Regulation of epithelial tubule formation by Rho family GTPases

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Eisen, Randi, Shereaf Walid, Don R. Ratcliffe, and George K. Ojakian. Regulation of epithelial tubule formation by Rho family GTPases. Am J Physiol Cell Physiol 290: C1297–C1309, 2006.—Previous work has established that the integrin signal transduction pathway plays an important role in the regulation of epithelial tubule formation. Furthermore, it has been demonstrated that Rho-kinase, an effector of the Rho signaling pathway, is an important downstream modulator of collagen-mediated renal and mammary epithelial tubule morphogenesis. In the present study, MDCK cells that expressed mutant dominant-negative, constitutively active Rho family GTPases were used to provide further insight into Rho-GTPase signaling and the regulation of epithelial tubule formation. Using collagen gel overlays on MDCK cells as a model system, we observed phosphorylated myosin light chain (pMLC) at the leading edge of migrating lamellipodia. This epithelial remodeling led to the formation of multicellular branching epithelial tubular structures with extensive tight junctions. However, in cells expressing dominant-negative RhoN19, MLC phosphorylation, epithelial remodeling, and tubule formation were inhibited. Instead, only small apical lumens with a solitary tight junctional ring were observed, providing further evidence that Rho signaling through Rho-kinase is important in the regulation of epithelial tubule formation. Because the present model for the Rho signaling pathway proposes that Rac plays a prominent but reciprocal role in cell regulation, experiments were conducted using cells that expressed constitutively active RacV12. When incubated with collagen gels, RacV12-expressing cells formed small apical lumens with simple tight junctions, suggesting that Rac1 signaling also has a prominent role in the regulation of epithelial morphogenesis. Complementary collagen gel overlay experiments with wild-type MDCK cells demonstrated that endogenous Rac1 activation levels decreased over a time course consistent with lamellipodia and tubule formation. Under these conditions, Rac1 was initially localized to the basolateral membrane. However, after epithelial remodeling, activated Rac1 was observed primarily in lamellipodia. These studies support a model in which Rac1 and RhoA are important modulators of epithelial tubule formation.

Rac signaling; Rho signaling; tight junction; adherens junction

MULTICELLULAR ORGANISMS CONTAIN a variety of differentiated epithelial cell types that exhibit specialized physiological functions. Epithelial cells are frequently organized into multicellular tubules that form distinct apical and basolateral compartments (28, 32). The apical membrane lining the tubule lumen is morphologically, biochemically, and physiologically distinct from the basolateral membrane that contacts the ECM. These differences in apical and basolateral membrane structure and function are termed epithelial cell polarity (32). To maintain epithelial cell polarity, epithelia have extensive lateral membrane specializations, including tight junctions that seal the extracellular space between adjacent cells and adherens junctions and desmosomes that function in cell-cell adhesion (29, 32).

The Madin-Darby canine kidney (MDCK) cell line has been used extensively as a model system for the study of cell signaling during biogenesis of cell polarity and epithelial tubule formation. MDCK cells cultured in three-dimensional (3D) collagen gels form polarized cysts with apical and basolateral membranes (38, 58, 59). The addition of hepatocyte growth factor (HGF) to these cultures induced the formation of polarized tubular extensions (30, 62). Another approach that has been used is to place collagen gel overlays on MDCK and mammary epithelial cell monolayers (19) to generate either polarized apical lumens or multicellular epithelial tubular structures (19, 40, 41, 54, 65). Mammary epithelial cells cultured on collagen gels attached to culture dishes form monolayers. Detachment of these gels from the culture dish allowed collagen gel contraction accompanied by rearrangement of mammary cells into differentiated epithelial tubules (2, 61).

Elucidation of the cellular mechanisms and signal transduction pathways involved in the regulation of epithelial tubule formation has been the focus of recent studies (15, 28, 39). Of considerable importance in this regulation are integrins, which are membrane receptors that reside primarily on the epithelial basolateral membrane and attach cells to ECM components (2, 9, 29, 44). There is evidence that collagen-mediated MDCK epithelial cell tubule formation is regulated by integrins (43, 51, 54). A major downstream component of integrin signaling is the Rho family GTPase signal transduction pathway (6, 15). Rho-GTPases have prominent roles in the regulation of cell adhesion, motility, polarity development, cytoskeletal dynamics, and intercellular interactions (15, 49). Rac, Rho, and Cdc42 are small Rho family GTPases that regulate the formation of actin stress fibers, lamellipodia, and filopodia (33, 34, 47, 48). Furthermore, Rac and Rho appear to be important in the modulation of adherens junction and tight junction assembly and function (4, 21, 22, 37, 52, 55, 63).

Rho family GTPases function in a reciprocal activation/deactivation cycle that regulates myosin II activity and subsequently cell-ECM and cell-cell interactions as well as nonmuscle cell motility (5, 6, 14, 15). Activated Rho-GTPases exist in a GTP-bound state, whereas inactive Rho-GTPases are bound to GDP (6, 14). Studies of the role of Rho family GTPases in cell regulation have greatly benefited from the availability of mutant forms that are continually bound to either GTP (constitutively active) or GDP (dominant negative). Constitutively active and dominant-negative Rac and Rho cDNA constructs have been used to provide new insights into the role...
of these GTPases in the regulation of adherens junction-mediated intercellular interactions and tight junction permeability (4, 18, 49, 53). Rho-kinase, a downstream effector of Rho signaling, is involved in the regulation of myosin light chain (MLC) phosphorylation and subsequent modulations of stress fiber assembly and non-muscle cell motility (15). Investigators at several laboratories have demonstrated that MDCK and mammary epithelial tubule formation is regulated by Rho-kinase (13, 62, 64). However, there is evidence that MLC kinase activity is not required (13, 62). These studies provided evidence that Rho-kinase signaling is important in the regulation of epithelial tubule biogenesis. In this article, we present further evidence that Rac and Rho signaling are involved in the regulation of epithelial tubule formation.

MATERIALS AND METHODS

Cell culture. MDCK II cells and MDCK II cells stably expressing Rac1- or RhoA-GTPases or Rac1-green fluorescent protein (Rac1-GFP) and E-cadherin-DsRed (provided by Dr. J. S. Eurlich and Dr. W. J. Nelson, Stanford University Medical School, Stanford, CA) were cultured in DMEM containing 10% FBS at 37°C in a 5% CO2 atmosphere as described previously (40, 41). For all experiments, MDCK cells were plated at 2.5 × 10^5 cells/ml on the following type I collagen-coated substrates: cover glasses for confocal microscopy, micropore filters (0.45-μm pores; Millipore, Bedford, MA) for trans-epithelial electrical resistance (TER) measurements, and 35-mm-diameter tissue culture wells for immunoblot analysis. Cells were cultured for 2 days to produce confluent monolayers. The MDCK cells expressing mutant forms of the Rho family GTPases used in these experiments were constitutively active Rac1 (RacV12) or RhoA (RhoV14) and dominant-negative Rac1 (RacN17) or RhoA (RhoN19). Stable expression of mutant Rac1 and RhoA occurred under regulation of the tetracycline-repressible transactivator (21).

Cells were cultured for 2 days in medium that either contained the tetracycline analog doxycycline (Dox; 20 ng/ml) to suppress mutant Rac1 and RhoA expression or lacked Dox to allow Rac1 or RhoA expression. Stock cultures were maintained in DMEM-FBS containing Dox. For collagen-regulated epithelial remodeling and tubule formation, cells were grown for 24 h in either +Dox or −Dox medium and then incubated with collagen gel overlays for an additional 24 h. For 2- and 6-h experiments, the cells were grown in medium with or without Dox for 48 h before incubation with collagen gel overlays. At the termination of the experiments, collagen gels were removed by aspiration and cell monolayers were prepared for confocal microscopy, TER measurement, or immunoblot analysis.

Antibodies and reagents. The primary antibodies used in these studies were mouse MAb 3F2 developed by our laboratory against MDCK apical membrane protein gp135 (42) and MAb against β-catenin (BD Transduction Laboratories, Lexington, KY), rabbit antibodies Ser19-phosphorylated MLC (Cell Signaling Technology, Beverly, MA), occludin and zonula occludens (ZO)-1 (Zymed Laboratories, San Francisco, CA), and Rac1 (Upstate Biotechnology, Lake Placid, NY). Phalloidin-Alexa Fluor 594, goat anti-rabbit (GAR) IgG-Alexa Fluor 488, and goat anti-mouse (GAM) IgG-Alexa Fluor 594 secondary antibodies were purchased from Molecular Probes (Eugene, OR).

Confocal microscopy. MDCK cells on cover glasses were fixed with either methanol at −20°C for 5 min (for 6- and 24-h experiments) or 4% formaldehyde-PBS for 20 min at 4°C (for pMLC staining) and then permeabilized with 0.1% Triton X-100 (TX-100) for 10 min at 4°C. Fixed cells were washed with PBS and blocked in 3% BSA-1% goat serum-PBS (BSA-GS-PBS). For antibody or phalloidin localization, primary and secondary antibodies were diluted in BSA-GS-PBS as follows: phalloidin (1:25 dilution), MAB 3F2 (1:5 dilution), myosin II (1:25 dilution), pMLC (1:20 dilution), occludin and ZO-1 (1:20 dilution), and GAR-Alexa Fluor 488 and GAM-Alexa Fluor 594 (1:100 dilution). Stained cells were mounted in 10% glycerol-PBS containing 12% triethyldiamine (Sigma Chemical, St. Louis, MO) as an antibleaching reagent. Confocal microscopy was

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![Fig. 1](http://ajpcell.physiology.org/) Expression of dominant-negative Rho inhibits lamellipodia formation and myosin light chain (MLC) phosphorylation. Madin-Darby canine kidney (MDCK) RhoN19 cells grown in the presence (a–c) or absence (d–f) of tetracycline analog doxycycline (Dox) were incubated with collagen gel overlays for 6 h and prepared for confocal microscopy. Cells in medium containing Dox (+Dox) exhibited extensive epithelial remodeling forming lamellipodia with actin filaments (a) and MLC phosphorylated on Ser19 (P-MLC) (b). Merged confocal images show extensive actin and pMLC colocalization (yellow staining, arrows) (c). Cells in medium without Dox (−Dox) expressing RhoN19 did not exhibit epithelial remodeling, and lamellipodia containing actin and pMLC were absent (d–f). Scale bar, 8 μm.
performed using a laser-scanning confocal microscope (model 1024; Bio-Rad Laboratories, Hercules, CA). Z series images were collected at 1-μm intervals from the apical to basal cell surfaces (~10 μm), combined into 3D projections, and assembled into figures using Adobe PhotoShop and Canvas software on Macintosh G4 and G5 computers.

SDS-PAGE and immunoblot analysis. Quantitation of β-catenin associated with the cytoskeleton was performed using differential detergent extraction (20, 41). Previously, we used this procedure as an index of epithelial remodeling (13). Briefly, cells were extracted with CSK buffer containing 0.5% TX-100 to produce a soluble fraction, followed by RIPA buffer (containing 1% TX-100, 1% sodium deoxycholate, and 0.1% SDS) for the cytoskeletal fraction. The CSK buffer was composed of 10 mM PIPES, pH 6.8, 3 mM MgCl2, 50 mM NaCl, and 300 mM sucrose. β-catenin levels were determined by performing SDS-PAGE and immunoblot analysis using NIH Image software, and data are presented as soluble-to-cytoskeletal fraction ratios. Statistical analysis was performed using Student’s t-test or paired two-tailed t-tests.

Rho-GTPase activation assay. The levels of Rac1 activation during epithelial tubule formation were determined using a quantitative pull-down assay (45). In this procedure, we used glutathione-S-transferase (GST) as a fusion protein with an NH2-terminal peptide from p21-associated kinase (PAK) that binds activated Rac1 (GTP loaded). Escherichia coli expressing the GST-PAK fusion protein were obtained from Wei Yu and Keith Mostov (University of California, San Francisco, San Francisco, CA; Ref. 63). The GST-PAK fusion protein was coupled to Sepharose-glutathione 4B beads and used in activated Rac1 pull-down assays according to procedures previously described for MDCK cells (17). Total and activated Rac1 were analyzed by performing SDS-PAGE and immunoblot analysis using a rabbit Rac1 antibody (1:500 dilution; Upstate Biotechnology), followed by sheep anti-mouse IgG coupled to horseradish peroxidase (1:2,000 dilution). Rac activation levels were determined by comparing levels of affinity-isolated Rac1 to total Rac1 using NIH Image software. Control experiments included GDP or GTPγS loading of Rac1 in total cell extracts and pull-down assays of activated Rac1 from RacV12 cells grown in the presence or absence of Dox. Only GTP-loaded Rac1 and RacV12 from –Dox cells bound to the GST-PAK columns demonstrated the specificity of this assay.

Localization of activated Rac1. The cellular distribution of activated Rac1 was determined using a modification of the method of Li et al. (26). Briefly, cells were fixed with paraformaldehyde, permeabilized with CSK buffer containing 0.5% TX-100, and incubated with GST-PAK fusion protein (1 mg/ml) for 16 h at 4°C. Localization of activated Rac1 was performed using rabbit anti-GST for 30 min, followed by GAR-Alexa Red 594.

TER measurements. Tight junction permeability of RacV12 MDCK monolayers grown on micropore filters was determined as described previously (41). TER measurements were performed at the start of each experiment, and then the cells were incubated in the presence or absence of collagen for 2–6 h before final TER measurements were conducted.

RESULTS

Rho-GTPase studies. In all experiments conducted in the present study, we used the collagen gel overlay model to form multicellular epithelial tubular structures (19, 54). The majority of these data were obtained after 6 h for epithelial remodeling (lamellipodia formation and cell migration) or after 24 h to evaluate epithelial tubule formation. Previously, our laboratory demonstrated that Rho-kinase, a downstream effector of Rho signaling, played a prominent role in the regulation of epithelial tubule morphogenesis using the Rho-kinase inhibitor Y-27632 (13). To further study the role of RhoA-GTPases in this regulatory process, tubulogenesis studies were initiated using MDCK cells expressing either constitutively active (GTP loaded) or dominant-negative (GDP loaded) forms of Rac1 or RhoA under the regulation of the tetracycline-repressible trans-activator (21, 22). When grown in the presence of Dox, these MDCK cell lines do not express mutant Rho family GTPases. In the absence of Dox, they express mutant Rac or Rho at levels equal to or greater than the endogenous proteins (21). MDCK cells expressing dominant-negative RhoN19 were grown in the absence or presence of Dox and then incubated with collagen gel overlays for 6 h. Cells cultured in the presence of Dox formed lamellipodia containing filamentous actin as determined using confocal microscopy (Fig. 1). Double labeling demonstrated that Ser19-pMLC colocalized with actin at the lamellipodial leading edge (Fig. 1,a–c) with a distribution identical to that observed in wild-type MDCK cells.

Fig. 2. Expression of dominant-negative RhoN19 affected adherens junctions but not tight junction function. RhoN19 cells grown in the presence or absence of Dox were incubated in the presence or absence of collagen gel overlays (Coll) for 6 h (n = 3). A: quantitation of β-catenin association with the actin cytoskeleton was performed using differential detergent solubility and immunoblot analysis. Expression of RhoN19 (–Dox) alone or incubation with collagen gel overlays with or without Dox produced statistically significant (P < 0.01) increases in β-catenin solubility compared with control (Dox) but not compared with each other. Data are soluble-to-insoluble β-catenin ratios (means ± SE). B: transepithelial electrical resistance (TER) measurements demonstrated that tight junction permeability was not affected by the presence of Coll. TER (Ω·cm⁻²) is presented as %change from initial readings (means ± SE).
Fig. 3. Expression of dominant-negative RhoN19 inhibited epithelial tubule formation. RhoN19 cells grown in the presence or absence of Dox were incubated with collagen gel overlays for 24 h. A: confocal microscopic images show that cells in Dox and collagen reorganized into large, multicellular branching tubular structures (A,a–c) as determined by distribution of apical membrane glycoprotein gp135 (A,a). Tight junctions were localized using occludin staining (A,b, arrows). RhoN19-expressing cells in collagen formed small tubules with gp135-positive lumens (A,d) and ringlike tight junctions (A,e, arrows). Merged confocal images (A,c and f) demonstrate the organization of apical lumens and tight junctions. B: using experimental conditions identical to those described in A, gp135 was used to demonstrate the morphology of apical lumens (B,a and d). Tight junctions (arrows) were localized using an antibody against the membrane protein zonula occludens (ZO)-1 (B,b and e). Scale bar, 10 μm.
(13). These images were collected across a 15-μm range above the cell monolayer focal plane. To view the overall distribution of actin filaments and myosin II during epithelial remodeling, see Eisen et al. (13) and Supplemental Fig. 1 in the online version of the present article (http://ajpcell.physiology.org/cgi/content/full/00287.2005/DC1). In collagen-treated cells expressing RhoN19, lamellipodia containing actin and pMLC were not observed (Fig. 1, d–f). These data strongly suggest that expression of RhoN19 inhibited MLC phosphorylation and the subsequent formation of lamellipodia. Epithelial remodeling was assessed using differential detergent solubility to determine the levels of β-catenin associated with the cytoskeleton (20, 41). RhoN19 cells grown in the absence or presence of Dox were incubated with collagen gel overlays for 6 h and then extracted using detergent. Quantitative immunoblot analysis demonstrated significantly increased β-catenin solubility in cells expressing RhoN19 incubated with or without collagen gel (Fig. 2A). However, increased β-catenin solubility was not significantly different from control cells in collagen (Fig. 2A). These data suggest that the expression of RhoN19 decreased β-catenin association with the cytoskeleton. Tight junction permeability was determined during epithelial remodeling by TER measurements. These studies show that tight junction permeability was not affected during incubation with collagen gel containing or lacking Dox (Fig. 2B).

In previous studies, we demonstrated that the Rho-kinase inhibitor Y-27632 did not inhibit epithelial lumen formation but prevented the morphogenesis of multicellular epithelial

![Fig. 4. Tight junctions in RhoV14 cells were not affected by incubation in collagen gel. A: RhoV14 cells grown in presence or absence of Dox were incubated without (A,a,d,e, and f) or with collagen gel overlays (A,c,d,g, and h) for 24 h. After fixation, tight junctions were labeled with antibodies against either occludin (A,a–d) or ZO-1 (A,e–h). Control cells in +Dox medium (A,a and e), cells in −Dox medium (A,b and f), cells incubated with collagen gel +Dox (A,c and g), or collagen gel −Dox (A,d and h) had intact tight junctions. Scale bar, 10 μm. B: β-catenin detergent solubility. RhoV14 cells grown in the presence or absence of Dox were incubated with or without Dox or in the presence of collagen gel overlays either containing (Coll/Dox) or lacking Dox (Coll/−Dox) for 6 h. Quantitation of β-catenin association with the actin cytoskeleton was performed using differential detergent solubility and immunoblot analysis. Expression of RhoV14 (−Dox) with or without collagen gel did not induce statistically significant increases in β-catenin solubility compared with control cells (Dox). However, incubation of −Dox cells with collagen gel overlays (−Dox/Coll) induced a small, statistically significant increase in β-catenin association with the cytoskeleton (P = 0.02) compared with −Dox control cells. Data are presented as soluble-to-insoluble β-catenin ratios (means ± SE). C: TER measurements demonstrated that tight junction permeability was not affected by the presence of collagen gel overlays for 6 h. TER (Ω·cm²) is expressed as %change from initial readings (means ± SE). AJP-Cell Physiol • VOL 290 • MAY 2006 • www.ajpcell.org](http://ajpcell.physiology.org/)}
tubules (13). Therefore, expression of RhoN19 would be predicted to inhibit downstream signaling through Rho-kinase and to affect epithelial tubulogenesis. RhoN19 cells in the absence or presence of Dox were incubated with collagen gel overlays for 24 h. In control RhoN19 cells, confocal microscopy demonstrated the presence of large, multicellular, branching epithelial tubular structures (termed tubulocysts by other workers; see Refs. 63, 65) with gp135-positive apical lumens and an intricate assembly of occludin-positive tight junctions (Fig. 3A). However, in cells expressing dominant-negative RhoN19, primarily small, gp135-positive apical lumens surrounded by a single ringlike tight junction were observed between cell pairs (Fig. 3A). Similar results were obtained when the tight junction protein ZO-1 was localized in 24-h tubular structures (Fig. 3B). The presence of small lumens suggested that the fundamental mechanisms regulating the rearrangement of epithelial cells into tubules had been compromised. These data are remarkably similar to those regarding Rho-kinase inhibition and support our hypothesis that Rho signaling has an important role in the regulation of epithelial tubule morphogenesis (13).

Reciprocal experiments were performed with cells that expressed constitutively active RhoV14. Cells expressing or not expressing RhoV14 (±Dox), as well as cells in the presence or absence of collagen, did not form multicellular tubules or apical lumens (Fig. 4A). The association of β-catenin with the cytoskeleton was determined on the basis of detergent solubility and immunoblot analysis after 6-h incubation in collagen. Cells that expressed (−Dox) RhoV14 did not respond to the collagen overlay, because β-catenin solubility was unchanged (Fig. 4B). However, a small, significant change in β-catenin solubility was observed in control cells incubated in collagen (Dox/Coll), which is evidence that signaling to the adherens junctions had occurred despite the lack of cellular rearrangement. Collagen-mediated changes in TER were not observed (Fig. 4C). These data suggest that cell interactions with collagen were not sufficient to activate the signal transduction pathways required for epithelial tubule morphogenesis.

**Rac-GTPase studies.** Numerous studies have demonstrated that Rac and Rho function cooperatively in a reciprocal cycle to regulate a variety of functions, including cell adhesion and motility (5, 6, 50, 53). To test whether this hypothesis could account for the cell rearrangements observed during tubulogenesis, studies were initiated with MDCK cells that expressed constitutively active RacV12 in the absence of Dox (24). After 6-h incubation with collagen gel overlays, control RacV12 cells (±Dox) exhibited epithelial remodeling and lamellipodia formation identical to both wild-type (13) and control RhoN19 cells (Fig. 1), whereas cells expressing RacV12 cells did not (Eisen R and Ojakian GK, unpublished data). The influence of Rac signaling on adherens junction β-catenin association with the cytoskeleton was determined using detergent solubility and immunoblot analysis. After 6-h incubation of RacV12 cells with collagen gel overlays, significant changes in β-catenin solubility were observed in both control RacV12 cells and those expressing RacV12 (Fig. 5A). Furthermore, RacV12-expressing cells in collagen exhibited an additive increase in β-catenin solubility (Fig. 5A), suggesting that adherens junction integrity was regulated by Rac1.

The role of activated RacV12 in tight junction function during epithelial remodeling was further investigated. RacV12 cells were grown in the absence or presence of Dox, and TER measurements were performed after 2-h incubation without or with collagen gel overlays. Under these conditions, no changes in TER were observed in the absence of collagen or in control RacV12 cells incubated with collagen (Fig. 5B). However, cells expressing activated RacV12 exhibited significantly increased tight junction permeability when incubated with collagen gel (Fig. 5B), suggesting that RacV12 had a regulatory effect on signaling to tight junctions.

To further study tubule formation, RacV12 cells were incubated with collagen gel overlays in the absence or presence of Dox for 24 h. Control cells formed multicellular branching tubular structures with gp135-positive lumens and extensive...
Fig. 6. Expression of constitutively active RacV12 inhibited epithelial tubule formation. RacV12 cells grown in the presence or absence of Dox were incubated with collagen gel overlays for 24 h. A: +Dox control cells organized into multicellular branching tubules with gp135-positive apical lumens (A,a). Localization of occludin demonstrated an extensive network of associated tight junctions (A,b). RacV12-expressing cells in collagen gel had only small gp135-positive apical lumens (A,d) with occludin staining demonstrating associated tight junctions (A,e). B: ZO-1 staining of tight junctions. In Dox-grown control cells, confocal microscopy demonstrated formation of multicellular branching tubules with gp135-positive apical lumens (B,a) and extensive ZO-1-containing tight junctions (B,b). In −Dox cells, expression of constitutively active RacV12 allowed formation of only small gp135-positive lumens (B,d). ZO-1 was associated with each apical lumen and was frequently observed in tangled arrays (B,e, arrows). Merged confocal images (A,c and f; B,c and f) demonstrate the organization of apical lumens and tight junctions for both occludin and ZO-1 staining. Scale bar, 10 μm.
occludin-positive tight junctions (Fig. 6A). However, in cells expressing RacV12, only small gp135 apical lumens with occludin-positive tight junctions were observed (Fig. 6A). Because RacV12 expression had such a prominent effect on tight junction organization in collagen, further studies were conducted. In RacV12 control cells (−Dox) incubated with collagen for 24 h, tight junctions localized with an antibody against ZO-1 had a tight junction morphology that was less complex than that of the occludin-stained cells. ZO-1 was associated only with the gp135-positive lumens and not with adjacent lateral membranes (Fig. 6B). After incubation of RacV12-expressing cells (−Dox) with collagen, small lumens with associated ringlike tight junctions were observed (Fig. 6B). Also present were numerous tangled arrays of ZO-1-positive strands that appeared to associate with but not surround the apical lumens (Fig. 6B). These lumens appeared to be identical to those observed in RhoN19-expressing cells incubated with collagen gel with or without Dox (Fig. 3).

Because there is evidence that occludin and ZO-1 interact (28), experiments were performed with RacV12 cells in collagen for 24 h to determine whether these proteins were present in the same tangled structures. Double labeling using the appropriate mouse and rabbit primary antibodies demonstrated that occludin and ZO-1 colocalized to the same tight junction rings and tangles (Fig. 7), providing further evidence that these proteins interact. These data also demonstrate that linear and tangled occludin staining patterns were found within the same cell.

Previous studies demonstrated that expression of dominant-negative RacN17 in MDCK cells inhibited the formation of properly oriented epithelial cysts in collagen gel (38). RacN17 cells grown in the presence or absence of Dox did not respond to collagen gel overlays and epithelial remodeling, and tubule formation was not observed (Fig. 8A). Similarly, we were unable to detect any collagen-mediated changes in either β-catenin associated with the cytoskeleton (Fig. 8B) or TER measurements (Fig. 8C). However, other researchers (63) have presented data demonstrating that RacV12 cells form tubular structures when incubated with collagen gel overlays for 5 days. Altogether, these results suggest that collagen-mediated signaling occurs at a suboptimal rate in the RacV12 clonal cell line.

Rac1 inactivation and redistribution in wild-type MDCK cells. Others have shown that Rac1 activation levels appear to be regulated by the formation of MDCK cell-cell contacts (35). These data are supported by studies demonstrating that Rac is recruited to sites of adherens junction formation (12, 25, 31). Therefore, we initiated GST fusion protein pull-down experiments with GST-PAK beads to determine Rac1 activation levels in wild-type MDCK cells during epithelial remodeling and tubule formation. Our studies have demonstrated that Rac1 activation levels did not change in the presence of collagen gel overlays during a time course of 0.5–4 h (Fig. 9). At 6 h, significant decreases (~40%) in Rac1 activation were detected, and these reduced activation levels persisted for 24 h (Fig. 9). These observations are not in complete agreement with those of Yu et al. (63), who recently demonstrated that wild-type MDCK cells incubated with collagen gel overlays exhibited transient increases in Rac activation that peaked at 15 min and then decreased to control levels after 1 h. One possibility for this discrepancy is that these investigators used a commercial collagen preparation, whereas our type 1 collagen was prepared from rat tail tendons. Under our experimental conditions, tendon collagen did not fully gel until 30–60 min after application, and it is possible that differences in collagen fibril polymerization time or composition could be responsible. Another possibility is that different wild-type MDCK cell lines were used. In this regard, Zuk and Matlin (65) previously demonstrated that only one of their wild-type MDCK cell lines responded to collagen gel overlays by forming tubular structures, whereas others did not. Finally, examination of the SDS-PAGE data of Yu et al. (63) suggests that Rac1 activation decreased at 6 h.

Our observations that Rac1 inactivation occurred during epithelial remodeling were unexpected, because Rac1 plays an important role in cell motility (6, 18, 49). To further study Rac and epithelial remodeling, we obtained MDCK cells that stably coexpressed Rac1-GFP and E-cadherin-DS Red. MDCK Rac1-GFP cells were previously used to monitor the dynamics of Rac1 redistribution during the formation of adherens junctions...
Rac1-GFP cells were incubated with collagen gel overlays for 6 h to induce epithelial remodeling and then analyzed using confocal microscopy. In control cells without collagen, Rac1-GFP colocalized with E-cadherin-DsRed on lateral membranes (Fig. 10, a–c). Rac1-GFP was also observed in the cytoplasm. After 6 h in collagen gel, lateral membrane Rac1-GFP had dispersed and Rac1-GFP was localized within lamellipodia as well as throughout the cell (Fig. 10, d–f).

To further these studies, the procedure of Li et al. (1, 26, 61a) was used to localize activated Rac1 during epithelial remodeling. After 6 h in collagen gel, MDCK cells were fixed, permeabilized, and incubated with GST-PAK, a fusion protein that binds to activated Rac1 (46). The distribution of activated Rac1 was determined using confocal microscopy. In control cells, activated Rac1 had a homogeneous cell distribution (Fig. 11a). After 6 h in collagen, intense staining of activated Rac1 was observed in the lamellipodia of migrating cells (Fig. 11b).

**DISCUSSION**

Our laboratory previously demonstrated that integrin function and Rho-kinase signaling are required for epithelial tubule formation (13, 40–42, 54). We proposed that regulation of myosin II activity by the Rho signaling pathway is important in the modulation of cell movement during the development of epithelial tubules (13). In the present study, MDCK cell lines expressing mutant Rac and Rho were used to confirm and extend these results. We have provided new evidence herein.
that the Rho family GTPase signal transduction pathway plays a significant role in the regulation of cell rearrangement, MLC phosphorylation, the maintenance of adherens junctions, and the targeting of tight junction proteins during epithelial tubule formation. Expression of either RhoN19 or RacV12 inhibited collagen-mediated multicellular epithelial tubule formation, allowing only the biogenesis small, gp135-positive apical lumens. These data suggest that the process of epithelial tubule formation, but not that of apical membrane targeting and tight junction assembly, is selectively inhibited by RhoN19 or RacV12 expression. Our laboratory also has demonstrated that epithelial remodeling in MDCK cells is accompanied by Rac1 inactivation and redistribution of activated Rac1 to lamellipodia. We propose that cell signaling due to collagen binding temporospatially modulated Rac1 activation levels, allowing the disassembly of tight and adherens junctions, increased cell migration, and epithelial remodeling and leading to tubule formation.

Rho signaling and regulation of epithelial tubule formation.

Specific inhibitors have been used to study the role of the Rho signaling pathway in the regulation of epithelial cell junction structure and function (4, 14, 15, 37, 57). Previously, we used a pharmacological approach to demonstrate that Rho-kinase had a prominent regulatory role in the biogenesis of epithelial tubules (13). To obtain more precise information on the regulation of epithelial tubule formation by the Rho signaling pathway, we used a molecular approach using MDCK cell lines that expressed mutant Rho family GTPases. The incubation of control RhoN19 cells with collagen gel overlays induced extensive epithelial remodeling, including the formation of lamellipodia containing actin filaments colocalized with Ser19-phosphorylated MLC at the leading edge, followed by assembly of multicellular branching tubular structures identical to those formed by wild-type cells (13, 63). However, cells expressing dominant-negative RhoN19 did not undergo collagen-mediated lamellipodia formation or MLC phosphorylation. These observations and those demonstrating that collagen gel overlays induced the formation of only small apical lumens during RhoN19 expression strongly suggest that collagen-mediated cell rearrangement was inhibited. Our findings regarding dominant-negative Rho inhibition are supported by previous observations demonstrating that Rho activation levels are regulated by cell-cell adhesion and cadherin engagement (35, 36, 60). The small apical lumens in RhoN19-expressing cells had associated tight junctions and appeared to be morphologically identical to those produced in wild-type cells incubated with collagen gel containing the Rho-kinase inhibitor Y-27632 (13). Our studies have demonstrated that the targeting mechanisms required for biogenesis of the polarized, gp135-positive apical compartment were not affected by the expression of dominant-negative RhoN19, suggesting that myosin II-regulated motility was specifically inhibited. Our data are consistent with those demonstrating that dominant-negative Rho expression inhibited dorsal closure of actomyosin-regulated migrating epithelial sheets in developing Drosophila embryos (3). These observations constitute strong supporting evidence that the Rho GTPase signal transduction pathway regulates epithelial remodeling and tubule formation. Therefore, in our kidney model system, Rho is positioned upstream of Rho-kinase, a hypothesis that is in agreement with that proposed for the biogenesis of mammary epithelial tubules (63).

On the basis of the model for Rac and Rho function (5, 6, 50, 53, 64), MDCK cells expressing constitutively active RhoV14 were expected to exhibit accelerated epithelial remodeling in collagen. However, we did not observe epithelial remodeling or tubule formation with collagen gel overlays, suggesting that RhoV14 cells lacked sufficient interaction with collagen to initiate these processes. This possibility is supported by previous observations that an MDCK clonal cell line did not respond to collagen gel overlays by forming tubular structures (65). However, we were able to detect small but significant changes in β-catenin solubility after incubation with collagen gel overlays for 6 h. These data suggest that some integrin signaling may have been initiated but that it was insufficient to support epithelial remodeling.

Rac signaling and regulation of epithelial tubule formation.

Several studies have demonstrated that Rac and Rho appear to have reciprocal functions in a variety of cells, including MDCK cells (6, 18, 50, 53, 64). To further test this hypothesis, collagen gel overlay studies were conducted with MDCK cells that expressed constitutively active RacV12. Under these conditions, control RacV12 cells (i.e., cells not expressing RacV12) reorganized into multicellular branching tubular structures with extensive apical lumens and associated tight junctions. These tubular structures were identical to those...
observed in wild-type MDCK cells (see Ref. 13), as well as in control RhoN19 cells in this study. However, when RacV12 expressing cells were incubated with collagen gel overlays, only small gp135-positive lumens were observed, suggesting that excessive Rac-regulated actin polymerization and lamellipodia formation did not provide a favorable environment for the assembly of epithelial tubules. This proposal is supported by time-lapse multiphoton microscopy demonstrating that although RacV12 overexpression in MDCK cells induced extensive lamellipodia formation and ruffling activity, these cells were considerably less mobile than those that did not express RacV12 (12). On the basis of observations presented herein as well as those described in our previous study (13), it is likely that coordinated Rac and Rho modulation of actin and myosin activity played a critical role in the regulation of epithelial tubule formation. This suggestion is supported by observations that activated RacV12 expression causes disruption of tight junction structure and function (22) and that Rac plays a critical role in the morphogenesis of endothelial cell tubule formation (8). However, these observations are not consistent

Fig. 10. Redistribution of Rac occurs during epithelial remodeling. MDCK cells expressing Rac1-green fluorescent protein (Rac1-GFP) and E-cadherin-DS Red were incubated in the absence (a–c) or presence (d–f) of collagen gel overlays for 6 h. In control cells (a–c), E-cadherin-DS Red had a lateral membrane distribution (a) and colocalized with Rac1-GFP (c). Rac1-GFP was localized to lateral membranes, and some cytoplasmic staining was observed (b). After epithelial remodeling in collagen (d–f), lateral membrane Rac1-GFP staining had dispersed and Rac1-GFP fluorescence was observed in lamellipodia (f, arrows). Scale bar, 10 μm.

Fig. 11. Localization of activated Rac1 to lamellipodia during epithelial remodeling. The distribution of activated Rac was evaluated using a Rac-Cdc42/Rac interaction binding (CRIB) domain-GST fusion protein. In control cells, Rac-CRIB demonstrated a homogeneous distribution within the cytoplasm (a). After epithelial remodeling in collagen for 6 h, Rac-CRIB was localized to lamellipodia (b, arrows). Scale bar, 8 μm.
with studies demonstrating that cells expressing RacV12 organized into polarized cysts within collagen gels (38, 39). This apparent inconsistency probably reflects the fact that polarized cyst formation in collagen gel takes 4 days and requires cell growth (38), whereas tubules formed using the collagen gel overlay model are a result of increased epithelial reorganization requiring minimal cell proliferation (13, 65).

Although the small gp135-positive lumens in RacV12-expressing cells appeared to be morphologically similar to those observed in cells expressing dominant-negative RhoN19, the tight junction protein ZO-1 did not always appear to assemble properly. Instead, many lumens had associated ZO-1 tangles that did not completely surround the lumen, an observation consistent with freeze-fracture data (22). Because occludin colocalized with ZO-1, these data suggest that constitutively active Rac expression does not affect tight junction assembly and targeting but is important in the regulation of tight junction organization during epithelial tubule formation.

Previous studies have demonstrated that Rac1 activity was increased during integrin signaling (7, 10, 11, 45). Although these results appear to be the opposite of those observed in the present study, the reported increases were transient and returned to baseline levels within 0.5–3 h (7, 10, 11, 45). However, our experiments with wild-type MDCK cells were conducted over a considerably longer time course (0.5–24 h), and we did not use suspension cells plated on ECM-coated substrates. Furthermore, our results are supported by findings reported in studies of MDCK adherens junction formation demonstrating that Rac1 activity increased during epithelial cell-cell adhesion and decreased under conditions favoring reduced cell contacts (35, 36). Our studies have demonstrated that reduced Rac1 activity coincided with decreased cell-cell adhesion and corresponding cell rearrangement (13, 40). They suggest that decreased Rac activity is coupled to the regulation of epithelial remodeling and tubule formation. These observations are consistent with those showing that Rac is involved in the biogenesis of adherens junctions in endothelial and epithelial cells (12, 25, 31, 60). It is possible that decreased Rac1 activity was confined to adherens junctions, whereas Rac1 activation persisted at the leading edge of migrating lamellipodia. This proposal is supported by fluorescence activation studies demonstrating that Rac1 cellular activity was confined to the direction of cell migration, including the lamellipodia (24). Our observation that Rac1 inactivation persisted after 24 h is consistent with findings reported previously that demonstrated epithelial tubule formation using collagen gel overlays in a continuous state of development for 72 h (56). Our hypothesis is that collagen-mediated signaling selectively activates Rac1 in a temporospatial manner, possibly through integrins, modulating the disassembly of junctional complexes and the redistribution of activated Rac1 to lamellipodia, allowing increased cell migration and epithelial tubule formation.

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