Rho GTPase signaling regulates tight junction assembly and protects tight junctions during ATP depletion

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Gopalakrishnan, Shobha, Narayan Raman, Simon J. Atkinson, and James A. Marrs. Rho GTPase signaling regulates tight junction assembly and protects tight junctions during ATP depletion. Am. J. Physiol. 275 (Cell Physiol. 44): C798–C809, 1998.—Tight junctions control paracellular permeability and cell polarity. Rho GTPase regulates tight junction assembly, and ATP depletion of Madin-Darby canine kidney (MDCK) cells (an in vitro model of renal ischemia) disrupts tight junctions. The relationship between Rho GTPase signaling and ATP depletion was examined. Rho inhibition resulted in decreased localization of zonula occludens-1 (ZO-1) and occludin at cell junctions; conversely, constitutive Rho signaling caused an accumulation of ZO-1 and occludin at cell junctions. Inhibiting Rho before ATP depletion resulted in more extensive loss of junctional components between transfected cells than control junctions, whereas cells expressing activated Rho better maintained junctions during ATP depletion than control cells. ATP depletion and Rho signaling altered phosphorylation signaling mechanisms. ZO-1 and occludin exhibited rapid decreases in phosphoamino acid content following ATP depletion, which was restored on recovery. Expression of Rho mutant proteins in MDCK cells also altered levels of occludin serine/threonine phosphorylation, indicating that occludin is a target for Rho signaling. We conclude that Rho GTPase signaling induces posttranslational effects on tight junction components. Our data also demonstrate that activating Rho signaling protects tight junctions from damage during ATP depletion.

One integral membrane component of the tight junction has been identified, called occludin (26). Occludin was localized to intramembranous strands, and data suggest that occludin is a major constituent of these intramembranous strands (25). Occludin regulates transepithelial resistance, paracellular permeability, and the lateral diffusion barrier for lipids between the apical and basolateral plasma membrane domains (12, 18, 41, 65). Serine/threonine phosphorylation regulates occludin assembly into the junctional complex (52).

Several other protein components of the tight junction have now been identified, but specific functions for these proteins have not been established (5). Most are thought to be structural components, but these proteins may also have signaling roles. Zonula occludens-1 (ZO-1) was the first component identified of the tight junction. ZO-1, together with the related tight junctional protein ZO-2, is a member of the membrane-associated guanylate kinase (MAGuK) gene superfamily (5). Several MAGuK family members are developmental signaling molecules (33), suggesting that ZO-1 and other MAGuK family members in the tight junction may have intracellular signaling functions (4). MAGuK proteins act to cluster membrane proteins in specialized plasma membrane subdomains (epithelial junctional complexes and neuronal synapses) (55). ZO-1 binds directly to the occludin cytoplasmic domain (27), and ZO-1 clusters occludin in the tight junction (41a).

Paracellular permeability is regulated by intracellular signaling and as a pathophysiological consequence of disease (9, 13, 15, 37, 53). Signaling pathways that affect tight junction function, and how these pathways are affected by pathophysiological events, remain unclear. Both protein tyrosine and serine/threonine phosphorylation mechanisms have been implicated in the regulation of tight junction assembly and paracellular permeability changes. Tyrosine kinase agonists and tyrosine phosphatase inhibitors were shown to affect phosphotyrosine levels in ZO-1 and ZO-2, and this was correlated with altered permeability properties and tight junction component redistribution in epithelial and endothelial cells (56, 59, 63). Protein kinase C activation results in cadherin-independent tight junction assembly and increased barrier function in Madin-Darby canine kidney (MDCK) cell monolayers (10), but protein kinase C activation in established epithelial monolayers increases permeability and inhibits tight junction assembly (19, 45, 37). Epithelial and endothelial cell permeabilities are compromised as a consequence of cell injury. Ischemic events in the kidney

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result in a rapid opening of tubular epithelial cell tight junctions (16, 39, 44). In the brain, injury (for example, stroke or head trauma) that causes cerebral ischemia may lead to disruption of endothelial cell tight junctions (see discussion in Ref. 54). Signaling mechanisms disrupted during ischemia that lead to tight junction dysfunction remain unknown.

Rho is a member of the Ras superfamily of small GTP-binding proteins that switch between GTP-bound (active) and GDP-bound (inactive) conformations (30). Activated (GTP-bound) Rho interacts with a growing list of effector molecules that propagate downstream signaling (3, 34, 50). Switching between GTP- and GDP-bound forms is regulated by GTP hydrolysis and nucleotide exchange, which requires guanine nucleotide exchange factors. Rho family GTPases include Rho, Rac, and Cdc42. In nonepithelial cells, various studies have demonstrated that Rho, Rac, and Cdc42 regulate distinct actin structures in response to growth factors and other signals (30). Like the other members of the ras gene superfamily, constitutively active and dominant negative mutations for Rho family GTPases have been characterized (30). For example, the constitutively active mutant, Rho-V14, has a low basal rate of GTP hydrolysis that is not stimulated by GTPase activation proteins and is thereby locked in an activated state. Rho-N19 has preferential affinity for GDP and exerts a dominant negative effect, probably by inhibiting the action of guanine nucleotide exchange factors. Also, C3 transferase from Clostridium botulinum ADP ribosylates Rho (but not Rac or Cdc42) and can be used to specifically inactivate Rho (17, 48).

Rho family GTPase regulation of actin assembly in nonepithelial cell types has been well characterized, but much less is known about the effects of Rho family GTPase signaling in epithelial cells. However, recent studies have shown that Rho family GTPases control junctional complex assembly (14, 21, 22, 31, 46, 58). Inactivation of Rho in Caco-2 epithelial cells using C3 transferase selectively disrupted tight junction structure and function, without apparent effects on the adherens junction (46). In MDCK cells, recent studies show that C3 transferase inhibits both tight junction and adherens junction assembly (58). Also, MDCK cell actin and adherens junction assembly mechanisms were affected by altered Rac signaling (31, 58). Braga et al. (14) demonstrated that keratinocyte adherens junction assembly was blocked when Rho or Rac signaling was inhibited. Therefore, growing evidence shows that Rho family GTPase signaling regulates cell-cell junctional complex assembly, but mechanisms for Rho family GTPase signaling in epithelial junctional complex assembly are not well characterized.

In the present study, we investigated targets for Rho GTPase signaling in tight junction assembly and the effects of ATP depletion on Rho-mediated tight junction assembly. We tested the hypothesis that ATP depletion inhibits Rho GTPase signaling, which contributes to junctional complex disassembly. We found that inhibiting Rho resulted in tight junction disassembly and that activated Rho expression led to accumulation of the tight junction components ZO-1 and occludin in MDCK cell junctional complexes. Analysis of redistribution of tight junction components after ATP depletion of MDCK cells expressing Rho mutant proteins suggests that these treatments affect the same tight junction assembly pathway. To examine the potential mechanisms affected by ATP depletion and Rho GTPase signaling that lead to tight junction disassembly, protein phosphorylation of tight junction components was examined. Both ATP depletion and mutant Rho expression in MDCK cells affect phosphorylation of tight junction components. These data suggest that tight junction disassembly in response to ATP depletion results from inactivation of Rho signaling, leading to posttranslational effects on tight junction components and then tight junction disassembly/dysfunction. This model may apply generally to other cellular injury events that lead to cytoskeletal disruption and junctional complex disassembly.

**MATERIALS AND METHODS**

Cell culture, antibodies, and reagents. MDCK type II cells were maintained in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum with penicillin, streptomycin, and glutamine (GIBCO BRL). Reagents were purchased from Sigma Chemical (St. Louis, MO) or Midwest Scientific (St. Louis, MO) unless otherwise indicated.

Polyclonal antibodies against ZO-1 and occludin were purchased from Zymed (South San Francisco, CA). The ZO-1 hybridoma, R26.4C (6, 57), was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, The University of Iowa (Iowa City, IA) under contract from the National Institute of Child Health and Human Development. Dr. Mark Wagner (Indiana University) provided the hybridoma 9E10 against the Myc epitope. Polyclonal antibodies against ZO-2 were a gift from Drs. Alan Fanning and Jim Anderson (Yale University). Horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody was purchased from Transduction Laboratories (Lexington, KY). Microinjection and transient transfection. MDCK cells were plated at 3 × 10^5 cells per 35-mm culture dish on collagen-coated glass coverslips and microinjected with C3 transferase (bacterial expression system generously provided by Dr. Alan Hal, Medical Research Council, University College, London, UK) purified as described (20). Cells were microinjected using Eppendorf (Hamburg, Germany) femptotips attached to an Eppendorf microinjector 5242 controlled by an Eppendorf micromanipulator 5170. Effective C3 transferase concentration was determined empirically by finding the concentration that causes stress fiber disassembly in Swiss 3T3 cells. Cells were coinjected with FITC-conjugated dextran for identification. Microinjected cells were incubated at 37°C for 45 min, and then cells were fixed and processed for indirect immunofluorescence using anti-ZO-1 antibodies (see Immunofluorescence, Image acquisition, and Image analysis, below). For transfections, 2 × 10^5 MDCK cells were plated per 35-mm culture dish. Cells were transfected 24 h later with 2 µg each of control vector or plasmids with Rho-V14 or Rho-N19 cDNAs expressed from SV40 promoters (generously provided by Dr. Marc Symons, Onyx Pharmaceuticals) using Lipofectamine (according to manufacturer’s protocol; GIBCO BRL). Cells were incubated with the transfection mixture for 5 h. Then, this mixture was replaced with normal growth.
medium. Cells were analyzed at various times, but the experiments shown were analyzed 48 h posttransfection.

ATP depletion. MDCK cells were plated at a density of 2 × 10^4 per 35-mm culture dish. After 72 h, cells were rinsed in prewarmed depletion medium; cells were ATP depleted for different times by incubating cells with depletion medium containing 0.1 µM antimycin A (16). For transfected cells, ATP depletion was performed 48 h posttransfection. ATP levels were assayed as described previously (16).

Immunoprecipitation and immunoblotting. Cells were rinsed with ice-cold PBS (in mM: 2.7 KCl, 1.5 K2HPO4, 137 NaCl, 8.1 Na2HPO4) and lysed in RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.05 M Tris, pH 8.0) for 15 min on ice. Cells were scraped, and lysates were collected and cleared by centrifugation in a microcentrifuge at 13,000 rpm for 5 min at 4°C. Primary antibody (anti-ZO-1, Zymed) was added to supernatants, and tubes were rotated at 4°C for 1 h. Immune complexes were collected with protein A Sepharose beads (Pharmacia, Piscataway, NJ) and washed three times in lysis buffer. Beads were resuspended in SDS-PAGE sample buffer for analysis and separated on 7.5% SDS polyacrylamide gels.

For immunoblotting total cellular proteins, cells were extracted with SDS-containing buffer (1% SDS, 10 mM Tris, pH 7.5, 2 mM EDTA). Cells were scraped, and lysates were collected, heated at 100°C for 5–10 min, and sonicated. Samples were cleared by centrifugation. Protein assays were performed on supernatants using the bicinchoninic acid kit (Pierce Chemical, Rockford, IL). Thirty micrograms of samples were separated on 10% SDS polyacrylamide gels.

Polyacrylamide gels were transferred to nitrocellulose (Bio-Rad, Hercules, CA) and blocked in TBST (10 mM Tris, pH 7.5, 0.1 M NaCl, 0.1% Tween 20) containing 3% BSA and 5% nonfat dry milk (Carnation), rocked at 4°C overnight, or in TBST containing 1% BSA (phosphotyrosine blotting). Membranes were incubated in primary antibody (occudin, 1:3,000; ZO-1, undiluted hybridoma supernatant; HRP-conjugated anti-phosphotyrosine antibody, 1:2,500) diluted in block (Amersham, Arlington Heights, IL). Membranes were washed as above, detected by enhanced chemiluminescence (Amersham), and exposed to film (Kodak Bio-Max ML, Eastman Kodak, Rochester, NY). For quantification, films were scanned using a Silverscanner III (LaCie, Beaverton, OR) and analyzed using ImageIQ software (Universal Imaging, West Chester, PA). This image was generated by mathematically summing the z-series images in one 16-bit image for quantification using Metamorph software (Universal Imaging, West Chester, PA). This image was background corrected by subtracting from each pixel the median pixel value from the surrounding 32 by 32 pixel neighborhood. Junctional regions were highlighted, and the total fluorescence and length of the junction region were calculated. Average fluorescence per unit length was calculated, and ratios described in RESULTS were calculated from these numbers. For each experimental condition, ~10 junctions between transfected cells and ~10 junctions between untransfected cells were analyzed. Three to four independent experiments were performed.

RESULTS

Rho signaling is required for tight junction assembly in MDCK cells. MDCK cells have been used extensively as a model for a polarized epithelial cell phenotype and to study junctional complex assembly (8, 39, 51) and used to model renal ischemia and recovery in vitro by ATP depletion and repletion (8, 39, 40, 61). We utilized the MDCK cell model system to examine roles of Rho GTPase signaling in tight junction assembly and in ATP depletion-induced tight junction dysfunction. Previous experiments used C3 transferase (a specific inhibitor of Rho GTPase) to show that Rho GTPase regulates tight junction assembly in Caco-2 cells and MDCK cells (46, 58). We also examined the effects of C3 transferase on tight junction assembly in MDCK cells. Bacterially expressed and purified C3 transferase was microinjected into MDCK cells in small colonies, and FITC-conjugated dextran was coinjected for identification of injected cells. Injected cell cultures were incubated for 45 min at 37°C before fixation and processing for immunofluorescence to determine ZO-1 distribution.

ZO-1 staining at junctions, either between pairs of injected cells, between an injected cell and an uninjected cell, or between pairs of uninjected cells, was analyzed by quantitative image analysis (see MATERIALS AND METHODS). In pairs of microinjected cells directly adjacent to one another, C3 transferase results in decreased ZO-1 localization at sites of cell-cell contact to 0.35-fold relative to uninjected cell pairs (differences were significant to P < 0.0001, t-test). In addition, junctions between injected cells and uninjected cells showed decreased ZO-1 localization at sites of cell-cell contact but to a lesser extent than junctions between pairs of injected cells: 0.49-fold relative to uninjected cell pairs (P < 0.0005, t-test). These data are in contrast...
to the conclusions of Takaishi et al. (58), who did not observe an effect of C3 transferase on tight junctions between injected cells and un.injected cells. Our data suggest that Rho family GTPase signaling is required for normal tight junction maintenance in MDCK cells and that Rho function is required in both cells that contribute to the junction.

To further investigate the effects of Rho on tight junction assembly, transient transfection was performed with plasmids encoding dominant negative Rho GTPase mutant (Rho-N19) and dominant active Rho GTPase mutant (Rho-V14) proteins. Both these constructs contain a Myc epitope tag engineered at the amino terminus that allowed detection of the exogenous proteins and identification of transfected cells using a monoclonal antibody (9E10). Subconfluent MDCK cell cultures were transfected, and mutant Rho expression was examined at various times following transfection. Transfected cell cultures achieved confluence, usually by 24 h posttransfection. Peak expression was observed at 48 h posttransfection, and this time point was used for all experiments reported here. However, effects of mutant Rho GTPases were observed at earlier and later time points (data not shown).

MDCK cells transfected with mutant Rho proteins were fixed and processed for double-label immunofluorescence to detect Myc-tagged Rho proteins and ZO-1 or occludin. Again, a z-series of x-y images through the entire volume of the cells was collected using a laser scanning confocal microscope and combined, to avoid missing fluorescence from other focal planes. Levels of fluorescence for tight junction components ZO-1 and occludin were reduced at junctions between Rho-N19-transfected cells, relative to junctions between untransfected cells (Fig. 1; see also Figs. 2 and 3). As with C3 transferase-mediated Rho inhibition, dominant negative Rho expression in MDCK cells inhibited tight junction assembly. Expression of dominant active Rho-V14 showed an opposite effect on tight junction assembly to that of Rho-N19. Rho-V14 expression resulted in increased amounts of tight junction components, reducing junction circumference and concentrating the same amount of protein in a shorter junction length. However, the amount of accumulation was different for ZO-1 and occludin (7.9-fold and 4.6-fold, respectively; see Figs. 4 and 5). Also, we measured the junction circumference for a set of transfected cells and untransfected cells, showing a reduction in circumference of 0.87-fold to that of control. This reduction could only account for a 1.15-fold accumulation in junction components.

Short-term inhibition of Rho by microinjecting MDCK cells with C3 transferase showed little or no change in E-cadherin distribution (data not shown), similar to that observed by Madara and colleagues (46) using Caco-2 cells. In contrast, expression of mutant Rho proteins by transient transfection produced effects on E-cadherin distribution (Gopalakrishnan and Marrs, unpublished observations). The incubation times for C3 transferase microinjection experiments were 45 min to 1 h, which may not have been sufficient time to elicit effects on the E-cadherin distribution, like those observed by Takaishi et al. (58). The possibility remains that Rho signaling effects on adherens junctions lead to alterations in tight junction structure. However, with the consideration that tight junction effects precede effects on adherens junctions, these data suggest that Rho signaling also affects tight junction independently of effects on adherens junctions.

To further investigate the effects of Rho on tight junction assembly in MDCK cells expressing Rho mutant proteins. Next, we tested whether Rho GTPase signaling affected tight junction assembly in the model system for renal ischemia, ATP depletion of MDCK cell monolayers. ATP depletion was accomplished by incubating cells with antimycin A, a reversible inhibitor of cytochrome reductive electron transport, which caused ATP levels to drop rapidly to <5% of control within 15 min following antimycin A treatment. Nearly all cells survive and recover from this injury, and ATP levels recover to ~50% of control levels 60 min after cells are returned to normal growth medium. The effects of ATP depletion on cultured epithelial cells strongly resemble the consequences for tubular epithelial cells during renal ischemia in vivo, in which ATP levels also decrease due to restricted perfusion in tissues. Both ATP depletion in
vitro and renal ischemia in vivo cause the disassembly of tight junctions (16, 23, 39, 44).

To test the effect of Rho GTPase signaling during ATP depletion, subconfluent MDCK cell cultures were transfected with constructs encoding mutant Rho GTPases, and, at 48 h posttransfection, these MDCK cell monolayers were ATP depleted for 60 min or untreated in controls. Cells were then fixed and processed for double-label immunofluorescence to detect Myc-tagged Rho proteins and ZO-1 or occludin. Effects of Rho GTPase signaling and ATP depletion were assayed using confocal microscopy and quantitative image analysis. Fluorescence intensity per unit length was measured using Metamorph image analysis software. To determine the consequences of expressing Rho mutant proteins, a fluorescence intensity ratio of the fluorescence intensity per unit length in junctions between two transfected cells divided by the fluorescence intensity per unit length in junctions between two untransfected cells was calculated. This ratio expresses the magnitude of decrease or increase in fluorescence intensity per unit length as a consequence of mutant Rho protein expression. This ratio also allows us to distinguish relative changes in fluorescence after ATP depletion because the ratio is internally normalized to untransfected cell-cell junctions.

MDCK cells expressing Rho-N19 that were ATP depleted for 60 min showed a more dramatic decrease in tight junction assembly compared with parallel cultures of MDCK cells expressing Rho-N19 that were not subjected to ATP depletion (Figs. 2 and 3). The ratio of fluorescence intensity for junctions between Rho-N19 transfected cell pairs divided by untransfected cell pairs stained for ZO-1 was reduced from 0.27 ± 0.05 (SD) in control cultures to 0.09 ± 0.02 (SD) in parallel cultures that were ATP depleted for 60 min (Fig. 2B) similar to that for ZO-1. Because it has been previously shown that the tight junction structure was disrupted by ATP depletion over a 60-min time course (8), the decreased fluorescence intensity ratio we observed in cells expressing Rho-N19 relative to cells not ATP depleted probably represents acceleration of disassembly mechanisms resulting from Rho signaling inhibition.

MDCK cells expressing dominant active Rho-V14 were ATP depleted for 60 min or untreated in controls, and quantitative image analysis was performed on confocal, extended focus images of double-label immunofluorescence images stained to detect Myc-tagged Rho proteins and tight junction component proteins (Figs. 4A and 5A). Rho-V14 expression in MDCK cells protected tight junctions from disassembly during ATP depletion. The ratio of fluorescence intensity for junctions between Rho-V14-transfected cell pairs divided by untransfected cell pairs was increased from 7.9 ± 1.0 (SD) in control cultures to 15.3 ± 1.8 (SD) in parallel cultures that were ATP depleted for 60 min (Fig. 4B). The ratio of fluorescence intensity for occludin staining in cell-to-cell contacts between Rho-V14-transfected cell pairs divided by untransfected cell pairs was increased from 4.6 ± 1.7 (SD) in control cultures to 16.3 ± 2.2 (SD) in parallel cultures that were ATP depleted for 60 min (Fig. 5B). This fluorescence intensity ratio increase in cells expressing Rho-V14 that were subjected to ATP depletion suggests that...
Rho-V14 expression inhibited the injury-induced disassembly mechanisms, thereby protecting cells from injury to tight junctions.

Protein phosphorylation pathways for tight junction components are disrupted by ATP depletion in MDCK cells. Both ATP depletion and Rho GTPase signaling affect tight junction assembly (16, 23, 39, 44, 46, 58), and data presented above suggest that Rho-mediated signaling mechanisms are affected by ATP depletion. Rho effectors are being identified and characterized, and protein phosphorylation mechanisms are common among these pathways (3, 34, 50). ATP depletion down-regulates protein phosphorylation signaling mechanisms (35). We tested whether alterations in phosphorylation states for ZO-1 and occludin accompany changes in tight junction assembly that were observed in re-

![Fig. 3. Effect of ATP depletion on distribution of occludin in cells expressing dominant negative Rho GTPase mutant protein. MDCK cells were transiently transfected with Myc-tagged dominant negative Rho [Rho-N19 (DN), arrows]. At 48 h posttransfection, cells were ATP depleted for 60 min or left untreated (control). A: cells were processed for double-label, indirect immunofluorescence using occludin antibodies, and mutant Rho GTPase expressing cells were detected using anti-Myc antibodies. Each image shown is 179.2 µm². B: mean fluorescence intensity was calculated for junctions between pairs of mutant Rho GTPase expressing cells (transfected) and between pairs of untransfected cells from the same experiment. Ratio decreased from 0.40 for Rho-N19 alone (n = 20) to 0.17 for Rho-N19 with 60-min ATP depletion (n = 20) (P = 3.3 × 10⁻², t-test). Dashed line shows mean fluorescence intensity ratio in untransfected junctions for reference. Data shown are representative of 3 independent experiments.](image1)

![Fig. 4. Effect of ATP depletion on distribution of ZO-1 in cells expressing dominant active Rho GTPase mutant protein. MDCK cells were transiently transfected with Myc-tagged dominant active Rho [Rho-V14 (DA), arrows]. At 48 h posttransfection, cells were ATP depleted for 60 min or left untreated (control). A: cells were processed for double-label, indirect immunofluorescence using ZO-1 antibodies, and mutant Rho GTPase expressing cells were detected using anti-Myc antibodies. Each image shown is 179.2 µm². B: mean fluorescence intensity was calculated for junctions between pairs of mutant Rho GTPase expressing cells (transfected) and between pairs of untransfected cells from the same experiment. Ratio increased from 7.9 for Rho-V14 alone (n = 25) to 15.3 for Rho-V14 with 60-min ATP depletion (n = 25) (P = 4.1 × 10⁻⁹, t-test). Dashed line shows mean fluorescence intensity ratio in untransfected junctions for reference. Data shown are representative of 4 independent experiments.](image2)
sponse to altered Rho signaling and ATP depletion. ZO-1 is reportedly serine/threonine and tyrosine phosphorylated (6, 56), and occludin was shown to be serine/threonine phosphorylated (52). Tyrosine phosphorylation of ZO-1 and serine/threonine phosphorylation of occludin correlate with tight junction assembly and function (52, 56, 59).

Confluent MDCK cell monolayers were ATP depleted and allowed to recover in normal growth medium. At various times, cells were assayed for protein phosphorylation changes. ZO-1 was immunoprecipitated from cell extracts. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted to detect phosphotyrosine or blotted to detect ZO-1 to show that comparable amounts of ZO-1 were immunoprecipitated (Fig. 6). A second protein of ~160 kDa was coimmunoprecipitated with ZO-1 and detected with the anti-phosphotyrosine monoclonal antibody (Fig. 6). Immunoblotting our ZO-1 immunoprecipitates with specific antibodies to ZO-2 (a 160-kDa protein that is structurally related to ZO-1, binds directly to, and coimmunoprecipitates with ZO-1) (32) showed that the coimmunoprecipitating protein was recognized by these antibodies (data not shown). ZO-1 and ZO-2 tyrosine phosphorylation decreased rapidly during ATP depletion. Within 30 min, phosphotyrosine content in ZO-1 and ZO-2 was nearly undetectable (Fig. 6).

We also examined whether phosphotyrosine content in ZO-1 returned during recovery and whether duration of ATP depletion affected the extent of ZO-1 phosphotyrosine recovery. MDCK cells were ATP depleted for 30 or 60 min, and cells subjected to injury for these different times were allowed to recover for 60 min. Both 30 and 60 min of injury were sufficient to reduce ZO-1 and ZO-2 phosphotyrosine levels to undetectable levels (Fig. 6B), as expected from the earlier time course experiment (Fig. 6A). However, the recovery of ZO-1 and ZO-2 phosphotyrosine levels after 60

Fig. 5. Effect of ATP depletion on distribution of occludin in cells expressing dominant active Rho GTPase mutant protein. MDCK cells were transiently transfected with Myc-tagged dominant active Rho (Rho-V14, arrows). At 48 h posttransfection, cells were ATP depleted for 60 min or left untreated (control). A: cells were processed for double-label, indirect immunofluorescence using occludin antibodies, and mutant Rho GTPase expressing cells were detected using anti-Myc tag antibodies. Each image shown is 179.2 µm². B: mean fluorescence intensity was calculated for junctions between pairs of mutant Rho GTPase expressing cells (transfected) and between pairs of untransfected cells from the same experiment. Ratio increased from 4.6 for Rho-V14 alone (n = 25) to 16.3 for Rho-V14 with 60-min ATP depletion (n = 25) (P = 8.1 × 10⁻⁸, t-test). Dashed line shows mean fluorescence intensity ratio in untransfected junctions for reference. Data shown are representative of 3 independent experiments.
min of recovery was less for cells subjected to 60 min of ATP depletion, compared with cells subjected to 30 min of ATP depletion (Fig. 6B). The level of phosphotyrosine in ZO-1 and ZO-2 that recovers was correlated with the duration of the initial injury, rather than the time of the recovery period.

To examine phosphorylation of occludin, we took advantage of the observation that 10 or more serine/threonine phosphorylation isoforms of occludin migrate differentially in SDS-PAGE; the slower-migrating isoforms are more highly serine/threonine phosphorylated than the faster-migrating isoforms, and phosphorylation status directly correlates with occludin's assembly state (52). Confluent MDCK cell monolayers were ATP depleted and allowed to recover in normal growth medium. At various times, extracts were prepared from these cells, and equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose, and blotted using occludin antibodies (Fig. 7). Occludin isoforms with slower relative migration in SDS-PAGE, representing more highly serine/threonine phosphorylated isoforms, were detected in control extracts. These slower-migrating occludin isoforms were progressively lost during ATP depletion. By 30 min of ATP depletion, occludin had shifted to predominantly the faster-migrating isoforms (Fig. 7A). There was a further decrease between 30 and 60 min of ATP depletion (Fig. 7B).

We next tested the effect of injury duration on recovery of serine/threonine phosphorylation occludin variants. MDCK cells were ATP depleted for 30 or 60 min and then allowed to recover for 60 min. Again, both 30 and 60 min of injury showed significant reduction in serine/threonine phosphorylated occludin isoforms (Fig. 7B). The extent that serine/threonine-phosphorylated occludin isoforms returned was less for cells subjected to 60 min of ATP depletion compared with cells subjected to 30 min of ATP depletion (Fig. 7B), suggesting that recovery of serine/threonine phosphorylation isoforms of occludin was affected by duration of the initial injury.

Protein phosphorylation pathways for occludin are affected by Rho signaling in MDCK cells. Quantitative image analysis showed that Rho GTPase signaling mechanisms and ATP depletion act together to affect tight junction assembly. ATP depletion also led to rapid reductions in phosphorylation of ZO-1 and occludin.

Based on our hypothesis that Rho signaling is inhibited during ATP depletion, we would predict that Rho GTPase inhibition would affect phosphorylation of ZO-1, occludin, or both. To test our prediction, subconfluent MDCK cell cultures were transiently transfected with plasmids encoding Rho mutant proteins. For these experiments, transfection efficiency was optimized to detect the effects of Rho signaling on tight junction components biochemically; we achieved ~50% transfection. Phosphorylation status for tight junction components in transfected cell monolayers was examined at 48 h posttransfection using methods described for Figs. 6 and 7.

ZO-1 tyrosine phosphorylation was not affected in cells expressing mutant Rho proteins (data not shown). At 48 h posttransfection with plasmids encoding Rho-V14, Rho-N19, or vector alone, cell extracts were immunoprecipitated using antibodies specific for ZO-1. These immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect phosphotyrosine and to detect ZO-1. Expressing Rho-V14 or Rho-N19 did not reproducibly change phosphotyrosine levels in ZO-1 relative to vector-transfected cells, suggesting that Rho signaling does not affect ZO-1 tyrosine phosphorylation. It is possible that effects were transient or not sufficiently robust to be detected in our system.

Levels of serine/threonine phosphorylation variants of occludin were affected by expressing mutant Rho protein in MDCK cells. Cells were transfected with vector and Rho-V14 and Rho-N19 expression plasmid constructs. Cell extracts were prepared 48 h posttransfection. Equal amounts of protein from these extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted to detect occludin. Expression of Rho-N19 in MDCK cells caused a reduction (0.42-fold relative to vector-transfected control cells per unit protein) of slower-migrating, more highly phosphorylated occludin isoforms (Fig. 8). This suggests that reductions in occludin phosphorylation during ATP depletion are a result of inhibiting Rho GTPase signaling mechanisms. In addition, Rho-V14 expression in
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MDCK cells caused a 1.80-fold accumulation (per unit protein) of slower-migrating, more highly serine/threonine phosphorylated occludin isoforms relative to vector-transfected control cells (Fig. 8), perhaps representing a Rho signaling target that alters tight junction assembly and protects junctions from cell injury.

DISCUSSION

Evidence indicates that Rho signaling regulates tight junction and adherens junction assembly in epithelial cells (14, 46, 58) and Rac also regulates adherens junction assembly (14, 31, 58). In this study, effects of Rho GTPase signaling on tight junction assembly were examined in normal MDCK cells and during ATP depletion processes (ATP depletion, a model of renal ischemia/acute renal failure). Our results suggest that Rho regulates tight junction assembly by affecting occludin protein phosphorylation states and that Rho-mediated tight junction assembly mechanisms are disrupted during ATP depletion. These studies provide novel insights into Rho GTPase actions in cell-cell junctional complex assembly and the role of Rho family GTPase signaling during cellular injury and recovery.

Rho signaling and mechanisms for tight junction regulation. Our results and those of others (46, 58) show that tight junctions are regulated by Rho GTPase signaling. We show that inhibiting Rho signaling decreased tight junction assembly and reduced levels of occludin phosphorylation, and activating Rho signaling stimulates tight junction assembly and increased levels of occludin phosphorylation. These data indicate that Rho signaling affects tight junction assembly via specific protein phosphorylation mechanisms.

Rho GTPase signaling mechanisms are generally mediated by protein kinases (50). In particular, Rho signaling activity affects myosin light chain phosphorylation. The Rho effector, Rho kinase, affects myosin light chain phosphorylation, and activation of Rho increases myosin activity (2, 3, 34). Effects on myosin light chain phosphorylation are well-characterized actions of Rho signaling, but Rho signaling mechanisms affect other kinase substrates (50). How Rho signaling pathways regulate tight junction component phosphorylation is unclear. Further experimentation will be necessary to define Rho signaling pathways that lead to altered tight junction assembly.

An alternative mechanism for Rho effects on tight junction assembly may be indirect, via Rho effects on cadherin-mediated adhesion. Tight junction assembly in epithelial cells requires cadherin function (29, 64). Blocking Rho or Rac function in keratinocytes inhibited cadherin-mediated adherens junction assembly (14, 31, 58). The effects of inhibiting Rho signaling on cadherin function may lead to disassembly of tight junctions in MDCK cells. Activated Rho did not seem to increase adherens junction assembly in keratinocytes (14), and consequences of strengthened cadherin-mediated adhesion for tight junction assembly are not well characterized. Like Madara and colleagues (46), we did not observe redistribution of E-cadherin as a consequence of short-term effects of inhibiting RhoA using C3 transferase proteins (where there was redistribution of ZO-1), suggesting that there are also cadherin-independent, Rho-mediated effects on tight junction assembly. Of course, the actin cytoskeleton regulates tight junction function and assembly (13, 38), and effects on tight junction assembly could also be consequences of actin cytoskeleton rearrangements.

ATP depletion causes tight junction disassembly, perhaps by inhibiting Rho. Renal ischemia is a consequence of renal injury and numerous renal diseases, resulting in a rapid decrease in cellular ATP levels (60). ATP-depleting epithelial cells in tissue culture are an in vitro model of renal ischemia; ATP levels recover rapidly following removal of the drug, and, with time, cells recover a normal epithelial phenotype (23). This model recapitulates numerous cellular features of renal ischemia in vivo that have been documented in animal models and human patients, including the effects on actin cytoskeleton, cell polarity, and junctional complexes (1, 8, 16, 23, 39, 40, 42-44). With the use of this model system to study the role of Rho GTPase signaling on tight junction disassembly during ATP depletion, our studies support the idea that Rho GTPase signaling was inhibited during ATP depletion. Furthermore, Rho signaling mechanisms protect tight junctions from injury in epithelial cells.

Renal ischemia in vivo and ATP depletion of cultured epithelial cells result in a rapid breakdown of the tight junction permeability barrier (16, 39, 44). ATP depletion of MDCK cells resulted in paracellular permeability barrier dysfunction within 10 min, but the lateral diffusion barrier function of the tight junction was not compromised until later times (8, 39). Also, with longer times of ATP depletion, ZO-1 distribution and freeze-fracture tight junction ultrastructure were disrupted (8). These data suggest that ATP depletion rapidly
disrupts signaling processes that result in paracellular barrier dysfunction, before dramatic structural effects on tight junctions, which occur later. Structural changes in tight junctions following ATP depletion are also subsequent to major rearrangements of the actin cytoskeleton, where normal actin structures disassemble and filamentous actin accumulates in perinuclear aggregates (8). Peripheral tight junction components (ZO-1, ZO-2, and cingulin) are redistributed to the cytoplasm and accumulate in large-molecular-weight complexes containing actin and fodrin with extended times of ATP depletion (61). We propose that a key event during the rearrangement of the tight junction is inhibition of Rho GTPase, triggering disassembly and dysfunction.

Tight junction disassembly during ATP depletion was more dramatic in MDCK cells expressing dominant negative Rho mutant proteins. We observed that tight junction components were rapidly dephosphorylated during ATP depletion. Consistent with our hypothesis that Rho signaling is inhibited during ATP depletion, we found that levels of occludin phosphorylation were reduced in MDCK cells expressing dominant negative Rho relative to vector transfected cells. These data suggest that inhibition of Rho during ATP depletion leads to dephosphorylation of occludin and subsequent disassembly and dysfunction of the tight junction. The observation that constitutively active Rho (Rho-V14) partially rescues cells from tight junction disassembly during ATP depletion strongly suggests that Rho signalling provides a specific protective mechanism from cellular injury. Ocludin phosphorylation may be the target of this protective signaling pathway.

Rho GTPase signaling may regulate junctional complex assembly in a variety of situations, not limited to cellular injury. Normal physiological responses lead to changes in tight junction permeability (36, 37, 49, 62). Future studies should address whether Rho GTPase signaling regulates tight junction permeability during these physiological events. Protection of tight junctions from disassembly during ATP depletion by Rho GTPase signaling reveals novel signalling events that are disrupted during renal ischemia, leading to pathophysiological consequences in this condition. Rho GTPase activation also protects cellular actin structures from disassembly during ATP depletion (Raman and Atkinson, unpublished observations), suggesting that protective effects of Rho signalling for epithelial cells are more widespread. Other Rho family GTPases may be inactivated during ATP depletion, and their effects will also require study. Analysis of Rho family GTPase signaling will provide a more complete understanding of cellular events that lead to rearrangement of the actin and junctional complexes.

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References


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Rho REGULATES TIGHT JUNCTION ASSEMBLY


