RhoA, Rac1, and Cdc42 exert distinct effects on epithelial barrier via selective structural and biochemical modulation of junctional proteins and F-actin

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INTESTINAL EPITHELIAL CELLS are physically linked by intercellular junctional complexes that regulate multiple functions, including polarity, mechanical integrity, and signaling (43). Epithelial paracellular permeability is regulated by the apical-most intercellular junctional complex referred to as the apical junctional complex (AJC). The major constituents of the AJC are tight junctions (TJs) and subjacent adherens junctions (AJs). The composition of TJs and AJs is similar, consisting of transmembrane and cytoplasmic linker proteins that affiliate with the underlying actin cytoskeleton (5, 14).

Proteins constituting the TJ complex include the transmembrane proteins occludin, junctional adhesion molecule (JAM), claudin family members, and linker proteins such as ZO-1 that affiliate with the underlying actin cytoskeleton (6, 9, 10, 25). It was shown previously that claudin-1 and -2 are important for TJ strand formation (8). The AJ is immediately subjacent to the TJ and is essential for cell-cell recognition and cell sorting (13).

TJ assembly and function can be modulated by a number of signaling molecules, including cAMP, Ca 2+, PKC, G proteins, phospholipase C, and diacylglycerol (1, 27, 36). Recently, the Rho family of small GTPases, comprising RhoA, Rac1, and Cdc42, has been shown to be important in the regulation of epithelial structure, function, and assembly (3, 18, 29, 45) and in the regulation of F-actin dynamics (15, 28, 33). Rho GTPases cycle between an active GTP-bound state and an inactive GDP-bound state (2, 16). Transitions between GTP- and GDP-bound forms of small GTPases are controlled by specialized regulators (2), and by cycling between GTP- and GDP-bound states RhoA family GTPases are able to transduce signals between cell surface receptors and intracellular target molecules (16).

Diverse pharmacological and molecular tools that interfere with Rho protein function have offered valuable insights into how Rho GTPases regulate epithelial permeability. Our initial investigations (29) utilized a modified cell-permeant chimeric toxin consisting of the Clostridium botulinum toxin C3 trans-ferase (to inhibit RhoA activity through ADP-ribosylation of Asp41) and the receptor binding domain of diphtheria toxin to perturb Rho protein function have offered valuable insights into how Rho GTPases regulate epithelial permeability. Our initial investigations (29) utilized a modified cell-permeant chimeric toxin consisting of the Clostridium botulinum toxin C3 trans-ferase (to inhibit RhoA activity through ADP-ribosylation of Asp41) and the receptor binding domain of diphtheria toxin to facilitate internalization. In this system, barrier function of T84 intestinal epithelial cells was compromised with reductions in transepithelial resistance (TER), enhancements in paracellular permeability, and redistribution of ZO-1 and occludin away from the TJ membrane (29). Moreover, ZO-1 and occludin, which are known to be affiliated with membrane microdomains or detergent-insoluble glycolipid rafts (DIGs), were redistrib-
RHO GTPASES AND EPITHELIAL BARRIER FUNCTION

inactivation or activation of RhoA, Rac1, or Cdc42 perturbs TJ gate and fence function (22, 34). These changes were associated with redistribution of occludin and ZO-1 away from the lateral membrane and abnormal TJ strand morphology after activation of the above GTPases, whereas TJ structure remained intact after inactivation of these GTPases. Similarly, cytotoxic necrotizing factor (CNF)-1, a bacterial toxin that constitutively activates Rho GTPases via inhibition of GTP hydrolysis (7, 24, 39), was shown to disrupt barrier function by displacement of occludin and ZO-1 and reorganization of JAM-1 away from the TJ membrane (19). In contrast, another study described decreased localization of occludin and ZO-1 at cell junctions during RhoA inactivation, whereas constitutive RhoA signaling caused an accumulation of these proteins at cell junctions (11). However, continuous expression of the same construct was reported to have no effect on ZO-1 localization at MDCK cell contact sites (41). Ambiguities such as those mentioned directly above highlight the importance of solid model systems in any investigation of the role of Rho proteins in barrier function. Cellular signaling from Rho proteins is likely to be tightly regulated at all times, especially in light of the fact that any imbalances in Rho activity (whether activation or inactivation) evoke similar disturbances in function. Thus our study utilized a tetracycline-repressible system to exert tight control over the expression of Rho, Rac, and Cdc42 GTPases in MDCK epithelial cells. We used this model system to dissect out in detail the effects of Rho GTPases on TJ structure/function and the contribution of the actin cytoskeleton to this process, to resolve ambiguities regarding the contribution of Rho proteins to barrier function in vitro. We present a number of novel aspects in our study including an important role for claudin-1 and -2 in the regulation of barrier function by Rho proteins. Although increased paracellular permeability in dominant-negative RhoA was associated with changes in the apical actin only, we observed a substantial internalization of TJ transmembrane proteins in constitutively active GTPases, dominant-negative Rac1, and dominant-negative Cdc42. These structural changes were associated with an increased detergent solubility of claudin-1 and -2 in all constitutively active GTPases, whereas constitutively active RhoA alone reduced claudin-2 and ZO-1 partitioning into detergent-insoluble membrane rafts and JAM-1 from membrane raft-containing fractions.

MATERIALS AND METHODS

Cell culture. MDCK epithelial cells (T23 clone) expressing either myc-tagged dominant-negative RhoAN19, Rac1N17, or Cdc42N17 or constitutively active RhoAV14, Rac1V12, or Cdc42V12 under the control of a tetracycline-repressible transactivator were grown as previously described (21). Briefly, cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 0.1 mM nonessential amino acids (Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin, and 20 ng/ml doxycycline (DC; Sigma, St. Louis, MO) at 37°C in a humidified atmosphere containing 5% CO2. Induction of gene expression in RhoAN19, RhoAV14, Rac1N17, Rac1V12, Cdc42N17, and Cdc42V12 cells was achieved as follows. Cells were trypsinized and seeded into T75 flasks at low density in medium lacking DC for 15–20 h (RhoAN19 36–40 h). At this time cells reached ~30–50% confluence. Cells were trypsinized and seeded at high density on permeable polycarbonate filters (0.4-µm pore size) with surface areas of 0.33, 5, or 45 cm2 (Costar, Cambridge, MA) for 18–48 h before experimental treatments as described previously (26). The parental cell line was transfected with an empty plasmid (pUHD) and treated in an identical manner served as a control. In addition, noninduced mutant cells (maintained in the presence of DC) produced similar results.

Electrophysiology and permeability assays. TER was checked in all monolayers before each experiment with an epithelial voltohmme-
ter (EVOM/EndOhm; World Precision Instruments, Sarasota, FL). Paracellular permeability to fluoresceinilated dextran (mol wt 3,000; FD-3) was assessed as previously described (38). Briefly, cells grown reached ~3.3% permeability for permeable supports washed in HBSS+ for 18–48 h in medium either containing 20 ng/ml DC or lacking DC were washed in Hanks’ balanced salt solution-10 mM HEPES (HBSS+) and equilibrated at 37°C for 10 min on an orbital shaker. Monolayers were loaded apically with 1 mg/ml FD-3 (Molecular Probes, Eugene, OR). Basolateral samples were collected at t = 0 and 120 min, and fluorescence intensity was analyzed on a fluorescence plate reader (Fluoscan; BMG Labtechnologies, Durham, NC). FD-3 concentrations transported were extrapolated from a standard curve and expressed as micromolar FD-3 transported per square centimeter per hour. Monolayers permeabilized with Triton X-100 (TX-100; 1%) for 10 min were used as positive controls.

Immunofluorescent localization of c-myc, functional proteins, and F-actin. Monolayers of MDCK cells grown 18 or 48 h on 0.33 cm2 permeable supports were washed in HBSS+, fixed/permeabilized with either ethanol at −20°C for 20 min or 3.7% paraformaldehyde [10 min, room temperature (RT)] and 0.5% TX-100. Nonspecific background was blocked with 5% BSA (1 h, RT). Monolayers were incubated with primary antibodies to occludin, ZO-1, claudin-1, claudin-2 (Zymed, San Francisco, CA), JAM-1 (J10.4; Ref. 25), E-cadherin, β-catenin (Transduction Laboratories, Lexington, KY), or c-myc (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Monolayers were washed and probed with Alexa (Molecular Probes)- or FITC (Jackson Labs, West Grove, PA)-conjugated secondary antibodies. All monolayers were visualized on an LSM510 confocal microscope (Carl Zeiss Microimaging, Thornwood, NY).

Immunoblotting for TJ/AJ proteins in epithelial cells. MDCK cells were grown on 5-cm2 permeable supports for 18 or 48 h as described above, washed in HBSS+, and scraped into extraction buffer (in mM: 100 KCl, 3 NaCl, 3.5 MgCl2, and 10 HEPES pH 7.4) containing 1% TX-100, protease inhibitors (250 µM PMSF, 5 µg/ml leupeptin, 10 µg/ml chymostatin, 0.25 µg/ml pepstatin, and 2 µg/ml aprotinin), and phosphatase inhibitors (in mM: 25 sodium fluoride, 10 sodium orthovanadate). Cell lysates were centrifuged (4,000 g, 5 min, 4°C), and equivalent protein concentrations (10 µg/lane) from induced and noninduced monolayers were subjected to SDS-PAGE. Western blots were analyzed for TJ/AJ proteins, Rac1, Cdc42, and RhoA.

Differential detergent extraction of AJC proteins. MDCK cells were grown on 5-cm2 permeable supports for 18 or 48 h as described above, washed, and incubated for 30 min at 4°C with 1% TX-100 extraction buffer as above. The TX-100-soluble fraction was subjected to low-speed centrifugation to remove cell debris and added to an equal amount of 2× sample buffer (3% SDS, 0.75 M Tris pH 8.8, 20% glycerol, and 20 mM DTT). The TX-100-insoluble fraction was scraped into an equal amount of SDS sample buffer. Equal volumes of each fraction were analyzed by SDS-PAGE and immunoblotting for TJ/AJ proteins as previously described (30). Densitometry was performed with the UN-SCAN-IT automated digitizing system (Silk Scientific, Orem, UT).

Isolation of DIGs by sucrose gradient fractionation. Transfected MDCK cell lines were grown on 45-cm2 permeable supports for 18–70 h and harvested into HBSS+−containing 1% TX-100 and protease inhibitors as above. The sucrose concentration of the cell lysate was adjusted to 40%, placed at the bottom of an ultracentrifuge tube, and overlaid with a 5–30% (wt/wt) linear sucrose gradient as previously described (31). Gradients were ultracentrifuged (19 h, 39,000 rpm, 4°C), fractionated, and analyzed for sucrose concentra-
tion, light scattering at 600 nm, protein concentration, and alkaline phosphatase activity as previously described (31, 32). TJ/AJ protein profiles in raft fractions were determined by SDS-PAGE and Western blotting.

Statistics. Results are expressed as means ± SE. Student’s t-tests or Welch tests were used to compare results, with statistical significance assumed at P < 0.05. Individual experiments were performed in triplicate or greater, and each experiment was performed independently three or more times.

RESULTS

Enhanced paracellular permeability in RhoA, Rac1, and Cdc42 mutants. The effects of mutant small GTPases on paracellular permeability were examined in MDCK cells under two different sets of growth conditions. First, cells were seeded on inserts in medium containing DC for 4–6 days to allow the establishment of mature TJs. At this time GTPase mutant expression was induced by incubating monolayers for 24 h in medium lacking DC but containing 2.5 mM sodium butyrate. Under these conditions, stable mutant expression was observed only in RacN17 and CdcN17, whereas all other GTPases lacked a constant mutant expression. However, both dominant-negative Rac1 and Cdc42 exhibited TER values and paracellular flux rates similar to those in control cells (data not shown).

In the second set of conditions, mutant GTPase expression was induced at early time points after cell-cell contact had occurred (18 h). Effects of each mutant on paracellular permeability are shown in Fig. 1. TER was significantly lower (P < 0.01) in all GTPases mutants relative to cells with an empty vector (pUHD) or GTPases without mutant induction (data not shown) 18 h after initiation of cell-cell contacts (Fig. 1A). After peaking at 18 h after seeding, the TER of control cells consistently dropped to low but stable values by 48 h (~90 Ω·cm²; data not shown). At this time point, TER was not affected by induction of any mutant GTPase, including Rac1N17.

The decrease in TER 18 h after induction of cell-cell contacts in GTPase mutants correlated with a significant increase (P < 0.01) in the paracellular flux of FD-3 in all mutant GTPases, consistent with an increase in paracellular permeability (Fig. 1B). Data from Rac1N17 are not shown at this time point because the monolayers never achieved confluence and thus paracellular fluxes were artificially high. Although TER was similar to that in control cells 48 h after seeding, we observed a significant increase in paracellular FD-3 flux in all constitutively active mutants (data not shown) as well as in Rac1N17 relative to control cells. The relative difference in TER vs. FD-3 flux is consistent with previous reports that showed exponential increases in flux at TER values <300 Ω·cm² (38). Thus, at these TER values, measurement of flux reveals greater sensitivity as a parameter of paracellular transport. It should be noted that 18 h after induction of cell-cell contacts Rac1N17 cells never reached confluence, whereas after 48 h a confluent monolayer had developed. Therefore, all further experiments in Rac1N17 cells were performed 48 h after seeding cells on inserts.

Expression levels of mutant GTPase induction remained constant during the experimental time course for each cell line and are shown by immunoblot in Fig. 2A. Uniformity of expression in epithelial monolayers was confirmed by immunofluorescence staining of c-myc-tagged mutant GTPases (Fig. 2B).

Fig. 1. Dominant-negative (DN) and constitutively active (CA) RhoA, Rac1, and Cdc42 disrupt gate function in Madin-Darby canine kidney (MDCK) cells. MDCK monolayers were induced to express mutant GTPases for 18–48 h. A: transepithelial resistance (TER) was measured in 3 filters in triplicate for each cell line, and the data are reported as means ± SE. B: paracellular flux of FITC-dextran 3,000 (FD-3) was examined as an index of passive paracellular transport. Graph represents an average of 3 experiments in triplicate for each cell line. pUHD, empty vector; BL, basolateral.
Deficits in brush border F-actin were also evident in monolayers expressing Cdc42V12 (Fig. 3, G and N); however, aggregation of F-actin in a subapical pool was not observed.

F-actin imaging in the reconstructed vertical or x-z plane revealed highly polarized monolayers with distinct pools of F-actin at the apical, lateral, and basolateral membranes. Actin polarity was generally preserved during expression of the dominant-negative or constitutively active GTPases, with the exception of RhoAV14, in which cells lost some of their columnar epithelial morphology and occasionally formed multilayers.

**Effects of mutant GTPases on distribution of TJ and AJ proteins.** Because we observed that all GTPase mutants showed an increase in paracellular permeability and this function is primarily regulated by epithelial TJs, we examined the distribution of TJ and AJ proteins with immunofluorescence confocal microscopy. In control polarized MDCK cells expressing only the empty vector (pUHD) or noninduced GTPases (data not shown) all of the TJ proteins (Fig. 4, A1, B1, C1, D1, and E1) were appropriately localized to their respective intercellular junctions. Thus, in the en face or horizontal plane, they presented the characteristic “chicken wire” staining pattern typical for TJ-associated proteins. Expression of dominant-negative RhoA (Fig. 4, A2, B2, C2, D2, and E2) did not influence distribution of TJ proteins despite an increase in paracellular permeability 18 h after plating on inserts. In Rac1N17 (Fig. 4, A3, B3, C3, D3, and E3) and Cdc42N17 (Fig. 4, A4, B4, C4, D4, and E4) cells, occludin and ZO-1 localization was similar to control cells. Moreover, Cdc42N17 cells showed intact ring structures at the TJ membrane for both claudin-1 and JAM-1 (Fig. 4, C4 and E4) with only a minor decrease in staining intensity for JAM-1 relative to control cells. In contrast, the characteristic ring structure of claudin-2 was disturbed at some tricellular corners in cells expressing Cdc42N17 and internalization of claudin-2 was observed (Fig. 4D4). Rac1N17-expressing cells displayed claudin-1 and -2 and JAM-1 reorganization in the TJ plane, manifested as decreased localization of the respective TJ protein at the TJ membrane as well as internalization of claudin-1 and -2 (Fig. 4, C3, D3, and E3).

Constitutively activated RhoA, Rac1, and Cdc42 caused pronounced redistribution of several TJ proteins. These effects were most dramatic in RhoAV14 cells (Fig. 4, A5, B5, C5, D5, and E5), with severe discontinuities in the ring structures for occludin, ZO-1, and claudin-1 and -2, whereas JAM-1 showed some submembranous diffusion away from the TJ membrane. Internalization in RhoAV14 cells was observed for occludin and claudin-1 and -2 (Fig. 4, A5, B5, C5, D5, and E5). In Rac1V12-expressing cells (Fig. 4, A6, B6, C6, D6, and E6), ring structures at the level of the TJ were mainly preserved for occludin, ZO-1, and JAM-1, whereas severe discontinuities and internalization were observed in the case of Claudins-1 and -2. In Cdc42V12-expressing cells (Fig. 4, A7, B7, C7, D7, and E7) several alterations were visible, namely, severe discontinuities for all TJ proteins and internalization for claudin-2 and JAM-1. As previously described by others (22), reorganization of TJ proteins on mutant GTPases induction was not associated with significant changes in the total levels of these proteins as determined by Western blotting, suggesting TJ protein redistribution rather than degradation (data not shown).

**Fig. 2.** Inducible expression of myc-tagged DN and CA RhoA, Rac1, and Cdc42 and their distribution in polarized MDCK cells. A: MDCK monolayers were induced to express mutant GTPases for 18–48 h. The filter-grown cells were lysed, and equal amounts of total cellular protein were separated by SDS-PAGE and Western blotted with antibodies to RhoA, Rac1, and Cdc42 to detect induction of the myc-tagged RhoA, Rac1, and Cdc42 mutant proteins (arrows) as well as the endogenous proteins. DC, doxycycline. B: MDCK cells grown as in A were fixed, permeabilized, and labeled with anti-c-myc antibody followed by FITC-conjugated secondary antibody and visualized by confocal microscopy. Images represent an overlay from the whole layer. Bar, 10 μm.

**Fig. 3.** Effect of DN and CA RhoA, Rac1, and Cdc42 on F-actin distribution in transfected MDCK cells. MDCK monolayers were induced to express mutant GTPases for 18–48 h. Cells were fixed and stained with rhodamine-phalloidin and examined by confocal microscopy. A–G: x-y images taken in the plane of the tight junction (TJ). Arrows in A indicate a circumferential band of F-actin at the peripheral level. Arrows in B indicate reduction in F-actin staining at the perijunctional ring. H–N: 3–4 images taken at the basal membrane. Bottom: x-z images taken through the full thickness of the layers. Ap, apical. Bar, 10 μm.
Immediately subjacent to the TJ, the AJ proteins β-catenin (Fig. 5A) and E-cadherin (Fig. 5B) were also visualized in a ring pattern in control cells by en face confocal imaging. Subtle diffusions of both β-catenin and E-cadherin away from the membrane were observed in cells expressing RhoAV14 (Fig. 5, A5 and B5) and Cdc42V12 (Fig. 5, A7 and B7). In contrast, all other induced GTPases evoked staining patterns similar to those in control cells. Analogous to TJ proteins, total cellular levels of AJ proteins were not changed by GTPases activation (data not shown).

**Effect of mutant GTPases on detergent solubility of AJC proteins.** In epithelial cells with mature cell-cell junctions, a significant fraction of TJ and AJ proteins partition into a TX-100-insoluble pool that may reflect affiliation with the cytoskeleton or protein oligomerization (46). Because inactivation as well as activation of GTPases enhanced paracellular

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**Fig. 4.** Effect of DN and CA RhoA, Rac1, and Cdc42 on distribution of TJ proteins in transfected MDCK cells. MDCK monolayers were induced to express mutant GTPases for 18–48 h. Cells were fixed and stained for occludin (A1–A7), ZO-1 (B1–B7), claudin-1 (C1–C7), claudin-2 (D1–D7), and junctional adhesion molecule (JAM)-1 (E1–E7) and examined by confocal microscopy. The en face (x-y) confocal images shown were taken in the plane of the TJ. Arrows, protein discontinuities; arrowheads, protein internalization. Bar, 10 μm.

**Fig. 5.** Effect of DN and CA RhoA, Rac1, and Cdc42 on distribution of adherens junction (AJ) proteins in MDCK cells. MDCK monolayers were induced to express mutant GTPases for 18–48 h. Cells were fixed and stained with β-catenin (A1–A7) or E-cadherin (B1–B7) and examined by confocal microscopy. The en face (x-y) confocal images shown were taken at the level of the AJ. Bar, 10 μm.
permeability and induced TJ/AJ protein redistribution, we analyzed the TX-100 solubility profiles of these proteins (Fig. 6). In control monolayers (pUHD), high-molecular-weight or junction-associated occludin and ZO-1 partitioned predominantly to the TX-100-insoluble pool. As shown previously (21), expression of RhoAN19, RhoAV14, Rac1N17, Rac1V12, Cdc42N17, or Cdc42V12 had no effect on the TX-100 solubility properties of occludin or ZO-1 (data not shown). In contrast, JAM-1 was predominantly found in the TX-100-soluble pool, as recently described in T84 cells (4), and was influenced neither by inactivation nor by activation of GTPases (data not shown). Claudin-1 and -2 were found in both the TX-100-insoluble and -soluble fractions of pUHD cells and all induced GTPases. However, activation of RhoA, Rac1, and Cdc42 did induce a shift into the TX-100-soluble pool. Den- sitsometric estimation of the ratio of TX-100-insoluble to -soluble pool further emphasized the significant shift of claudin-1 and -2 into the TX-100-soluble pool in all constitutively activated GTPases (data not shown).

The AJ proteins E-cadherin and β-catenin were found in both the TX-100-insoluble and -soluble fractions of control and all induced GTPases (Fig. 6). We observed a significant shift of E-cadherin toward the TX-100-soluble pool after inactivation of Rac1. These results suggest that a significant fraction of TX-100-insoluble E-cadherin is affiliated with the actin cytoskeleton and is influenced by inactivation of Rac1, causing a decrease in cell-cell adhesion.

Influence of GTPase inactivation/activation on affiliation of AJC proteins with membrane rafts. TX-100 insolubility has also been described for proteins partitioning to membrane microdomains or DIGs (31, 46). Because we previously showed affiliation of TJ proteins with membrane rafts (31) and inactivation of Rho GTpases by Clostridium difficile toxins induced redistribution of occludin and ZO-1 from detergent-insoluble membrane microdomains (32), we analyzed the influence of inactivation/activation of GTpases on TJ/AJ protein affiliation with membrane rafts. Light scattering at 600 nm, alkaline phosphatase activity, and protein profiles were not significantly different in GTPase-inactivated and -activated cells, suggesting that the overall biophysical properties of membrane rafts were not altered (data not shown). In cells with an empty vector (pUHD) or in noninduced cells (data not shown), hyperphosphorylated occludin, ZO-1, and claudin-2 were predominantly localized in membrane raft-containing light fractions (Fig. 7). In contrast, a significant pool of claudin-1, β-catenin, and E-cadherin localized in non-DIG fractions at the bottom of the gradient. Induction of RhoAV14 promoted an increase in the proportion of ZO-1 and claudin-2 in high-density fractions at the bottom of the gradients. In contrast, associations of other TJ or AJ proteins with DIG fractions were minimally affected by activation or inactivation of RhoA, Rac1, or Cdc42 (data not shown).

![Fig. 6. Triton X-100 (TX-100) solubility profiles of TJ proteins claudin-1 and claudin-2 and AJ proteins β-catenin and E-cadherin in MDCK-cells expressing RhoA, Rac1, or Cdc42 mutants.](image-url)

![Fig. 7. Effect of DN and CA RhoA on the distribution of TJ/AJ proteins in membrane microdomains.](image-url)
DISCUSSION

The precise regulation of intestinal epithelial TJs is crucial to maintaining barrier function between the luminal milieu and the internal environment. Recent studies have revealed an important role for Rho GTPases in regulating TJ structure/function (22, 29). In particular, TJ strand organization has been shown to be altered by constitutively active RhoA and Rac1 mutants (22) and inactivation of GTPases by C. difficile toxins is known to cause redistribution of occludin and ZO-1 from membrane microdomains or membrane rafts (32). As a result, we have further explored the mechanisms whereby paracellular permeability is influenced by this family of mediators and investigated whether the inactivation of a single GTPase (RhoA, Rac1, or Cdc42) has an effect on TJ distribution in such membrane rafts and whether TJ proteins involved in strand formation (such as claudin-1 and -2) are altered in this setting.

Using MDCK cell lines that express constitutively active or dominant-negative RhoA, Rac1, or Cdc42 under the control of the tetracycline-repressible transactivator (12, 21), we show that activation as well as inactivation of each GTPase enhances paracellular permeability. Because TJs are key structural components that regulate epithelial paracellular permeability, we analyzed mechanisms by which activation or inactivation may regulate such properties in epithelial cells. It was previously documented that the apical perijunctional F-actin ring plays a central role in regulating TJ function (26). F-actin filaments in this ring are themselves intimately associated with the actin filaments of the terminal web into which the F-actin-rich microvillus core rootlets are linked in a myosin-dependent manner (20, 42). In our study, enhanced paracellular permeability induced by both activation and inactivation of RhoA, Rac1, or Cdc42 was paralleled by profound reorganization of the F-actin cytoskeleton and/or TJ proteins. RhoAN19 expression evoked significant reductions in F-actin staining intensity at the level of both the perijunctional ring and the basal stress fibers without affecting TJ protein localization and distribution in membrane rafts. Our previous results (29) showed that inactivation of RhoA with C. botulinum transferase was associated with reorganization of F-actin in the perijunctional F-actin ring as well as redistribution of ZO-1 from the TJ membrane. Moreover, using C. difficile toxins A or B to inactivate RhoA, Rac1, and Cdc42, we showed that enhanced paracellular permeability was associated with reduction of hyperphosphorylated occludin and displacement of ZO-1 from membrane rafts (32). In contrast, disturbances in barrier function induced on inhibition of Rho kinase, a downstream effector of Rho GTPases, were characterized by reorganization of apical F-actin without alterations in TJ protein localization (44). Discrepancies in these results may be due to several factors. First, effects of different toxins, pharmacological agents, or GTPase mutants can be different. In this regard, it should be noted that the expression level of dominant-negative RhoA in our study was relatively low, inducing a leaky TJ gate function without any apparent effects on TJ structure/function. In contrast, inactivation of RhoA by the above-mentioned toxins induced a dramatic increase in paracellular permeability after short-term incubation that was more profound than the respective changes observed after inhibition of Rho kinase signaling (29, 44). Second, activated Rho can engage several different efforts in addition to Rho kinase, thereby accounting for this difference in effect on TJ protein organization. Third, the combined inactivation of RhoA, Rac1, and Cdc42 by C. difficile toxins is likely to affect TJ structure/function more dramatically than inactivation of RhoA alone. Therefore, it seems likely that only dramatic or toxic effects on barrier function induce displacement of TJ proteins from membrane rafts.

However, RhoA inactivation could be clinically relevant as a therapeutic approach for inflammatory disorders such as inflammatory bowel disease (IBD), in which TJ proteins are disrupted and proinflammatory cytokines such as tumor necrosis factor-α and interferon-γ are elevated (4, 23). It was shown recently that RhoA activation levels are increased in the inflamed mucosa of IBD patients (40). Our in vitro results as well as a recent report by Hopkins et al. (19) showing dramatic effects of CNF-1 on the localization of the TJ proteins occludin, ZO-1, and JAM-1 support the above hypothesis.

Furthermore, internalization of claudin-1 and -2 and changes in their biochemical properties induced by constitutively active GTPases could explain alterations in the formation of TJ strands, although TX-100 solubility for other TJ proteins such as occludin and ZO-1 remained unaltered in our model and in a previous report (22). Disruption of the occludin gene in embryonic stem cells has revealed functional TJ strand formation in the absence of occludin (37), whereas claudin-1 and -2 have been shown to be essential for TJ strand formation (8). In our model, JAM-1 localization was also disturbed but, unlike the other TJ proteins, did not virtually “disappear” from the plane of the TJ membrane in response to RhoA activation. Instead, some diffusion of JAM-1 away from the membrane was evident in en face confocal images. It is intriguing to speculate why the disruption of JAM-1 is not as drastic as that of the other TJ proteins. This might relate to a necessity for JAM-1 localization at the TJ membrane for correct assembly of the TJ protein complex (25). Loss of this protein could prove detrimental for the reestablishment of barrier function after transient insult by RhoA activation. Another feature in RHOV14-expressing cells was loss of polarity, in which cells lost their parallel orientations relative to each other and occasionally formed multilayers. This may suggest that dominant-active RhoA expression interferes not only with cell-cell but also with cell-matrix adhesion.

Another interesting observation from our study was the delay in formation of a confluent monolayer in cells expressing dominant-negative Rac1. It was shown previously that Rac1 inhibition in fibroblasts completely prevents cell movement (17). However, because cells in our epithelial model did eventually achieve confluence, it seems that Rac1 is not the only participant in the complex sequence of events leading to coordinated spreading/migration of epithelial sheets. The importance of Rac1 in epithelial sheet movement is demonstrated by the fact that sustained Rac1 activation has been implicated in keratinocyte migration induced by epidermal growth factor (35). However, we cannot exclude the possibility that Rac1 inhibition also interferes with cell-cell (as well as cell-matrix) attachment in our model. Our results do conflict with a previous report by Jou et al. (22) in the same inducible GTPase system, where Rac1N17-expressing cells formed a monolayer of uniformly shaped cells 18 h after induction of cell-cell contacts. This discrepancy may arise from differences in cell
seeding density, because the same group showed that Rac1N17-expressing cells grown at low density rounded up and spread less on the substratum, a feature that we also observed in our immunofluorescence staining for F-actin. Moreover, Rac1N17 expression resulted in substantial reductions in the microvillous F-actin pool and some submembranous diffusion away from the perijunctional ring. These changes were associated with alterations in claudin-1, claudin-2, and JAM-1 localization, whereas occludin and ZO-1 remained unaltered in cells expressing Rac1N17 as described previously (22).

In conclusion, we have shown that Rho family GTPases regulate epithelial intercellular junctions via distinct morphological and biochemical mechanisms and that perturbations in barrier function reflect any imbalance in active vs. resting GTPase levels rather than simply loss or gain of GTPase activity.

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