Rho/Rho-associated Kinase-II Signaling Mediates Disassembly of Epithelial Apical Junctions

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Apical junctional complex (AJC) plays a vital role in regulation of epithelial barrier function. Disassembly of the AJC is observed in diverse physiological and pathological states; however, mechanisms governing this process are not well understood. We previously reported that the AJC disassembly is driven by the formation of apical contractile acto-myosin rings. In the present study, we analyzed the signaling pathways regulating acto-myosin-dependent disruption of AJC by using a model of extracellular calcium depletion. Pharmacological inhibition analysis revealed a critical role of Rho-associated kinase (ROCK) in AJC disassembly in calcium-depleted epithelial cells. Furthermore, small interfering RNA (siRNA)-mediated knockdown of ROCK-II, but not ROCK-I, attenuated the disruption of the AJC. Interestingly, AJC disassembly was not dependent on myosin light chain kinase and myosin phosphatase. Calcium depletion resulted in activation of Rho GTPase and transient colocalization of Rho with internalized AJC proteins. Pharmacological inhibition of Rho prevented AJC disassembly. Additionally, Rho guanine nucleotide exchange factor (GEF)-H1 translocated to contractile F-actin rings after calcium depletion, and siRNA-mediated depletion of GEF-H1 inhibited AJC disassembly. Thus, our findings demonstrate a central role of the GEF-H1/Rho/ROCK-II signaling pathway in the disassembly of AJC in epithelial cells.

INTRODUCTION

The intercellular apical junctional complex (AJC) (Matter and Balda, 2003) regulates epithelial barrier function, permitting the passive entry of nutrients, ions, and water, while restricting pathogen access to underlying tissue compartments. The most apical tight junction (TJ) and its subjacent adherens junction (AJ) constitute the AJC. While TJs are responsible for maintaining the seal between epithelial cells (Tsukita *et al.*, 2001), AJs are vital for initiating and maintaining cell–cell contacts (Yap *et al.*, 1997).

Both TJs and AJs represent multiprotein complexes composed of transmembrane proteins that affiliate with cytoplasmic plaque proteins. The former proteins mediate cell–cell adhesion, whereas the latter link TJs and AJs to the cytoskeleton and participate in intracellular signaling. Transmembrane proteins in TJs include occludin, claudin family of proteins, coxsackie adenovirus receptor and junctional adhesion molecule (JAM)-A, whereas cytoplasmic

plaque proteins consist of a number of scaffolding and signaling molecules, such as zonula occludens (ZO) family proteins and cingulin (Tsukita *et al.*, 2001). In AJs, the transmembrane protein E-cadherin associates with α -, β -, and p120 catenin cytoplasmic proteins (Yap *et al.*, 1997). Several AJ and TJ cytosolic plaque proteins have been shown to directly interact with F-actin (Mege *et al.*, 2006) and myosin (Cordenonsi *et al.*, 1999). Such interactions are likely to mediate the attachment of the AJC to the perijunctional acto-myosin bundles, and they are thought to stabilize apical junctions and regulate their dynamics (Turner, 2000).

The AJC is a highly dynamic structure and may be rapidly and reversibly disassembled in different physiological and pathological circumstances, such as spermatogenesis (Lui and Lee, 2006), epithelial–mesenchymal transition (Grunert *et al.*, 2003), during bacterial and viral invasion (Hecht *et al.*, 1988; Spitz *et al.*, 1995), and diapedesis (Sandig *et al.*, 1997; Xu *et al.*, 2005). Nevertheless, the signaling pathways regulating AJC disassembly remain enigmatic.

We recently reported that rapid disassembly of the AJC in calcium-depleted intestinal epithelial cells was accompanied by a dramatic reorganization of perijunctional F-actin bundles into centrally located ring-like structures (Ivanov *et al.*, 2004a). The contraction of these newly formed F-actin rings resulted in retraction of plasma membranes of adjacent cells, thereby providing the force for the disruption of their intercellular junctions. Contractile F-actin rings contained activated (phosphorylated) mammalian nonmuscle myosin (MNMM) II. Furthermore, the formation of contractile F-actin rings and disruption of AJCs were blocked by a selective MNMM II inhibitor, blebbistatin (Ivanov *et al.*, 2004a).

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Abbreviations used: AJ, adherens junction; AJC, apical junctional complex; GEF, guanine nucleotide exchange factor; JAM, junctional adhesion molecule; MLCK, myosin light chain kinase; MNMM, mammalian non-muscle myosin; MYPT, myosin phosphatase target subunit; RMLC, regulatory myosin light chain; ROCK, Rho-associated kinase; TEER, transepithelial electrical resistance; TJ, tight junction.

Based on these data, we concluded that acto-myosin contraction is a critical mediator of epithelial AJC disassembly.

The present study was designed to identify signaling pathways that regulate acto-myosin contraction to drive disassembly of apical junctions. Using T84 and SK-CO15 intestinal epithelial cell lines and a classical extracellular Ca^{++} depletion model (Gonzalez-Mariscal *et al.*, 1985; Siliciano and Goodenough, 1988; Ivanov *et al.*, 2004a,b, 2006), we report that the Rho exchange factor, guanine nucleotide exchange factor (GEF)-H1 acts upstream of Rho/ROCK-II signaling, resulting in acto-myosin contraction and disassembly of apical junctions.

MATERIALS AND METHODS

Antibodies

Anti-RhoA (119) antibody recognizing RhoA, RhoB, and RhoC; anti-ROCK-I (H-85); anti-ROCK-II (H-85 and C-20); anti-myosin phosphatase target subunit (MYPT)1 (H-130); anti-phospho(Thr696)-MYPT1 (R) and anti-myosin light chain kinase (MLCK) (H-195) polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-occludin rabbit polyclonal and anti-E-cadherin monoclonal antibodies were obtained from Zymed Laboratories (San Francisco, CA). Anti-GEF-H1 rabbit polyclonal antibodies were obtained from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal anti-actin antibodies were from Sigma-Aldrich (St. Louis, MO). Alexa 488/546/633-conjugated goat anti-mouse, goat anti-rabbit, and donkey anti-rabbit antibody were purchased from Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cells and Transepithelial Electrical Resistance (TEER) Measurement

T84 (American Type Culture Collection, Manassas, VA) and SK-CO15 (gift from Dr. E. Rodriguez-Boulan, Weill Medical College of Cornell University, NY) human colonic epithelial cells were grown as described previously (Le Bivic *et al.*, 1989; Utech *et al.*, 2005; Ivanov *et al.*, 2006). Cells were plated on collagen-coated polycarbonate Transwell filters (Corning Life Sciences, Acton, MA) with 0.4- μm pore size and either 0.33- or 5- cm^2 surface area for immunofluorescence staining and biochemical experiments, respectively, and grown for 6–10 d to confluence. The confluence of epithelial monolayers was controlled by TEER measurements (usually to be 1500–3000 Ωcm^2 for both T84 and SK-CO15 cells). TEER of epithelial monolayers was measured using EVOM electrovoltmeter (WPI, Sarasota, FL). The resistance of cell-free collagen-coated Transwell filters was used as a reference.

Depletion of Extracellular Ca^{++} and Pharmacological Modulation of AJC Disassembly

The depletion of extracellular Ca^{++} in T84 and SK-CO15 cells was performed as described previously (Ivanov *et al.*, 2004a,b, 2006). Confluent monolayers were washed twice with calcium-free Eagle's minimum essential medium (Sigma-Aldrich) supplemented with 15 mM HEPES, pH 7.4, 2 mM EGTA, and containing either 10% fetal bovine serum dialyzed against calcium-free phosphate-buffered saline for SK-CO15 cells, or 5% dialyzed newborn calf serum for T84 cells. Then, cells were incubated in the same Ca^{++} -free medium for either 60 min (SK-CO15 cells) or 30 min (T84 cells) at 37°C. Control monolayers were washed two times in and incubated with normal cell culture medium only.

For pharmacological inhibition of MLCK and ROCK, T84 and SK-CO15 cells were preincubated in the normal cell culture medium containing either 30 μM ML-7 or 20 μM Y-27632, or H-1152 (all from EMD Biosciences, San Diego, CA), respectively, for 60 min followed by incubation in calcium-free medium containing the same concentration of inhibitor for indicated time. DC3B (gift from Dr. P. Boquet, Institut National de la Santé et de la Recherche Médicale, Nice, France) was carefully titrated to achieve the efficient inhibition of AJC disassembly in calcium-depleted cells without inducing alterations in the staining patterns of F-actin and AJC proteins in the cells growing in complete media. Cells were preincubated with DC3B (2 $\mu\text{g}/\text{ml}$) for 4 h. Then, cells were transferred in Ca^{++} -free medium containing the same concentration of inhibitor and incubated for either 60 or 30 min for SK-CO15 and T84 cells, respectively.

Immunofluorescence Labeling and Confocal Microscopy

Immunolabeling and confocal microscopy of fixed T84 and SK-CO15 cells was described in detail previously (Utech *et al.*, 2005; Ivanov *et al.*, 2006). Briefly, cells were fixed/permeabilized either in absolute ethanol or ethanol:acetone (1:3) mix for 20 min at -20°C . For Rho staining, cells were pre-extracted with cytoskeleton-stabilizing buffer [10 mM 2-(N-morpholino)ethanesulfonic acid, 138 mM KCl, 3 mM MgCl_2 , 2 mM EGTA, 0.32 M sucrose, 0.5% Triton X-100,

and 1 $\mu\text{g}/\text{ml}$ phalloidin, pH 6.1) for 10 min at 4°C before the fixation (Cramer *et al.*, 2002; Ivanov *et al.*, 2005). For visualization of F-actin, cells were fixed in 3.7% paraformaldehyde for 15 min and subsequently permeabilized with 0.5% Triton X-100 for 10 min at room temperature (RT). Cells were blocked in Hanks' balanced salt solution (HBSS+) (Sigma-Aldrich) containing 1.5% bovine serum albumin, and they were sequentially incubated with primary and Alexa-conjugated secondary antibodies or Alexa-phalloidin (Invitrogen) for 60 min at RT, followed by mounting on slides using ProLong Antifade medium (Invitrogen). Stained monolayers were analyzed using Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging, Thornwood, NY) equipped with Zeiss 40 $\times$ Pan-Neofluar or 100 $\times$ Pan-Apochromat oil lenses. Images shown are representative of at least three independent experiments with multiple images taken per slide.

Immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Na deoxycholate, 2 mM EGTA, 2 mM EDTA, and 0.1% SDS) containing protease inhibitor cocktail (1:100; Sigma-Aldrich) and phosphatase inhibitor cocktails 1 and 2 (both at 1:200; Sigma-Aldrich). Lysates were spun at 14,000g for 10 min at 4°C to remove cellular debris and equalized for total protein concentration by using BCA protein assay (Pierce Chemical, Rockford, IL). Then, samples were boiled in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis and Western blotting with 10–30 μg of total equalized protein per lane. The results shown are representative immunoblots of three independent experiments.

Rho Activation Assay

The activation status of Rho was accessed using Rho activation assay kit (Upstate Biotechnology, Charlottesville, VA) according to manufacturer's instructions and previously published protocols (Hopkins *et al.*, 2003; Utech *et al.*, 2005). Samples were analyzed by immunoblotting using rabbit polyclonal anti-RhoA antibody (Santa Cruz Biotechnology). Quantification of the protein expression was carried out by densitometric analysis of at least three immunoblots each representing independent experiment using the UN-SCAN-IT gel automated digitizing software (Silk Scientific, Orem, UT) and Scion Image software (Scion, Frederick, MD).

siRNA Silencing

p160ROCK duplex 2, Hs_ROCK2 duplex 1 (both from QIAGEN, Valencia, CA), ARHGEF2 siGENOME duplex, and MLCK siGENOME SmartPool (Dharmacon RNA Technologies, Lafayette, CO) siRNAs have been used to down-regulate ROCK-I, ROCK-II, GEF-H1, and MLCK, respectively. SK-CO15 cells were plated on Transwell filters (Corning Life Sciences) at ~75% confluence, and the next day they were transfected in Opti-MEM I media (Invitrogen) with 50 nM siRNA using Lipofectamine 2000 (Invitrogen). Scramble duplex and Lamin A/C siRNA (Dharmacon RNA Technologies) have been used as a control. Seventy-two hours posttransfection, cells were analyzed by immunoblotting or used for immunofluorescence labeling.

Statistical Analysis of TJs Disassembly

Cells were stained for occludin, and fluorescent confocal images with 512×512 pixels resolution were acquired at the level of TJs by using Zeiss 40 $\times$ Pan-Neofluar oil objective. To ensure that siRNA transfection does not cause the detachment of cells from the substrate, the same microscopic fields were analyzed by differential interference contrast microscopy. Cells maintaining TJs with their neighbors were identified as cells with discontinuous (showed up on confocal images bigger than a visual dot) occludin localization pattern common between two or more neighbor cells. If in one cell occludin showed diffuse cytoplasmic localization or peripheral ring-like pattern and/or had no discontinuous contact with the other cells, these cells were considered as cells with disassembled TJs (Supplemental Figure 1).

RESULTS

Pharmacological Inhibition of ROCK Prevents the Disruption of AJC

The assembly of acto-myosin complexes and its contractility both require the activation of MNMM II through serine/threonine phosphorylation of the regulatory myosin light chain (RMLC) (Tan *et al.*, 1992; Bresnick, 1999). Multiple protein kinases have been shown to phosphorylate RMLC (Matsumura, 2005). Among them, Rho-associated kinase (ROCK) (Amano *et al.*, 1996; Totsukawa *et al.*, 2000) and MLCK (Adelstein, 1982) are thought to be key activators of myosin II in nonmuscle cells. Therefore, we first investigated whether ROCK is involved in the formation of contractile F-actin rings and subsequent disassembly of AJC in Ca^{++} -

depleted cells by using two chemically unrelated selective ROCK inhibitors, Y-27632 (Uehata *et al.*, 1997) and H-1152 (Sasaki *et al.*, 2002). Confluent T84 cells were pretreated with either 20 μ M Y-27632 or vehicle for 60 min, followed by 30 min of calcium depletion in the presence of the inhibitor. The formation of contractile F-actin rings and disassembly of AJs and TJs were analyzed by immunolabeling and confocal microscopy.

In agreement with our previous studies (Ivanov *et al.*, 2004a), calcium depletion of vehicle-treated T84 monolayers resulted in dramatic changes in staining patterns for F-actin and AJC proteins, from a characteristic cobblestone-like staining pattern in normal calcium (~ 2 mM) conditions (Figure 1A) to subapical ring-like structures in calcium-depleted cells. Inhibition of ROCK with Y-27632 completely blocked formation of contractile F-actin rings and translocation of AJ proteins E-cadherin (Figure 1A) and β -catenin (data not shown) and TJ proteins occludin (Figure 1A) and JAM-A (data not shown) from the areas of cell-cell contacts into cytosolic ring-like structures in calcium-depleted cells. Similar inhibition of F-actin reorganization and AJC disassembly was observed in SK-CO15 cells (data not shown). Moreover, similar results were obtained in both cell lines by using 20 μ M H-1152 (data not shown). Importantly, ROCK inhibition not only prevented gross morphological changes in AJC structure but also attenuated the loss of paracellular barrier function in calcium-depleted cells. Indeed, although the depletion of extracellular calcium in vehicle-treated T84 monolayers caused the dramatic drop in TEER from ~ 2500 Ohm $\cdot$ cm 2 down to resistance of cell-free Transwell filters, the TEER values of calcium-depleted Y-27632- and H-1152-treated cells remained significantly higher at ~ 1230 and ~ 1770 Ohm $\cdot$ cm 2 , respectively (Figure 1B). Together, these data strongly suggest that ROCK plays a critical role in the regulation of AJC disassembly in Ca $^{++}$ -depleted epithelial cells.

Rho-associated Kinase-II, but Not ROCK-I, Regulates the Disassembly of AJC in Ca $^{++}$ -depleted Cells

Two isoforms of Rho-associated kinase termed ROCK-I (p160ROCK or ROK β) and ROCK-II (p150ROCK or ROK α) (Riento and Ridley, 2003) have been recently reported to have distinct cellular functions and different modes of regulation of myosin II activity (Yoneda *et al.*, 2005). Therefore, we sought to determine the isoform of ROCK that mediates disassembly of the AJC in calcium-depleted epithelial cells.

Because the disassembly of the AJC in calcium-depleted epithelial cells is driven by the contraction of apical actomyosin rings, which requires activity of myosin II (Ivanov *et al.*, 2004a), responsible kinases and other signaling molecules must either physically interact with acto-myosin rings, or accumulate in the vicinity of the rings. Therefore, we analyzed localization of ROCK-I and ROCK-II in control and calcium-depleted T84 cells. In confluent T84 monolayers grown in normal calcium conditions, both ROCK-I and ROCK-II showed diffuse cytoplasmic staining pattern without significant colocalization with AJC proteins occludin (Figure 2, A and B, top) or E-cadherin (data not shown). In contrast, after 30 min of calcium depletion, both ROCK-I and ROCK-II were found in subapical ring-like structures where they colocalized with occludin (Figure 2, A and B, bottom). Similar colocalization of ROCK-I and ROCK-II with internalized occludin was observed in Ca $^{++}$ -depleted SK-CO15 cell monolayers (Supplemental Figure 2).

Because pharmacological inhibitors do not discriminate between two ROCK isoforms, we next selectively down-regulated the expression of ROCK-I and ROCK-II by using

Figure 1. Pharmacological inhibition of ROCK prevents AJC disassembly. T84 cells were preincubated with either vehicle or 20 μ M Y-27632 or 20 μ M H-1152 for 1 h before incubation in Ca $^{++}$ -free medium containing the same drugs for 30 min. (A) Both vehicle- and Y-27632-treated cells were fixed with ethanol and costained for occludin and E-cadherin. Note that Y-27632 prevented the formation of contractile F-actin rings and internalization/disassembly of TJs and AJs in calcium-depleted cells. Bar, 10 μ m. (B) TEER was measured in vehicle-, Y-27632-, and H-1152-treated cells. Each bar represents the average $\pm$ SE of three independent experiments with at least four Transwell filters per experiment. Both Y-27632 and H-1152 significantly attenuated the drop in TEER induced by calcium depletion (* p < 0.05 compared with calcium-depleted vehicle-treated control).

isoform-specific siRNAs and analyzed the effects of such a down-regulation on the disassembly of the AJC in calcium-depleted cells. Given that T84 cells are not amenable to transient transfection with siRNA, we chose to work with SK-CO15 cells for these series of experiments. Transfection of SK-CO15 cells with ROCK-I siRNA duplex dramatically (up to 85%) down-regulated protein expression of ROCK-I without changing the expression levels of ROCK-II and vice versa (Figure 3A). Additionally, down-regulation of ROCKs did not alter the expression levels of the AJC proteins (occludin, claudin-1, ZO-1, E-cadherin, and β -catenin) as well as the molecules involved in the regulation of actomyosin contractility (RMLC, Rho, and MYPT) (data not shown). Both ROCK-I- and ROCK-II-deficient SK-CO15 cells

Figure 2. Colocalization of ROCK-I and ROCK-II with disassembled TJs. Control T84 cells and T84 monolayers incubated in Ca^{++} -free medium for 30 min were either fixed with ethanol and costained for occludin and ROCK-I (A) or fixed in acetone:ethanol mix and costained for occludin and ROCK-II (B). Although ROCK-I and ROCK-II showed diffuse cytoplasmic staining and did not localize with TJ proteins in control cells, they both colocalized with occludin in contractile rings (arrowheads) upon calcium depletion. Bar, 10 μm .

formed structurally normal TJs as was observed by immunofluorescence labeling for occludin after 72–96 h posttransfection (data not shown). However, we observed a different sensitivity of apical junctions to calcium depletion-triggered disassembly in ROCK-I and ROCK-II siRNA-transfected cells. In scramble siRNA-transfected monolayers $73.3 \pm 2.4\%$ of the total cell population lost their occludin staining from cell–cell contacts after 1 h of calcium depletion (Figure 3, B and C). A similar number of ROCK-I-deficient cells ($71.3 \pm 4.6\%$) disassembled their apical junctions in calcium-free conditions (Figure 3, B and C). In contrast, depletion of ROCK-II reduced the number of cells with disassembled TJs up to $42.3 \pm 4.7\%$, i.e., 1.7 times less than that in scramble and ROCK-I siRNA-transfected cells (Figure 3, B and C). Together, these data suggest that ROCK-II, but not ROCK-I, is responsible for activation of acto-myosin contractility and disassembly of the AJC in calcium-depleted epithelial cells.

MLCK Does Not Regulate the AJC Disassembly in Ca^{++} -depleted Cells

To analyze the role of MLCK in the formation of contractile F-actin rings and disassembly of the AJC, confluent T84 cells were pretreated with either 30 μM ML-7, cell-permeable selective inhibitor of MLCK (Saitoh *et al.*, 1986; Morel *et al.*,

Figure 3. Effect of ROCK-I and ROCK-II siRNA knockdown on TJ disassembly in Ca^{++} -depleted cells. SK-CO15 cells were transfected with either control scramble duplex, or ROCK-I duplex 2, or ROCK-II duplex 1 siRNA. Cells were analyzed 72 h posttransfection. (A) Cells were lysed and analyzed by Western blotting by using anti-ROCK-I and anti-ROCK-II antibodies. To ensure equal protein loadings the membranes were stripped and reprobed for actin. Transfection of cells with either ROCK-I or ROCK-II siRNA duplex dramatically ($\sim 85\%$) and specifically reduced the expression level of the corresponding ROCK isoform. (B and C) Cells were incubated either in complete or Ca^{++} -free medium for 60 min, fixed, and stained for occludin. (B) Representative en face confocal images showing the distribution occludin in scramble duplex-, ROCK-I duplex 2-, or ROCK-II duplex 1-transfected cells. Down-regulation of ROCK-I protein level did not significantly alter the number of cells with disassembled occludin junctions, whereas siRNA knockdown of ROCK-II significantly preserved TJs from disassembly in calcium-depleted medium. Bar, 50 μm . (C) Quantitative analysis of TJ disassembly derived from images in B. Each bar represents the average $\pm$ SE from three independent experiments with at least five randomly selected microscopic fields taken in each experiment. * $p < 0.05$ compared with scramble duplex and ROCK-I duplex 2-transfected cells.

Figure 4. MLCK does not mediate AJC disassembly in Ca⁺⁺-depleted cells. (A) T84 cells were preincubated either with vehicle or 30 μ M ML-7 for 1 h followed by incubation in Ca⁺⁺-free medium containing the same drug for 30 min. Cells were fixed and stained for F-actin, occludin, and E-cadherin. Depletion of extracellular calcium induced dramatic reorganization of apical actin into contractile F-actin rings and internalization of AJ/TJ proteins from cell-cell contacts to contractile rings. ML-7 did not affect the formation of F-actin rings and AJC disassembly. Bar, 10 μ m. (B–D) SK-CO15 cells were transfected with either control scramble duplex or MLCK SmartPool siRNA and analyzed 72 h posttransfection. (B) Cells were lysed and analyzed by Western blotting by using anti-MLCK antibodies. To ensure equal protein loadings, the membranes were stripped and re-probed for actin. Transfection of cells with MLCK

SmartPool siRNA dramatically (~85%) reduced the expression level of MLCK. (C and D) Cells were incubated either in complete or Ca⁺⁺-free medium for 60 min, fixed, and stained for occludin. (C) Representative en face confocal images showing the distribution of occludin in scramble duplex- and MLCK SmartPool-transfected cells. Bar, 50 μ m. (D) Quantitative analysis of TJ disassembly derived from images in B. Each bar represents the average $\pm$ SE from three independent experiments with at least five randomly selected microscopic fields taken in each experiment. Depletion of extracellular calcium resulted in the loss of occludin staining from cell-cell contacts in the majority of cell population. Note that depletion of MLCK did not significantly change the number of cells with disassembled occludin junctions compared with the scramble siRNA-transfected cells ($p > 0.5$). (E) Control T84 cells and T84 cells incubated in Ca⁺⁺-free medium for 30 min were fixed and stained for MLCK and F-actin. Although MLCK clearly colocalized with perijunctional F-actin belt in control cells (arrowheads), no colocalization of MLCK with contractile F-actin rings was observed in calcium-depleted cells. Bar, 10 μ m.

1990), or vehicle followed by 30 min of calcium depletion in the presence of the drug. The formation of contractile F-actin

rings and disassembly of the AJC were analyzed in fixed cells by fluorescent staining for F-actin and immunolabeling

for E-cadherin and occludin, respectively. Compared with the vehicle-treated control, incubation with ML-7 did not affect the formation of F-actin rings containing E-cadherin and occludin in calcium-depleted T84 cells (Figure 4A). To verify that ML-7 is active in our experimental conditions, we analyzed the phosphorylation status of myosin II by Western blotting by using phospho-specific anti-RMLC antibody in cells preincubated either with vehicle or ML-7. ML-7 significantly decreased RMLC phosphorylation in T84 cells at a concentration (30 μ M) that had no effect on junctional disassembly (data not shown).

To confirm the above-mentioned ML-7 results, we used siRNA silencing technique to deplete MLCK from epithelial cells, and we analyzed the effect of such a depletion on the AJC disassembly. Transfection of SK-CO15 cells with MLCK SmartPool siRNA dramatically (up to 85%) down-regulated the expression of MLCK (Figure 4B), but it had no effect on the sensitivity of TJs to calcium depletion (Figure 4C). We found that $68.7 \pm 6.9\%$ of scramble siRNA-transfected cells lost their occludin staining from the areas of cell-cell contacts after 1 h of calcium depletion, which was not significantly different from that observed in MLCK-deficient cells ($66.7 \pm 2.9\%$) (Figure 4D).

Next, we analyzed localization of total MLCK, as well as two phosphorylated forms of MLCK [phospho-(Tyr464)-MLCK and phospho-(Tyr471)-MLCK] known to regulate acto-myosin contractility (Birukov *et al.*, 2001) in control T84 monolayers and T84 cells subjected to Ca^{++} depletion. In confluent cell monolayers growing at normal extracellular calcium conditions, both MLCK (Figure 4E, arrowheads) and fraction of phospho-(Tyr464)-MLCK (data not shown) were enriched at the perijunctional F-actin belt and AJC. However, no colocalization of MLCK (Figure 4E) or either of its phospho-forms (data not shown) with contractile F-actin rings and disassembled AJC was observed in Ca^{++} -depleted cells. Similar labeling patterns of MLCK were obtained in SK-CO15 colonic epithelial cells (data not shown). Collectively, our pharmacological, siRNA and immunolabeling data argue against the involvement of MLCK in the formation of contractile F-actin rings and disassembly of the AJC during calcium depletion in intestinal epithelial cells.

Myosin Phosphatase Does Not Regulate Disassembly of the AJC in Ca^{++} -depleted Cells

ROCK can induce acto-myosin contractility via two different mechanisms. One is a direct phosphorylation of RMLC by ROCK (Amano *et al.*, 1996; Totsukawa *et al.*, 2000). Another involves phosphorylation of the so-called MYPT, resulting in the inhibition of myosin phosphatase (Kimura *et al.*, 1996) and thereby hyperphosphorylation of RMLC. Thus, we investigated whether MYPT-mediated signaling is responsible for the activation of acto-myosin contractility and disassembly of the AJC in calcium-depleted epithelial cells.

The amount of total and inactive (phosphorylated) MYPT was compared in T84 cells incubated in either complete or calcium-free medium. No significant changes were observed in either MYPT or phospho-MYPT protein levels after 30 min of incubation in Ca^{++} -free medium (Figure 5A). Next, we analyzed localization of total MYPT and phospho-MYPT in control and Ca^{++} -depleted cells. Although both active nonphosphorylated (data not shown) and inactive phosphorylated (Figure 5B, top, arrowheads) forms of MYPT colocalized with occludin in intact TJs, neither MYPT (data not shown) nor phospho-MYPT accumulated with occludin in cytosolic ring-like structures in calcium-depleted cells (Figure 5B, bottom). Collectively, these data suggest that the activation of acto-myosin

Figure 5. MYPT does not regulate AJC disassembly in Ca^{++} -depleted cells. (A) Control T84 cells and T84 cells incubated in Ca^{++} -free medium for 30 min were lysed and probed for nonphosphorylated MYPT and phospho-MYPT, respectively. Equal protein loading was controlled by stripping the membrane and reprobing for actin. Calcium depletion does not affect the protein level of both MYPT and phospho-MYPT. (B) Control T84 monolayers and T84 cells incubated in Ca^{++} -free medium for 30 min were fixed and coimmunolabeled for occludin and phospho-MYPT. Although phospho-MYPT colocalizes with occludin in native TJs in control cells (arrowheads), no such colocalization was observed in occludin-containing contractile rings formed in calcium-depleted cells. Bar, 10 μ m.

contractility in calcium-depleted cells is not mediated by the inhibition of myosin phosphatase.

Rho Mediates the Disassembly of the AJC in Ca^{++} -depleted Cells

Because ROCK is a downstream effector for Rho GTPase (Leung *et al.*, 1995; Matsui *et al.*, 1996), we next sought to investigate the role of Rho in the regulation of the AJC disassembly in Ca^{++} -depleted cells. First, we compared the activation status of Rho in T84 cells incubated in high-calcium and calcium-free medium by using a rhotekin pull-down assay. Thirty minutes of calcium depletion significantly (~ 3 -fold) increased the amount of active GTP-bound Rho compared with control cells (Figure 6, A and B). Next, we analyzed the localization of Rho in control nontreated and Ca^{++} -depleted cells. Rho significantly colocalized with occludin in intact AJCs of control monolayers. Depletion of extracellular calcium resulted in disruption of Rho junctional staining pattern and translocation of Rho in occludin-containing contractile F-actin rings (Figure 6C).

Finally, we selectively inhibited Rho in T84 cells by using a cell-permeable chimeric toxin DC3B (Aullo *et al.*, 1993; Nusrat *et al.*, 1995; Walsh *et al.*, 2001), and we examined the effect of such inhibition on AJC disassembly in calcium-depleted cells. DC3B is a fusion protein consisting of *Clostridium botulinum* exoenzyme C3 and diphtheria toxin B fragment. The former inhibits Rho activity, whereas the latter facilitates cellular entry of the toxin (Aullo *et al.*, 1993). Confluent T84 monolayers were pretreated with either DC3B (2 μ g/ml) or vehicle for 4 h followed by 30 min of calcium depletion in the presence of the drug and analyzed by immunolabeling and confocal microscopy. DC3B com-

Figure 6. Rho is activated during AJC disassembly and colocalizes with native and disassembled TJs. (A) Rho activation status was assayed in control T84 cells and cells incubated for 30 min in Ca⁺⁺-free medium as described in *Materials and Methods*. Representative Western blot demonstrating total Rho in cell lysates and GTP-Rho pulled-down from control and Ca⁺⁺-depleted cells is shown. Calcium depletion significantly increased the amount of GTP-bound Rho pulled down with RBD-agarose. (B) Quantification of data on Rho activation assay. Shown are average integrated intensities $\pm$ SE from three independent experiments. * $p < 0.05$ compared with control. (C) Control T84 cells and cells exposed for 30 min in Ca⁺⁺-free medium were pre-extracted with cytoskeleton-stabilizing buffer, fixed, and costained for occludin and Rho. Note significant colocalization of Rho with occludin in native TJs in control cells as well as in contractile rings (arrowheads) in calcium-depleted cells. Magnified image demonstrating colocalization of Rho with occludin in the contractile rings is shown in insert. Bar, 10 μ m.

pletely inhibited the formation of contractile F-actin rings and translocation of AJ proteins E-cadherin (Figure 7) and β -catenin (data not shown) and TJ proteins occludin (Figure 7) and JAM-A (data not shown) from apical junctions in calcium-depleted cells. Together, these data suggest an important role of Rho in the disassembly of the AJC triggered by calcium depletion.

Rho Guanine Nucleotide Exchange Factor GEF-H1 Regulates AJC Disassembly in Ca⁺⁺-depleted Cells

Although the activity of Rho GTPases may be regulated by a variety of signaling molecules, GEFs are considered as major upstream activators of Rho (Schmidt and Hall, 2002). Because Rho guanine nucleotide exchange factor GEF-H1 (Ren *et al.*, 1998) has been recently implicated in the regulation of the AJC in epithelial and endothelial cells (Benaïss-Pont *et al.*, 2003; Birukova *et al.*, 2006), we investigated whether GEF-H1 plays a role in the disassembly of apical junctions induced by calcium depletion. Immunolabeling and confocal microscopy analysis revealed significant colo-

Figure 7. Pharmacological inhibition of Rho prevents AJC disassembly. T84 cells were preincubated either with vehicle or 2 μ g/ml DC3B for 4 h after the incubation in Ca⁺⁺-free medium containing the same drugs for 30 min. Cells were fixed and double-immunolabeled for occludin and E-cadherin. Although DC3B did not affect perijunctional F-actin and AJC in control cells, it completely inhibited formation of contractile F-actin rings and disassembly/internalization of both occludin and E-cadherin junctions in calcium-depleted cells. Bar, 10 μ m.

calization of GEF-H1 and occludin in intact TJs of control T84 monolayers (Figure 8, top). Furthermore, in calcium-depleted cells, GEF-H1 colocalized with internalized occludin in contractile F-actin rings (Figure 8, bottom).

Next, we up to 75% down-regulated expression of GEF-H1 in SK-CO15 cells by using siRNA silencing technique (Figure 9A), and analyzed the effect of such a GEF-H1 knockdown on AJC disassembly. Scramble siRNA-transfected cells responded to 60-min calcium depletion by dis-

Figure 8. Colocalization of GEF-H1 with native and disassembled TJs. T84 monolayers were incubated either in complete or Ca⁺⁺-free medium for 30 min, fixed, and costained for occludin and GEF-H1. Note strong colocalization of GEF-H1 with occludin in both native junctions in control cells and contractile rings in calcium-depleted cells (arrowheads). Bar, 10 μ m.

Figure 9. Effect of GEF-H1 knockdown on TJ disassembly in Ca^{++} -depleted cells. SK-CO15 cells transfected with either control scramble duplex or GEF-H1 duplex siRNA were analyzed 72 h posttransfection. (A) Cells were lysed and analyzed by Western blotting using anti-GEF-H1 antibody. To control equal protein loading the membranes were stripped and reprobed for actin. Transfection of cells with GEF-H1 siRNA dramatically ($\sim 75\%$) reduced expression level of GEF-H1. (B and C) Cells were incubated either in complete or Ca^{++} -free medium for 60 min, and then they were fixed and stained for occludin. (B) Representative en face confocal images showing occludin staining in cells transfected with scramble duplex or GEF-H1 siRNA. Note that knockdown of GEF-H1 significantly attenuated the disassembly of occludin junctions in calcium-free medium. Bar, 50 μm . (C) Quantitative analysis of cells with disassembled occludin junctions derived from images in B. Each bar is the average $\pm$ SE from three independent experiments with at least five randomly selected microscopic fields taken in each experiment. * $p < 0.05$ compared with scramble duplex siRNA-transfected cells.

assembling $73.3 \pm 2.4\%$ of their occludin-containing TJs. A significant reduction in number of disassembled junctions ($37.6 \pm 7.0\%$) was observed in GEF-H1-deficient cells (Figure 9, B and C). To confirm the specificity of the effect of GEF-H1 knockdown we first verified that GEF-H1 depletion did not influence the expression of other molecules involved in acto-myosin contractility (RMLC, Rho, MYPT) or AJC proteins (occludin, claudin-1, E-cadherin, and β -catenin) (data not shown). Second, we analyzed the effect of siRNA-mediated knockdown of another Rho GEF, GEFp115 (Hart *et al.*, 1996), on TJ disassembly in calcium-depleted SK-CO15 cells. The depletion of GEFp115 did not affect the sensitivity of TJs to calcium depletion compared with scramble siRNA-transfected cells (data not shown). Thus, both our colocalization and siRNA knockdown data support a critical role

for GEF-H1 in the regulation of AJC disassembly in Ca^{++} -depleted epithelial cells.

DISCUSSION

Contractile forces created by acto-myosin complexes have long been implicated in the remodeling of apical junctions during epithelial morphogenesis as well as during the disruption and repair of epithelial barriers (Lecuit, 2005; Mege *et al.*, 2006). Recently, we and others provided the evidence that acto-myosin contraction mediates the formation of epithelial junctions (Ivanov *et al.*, 2005; Shewan *et al.*, 2005; Zhang *et al.*, 2005; Miyake *et al.*, 2006) as well as the disassembly of the AJC (Ma *et al.*, 2000; Ivanov *et al.*, 2004a; de Rooij *et al.*, 2005).

In the present study, we identified a cascade of regulatory molecules that signal to activate acto-myosin contractility and induce epithelial AJC disassembly. Our present data strongly suggest that such acto-myosin reorganization and subsequent disruption of AJC are mediated by ROCK. This conclusion is based on 1) significant colocalization of ROCK with contractile F-actin rings containing internalized junctional proteins (Figure 2), 2) inhibition of the formation of contractile F-actin rings and disassembly of the AJC in Y-27632- and H-1152-treated cells (Figure 1A), and 3) dramatic attenuation of the drop in TEER upon pharmacological inhibition of ROCK (Figure 1B).

We not only identified ROCK as a critical activator of acto-myosin contractility that disrupts AJC in calcium-depleted epithelial cells but also demonstrated that such an activation is mediated by a single ROCK isoform, ROCK-II. ROCK family of protein kinases consists of two highly homologous members, ROCK-I and ROCK-II, which show 65% overall identity and 92% identity in their kinase domain (Riento and Ridley, 2003). Both ROCKs have been previously implied in myosin-mediated formation of stress fibers and focal adhesions (Leung *et al.*, 1996; Totsukawa *et al.*, 2000; Katoh *et al.*, 2001) as well as in the regulation of AJC (Walsh *et al.*, 2001; Sahai and Marshall, 2002). Nevertheless despite their sequence similarity, ROCK-I and ROCK-II may regulate different myosin-dependent processes (Yoneda *et al.*, 2005).

Although in our experiments both ROCK isoforms seem to be recruited to contractile F-actin rings in calcium-depleted cells (Figure 2), selective expressional down-regulation of ROCK-I and ROCK-II revealed the involvement of only the latter isoform in the disassembly of the AJC (Figure 3). None of the previous studies attempted to dissect the putative roles of ROCK-I and ROCK-II in the regulation of epithelial junctions. Indeed, previous reports focused on a single ROCK isoform only (Leung *et al.*, 1995; Sahai and Marshall, 2002). Therefore, our data are the first to simultaneously analyze both ROCK isoforms and determine their role in the regulation of the AJC disassembly in epithelial cells.

Interestingly, we did not find evidence supporting a role for MLCK in disruption of apical junctions in calcium-depleted cells. MLCK is thought to be a major kinase, which phosphorylates RMLC and thus regulates acto-myosin contractility in nonmuscle cells (Adelstein, 1982), and it also is implicated in the AJC regulation in epithelial cells (Hecht *et al.*, 1996; Turner *et al.*, 1997; Zolotarevsky *et al.*, 2002; Shen *et al.*, 2006). Nevertheless, in our experiments both classical MLCK inhibitor ML-7 (Figure 4A) and siRNA-mediated knockdown of MLCK (Figure 4, B–D) did not prevent the formation of contractile F-actin rings and disintegration of the AJC in calcium-depleted cells. Another argument against

Figure 10. A model for the regulation of AJC disassembly in calcium-depleted cells. Depletion of extracellular calcium causes the disruption of homotypic E-cadherin interactions between neighbor cells (Koch *et al.*, 2004). GEF-H1 is activated by unknown signaling molecules either downstream of E-cadherin or by a putative E-cadherin-independent pathway. Activated GEF-H1 stimulates the exchange of ADP to ATP on Rho and thereby activates Rho. In its turn, Rho signals on its downstream kinase ROCK-II, resulting in assembly and contraction of apical actomyosin rings. The contraction of actomyosin rings provides the mechanical force for retraction of plasma membranes of adjacent cells resulting in the disassembly of AJC.

the involvement of MLCK in the AJC disassembly was obtained in the immunolabeling experiments that did not show accumulation of MLCK at contractile F-actin rings (Figure 4E).

We also attempted to clarify the mechanism by which ROCK-II activates actomyosin contractility to trigger the disassembly of AJC. Two different modes of ROCK-dependent activation of RMLC have been reported: 1) a direct phosphorylation of RMLC by ROCK (Amano *et al.*, 1996; Totsukawa *et al.*, 2000) and 2) indirect increase in RMLC phosphorylation by the inhibition of ROCK-dependent myosin phosphatase through the phosphorylation of its regulatory target subunit (MYPT) (Kimura *et al.*, 1996). Our data strongly support the former mechanism. Indeed, we observed neither hyperphosphorylation of MYPT nor the translocation of phospho-MYPT in contractile F-actin rings in calcium-depleted cells (Figure 5).

Because ROCK-I and ROCK-II represent classical downstream effectors for Rho GTPase (Leung *et al.*, 1995; Matsui *et al.*, 1996), we hypothesized that Rho is involved in the AJC disassembly during calcium depletion. This hypothesis was tested by a set of experiments that showed a rapid activation of Rho and its colocalization with disassembled apical junctions in calcium-depleted cells (Figure 6). Furthermore, the

selective inhibition of Rho significantly attenuated the formation of contractile actomyosin rings and disassembly of the AJC (Figure 7).

A critical role of Rho in assembly and maintenance of AJC has been previously demonstrated by our group (Nusrat *et al.*, 1995; Bruewer *et al.*, 2004; Utech *et al.*, 2005) and others (Takaishi *et al.*, 1997; Jou *et al.*, 1998) in different epithelial cell lines. Nevertheless, our results provide the first direct evidence that activated Rho signals to disassemble the AJC in calcium-depleted cells, and they are in a good agreement with the existing literature describing the antagonism between Rho activity and cell-cell adhesion (Tokman *et al.*, 1997; Jou *et al.*, 1998; Noren *et al.*, 2001; Bruewer *et al.*, 2004). Remarkably, we recently reported the involvement of Rho/ROCK signaling in interferon- γ -induced endocytosis of junctional proteins in T84 cells (Utech *et al.*, 2005). These data strongly argue that Rho/ROCK-mediated junctional disassembly is a common mechanism regulating the epithelial AJC rather than a peculiarity of a calcium depletion model.

To identify a mechanism responsible for Rho activation during calcium depletion, we focused on GEF-H1, a guanine nucleotide exchange factor of the Dbl family that exhibits Rho-specific GDP/GTP exchange activity (Ren *et al.*, 1998). GEF-H1 has been shown to localize in TJs and to activate Rho in TJ-restricted manner with simultaneous increase in paracellular permeability in Madin-Darby canine kidney cells (Benais-Pont *et al.*, 2003). In good agreement with these data, we found that GEF-H1 selectively colocalized at TJs in human T84 (Figure 8) and SK-CO15 cells (data not shown). More importantly, GEF-H1 was enriched in contractile actomyosin rings (Figure 8), and knockdown of GEF-H1 significantly attenuated the disassembly of the AJC in calcium-depleted cells (Figure 9).

These results provide the first evidence that GEF-H1 regulates the AJC disassembly in epithelial cells. Interestingly, GEF-H1 is known to be associated with microtubules (Ren *et al.*, 1998) and such an association has been shown to inhibit its activity (Krendel *et al.*, 2002). We recently reported that microtubules become reorganized and less stable in calcium-depleted epithelial cells and that microtubule reorganization controls the formation of contractile actomyosin rings and AJC disassembly (Ivanov *et al.*, 2006). Additionally, destabilization of microtubules with 2-methoxyestradiol has been shown to mediate ROCK-II-dependent disruption of barrier function in endothelial cells (Bogatcheva *et al.*, 2007). Therefore, we speculate that an early reorganization of apical microtubules causes the release and activation of microtubule-bound GEF-H1, which stimulates Rho to induce the formation of contractile actomyosin rings and the AJC disassembly.

In conclusion, our results suggest a novel mechanism regulating the rapid disassembly of apical junctions in calcium-depleted epithelial cells. This mechanism involves sequential activation of the cascade of signaling molecules consisting of GEF-H1, Rho, and ROCK-II (Figure 10). These signaling events trigger the formation of contractile actomyosin rings, which provide the force required for disruption of the AJC. We propose that similar signaling mechanisms may regulate the dynamics of epithelial apical junctions during normal morphogenesis and in pathological conditions.

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