Exocyst Sec10 protects epithelial barrier integrity and enhances recovery following oxidative stress, by activation of the MAPK pathway

Kwon Moo Park,1,4 Ben Fogelgren,1 Xiaofeng Zuo,1 Jinu Kim,1 Daniel C. Chung,2 and Joshua H. Lipschutz1,3

Departments of 1Medicine and 2Ophthalmology and 3Department of Medicine and Cell and Molecular Biology Graduate Group, University of Pennsylvania, and the Veterans Administration Medical Center, Philadelphia, Pennsylvania; and 4Department of Anatomy, Kyungpook National University School of Medicine, Daegu, Korea

Submitted 17 October 2009; accepted in final form 31 December 2009

Park KM, Fogelgren B, Zuo X, Kim J, Chung DC, Lipschutz JH. Exocyst Sec10 protects epithelial barrier integrity and enhances recovery following oxidative stress, by activation of the MAPK pathway. Am J Physiol Renal Physiol 298: F818–F826, 2010. First published January 6, 2010; doi:10.1152/ajprenal.00596.2009.—Cell-extracellular matrix interactions are essential for cell polarization and differentiation. The exocyst, a highly conserved eight-protein complex that targets secretory vesicles carrying membrane proteins, is involved in maintaining renal epithelial barrier integrity. Accordingly, increasing exocyst expression in renal tubule cells may protect barrier function from oxidative stress resulting from ischemia and reperfusion (I/R) injury. When cultured on plastic, Madin-Darby canine kidney (MDCK) cells overexpressing Sec10, a central exocyst component, formed domes showing increased resistance to hydrogen peroxide (H₂O₂). Transepithelial electric resistance (TER) of Sec10-overexpressing MDCK cells grown on Transwell filters was higher than in control MDCK cells, and the rate of TER decrease following H₂O₂ treatment was less in Sec10-overexpressing MDCK cells compared with control MDCK cells. After removal of H₂O₂, TER returned to normal more rapidly in Sec10-overexpressing compared with control MDCK cells. In collagen culture MDCK cells form cysts, and H₂O₂ treatment damaged Sec10-overexpressing MDCK cell cysts less than control MDCK cell cysts. The MAPK pathway has been shown to protect animals from I/R injury. Levels of active ERK, the final MAPK pathway step, were higher in Sec10-overexpressing compared with control MDCK cells. U0126 inhibited ERK activation, exacerbated the H₂O₂-induced decrease in TER and cyst disruption, and delayed recovery of TER following H₂O₂ removal. Finally, in mice with renal I/R injury, exocyst expression decreased early and returned to normal concomitant with functional recovery, suggesting that the exocyst may be involved in the recovery following I/R injury.

Exoc5; ischemia; reperfusion

IN MAMMALIAN EPITHELIAL cells, the cell junctions regulate movement of substances across cell layers as well as act as physical barriers between the apical and basolateral plasma membranes (39). Changes in the integrity of cell-cell contacts in kidney tubule epithelial cells have been associated with a number of pathological conditions, including acute kidney injury (AKI) induced by ischemia and reperfusion (I/R) (24–26), which is a significant and increasing problem. I/R injury occurs in various clinical settings, including transplantation and vascular surgery. One of the major contributors to kidney I/R injury is oxidative stress. Kidney I/R injury produces oxidative stress, by activation of the MAPK pathway.

reactive oxygen species (ROS) beyond its scavenging capacity and simultaneously impairs antioxidant enzymes. The ROS cause cell injury by lipid peroxidation, disruption of cellular cytoskeleton, and breakdown of DNA (1a, 5, 16, 29). Numerous studies have demonstrated that oxidative stress is associated with increases in intercellular permeability (6, 14, 23, 32).

Cell damage due to AKI or renal I/R can be subclassified into two groups: sublethal damage that disrupts epithelial barrier integrity, and lethal damage through apoptotic or necrotic mechanisms (36, 38). In states of sublethal damage, epithelial cells of the nephron lose polarization and show dissolution of cell-to-cell adhesions, resulting in the breakdown of barrier function and increased epithelial permeability. Following the ischemic event, the recovery process involves reestablishing polarity and cell-cell contacts in cells that sustained sublethal damage, so that nephron tubules can properly resume their physiological functions.

The exocyst is a highly conserved 750-kDa complex composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84, which was originally identified in Saccharomyces cerevisiae (9, 35). Mammalian homologs of all eight exocyst proteins have been identified (8, 12), and the exocyst is well known for targeting and docking secretory vesicles carrying proteins to the plasma membrane (2, 7, 17, 27). The mammalian exocyst localizes largely, although not exclusively, in the tight junction area, which is thought to be a “hotspot” for membrane protein incorporation (7).

Sec10 is a central component of the eight-protein exocyst complex (40), and we have previously shown that Sec10 overexpression in MDCK type II cells grown in a collagen matrix resulted in increased tubulogenesis compared with control MDCK cells (17). This was likely due to increased protein delivery in the Sec10-overexpressing cells (17), as we and others have shown that the exocyst is responsible for delivery and incorporation of basolateral plasma membrane proteins (17, 19, 27, 34). Thus we hypothesized that Sec10 overexpression could strengthen epithelial cell-cell adhesions and confer resistance to, and/or enhance recovery from, cell damage to kidney tubular epithelia due to I/R injury.

Here, we investigated the role of Sec10 in vitro in ROS-damaged cultured renal tubular epithelial cells, and in vivo following renal I/R injury in mice. We found that Sec10 overexpression in vitro led to increased resistance of renal tubular epithelial cells to oxidative stress, as well as an increase in the rate of epithelial barrier recovery following a period of oxidative damage. MAPK pathway activation was associated with this increased resistance and enhanced recovery. In vivo, exocyst expression in kidneys subjected to I/R injury decreased...
early after induction and gradually returned to normal along with functional recovery, suggesting that the exocyst may be involved in the recovery following I/R injury.

MATERIALS AND METHODS

Cell culture. Low-passage type II Madin-Darby canine kidney (MDCK) cells were obtained from Dr. K. Mostov (UCSF, San Francisco, CA) and used between passages 3 and 10. These cells were originally cloned by Daniel Louvard at The European Molecular Biology Laboratory (EMBL) and came to Keith Mostov via Karl Matlin. We previously generated MDCK type II cell lines, from these parent MDCK cells, that stably overexpressed human Sec10 (Sec10-overexpressing cells) (17). Cells were grown in modified Eagle’s MEM containing Eagle’s balanced salt solution and glutamine supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin on plastic culture dishes. Some cells were grown on Transwell 0.45-μm polycarbonate filter units coated with collagen (Corning Life Sciences, Lowell, MA). The culture medium was changed daily. To analyze MDCK dome formation and damage, cells were grown to confluency on plastic culture dishes and observed with a Nikon TS100 inverted microscope. Domes were scored as having a diameter of >50 μm and being clearly visible with a spherical shape above the plane of focus of the rest of the cell monolayer. Collapse, or damage, of domes, was determined by a visible dense clumping of cells that were slightly out of focus with the rest of the monolayer of cells. To culture cells in a three-dimensional collagen matrix, cells grown on plastic culture dishes were harvested using trypsin-EDTA, suspended in a mixture of type I collagen gel (Invitrogen, Carlsbad, CA), and seeded on a chamber slide. After allowing the gel to solidify at 37°C, the matrix was overlaid with culture medium. Cells were grown for 14 days, with the medium changed daily, and then used for experiments. To mimic oxidative stress caused by I/R, we added various concentrations of hydrogen peroxide (H₂O₂) to the cell culture medium for various time periods.

Measurement of transepithelial electric resistance. Control cells and Sec10-overexpressing cells were grown on Transwell filters until three days postconfluency, with confluency being determined by a hydrostatic water test, i.e., being able to maintain 1 ml of medium in the apical space for 24 h (20). Transepithelial electric resistance (TER) was measured using an epithelial volt-ohmmeter (model EVOM, World Precision Instruments). Absolute TER values were determined by subtracting the TER of blank filters with medium from all samples and presented as the measured resistance in ohms divided by the surface area of the Transwell filter.

Western blot analysis. Cells grown on plastic, on Transwell filters, in collagen, and whole kidneys were harvested in RIPA buffer (Sigma-Aldrich, St. Louis, MO) containing a proteinase inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich), and the lysates were centrifuged at 14,000 rpm for 20 min at 4°C. Supernatants were collected, and protein concentration was determined using the BCA method.

Fig. 1. Sec10 overexpression attenuated the loss of dome formation induced by hydrogen peroxide. Control (Madin-Darby canine kidney; “MDCK”) and Sec10-overexpressing (“Sec10-MDCK”) MDCK cells were grown on plastic culture dishes and formed typical domes. A: to determine the levels of Sec10 overexpression in one Sec10-overexpressing clonal cell line, Western blotting was performed using antibodies that we generated against Sec10 (40). Equal amounts of protein loading were determined by GAPDH antibody. Using Kodak Image Analysis software, we found a 4.34-fold increase in Sec10 expression in this cell line. B: dome-forming MDCK cells were treated with 1 mM H₂O₂ for 30 min. After H₂O₂ treatment, some domes were collapsed. Arrowheads and arrows indicate intact and damaged domes, respectively. C: numbers of intact domes before treatment with H₂O₂ were counted as described in MATERIALS AND METHODS. D: values represent % damaged domes [(number before H₂O₂ treatment) − (number after H₂O₂ treatment)/number before H₂O₂ treatment] × 100. Values are means ± SE of 3 independent experiments done in duplicate and were scored by an investigator who was blinded to the identity of the cell origin. *P < 0.05 for 1 mM H₂O₂ vs. respective 0 mM H₂O₂-treated cells. #P < 0.05 for Sec10-overexpressing vs. control MDCK cells.
protein assay (Thermo Scientific, Waltham, MA). Protein samples were mixed in an SDS-sample buffer and denatured by 5 min of boiling at 95°C. To prepare protein samples of cells grown in collagen matrix, cells were incubated in 100 unit/ml collagenase VII (Sigma-Aldrich) in MEM for 30 min at 37°C and centrifuged at 3,000 rpm for 3 min. The supernatant was discarded, and protein from the cellular pellet was then prepared as described above. The protein samples were separated on 4–12% SDS-PAGE gels (Invitrogen) and then transferred to an Immobilon membrane (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBS-T) and incubated with anti-phospho-ERK (1:1,000, Cell Signaling, Beverly, MA), -total-ERK (1:5,000; Cell Signaling), -Sec8 (1:5,000; Stressgen, Victoria, Canada), -Sec10 (1:2,000) (40), -Na-K-ATPase (1:2,500; Santa Cruz Biotechnology, CA), -GAPDH (1:2,500; Santa Cruz Biotechnology, CA), and -proliferating cell nuclear antigen (1:4,000; DAKO, Carpinteria, CA) antibodies overnight at 4°C. After a washing with PBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, the membranes were exposed to a Western blotting Chemiluminescence Reagent (Pierce, Rockford, IL) and developed on X-ray film. To determine fold-changes in Sec10 expression, Kodak 1D Imaging Analysis software was used.

**Immunofluorescence staining.** To analyze cyst morphology in the collagen gels, cells were fixed with 4% paraformaldehyde for 30 min at 4°C after digesting with collagenase VII (100 U/ml; Sigma-Aldrich) for 10 min at 37°C. The cyst cells were permeabilized with 0.025% saponin in PBS containing 0.7% fish skin gelatin (PFS buffer) for 30 min at room temperature. The collagen gel was blocked with PFS buffer and incubated in rhodamine-phalloidin (for actin staining, Cytoskeleton, Denver, CO) for 2 h at room temperature, washed, counterstained with 4,6-diamidino-2-phenylindole (DAPI; for staining of cell nuclei), and then mounted with mounting medium (Vectashield). To determine apoptotic cell death, cells grown on Transwell filters were stained with DAPI, a nuclear stain.

**Renal I/R in mice.** Experiments were performed in 8-wk-old C57BL/6 mice. Mice were allowed free access to water and a standard mouse diet. In all cases, studies were conducted according to the animal experimental procedures approved by the Kyungpook National University Institution Animal Care and Use Committee. Kidney ischemia was carried out as described previously (30). Briefly, animals were anesthes-
tized with pentobarbital sodium (60 mg/kg body wt ip) before surgery. Animals were subjected to either 30 min of bilateral renal ischemia or sham operation on day 0. Body temperature was maintained at 36.6–37.5°C throughout the procedure. To induce ischemia, renal pedicles were occluded using nontraumatic microaneurism clamps (Roboz, Rockville, MD). Kidneys were harvested at day 0 (before surgery), 1, 8, and 16 following I/R and were snap-frozen following surgical removal by immediate placement in liquid nitrogen. Lysate was generated from the whole kidney for Western blot analysis. Each animal group consisted of more than four mice.

Renal functional parameters. To evaluate the concentration of plasma creatinine (PCR), 70 μl of blood was taken from the orbital sinus at the indicated time (see Fig. 7A). PCR concentration was measured using the Beckman Creatinine Analyzer II (Beckman, Brea, CA).

Statistics. Results are expressed as means ± SE. Statistical differences among groups were calculated using ANOVA followed by a least significant difference post hoc comparison using the SPSS 12.0 program. Differences between groups were considered statistically significant at a P value of < 0.05. To determine the rate of decrease of TER (see Figs. 2 and 4), linear regression analysis was performed on the collected data, and the slopes of different samples were compared for statistical significance. To determine recovery rates following injury (see Fig. 5 and Supplemental Fig. 1; all supplementary material for this article is available on the journal web site), TER from the nadir onward was used for regression analysis, except for the Sec10-overexpressing cells, where data after the nadir were included only until the initial level of TER was achieved (i.e., recovered). Regression analysis and testing of slopes for statistical significance were performed using GraphPad Prism statistical software. Student’s t-test was used to determine the significance of the fold-change of Sec10 expression in Sec10-overexpressing MDCK cells compared with control MDCK cells.

RESULTS

Sec10 overexpression reduces the loss of dome formation from treatment with hydrogen peroxide. We previously generated MDCK type II cell lines stably overexpressing human Sec10 (Sec10-overexpressing cells) (17). We first reconfirmed that the levels of Sec10 protein expression in the stable Sec10-overexpressing MDCK cells were higher than in control MDCK cells, and the increase was 4.34-fold (P < 0.0025) (Fig. 1A). Confluent MDCK cells grown on plastic culture dishes form typical domes due to active secretion and tight cell-cell contacts, which results in a lifting of the cells off the plastic culture dish (Fig. 1, arrowhead). Dome formation is a long-appreciated characteristic of MDCK cells grown past confluency on plastic culture dishes and serves as a useful marker of epithelial barrier function as well as fluid and electrolyte secretion. To investigate the involvement of Sec10

Fig. 4. Inhibition of ERK activation accelerated the decrease of TER induced by hydrogen peroxide. Control ("MDCK") and Sec10-overexpressing ("Sec10-MDCK") MDCK cells were grown on Transwell filters until TER levels reached steady state, incubated in either vehicle or 10 μM U0126 for 30 min, and then treated with 1 mM H2O2 for 30 min. A: after 30 min of H2O2 treatment, with or without U0126 pretreatment, cells were harvested and then lysed in SDS buffer. Equal amounts of protein were loaded in each lane as determined by BCA assay, and Western blotting was performed using antibodies against p-ERK and t-ERK. U0126 prevented ERK activation. B and C: TER was measured at the indicated time points. Values are means ± SE of 3 independent experiments performed in duplicate (n = 6). *P < 0.05 for the TER at that time point vs. the TER at 0 min for that condition. #P < 0.05 for U0126- vs. control (vehicle alone) treated cells at that time point. In both control and Sec10-overexpressing cells, U0126 accelerated the decrease in TER following H2O2 treatment.
in the integrity of cell-cell contacts, we treated control and Sec10-overexpressing MDCK cells with 1 mM H$_2$O$_2$ for 30 min. Domes that lost barrier function from this treatment appear collapsed, but remain easily distinguishable from nearby attached cells (Fig. 1B). Before H$_2$O$_2$ treatment, the average number of domes per square millimeter in the Sec10-overexpressing MDCK cells was 7 vs. 20 in the control MDCK cells (Fig. 1C). We believe that this is due to Sec10-overexpressing MDCK cells having a higher TER than control MDCK cells (see Fig. 2) and, therefore, requiring a greater force to lift cells off the plastic culture dish, with a resultant decrease in dome number per area measured. H$_2$O$_2$ treatment disrupted dome formation to a significantly smaller degree in Sec10-overexpressing MDCK cells compared with control MDCK cells (Fig. 1B, arrow, and D).

Sec10 overexpression attenuates the decrease in TER caused by treatment of hydrogen peroxide. TER is a sensitive parameter in determining the integrity of cell-cell contacts in an epithelial monolayer. Disruption of cell-cell contacts is highly associated with various kidney disorders, including the breakdown of tubular epithelium from I/R injury (25, 36). When MDCK cells were seeded on Transwell filters, TER steadily increased and reached a steady state at 3 days postconfluency (data not shown). When TER was maintained at steady state, TER of Sec10-overexpressing cells was significantly greater than that of control MDCK cells (Fig. 2A). Hydrogen peroxide treatment (1 mM for 30 min) resulted in a steady decrease in TER over time (Fig. 2B). However, the TER decrease induced by hydrogen peroxide was significantly less in the Sec10-overexpressing cells compared with control MDCK cells (Fig. 2B). The rates of TER decrease to 30 min following hydrogen peroxide treatment were 0.75 $\pm$ 0.11 and 1.18 $\pm$ 0.1 $\Omega$/cm$^2$/min in Sec10-overexpressing MDCK cells and control MDCK cells, respectively ($P < 0.01$). These data suggest that Sec10 overexpression increases TER and recovery from I/R injury and oxidative stress (15, 28, 31). Furthermore, the exocyst is centrally involved in tubulogenesis (17), and tubulogenesis, in turn, is initiated by ERK activation (10, 11, 28). These data raised the possibility that ERK activation is involved in the increased resistance of Sec10-overexpressing cells to H$_2$O$_2$ insult. Therefore, we determined the levels of phosphorylated ERK in control MDCK cells and Sec10-overexpressing MDCK cells. When grown on either plastic culture dishes or Transwell filters, phosphorylated ERK levels were significantly higher in Sec10-overexpressing cells compared with control MDCK cells (Fig. 3, A and C). In agreement with previous studies (6), H$_2$O$_2$ treatment itself resulted in ERK activation in cells grown on both plastic (Fig. 3B) and Transwell filters (Fig. 3C).

ERK inhibition exacerbates the decrease in TER caused by hydrogen peroxide treatment. To determine whether the higher level of active ERK found in Sec10-overexpressing cells contributed to the higher resistance of Sec10-overexpressing MDCK cells to H$_2$O$_2$, we blocked ERK phosphorylation using U0126 (10 $\mu$M), a specific inhibitor of MEK phosphorylation (MEK is directly upstream on ERK). After a 30-min pretreatment with U0126, cells grown on Transwell filters were then treated with 1 mM H$_2$O$_2$. As seen in Fig. 4A, U0126 treatment completely blocked ERK phosphorylation caused by H$_2$O$_2$ treatment in both Sec10-overexpressing MDCK cells and control MDCK cells. Sec10 overexpression increases MAPK pathway activation. Phosphorylation (activation) of ERK is the final step in the activation of the MAPK pathway. It has been reported that ERK activation in epithelial cells is associated with both increased TER and recovery from I/R injury and oxidative stress (15, 28, 30, 31). How-
Sec10 OVEREXPRESSION PROTECTS EPITHELIA

Sec10 overexpression accelerates recovery of TER from H2O2 injury, whereas ERK inhibition delays recovery. We next investigated the involvement of Sec10 expression and ERK activation in recovery of barrier function after a period of oxidative stress. TER was measured across Sec10-overexpressing MDCK and control MDCK cells that were treated with U0126 for 30 min, then U0126 plus 1 mM H2O2 for 30 min, and followed for 8 h (Fig. 5). In agreement with Fig. 4, B and C, pretreatment of U0126 accentuated the decrease in TER induced by H2O2 in both control MDCK cells and Sec10-overexpressing MDCK cells (Fig. 5A). TER decreased continuously until ~30 min after removal of H2O2 in both cells and then started increasing as the epithelial barrier started to recover its integrity (Fig. 5A). In the absence of U0126, the recovery of TER was significantly faster in Sec10-overexpressing MDCK cells compared with control MDCK cells. The recovery of TER by Sec10-overexpressing MDCK cells started at 45 min after removal of the H2O2 compared with control MDCK cells, where the recovery of TER started at 2 h following the removal of H2O2 (Fig. 5A). In addition, in Sec10-overexpressing MDCK cells, it took only 2 h for TER to return to normal levels following removal of H2O2, whereas it took 7 h following removal of H2O2 for TER to return to normal in control MDCK cells (Fig. 5). The rates of TER recovery after reaching nadirs were significantly faster in the Sec10-overexpressing MDCK cells compared with control MDCK cells (Fig. 5B), indicating that Sec10 overexpression in renal tubular epithelial cells significantly accelerates recovery of epithelial barrier function following oxidative stress.

When cells were treated with U0126, TER decreased continuously until 90 min after removal of H2O2 in both Sec10-overexpressing and control MDCK cells (Fig. 5), indicating that ERK activation is important for recovery of TER. Indeed, with U0126, 8 h after removal of H2O2 was not enough time for TER to return to normal levels for either cell line. Although, as shown previously with U0126 (Fig. 4), the two cell lines had a different overall decrease in TER following H2O2.

Fig. 6. Sec10 overexpression attenuated the damage of cysts induced by hydrogen peroxide treatment. Control ("MDCK") and Sec10-overexpressing ("Sec10-MDCK") MDCK cells were grown in a type I collagen matrix for 12–14 days as described in MATERIALS AND METHODS. A: cells were harvested as described in MATERIALS AND METHODS and then lysed in SDS buffer. Equal amounts of protein were loaded in each lane as determined by BCA assay, and Western blotting was performed using antibodies against p-ERK and t-ERK. B and C: cells were treated with 1 mM H2O2 for 30 min. One group of cysts was treated with 10 μM U0126 for 30 min before addition of the 1 mM H2O2. After treatment, cysts were fixed with 4% paraformaldehyde and stained with phalloidin-rhodamine for actin and 4,6-diamidino-2-phenylindole (DAPI) for cell nuclei. Numbers of damaged cysts were counted. B: damaged cysts were evaluated by collapse of cysts and/or loss of actin staining. Red represents phalloidin-rhodamine-stained actin, and blue represents DAPI-stained nuclei. C: numbers of damaged and intact cysts were counted using a fluorescence microscope. Sec10 overexpression attenuated the damage to cysts from H2O2. Values are means ± SE (the experiment was repeated 3 times, and 100 cysts were counted in each experiment). *P < 0.05 for H2O2-treated vs. untreated cells. #P < 0.05 for Sec10-overexpressing vs. control MDCK cells.
treatment, the two cell lines had a similar rate of recovery when U0126 was present. Taken together, these data suggest that Sec10 overexpression both protects cells from ischemic injury and accelerates recovery of epithelial barrier integrity after oxidative stress injury, and ERK activation is the mechanism by which this occurs.

Sec10 overexpression prevents cyst damage by hydrogen peroxide. MDCK cells form cysts in a three-dimensional collagen matrix (40). Sec10-overexpressing MDCK cells in cysts had a 1.39-fold higher level of active ERK expression ($P = 0.09$) than did control MDCK cells (Fig. 6A). Although this did not reach statistical significance, the reliability of this assay may have been limited by the large excess of type I collagen protein from the collagen gel and could represent an underestimation of active ERK expression. It should also be noted that we previously showed that Sec10-overexpressing MDCK cells form cysts and tubules at a faster rate than control MDCK cells (17), which is consistent with higher levels of active ERK expression. 

H$_2$O$_2$ treatment resulted in cyst damage (Fig. 6, B and C), as determined by a loss of spherical morphology with a change in actin localization and a decrease in the intensity of actin staining. After 30 min of 1 mM H$_2$O$_2$ treatment, the proportion of damaged cysts was significantly less for Sec10-overexpressing MDCK cells compared with control MDCK cells (Fig. 6C). To investigate the role of ERK activation in mediating cyst damage, some cysts were incubated in medium containing U0126 for 30 min and then treated with 1 mM H$_2$O$_2$ plus U0126 for 30 min (Fig. 6C). Blocking ERK activation with U0126 increased cyst damage for both control MDCK and Sec10-overexpressing MDCK cell cysts (Fig. 6B).

**Transient I/R changes exocyst expression in murine kidneys.** To investigate a correlation of exocyst expression and damage and repair following in vivo I/R injury, we examined exocyst Sec8 expression in kidneys subjected to 30 min of ischemia followed by reperfusion. Renal function dramatically decreased early after I/R injury and then recovered over 2 wk (Fig. 7A). Exocyst expression, determined by the level of Sec8 protein, decreased early after reperfusion and then increased during the recovery phase (Fig. 7B). The expression pattern of the polarity marker Na-K-ATPase was similar to that of exocyst Sec8 (Fig. 7B). The increase in proliferative cell nuclear antigen expression started at 1 day after reperfusion, peaked at 2 days, and then decreased (Fig. 7B). This suggests that the exocyst may be associated with renal tubule epithelial cell recovery following I/R injury.

**DISCUSSION**

We report three principal findings here, all of which are quite interesting. First, we show that exocyst Sec10 overexpression in renal tubular epithelial cells leads to increased resistance of the epithelial barrier to oxidative stress, a major mediator of I/R injury. In addition, the increased expression of Sec10 in these cells accelerates the recovery of proper epithelial barrier function following oxidative stress. Second, we show that this resistance, and the increased rate of recovery, is likely mediated via activation of the MAPK pathway. Finally, in mice subjected to renal I/R injury, exocyst expression levels in the kidney decreased early after induction of I/R and returned to normal concomitant with functional recovery, suggesting that the exocyst could be involved in the recovery of tubular epithelium following I/R injury. A strength of this study is that we demonstrate these findings in multiple experimental systems of increasing complexity, including: renal tubule epithelial cells grown as a monolayer on plastic, as a monolayer on Transwell filters, as cysts cultured in a three-dimensional collagen matrix, and in vivo in mice.

Sec10 is a crucial component of the exocyst complex (40) that, along with Sec15, binds the Rab GTpase Sec4/Rab8, found on the surface of the protein-laden vesicles, to the rest of the exocyst complex that is in contact with the plasma membrane (9). Perturbation of Sec10 function has significant inhib-

---

**Fig. 7.** Ischemia and reperfusion (I/R) changed plasma creatinine concentrations and levels of Sec8, proliferating cell nuclear antigen (PCNA), and Na-K-ATPase expression in the kidneys. Mice were subjected to 30 min of I/R for indicated time periods. Blood (A) and kidneys (B) were harvested to determine concentration of plasma creatinine ($n = 4–7$ mice/time point; A) and levels of Sec8, PCNA, Na-K-ATPase and GAPDH expression (B) by Western blot analysis. GAPDH was used as a marker of equal loading. Sec8 and Na-K-ATPase expression followed a similar pattern. Values are means ± SE. *$P < 0.05$ for that time point vs. baseline at 0 days.
Sec10 OVEREXPRESSION PROTECTS EPITHELIA

F825

F825

in the present study, when dome-forming MDCK cells, grown on plastic culture dishes, were treated with hydrogen peroxide, an in vitro model of I/R injury involving oxidative stress, disruption of domes was significantly greater in control MDCK cells compared with Sec10-overexpressing MDCK cells. Domes in MDCK cells are formed by tight cell-cell contacts and secretion that lifts the cells off the surface of the plastic culture dishes. This implies, therefore, that cell-cell contacts in Sec10-overexpressing cells are enhanced and more resistant to disruption by H₂O₂.

TER is known to be a sensitive measure of barrier function and the integrity of cell–cell contacts. In MDCK cells grown on Transwell filters, TER increases as the cells become confluent, and a steady state is reached by 3 days postconfluence when the cells form a tight monolayer. In support of the idea that Sec10 overexpression leads to tighter cell-cell contacts and a stronger epithelial barrier, we found steady-state TER in Sec10-overexpressing cell monolayers to be higher than in control MDCK cell monolayers. In addition, recovery of normal TER after removal of hydrogen peroxide in Sec10-overexpressing cells was faster than in control MDCK cells. This is particularly interesting in light of a recent paper by Anderson and Yeaman showing that the exocyst is centrally involved in the formation of desmosomal junctions (1).

We and others have shown that the MAPK pathway is crucial for the survival of kidney tubular epithelial cells exposed to both I/R injury (30, 31) and oxidative stress (4, 13) and that the MAPK pathway is necessary and sufficient for the initiation of tubulogenesis in MDCK cells grown in collagen (10, 11, 28). MAPK pathway activation is also required for the recovery of cells from injury (6, 22). Kiley et al. (15) reported that MAPK inhibition significantly decreased TER in MDCK cells, and we previously showed that MAPK activation increased TER in MDCK cells (18, 21). Finally, we previously demonstrated that the exocyst is centrally involved in renal tubulogenesis (17, 33, 40). This was the rationale for investigating the role of the MAPK pathway in the increased TER seen in exocyst Sec10-overexpressing cells. We report here that steady-state levels of phosphorylated ERK in Sec10-overexpressing MDCK cells were significantly higher than in control MDCK cells, while levels of total ERK were equivalent. Pretreatment with U0126, an inhibitor of ERK phosphorylation, accentuated the decrease in TER and inhibited recovery following treatment with hydrogen peroxide. These data implicate the MAPK pathway in the enhanced resistance to and recovery from oxidative stress seen in Sec10-overexpressing cells. We previously reported that Sec10-overexpression accelerated ciliogenesis, cystogenesis, and tubulogenesis in kidney tubule epithelial cells (17, 40), and the results shown here are an important mechanistic compliment to those studies.

In mice subjected to renal I/R injury, exocyst expression levels in the kidney decreased early after induction of I/R and gradually returned to normal along with functional kidney recovery. This is consistent with our in vitro data showing that the exocyst is involved in the recovery following I/R injury, although it should be emphasized that the in vivo data demonstrate only an association and not causation. We previously reported that kidneys recovering from I/R injury have higher levels of active ERK compared with basal levels (30). Taken together, these data suggest that the exocyst may be associated with the restoration of damaged kidney tubular epithelium via activation of the MAPK pathway, although this remains to be proven.

AKI in hospitalized patients is a significant and increasing problem. Medical management of AKI/acute tubular necrosis has traditionally consisted of supportive care, with renal replacement therapy implemented for the most severe cases. Despite such interventions, however, mortality rates in affected patients remain very high (>50% in some series) (3). The exocyst, which is involved in the synthesis and delivery of secreted and membrane proteins, is absolutely essential for the establishment of many cellular functions, including cell polarity. Here, in cultured renal tubule MDCK cells, we show that exocyst Sec10 overexpression reduces damage to tubular epithelial barriers caused by hydrogen peroxide and speeds the recovery of normal epithelial barrier function after such an injury. Furthermore, our data indicate that the mechanism is via activation of the MAPK pathway. Finally, we show that exocyst reexpression is associated with the recovery that follows I/R injury, raising the possibility that the exocyst could be a rescue and/or resistance factor for ischemic AKI.

GRANTS

This work was supported in part by grants from the National Institutes of Health (DK069909 to J. H. Lipshutz), Satellite Healthcare (Norman S. Coplon Extramural Research Grant to J. H. Lipshutz), the University of Pennsylvania Translational Medicine Institute (to J. H. Lipshutz), the National Institutes of Health (EY017024 to D. L. Chung) and the National Research Foundation of Korea Grant funded by the Korean Government (M1064155001-06N1414-0011 to K. M. Park). The University of Pennsylvania Biomedical Imaging Core Facility of the Cancer Center is gratefully acknowledged for providing imaging services and the Morphology Core of the Center for the Molecular Studies of Liver and Digestive Diseases (Center Grant P30 DK50306) for providing microscopy services.

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES

Sec10 OVEREXPRESSION PROTECTS EPITHELIA


