F-actin organization was similar in leading edge cells overexpressing LIMK-1 (Fig. 6Bc, red). In contrast, exposure to Y-27632 induced F-actin aggregation (Fig. 6Bd, open arrows) and numerous long “filopodial-like” protrusions (Fig. 6B, arrowheads) in addition to broad protrusions (Fig. 6B, dashed arrow). F-actin aggregates (Fig. 6Be, arrow) were not normalized by overexpression of LIMK-1 in ROCK-inhibited cells (Fig. 6Be, red cell); however, no protrusions were observed in ROCK-inhibited cells overexpressing LIMK-1. Thus, our data are consistent with the model that actin-rich protrusions downstream of ROCK inhibition result from reduced cofilin phosphorylation, whereas abnormal actin aggregates in the same system relate to changes in MLC phosphorylation (Fig. 5).

DISCUSSION

Migration of epithelial cells is fundamental to both physiological and pathophysiological processes. Since epithelia by nature form biological barriers in which individual cells must tightly associate with each other, epithelial cell migration is thus distinct from that of unattached/single cells (e.g., leukocytes and fibroblasts) due to the propensity of epithelial cells to migrate as a cohesive unit (12).

Migration is dependent on dynamic rearrangements of the F-actin cytoskeleton, such as those mediated by the Rho family of small GTPases. The objective of our study was to evaluate the role of the Rho downstream effector protein ROCK in the organized motility of epithelial cells. Previously published reports have presented intriguing evidence that ROCK inhibitors exerted both stimulatory and inhibitory effects on cell motility (20, 28, 32, 40, 43). Thus, given the relevance of epithelial cell motility to the development or progression of solid carcinomas, we decided to further explore the mechanisms involved in the ROCK-mediated regulation of epithelial cell spreading and motility. Although it is difficult to separate the interrelated concepts of epithelial spreading and motility, it
can be considered that active migration of epithelial cells must at an early stage involve the extension and forward spreading of cells at the motile front. Thus, the terms spreading and migration are often used interchangeably here.

Superficially, ROCK inhibition with the pyridine derivative Y-27632 appeared to increase epithelial motility in wound healing models. However, we attribute this to a false enhancement of cell spreading, achieved through stimulation of excessive protrusiveness at the leading edge. This is consistent with the observation of uncharacteristic filopodial extensions in ROCK-inhibited epithelial cells struggling to close small circular wounds (39). In our experiments, modelling the closure of larger wounds, protrusiveness associated with the loss of ROCK activity correlated with overstretching of migrating sheets into the wound margins. However, promigratory effects at the leading edge were not accompanied by net overall movement of migrating sheets, as holes were frequently seen to form behind the leading edge (suggestive of sheet tearing).

One possible interpretation for our observations of hole formation in response to ROCK inhibition relates to the interesting recent description of transcellular tunnels (or macroapertures) induced in endothelial cells upon ADP ribosylation and thus inhibition of Rho activity by the Staphylococcal toxin EDIN (4). However, in many cases, the holes we observed in response to ROCK inhibition were larger and more irregular than might be predicted for reversible structures. This may relate in part to the strong mechanical forces generated in wounded epithelial sheets, potentially causing sheet tearing.

In addition to excessive monolayer stretching, ROCK inhibition also induced the formation of F-actin-rich aggregates close to the basal membrane of migrating or spreading cells. This was observed both after treatment with the ROCK inhibitor and also in cells expressing DN ROCK. This effect could impact cell-matrix adhesion by influencing the assembly of focal cell-matrix adhesion complexes through membrane-cytoskeletal linker proteins such as those of the ERM family.

Fig. 5. Inhibition of regulatory light chain of myosin II (MLC) phosphorylation with ML-7 impairs wound closure and induces F-actin aggregation in migrating monolayers. A: wounded T84 monolayers treated with vehicle control or ML-7 were allowed to migrate for 18 h. Localization of F-actin by immunofluorescence/confocal microscopy in both xy (en face) and xz planes revealed the formation of F-actin aggregates behind the leading edge of ML-7-treated (arrowheads) but not control monolayers. B: wounded T84 monolayers were allowed to migrate for 18 h in the presence of vehicle or the specific MLC kinase inhibitor ML-7 (20 μM). Wound closure was significantly impaired (>3-fold) by ML-7 relative to the vehicle control.
Fig. 6. Protrusions induced upon ROCK inhibition are dependent on cofilin phosphorylation. Whole cell lysates from wounded T84 cells migrating for 18 h in the presence or absence of Y-27632 were Western blotted for active or inactive cofilin [unphosphorylated and phosphorylated (p-), respectively], active or inactive LIMK-1 (phosphorylated and unphosphorylated, respectively), and total actin. Band intensities of Y-27632-treated samples were densitometrically quantitated and expressed as a percentage of matched control bands for each protein. As shown in the graph (A, right), ROCK inhibition reduced levels of active (phosphorylated) LIMK-1 and inactive (phosphorylated) cofilin. There was a concomitant increase in inactive (unphosphorylated) LIMK-1.

B: spreading Caco-2 epithelial colonies were transfected with myc-tagged LIMK-1 or a dominant negative form of LIMK-1 (LIMK-1s) and incubated with vehicle or Y-27632 (50 μM). Transfected cells were identified by immunolabeling with antibodies to the myc tag (red), whereas F-actin was highlighted with Alexa-488-phalloidin (green). Colonies expressing dominant negative LIMK-1 acquired some features characteristic of ROCK inhibition, namely, the outgrowth of abnormal protrusions (a, arrows). Meanwhile, control colonies (b) and those overexpressing LIMK-1 (c) exhibited regular spreading edges without abnormal protrusiveness. Aberrant F-actin-rich protrusions were observed at the leading edge of ROCK-inhibited epithelial cells (d, arrowheads). Characteristic F-actin aggregates (d, open arrows) were also observed just behind the spreading edge of Y-27632-treated colonies. Restoration of cofilin phosphorylation by overexpression of LIMK-1 in the presence of Y-27632 did not correct the formation of F-actin aggregates (e, arrow), but no aberrant protrusions were observed to form at the leading edge.
Ezrin links F-actin with the extracellular matrix in a Rho-dependent manner (29, 45), and dynamic extracellular matrix-cytoskeletal interactions generate propulsive forces driving migration. Thus, the abnormal ezrin/actin aggregates observed following ROCK inhibition could influence epithelial cell spreading and migration. Although ezrin was largely cytosolic in our model system as has been described in the past (15, 46), ROCK inhibition increased the partitioning of ezrin into membrane-bound and detergent-insoluble cellular pools. This was suggestive of the formation of large complexes affiliated with either (or both) the cytoskeleton and membrane.

Indeed, our results suggest enhanced association of ezrin and the membrane (following ROCK inhibition) via increased binding to the hyaluronic acid receptor CD44. Interestingly, direct association of CD44 variants and RhoA have been reported in metastatic breast cancer cells (3), perhaps regulating cell-matrix adhesion through ROCK signaling. Our data suggest that ROCK inhibition increases cell-matrix anchorage through actin/ezrin/CD44 complexes. Focal increases in matrix adhesion, when coupled with enhanced protrusiveness at the leading edge of migrating epithelial sheets, would likely interfere with cohesive forward movement. This could in part account for the formation of gaps and tears observed in migrating epithelial cells exposed to Y-27632.

Having described two main phenomena in response to ROCK inhibition in migrating epithelia, namely, enhanced protrusiveness and enhanced aggregation of F-actin-enriched complexes, it was next necessary to investigate which elements downstream of ROCK could be important in regulating both these processes. We first considered the relationship between aggregate formation and altered cell-matrix adhesiveness, a phenomenon often linked to phosphorylation of MLC (14, 22). MLC phosphorylation at the rear of migrating cells has been shown to generate tension, which allows bound F-actin filaments to contract and drag the cell rear forward (11). Therefore, we reasoned that reductions in MLC phosphorylation downstream of ROCK inhibition, by influencing tension and cell-matrix adhesion, could cause actin aggregate formation. This is relevant since Y-27632 is known to not only inhibit the ability of ROCK to directly phosphorylate MLC but, more importantly, to enhance MLC dephosphorylation by preventing the inactivation of myosin phosphatase (13, 14, 22).

Our experiments found that direct inhibition of MLC phosphorylation by targeting MLCK did not recapitulate the increased protrusiveness or enhanced migratory capacity of epithelial sheets. However, ML-7-induced formation of F-actin aggregates (similar to those seen in ROCK-inhibited cells) supported a role for MLC phosphorylation in inducing abnormal actin-based adhesion complexes. Our data (not shown), like those of a report (23) using alternative methodology, implicate reductions in di-phospho-MLC as a critical outcome of ROCK inhibition. As acto-myosin tension is influenced by the phosphorylation status of MLC, it is possible that reductions in di-phospho-MLC impair the generation of tension and thus impair retraction at the rear of migrating sheets.

MLC phosphorylation is involved not only in retraction of large wounds but also contraction of small wounds. Elegant work has shown discrete temporal and spatial requirements for ROCK and MLCK in the closure of small circular wounds (39). While ROCK activity is first required to assemble an F-actin ring around the perimeter of a small wound, MLCK activity is subsequently required to mediate purse string contraction of this ring (39). In this latter study, the wounds were small enough to reseal completely in less than 1 h, whereas, in our study, the wounds were larger and had not resealed by 24 h. Thus, in our model, it is likely that changing MLC phosphorylation status (whether indirectly through ROCK or directly through MLCK) would impact primarily on epithelial sheet retraction rather than purse string contraction.

Further evidence that ROCK has an important impact on retraction has come from elegant single cell migration models, from which lessons can be learned about coordinated migration of epithelial cells. ROCK activation has been shown to down-regulate integrin-based adhesive events, allowing tail retraction in transmigrating monocytes (53), and Y-27632 reportedly enhances adhesion and reduces death of neutrophils (27). In addition, the RhoA inhibitor C3-transferase has been shown to inhibit monocyte spreading and cause neutrophil tearing following incomplete rear retraction (54). In vivo, Drosophila embryos null for Rho1 have difficulty retracting the tails of migrating hemocytes (neutrophil/macrophage equivalents) due to incomplete retraction of cell-cell and cell-matrix contacts (44).

As our experiments indicated a possible role for MLC phosphorylation status in influencing abnormal adhesiveness but not protrusiveness in migrating epithelial sheets, we next moved on to investigate other ROCK effector pathways that could be responsible for the excessive protrusiveness observed. We considered the hypothesis that ROCK-mediated alterations in F-actin chain stability could be responsible for the excessive protrusiveness observed during organized epithelial spreading or migration. F-actin chain stability is modulated in part by actin-depolymerizing factors such as cofilin. Cofilin both depolymerizes F-actin and promotes F-actin turnover, which facilitates treadmilling at the leading edge (5, 6). Thus, high levels of active (unphosphorylated) cofilin could facilitate protrusion by freeing G-actin monomers to incorporate into growing filaments at leading edges. In accordance with the latter, dephosphorylation of actin-depolymerizing factor/cofilin has been implicated in growth factor-stimulated neurite outgrowth from neuroendocrine cells (30). Our results are consistent with this, since reductions in phospho-cofilin levels paralleled increased protrusive activity in ROCK-inhibited models. Furthermore, DN LIMK-1 (which also prevents cofilin phosphorylation) had a similar phenotypic effect on epithelial protrusiveness to that of the ROCK inhibitor. This was further backed up by our observations that overexpression of LIMK-1 (to restore cofilin phosphorylation) rescued spreading epithelial colonies from abnormal protrusiveness associated with Y-27632 treatment.

While we did not observe significant effects of WT LIMK-1 on cell polarity, others have observed that expression of constitutively active LIMK-1 interferes with cell polarity and induces the formation of multiple competing lamellipodia in migrating fibroblasts (9). In that system, overexpression of a nonphosphorylatable cofilin mutant rescued the phenotype and restored the formation of single uncompetiting lamellipodia (9). This may relate to differential effects on cofilin phosphorylation levels or perhaps to differential effects in models of single cell migration versus coordinated migration. However, these effects serve to underline the complexities of LIMK-1 signal-
ing and illustrate the potential for changes in cofilin phosphorylation/dephosphorylation to rapidly cell regulate migration.

Nonetheless, other recent reports and our findings favor that the promigratory effects of Rho/ROCK involve the enrichment of select protrusive fronts rather than multiple competing ones (52). This is especially relevant since the effective migration of epithelial sheets requires cells to commit to moving forward in a single coordinated direction rather than in many competing directions. However, it must be remembered that cell motility in wound closure models is essentially a two-dimensional process, whereas cell motility in vivo can occur in two dimensions or into a three-dimensional matrix where, in addition to matrix degradation, cells receive stimuli from the entire surface. Thus, the effects of interfering with F-actin cytoskeletal organization through manipulation of ROCK may differ depending on the site and type of cells. Indeed, ROCK inhibition has been reported to increase the invasion and motility of astrocytoma cells through activation of Rac1 (41), but the balance of published evidence suggests that inhibition of ROCK greatly reduces invasion in diverse cancerous epithelial cell types in Boyden chamber-based assays (2, 20, 26, 31, 42).

Thus, our overall model of the regulatory influence of the RhoA effector ROCK in coordinated epithelial cell motility can be summarized as follows. ROCK activity activates LIMK-1 by phosphorylation, which, in turn, inactivates cofilin by phosphorylation and limits treading of events that would otherwise promote the formation of abnormal unpolarized protrusions at spreading edges. Simultaneously, ROCK-mediated influence upon MLC phosphorylation status limits the accumulation of abnormal actin-based focal adhesion complexes that might promote excessive adhesion or impair adhesion. Thus, the net effect of ROCK inhibition is a detrimental combination of misdirected protrusiveness at the leading edge accompanied by excessive anterior adhesion, which uncouples the coordination necessary for organized epithelial cell migration. To conclude, our study yielded new insights into the importance of ROCK as a coordinator of actin remodeling during organized epithelial migration and endorse the potential of ROCK inhibitors as antitumor agents through their ability to collapse the dynamic coordination of epithelial cell motility.

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