TDAG51 mediates epithelial-to-mesenchymal transition in human proximal tubular epithelium

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CHRONIC KIDNEY DISEASE (CKD) is a major contributor to morbidity and mortality with an estimated prevalence of 11% in the United States (9). However, many individuals who have early-stage CKD are asymptomatic. There are many risk factors for the progression of CKD, including hypertension (17), proteinuria (8, 34), and poor glucose control in diabetes (35). Features of CKD progression remain consistent regardless of cause, including declining glomerular filtration rate, peritubular capillary loss resulting in tubular ischemia, and interstitial fibrosis (42). It has been suggested that epithelial-to-mesenchymal transition (EMT) is not a source of fibroblasts in renal fibrosis (55); however, recent lineage studies have provided direct evidence proving epithelial cell involvement in producing the fibroblasts found in renal interstitial fibrosis (29, 33, 54). EMT can cause interstitial fibrosis by transition of the tubular epithelium to collagen-producing fibroblasts (33). When the basement membrane is altered or damaged, the epithelium expresses cytokines, such as transforming growth factor (TGF)-β, that promote EMT (33). TGF-β1 is an important mediator of EMT (37); however, it is becoming clear that epithelial adherens junction detachment primes the cell for the TGF-β1 effect (39). The transformation from an epithelial to a mesenchymal phenotype results in local formation of fibroblasts. When this occurs, epithelial cells decrease the expression of typical epithelial cell proteins such as E-cadherin, show β-catenin cytoplasmic-to-nuclear translocation, and express mesenchymal proteins, such as type I collagen and vimentin (7, 37). This can occur via Wnt/β-catenin signaling. Wnt/β-catenin signaling is activated when β-catenin is released from its E-cadherin anchor in epithelial cell adherens junctions and accumulates in the cytoplasm. The β-catenin/TGF/LEF complex is then formed and translocated to the nucleus, resulting in the upregulation of specific genes, such as fibronectin, vimentin, matrilysin, and SNAIL2 (7).

Recent studies have shown that endoplasmic reticulum (ER) stress is a common feature of CKD of diverse etiology (12, 15). When there is a disruption in the protein-folding process, ER stress occurs (49). Transmembrane, ER luminal resident, and secretory proteins are synthesized in the ER, including proximal tubular cell transporter proteins such as the Na+-K+-ATPase that are critical for the reabsorption of ultrafiltrate components (1, 15). ER stress activates the unfolded protein response (UPR), thereby leading to the phosphorylation of eIF2α and inhibition of general translation (12). The drug salubrin (Sal) is a selective inhibitor of the dephosphorylation of eIF2α that protects from ER stress. Its mode of action involves the reduced formation of the PPP1/GADD34 dephosphatase complex. This has been demonstrated by the inhibition of ER stress-mediated apoptosis induced by tunicamycin (Tm) in PC12 cells (6). Sal has also been shown to protect against cyclosporine A (CsA)-induced nephrotoxicity, which may involve ER stress (44).

We have previously reported that T cell death-associated gene 51 (TDAG51), also known as the pleckstrin homology-like domain (PHLD), family A member 1 protein, is induced by certain ER stressors, including peroxynitrite, which inhibits the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) (14, 31). Others have shown that the ER stress inducer farnesol causes TDAG51 upregulation through the MEK/ERK/MAPK pathway (36). Initially, it was determined that TDAG51 is...
necessary for Fas-induced apoptotic cell death in T cells (45). However, TDAG51 knockout in the whole animal did not prevent the induction of T cell apoptosis (47). Subsequently, it was demonstrated that TDAG51 has significant homology to a tumor suppressor gene, \textit{Ipf}/\textit{Tssc} (21). We previously demonstrated that overexpression of TDAG51 leads to cell shape change and decreased cell adhesion, resulting in anoikis (detachment-mediated apoptosis), in human vascular endothelium (31). The mechanism by which TDAG51 generates cell shape change may be related to its colocalization with focal adhesion kinase in focal adhesions (31). Additionally, TDAG51 contains proline-histidine and proline-glutamine repeat sequences similar to apoptotic-promoting genes (25), transcriptional activators (45), and a pleckstrin homology-like domain similar to proteins regulating cytoskeletal function (27, 32). Furthermore, it has been confirmed by the Human Protein Atlas that human renal proximal tubular epithelial cells (hRPTEC) express a moderate amount of TDAG51 protein (4).

Since EMT and ER stress are both important in the pathology of CKD, we hypothesized that EMT can result from Ca\textsuperscript{2+} dysregulation-induced ER stress and is mediated by TDAG51 upregulation. Different ER stress inducers were utilized to determine whether ER stress results in hRPTEC EMT. Treatment with ER stress inducers Tg and CsA, which generated Ca\textsuperscript{2+} dysregulation-induced ER stress and TDAG51 overexpression, resulted in EMT. Sal, an inhibitor of TDAG51 overexpression during Tg-mediated ER stress, inhibited the EMT response. This response was also inhibited by buffering Ca\textsuperscript{2+} with the intracellular Ca\textsuperscript{2+} chelator BAPTA-AM. Furthermore, TDAG51 overexpression was found to induce proximal tubular cell shape change and the disruption of epithelial cell junctions, priming the proximal tubular cells for EMT. Moreover, TDAG51 knockout in the whole animal inhibited TGF-\beta1-induced peritoneal fibrosis, a response involving mesothelial cell EMT. Taken together, our findings provide evidence that TDAG51 is a novel mediator of EMT.

METHODS

Cell culture. hRPTECs (passages 2–5; Lonza, Walkersville, MD) were cultured on coverslips in REBM containing 0.5 ml hEGF, 0.5 ml hydrocortisone, 0.5 ml epinephrine, 0.5 ml insulin, 0.5 ml triiodothyronine, 0.5 ml transferrin, 0.5 ml GA-1000, and 2.5 ml fetal bovine serum SingleQuots per 500 ml of medium (Lonza), as indicated by the manufacturer. HK-2 cells were cultured in a 1:1 ratio of DMEM 1 g/l glucose media (Invitrogen; Carlsbad, CA) and F12 GlutaMAX nutrient mix (Invitrogen). Twenty-four hours before experiments, HK-2 cells were transferred into DMEM containing 4.5 g/l glucose (Invitrogen), unless otherwise stated.

Reagents. Tm, Tg, CsA, and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). Sal was purchased from Calbiochem (EMD; Gibbstown, NJ). Recombinant human TGF-\beta1 was purchased from R&D Systems (Minneapolis, MN) and activated according to the manufacturer’s instructions. Non-water-soluble reagents were dissolved in DMSO (Sigma-Aldrich) as a transitional solvent, and DMSO was used as a vehicle control. Rhodamine phalloidin, fura 2-AM, and BAPTA-AM were purchased from Invitrogen. Parafomaldehyde was obtained as a 4% solution in PBS (BioLynx, Brockville, ON).

Gel electrophoresis. Total cell lysates were generated in 4× SDS lysis buffer with protease inhibitor cocktail added (complete Mini; Roche; Laval, Quebec). Protein levels were determined using a Bio-Rad DC Protein Assay for control of protein loading. Cell lysates were subjected to electrophoretic separation in a 10% SDS-PAGE reducing gel (Bio-Rad).

Quantitative analysis of protein expression. Primary antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies and ECL Western Blotting Detection Reagents (GE Healthcare, Mississauga, ON), as previously reported (31). Results were densitometrically quantified using ImageJ software (version 1.43, National Institutes of Health, Bethesda, MD) and expressed as a ratio of \beta-actin loading control, unless otherwise stated, to blots developed on X-ray film.

Protein expression was quantified using quantum dots for fluorescence detection. To achieve this, low fluorescence polyvinylidene difluoride membranes (Immobilon-FL, Millipore, Billerica, MA) were blocked in WesternDot blocking buffer (Invitrogen). Membranes were incubated with primary antibodies for \beta-actin (A-5316, 1:5,000; Sigma), TDAG51 (sc-23866, 1:200; Santa Cruz, Biotechnology, Santa Cruz, CA), phospho-\alpha-tubulin (97215, 1:1,000; Cell Signaling, Danvers, MA), eGFP (sc-11386, 1:200; Santa Cruz Biotechnology), or KDEL, which detects both GRP78 and GRP94 (SPA-827, 1:1,000; Stressgen, Enzo Life Sciences; Plymouth Meeting, PA). Subsequently, membranes were incubated with biotinylated secondary antibodies (Biotin-XX-goat anti-mouse, 1:2,000) and then Qdot 625 streptavidin conjugate (1:2,000). Qdot conjugates were used no more than three times. Membranes were imaged using Bio-Rad ChemiDoc XRS+ through a specific filter (630BP30), and the results were densitometrically quantified using Image Lab software, version 2.0 (Bio-Rad), where the signal for the primary antibody was expressed as a ratio to the \beta-actin loading control, as previously (15a).

Fluorescence microscopy. An Olympus IX81 Nipkow scanning disk confocal microscope was used for fluorescence microscopy. Living cells were imaged in white light using differential interference contrast. HK-2 or hRPTECs were stained using anti-GRP78 antibodies (1:100, sc-1050; Santa Cruz Biotechnology), anti-TDAG51 antibodies (1:100, sc-23866; Santa Cruz Biotechnology), anti-\beta-catenin antibodies (1:200, no. 2677; Cell Signaling), anti-\alpha-smooth muscle actin (\alpha-SMA) antibodies (1:200, 1A4 clone; Thermo Scientific, Nepean, ON), or anti-vinculin antibodies (1:100, V4505; Sigma). This was followed by the addition of a species-specific secondary antibody conjugated to an Alexa dye at 488-or 594-nm excitation (1:200; Invitrogen, Molecular Probes). The DNA-specific dye DAPI was used to label the nuclei of cells. Perlmallor (Thermo Scientific) was used to mount the coverslips on microscope slides. Individual wavelengths for fluorophore excitation were as follows: DAPI: 377 nm/50 nm band-pass; Alexa 488: 482 nm/50 nm band pass; Alexa 594: 562 nm/40 nm band pass.

Cytosolic Ca\textsuperscript{2+} measurements. Cytosolic Ca\textsuperscript{2+} measurements were performed as previously described (16). Briefly, the Ca\textsuperscript{2+}-sensitive dye fura 2-AM was used to measure cytosolic Ca\textsuperscript{2+} concentrations as a ratio of the fluorescent signal stimulated by 340 (dye bound to Ca\textsuperscript{2+})- or 380 (free dye)-nm excitation with emission collected at 510 nm on a SpectraMax Gemini spectrofluorometer ( Molecular Devices). This was accomplished in cell populations of HK-2 cells cultured in 96-well plates (BD-Falcon, Black/Clear bottom, Optilux), maintained at 37°C in Hanks’ balanced salt solution (Invitrogen) containing 20 mM HEPES buffer at pH 7.4.

Construction and transfection of expression plasmids. TDAG51 cDNA was amplified via PCR and subsequently digested using the restriction enzymes HindIII and XbaI. Following digestion, the cDNA was then cloned into an enhanced green fluorescent protein (eGFP; Clontech, Mountain View, CA) vector creating a plasmid where eGFP was linked to TDAG51 protein (termed eGFP-TDAG51), as previously described (31). The eGFP-PHLD fusion protein was generated using an eGFP-C1 plasmid (Clontech). TDAG51 cDNA encoding the PHLD amino acid (aa) sequence (10-LKEGVLEKRS DGLQGLWKKK CCLTLEEGL LILPKOLQHQ QQQQQQQQ QQQQPPQGGPAE PQPSPGPAVA SLEPPVVLKE LHFNSMKTVD CVERGKGKYM FTVVMAEGKE IDFRCPQDG VNAEITLQMV QY-132 aa) was amplified by PCR before subcloning into a T-ovhang pGEM-T vector (Promega). Primers were designed with BglII and KpnI terminal restrict-
tion sites (forward 5'-AGATCTCTGAAGGAGGCGTG; reverse 5'-GGTACCGTACTGACCATTGCAGC) and synthesized by Integrated DNA Technologies (Skokie, IL). The pGEM-T transition vector containing PHLD was digested with BglII and KpnI, and the PHLD fragment was purified by gel extraction before cloning into the BglII/KpnI sites of pEGFP-C1. Construct identity was confirmed by DNA sequencing (MOBIX, Hamilton, ON). To replicate the plasmids, competent DH5α bacteria were transformed and grown overnight on kanamycin-resistant agar plates. Single colonies were selected and placed into kanamycin-selective LB broth and grown to the logarithmic growth phase. Plasmid-expressing bacteria were isolated using the EndoFree Plasmid Maxi Kit (Qiagen) per the manufacturer’s instructions. The amount and purity of plasmid recovered (which ranged between 1.8 and 1.9, as a 260/280 nm ratio) was determined by the SmartSpec 3000 (Bio-Rad). HK-2 cells or hRPTECs were transfected with eGFP plasmid alone, as a control, or with the eGFP-TDAG51 plasmid using FuGENE 6 transfection reagent (Roche) at a 6:1 ratio. The transfection efficiency between eGFP and eGFP-TDAG51 (29.7 ± 4.7 vs. 25.3 ± 2.2%, respectively) did not differ significantly.

Measurement of TDAG51’s effect on cell shape change. ImageJ software was used to measure the area and perimeter of eGFP- and eGFP-TDAG51-transfected HK-2 cells. These measurements were accomplished by selecting the perimeter of eGFP- or eGFP-TDAG51-transfected HK-2 cells using ImageJ software, which then calculated the perimeter and area of each individual cell. Graphical and statistical analysis was performed using Microsoft Excel software.

Measurement of TGF-β1 by sandwich ELISA. The TGF-β ELISA (R&D Systems) was used to measure active TGF-β levels in the conditioned media of HK-2 cells per the manufacturer’s instructions. Cells were grown to confluence in 1:1 DMEM (Invitrogen) and F12 GlutaMAX nutrient mix (7.75 mM d-glucose, Invitrogen) and were treated with drug vehicle DMSO or Tg (200 nM) for 24 h. The reaction for active TGF-β1 was developed with tetramethyl benzadine (Invitrogen, Molecular Probes), and nuclei were counterstained using DAPI (100 ng/ml) for 30 min.

RESULTS

Classic ER stressors and CsA induce the UPR and differentially affect TDAG51 protein expression. Western blot analysis demonstrated a significant increase in the UPR marker GRP78 at 18 h in response to all ER stress inducers (Fig. 1). TDAG51 protein levels were increased in response to the SERCA inhibitor Tg and the calcineurin inhibitor CsA but not the N-linked glycosylation inhibitor Tm in HK-2 cells at both 7 and 18 h (Fig 1) and primary hRPTECs at 18 h (data not shown). Treatment of cells with Tg or Tm was combined with Sal to determine whether dephosphorylation of eIF2α alters the levels of ER stress markers in HK-2 cells. However, Sal treatment did not reduce the expression of GRP78 or TDAG51 in HK-2 cells. To determine the effect of ER stress on cell shape change, HK-2 cells were treated with DMSO (vehicle), Tm, or Tg. GRP78 (green) upregulation was observed for both Tm and Tg (Fig 2A); however, TDAG51 (green) levels were increased in response to Tg, but not Tm by indirect immunofluorescence (Fig 2B). This confirms the Western blotting results showing no change in Tm-treated cells and increased TDAG51 expression in Tg-treated cells (Fig 1). Unlike vehicle- and Tm-treated cells, Tg-treated cells showed a lack of F-actin (red) in the periphery as well as cellular elongation, indicative of EMT. CsA treatment also produced F-actin rearrangement and shape change indicative of EMT (Fig 2C), as shown in other studies (41, 44, 47, 51).

ER stress induction and inhibition of thapsigargin-induced TDAG51 upregulation by Sal in HK-2 cells. At 4 h, HK-2 cells showed an increase in phospho-eIF2α in response to Tm, Sal, Tm plus Sal, and Tg plus Sal (Fig. 3A). To determine the effect of Sal alone on TDAG51 expression, HK-2 cells were treated with Sal for 18 h. Treatment with Sal alone exhibited a significant decrease in TDAG51 expression in HK-2 cells (Fig. 3C). Combined treatment with Tg plus Sal inhibited Tg-induced increases in TDAG51 expression at 48 h (Fig. 3C). Thus, we used Sal to determine whether it would inhibit EMT. Additionally, GRP78 expression was significantly increased in response to treatment with Sal alone for 18 h (Fig. 3D).

Cytosolic β-catenin accumulation and nuclear translocation induced by thapsigargin lead to EMT. To demonstrate the localization of β-catenin in response to ER stressors and Sal, proximal tubular cells were treated for 24 h and stained for β-catenin (Fig. 4A). Primary hRPTECs show β-catenin staining around the periphery of the cell in response to vehicle, Tm, and Sal treatments. Cells treated with Tg showed an accumulation of β-catenin in the perinuclear and nuclear regions, with reduced β-catenin in the periphery of the cell. β-Catenin staining was quantified and shows that, unlike the other treatments, Tg-treated cells contained significantly more β-catenin in the nuclear and perinuclear area, and less β-catenin in the periphery of the cell (Fig. 4A). This is indicated by a significant increase in the cytoplasmic-to-peripheral ratio of Tg treatment (2.37 ± 0.39) over vehicle (0.55 ± 0.08). Neither Sal (0.66 ± 0.06) nor Tm (0.67 ± 0.07) caused a significant change in this ratio compared with the vehicle. HK-2 cells were treated and stained as in Fig. 4A, and similar results were obtained (data not shown; see supplemental video for 3-dimensional reconstruction; supplementary material for this article is available on the journal website). To determine the effect of cadherin junction disruption on β-catenin staining, HK-2 cells were
treated with the Ca\(^{2+}\) chelator EGTA for 18 h. EGTA treatment resulted in translocation of \(\beta\)-catenin (red) to the perinuclear and nuclear region, indicating that Ca\(^{2+}\)-mediated cadherin disruption leads to \(\beta\)-catenin cytoplasmic-to-nuclear translocation. Tg-treated cells showed \(\beta\)-catenin cytoplasmic-to-nuclear translocation as well; however, they developed a distinct fibroblast-like morphology (Fig. 4B).

**Ca\(^{2+}\) chelator prevents thapsigargin-mediated effects.** Cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) was measured ratiometrically to determine whether treatment with various ER stressors resulted in Ca\(^{2+}\) disequilibrium, which is associated with epithelial junction disruption (39). Epithelial junction disruption, particularly the breaking of cadherin junctions, releases \(\beta\)-catenin from its anchorage on the plasma membrane, allowing

Fig. 1. Unfolded protein response (UPR) activation and T cell death-associated gene 51 (TDAG51) expression in response to endoplasmic reticulum (ER) stress. HK-2 cells were treated with vehicle, thapsigargin (Tg), tunicamycin (Tm), cyclosporine A (CsA), or combined treatments of Tm plus salubrinal (Sal; Tm+Sal) or Tg plus Sal (Tg+Sal) for 7 or 18 h. Cell lysates were assessed by Western blotting and probed for GRP94, GRP78, TDAG51, and \(\beta\)-actin. Densitometry showed a significant upregulation of GRP78 protein in response to Tg at 7 h. At 18 h, GRP78 protein levels were upregulated by all treatments, indicating ER stress induction. Densitometry also indicated a significant upregulation of TDAG51 in response to Tg, Tg+Sal, and CsA treatments at 7 and 18 h (n = 4, P < 0.05). Tg = 200 nM; Tm = 1 \(\mu\)g/ml; Sal = 30 \(\mu\)M; CsA = 5 \(\mu\)M.

Fig. 2. GRP78 and TDAG51 expression in response to ER stress. A: HK-2 cells were treated for 18 h with vehicle, Tm, or Tg and then stained for F-actin (red, arrowheads), GRP78 (green, arrows), and cell nuclei (blue). Vehicle- and Tm-treated cells displayed F-actin fibrils at the periphery of the cells (arrowheads), typical of the epithelial phenotype. Tg-treated cells show F-actin filaments at the periphery of cells undergoing change from an epithelial to mesenchymal phenotype (arrowheads). GRP78 staining appears to be more abundant in the perinuclear region of Tm- and Tg-treated cells, indicating activation of the UPR (arrows). B: HK-2 cells were stained for F-actin (red, arrowheads), TDAG51 (green), and cell nuclei (blue). F-actin staining showed similar results as in A. Tm treatments show no increase in TDAG51 levels; however, Tg-treated cells demonstrated abundant TDAG51 expression in cells that had undergone shape change (*). C: treatment with CsA for 24 h induces translocation of \(\beta\)-catenin (red) from the periphery of the cell to the perinuclear and nuclear area; nuclei are stained in blue (4,6-diamidino-phenylindole). Bars: A and B = 50 \(\mu\)m; C = 25 \(\mu\)m; Tg = 200 nM; Tm = 1 \(\mu\)g/ml; CsA = 5 \(\mu\)M.
ing it to accumulate in the cytoplasm and translocate to the nucleus (43). Tm treatment showed no change in cytosolic [Ca$^{2+}$], while both Tg and CsA demonstrated significant increases in cytosolic [Ca$^{2+}$] (Fig. 5A). Since only ER stressors associated with Ca$^{2+}$ disequilibrium resulted in TDAG51 upregulation, the intracellular Ca$^{2+}$ chelator BAPTA-AM was used to inhibit cytosolic [Ca$^{2+}$] increase and determine the effect on TDAG51 induction. HK-2 cells were treated with DMSO (vehicle), Tg (200 nM), or Tg with BAPTA-AM (100 μM). Cytosolic [Ca$^{2+}$] was measured ratiometrically to determine whether cotreatment with BAPTA-AM would suppress Tg-mediated Ca$^{2+}$ dysregulation. As expected, Tg treatment resulted in increased [Ca$^{2+}$]. Cotreatment with BAPTA-AM prevented the Tg-mediated increase in cytosolic [Ca$^{2+}$] (Fig. 5B). HK-2 cells were treated with DMSO, Tg, or Tg with BAPTA-AM for 18 h. Western blot analysis demonstrated that treatment with BAPTA-AM inhibited the Tg-mediated increase in GRP78 and TDAG51 expression (Fig. 5C).

Mesenchymal marker upregulation in response to thapsigargin- and TGF-β1-induced EMT. Increased expression of α-SMA and vinculin are markers of EMT and are induced by factors such as TGF-β1 (3, 37). After HK-2 cells were treated with DMSO (vehicle) or Tg (200 nM) for 24 h, active TGF-β1 was measured from the supernatant. Tg was
found to cause a significant increase in the activation of TGF-β1 (Fig. 6A). HK-2 cells were then treated for 48 h with vehicle, Tg, or Tg plus Sal and subsequently stained for F-actin and α-SMA (Fig. 6B) or vinculin (Fig. 6C). Most Tg-treated cells demonstrated an increase in α-SMA, decreased peripheral F-actin staining, and the formation of stress fibers, indicative of a fibroblastic phenotype (Fig. 6B). HK-2 cells treated with Tg plus Sal showed less α-SMA expression and maintained an epithelial morphology compared with Tg alone-treated cells. This indicates the eIF2α dephosphorylation inhibitor Sal partially prevents the progression of Tg-induced EMT (Fig. 6B), as well as TDAG51 expression at 48 h (Fig. 3C). Tg-treated cells show vinculin induction, another marker of EMT (7), which is inhibited by Sal (Fig. 6C). To establish that TGF-β1 induces EMT in subconfluent primary hRPTECs, cells were treated with 1 or 5 ng/ml of human recombinant TGF-β1. Immunofluorescent staining determined both doses of TGF-β1 induced an EMT response in these cells, as shown by changes in cell phenotype and increased expression of α-SMA (Fig. 6D).
**TDAG51 overexpression associated with epithelial phenotypic changes.** To assess the phenotypic changes associated with TDAG51 expression, TDAG51 was overexpressed in HK-2 cells (Fig. 7). HK-2 cells were transfected with the expression plasmids for eGFP (Fig. 7A) or eGFP-TDAG51 fusion protein expression (Fig. 7B) for 48 h. Rounded up and detached cells were observed after eGFP-TDAG51 transfection, but not eGFP transfection. eGFP-TDAG51-transfected cells had a significantly smaller area (Fig. 7C) and perimeter (Fig. 7D), indicative of shape change. To determine whether eGFP-TDAG51 resulted in apoptotic cell death, eGFP (Fig. 7E)- and eGFP-TDAG51-transfected cells (Fig. 7F) were stained with terminal uridine deoxynucleotidyl transferase-mediated dUTP nick-end labeling. Statistical analysis determined that eGFP-TDAG51 transfection resulted in significantly more apoptosis than eGFP transfection (Fig. 7G). It was demonstrated using F-actin and eGFP staining that cells transfected with eGFP did not undergo shape change or F-actin rearrangement (Fig. 7H), while eGFP-TDAG51-transfected cells appeared rounded (Fig. 7I) or elongated (Fig. 7J), breaking epithelial adherens junctions. Cells transfected with the PHLD of TDAG51 underwent shape change and β-catenin disruption. This indicates that the PHLD of TDAG51 may be the structural motif of the protein that causes cellular shape change, adherens junction dissolution, β-catenin nuclear translocation, and EMT induction.

**TDAG51 deficiency inhibits TGF-β1-mediated peritoneal fibrosis in vivo.** To determine whether TDAG51 mediates EMT in the whole animal, the TGF-β1 adenovirus-induced model of EMT was utilized (38) (Fig. 8). To determine the effect of TDAG51 on peritoneal fibrosis, C57BL/6 wild-type and TDAG51−/− mice were treated with a control vector (AdDL) or TGF-β1 adenovirus (AdTGFβ1). Untreated C57BL/6 wild-type mouse parietal peritoneum is illustrated as a comparison to viral-treated mice. After treatment with the AdTGFβ1, the submesothelial thickness of the parietal peritoneum from the anterior abdominal wall was measured. Results indicate that wild-type mice were significantly more fibrotic than TDAG51−/− mice, suggesting that TDAG51 plays a role in EMT-mediated fibrosis in the whole animal.

**DISCUSSION**

Tubulointerstitial fibrosis is a common factor in the progression of CKD, caused by a variety of renal pathologies (40, 55). Fibroblast accumulation leading to tubulointerstitial fibrosis is partly derived from epithelial cells comprising the proximal tubule modified through a process of EMT. There are three
types of EMT: type 1, EMT in embryogenesis; type 2, EMT in organ fibrosis; and type 3, EMT in cancer progression and metastasis. Type 2 EMT, under conditions of chronic organ injury, contributes to organ fibrosis (7). Organ fibrosis is mediated by fibroblasts and myofibroblasts. Although some debate exists whether EMT is a source of fibroblasts in renal fibrosis (55), recent lineage tracing studies have provided direct evidence (29, 33, 54). EMT involves the cells losing epithelial adhesions, expressing stress fibers and α-SMA, and producing extracellular matrix components, including type I

Fig. 6. Tg treatment reduces epithelial cell phenotype, while inducing the expression of myofibroblast markers. A: 24-h treatment with Tg was found to significantly increase the levels of active transforming growth factor (TGF)-β1 in the supernatant of cultured HK-2 cells. P < 0.05. B: HK-2 cells were treated for 48 h with vehicle, Tg, or Tg pretreated with Sal for 1 h. Cells were stained for F-actin (red), nuclei (blue), and α-smooth muscle actin (α-SMA; green). Vehicle treatment showed a typical epithelial cell phenotype, with prominent F-actin filaments at the cell periphery (arrowheads). Tg treatment induced α-SMA expression in cells that had undergone shape change, typical of mesenchymal transformation (arrows). Sal pretreatment appeared to reduce the Tg-mediated expression of α-SMA. C: HK-2 cells were treated as in B. Cells were stained for F-actin (red), nuclei (blue), and vinculin (green). Vehicle-treated cells show prominent F-actin filaments in the cell periphery, and few cells expressing vinculin (arrows). Tg treatment showed an increase in vinculin staining (arrows) in cells that underwent shape change. Sal pretreatment (1 h) appeared to reduce the effect caused by Tg, resulting in reduced vinculin staining at the cell periphery (arrows). F-actin filaments were also visualized in the cell periphery (arrowheads). D: immunofluorescent staining demonstrating that low and high doses of human recombinant TGF-β1 induced an epithelial-to-mesenchymal transition (EMT) response in primary human renal proximal tubule epithelial cells, as shown by change in cell phenotype (F-actin, red) and increased expression of α-SMA (green). Bar = 50 μm. Tg = 200 nM; Sal = 30 μM; low-dose TGF-β1 = 1 ng/ml; high-dose TGF-β1 = 5 ng/ml.
collagen (18). Additionally, focal adhesion proteins, such as vinculin, are expressed in human renal tubular epithelial cells in response to CsA-induced EMT (44). Vinculin facilitates cell spreading and lamellipodia formation (24). ER stress appears to be an important feature of CKD (10, 12, 15), including as a response to proteinuria (11). Loss of the epithelial cell phenotype involves cytoskeletal rearrangement and changes in cell adhesion, both of which are effects mediated by TDAG51. Thus we tested whether ER stress induces EMT through TDAG51 expression.

We found that Tm, Tg, and CsA induced a UPR, indicative of ER stress, in hRPTECs. Tm prevents the synthesis of N-linked glycoproteins, Tg blocks the SERCA pump, and CsA inhibits calcineurin, a Ca\(^{2+}\)/H\(^{+}\)-activated phosphatase; each of these different actions causes ER stress. However, only Tg and CsA elicited an increase in the expression of TDAG51, a protein previously shown to be induced by Ca\(^{2+}\)/H\(^{+}\) disequilibrium-mediated ER stress (14, 30). This suggests a Ca\(^{2+}\)/H\(^{+}\)-mediated mechanism, as treatment with Tg and CsA (but not Tm) resulted in increased intracellular Ca\(^{2+}\)/H\(^{+}\). Additionally, in cells cotreated with Tg and the Ca\(^{2+}\)/H\(^{+}\) chelator BAPTA-AM, BAPTA-AM prevented ER stress, as shown by the inhibition of GRP78 upregulation and TDAG51 overexpression. Taken together, these data indicate that Ca\(^{2+}\)/H\(^{+}\)-mediated ER stress...
results in TDAG51 overexpression in HK-2 cells. Tg-induced TDAG51 expression was associated with hRPTEC shape change, indicative of EMT, whereas Tm did not induce TDAG51 expression or result in shape change. Treatment with the ER stress inhibitor Sal reduced TDAG51 expression at 18 h in nonstressed cells and inhibited Tg-induced TDAG51 expression and EMT at 48 h. It should be noted, however, that Sal did not inhibit Tg-induced TDAG51 expression at 18 h. This effect may be related to the ability of Sal to increase GRP78 expression at 18 h and the action of GRP78 as a Ca\textsuperscript{2+}-binding ER luminal protein. We have demonstrated that TDAG51 expression is dependent on ER Ca\textsuperscript{2+} disruption, as shown by our BAPTA-AM results. Furthermore, GRP78 overexpression has been shown to retain ER Ca\textsuperscript{2+} (16). This may have caused Sal to act much like BAPTA-AM to buffer the ER Ca\textsuperscript{2+} disruption induced by Tg and thus TDAG51 overexpression. However, this effect shows time dependence due to the time required for Sal to induce new GRP78 protein synthesis.

Tg also caused disruption of epithelial junctions, shown by β-catenin cytoplasmic-to-nuclear translocation. Transient overexpression of TDAG51 directly caused hRPTEC shape change and β-catenin translocation in HK-2 cells. TDAG51 contains a PHLD, which is found in proteins that are targeted to the membrane and interact with the cytoskeleton (27). This is the structural motif in the TDAG51 protein that may cause hRPTEC shape change and β-catenin signaling. To test this hypothesis, we constructed an expression plasmid containing the PHLD of TDAG51 in a GFP fusion protein to track its subcellular localization and its effect on hRPTEC shape change. It appears that the PHLD domain of TDAG51 interacts with the cytoskeleton and induces hRPTEC shape change. Thus this seems the region in the TDAG51 protein that induces cell shape change and initiates the canonical Wnt/β-catenin signaling pathway.

One important form of kidney disease associated with tubulointerstitial fibrosis is CsA-induced nephropathy. CsA-induced nephropathy consists of an early and a late phase. The acute phase is mediated by increased levels of endothelin and angiotensin II and reduced nitric oxide bioavailability (19). This leads to increased vascular resistance and decreased renal blood flow and glomerular filtration rate. The chronic phase is mediated by TGF-β, platelet-derived growth factor, fibroblast growth factor, and TNF-α (19) and can be diagnosed by typical histological findings such as striped fibrosis (20, 46) and arteriolar hyalnosis (46).

Previously, it has been demonstrated that CsA causes ER stress while concurrently inducing phenotypic changes in human renal epithelial cells, indicative of EMT (44). Other ER stress inducers, such as Tg, were shown to produce human renal epithelial cell shape change (12, 44). Additionally, the eIF2α dephosphorylation inhibitor Sal inhibited the CsA-induced epithelial cell shape change in vitro and tubulointerstitial fibrosis associated with CsA-induced nephrotoxicity in rats in vivo (44). We found a similar effect of Sal on epithelial cell shape change in vitro and also demonstrate that Sal alone increases GRP78 expression. This is a potential mechanism for the action of Sal, since GRP78 overexpression can inhibit UPR activation by binding to the ER transmembrane UPR transducers Ire1 and perk and preventing their activation (5). Furthermore, GRP78 is an ER luminal Ca\textsuperscript{2+} binding protein (12), whose overexpression has been found to inhibit ER Ca\textsuperscript{2+} disequilibrium (14, 16). Thus Sal may have acted to inhibit TDAG51 expression through its effects on GRP78 expression.

Other studies, using the HK-2 cell model of hRPTECs, have also shown that CsA induces an EMT response characterized by F-actin stress fiber formation, β-catenin cytoplasmic and nuclear translocation, and extracellular matrix component production (41). These effects appear to have been mediated by TGF-β1, as they were inhibited by an anti-TGF-β1-neutralizing antibody (41). CsA-treated renal epithelial cells showed the induction of α-SMA mRNA and protein (41, 51). This induction was associated with an increase in TGF-β1 release, inhibited by the protein kinase C-β inhibitor hspidin (51). Protein kinase C-β requires cytosolic Ca\textsuperscript{2+} upregulation for activation, which we have demonstrated is induced by CsA (Fig. 5A). One previous report has indicated that ER stress leads to an EMT-like phenotype in epithelial cells. In PC13 thyroid cells, this EMT response was accompanied by changes in the epithelial monolayer, downregulation of E-cadherin, and upregulation of vimentin, α-SMA, type I collagen, and SNAI1/SIP1 (52). However, it is unknown whether ER stress induction in this cell type results from TDAG51 expression.

ER stress-induced EMT appears to be mediated by changes in cytosolic Ca\textsuperscript{2+}, since both CsA and Tg resulted in these changes whereas the N-linked glycosylation inhibitor Tm showed no changes in cytosolic Ca\textsuperscript{2+} and did not induce EMT. These data are in agreement with previous reports that showed Tm did not change cytosolic Ca\textsuperscript{2+} (2, 13). It has been demonstrated that contact disassembly of proximal tubular cells mediated by Ca\textsuperscript{2+} removal primes cells for TGF-β1-induced EMT (39). These experiments suggested a “two-hit” mechanism where contact disassembly primes the cells for TGF-β1-induced EMT via β-catenin signaling (39). Furthermore, the Wnt/β-catenin signaling pathway is important in tubular EMT (37). Using the unilateral ureter obstruction model of interstitial renal fibrosis, it has been demonstrated that β-catenin target genes, including Twist, lymphoid enhancer-binding factor 1, and fibronectin, were induced, correlating with renal β-catenin abundance (28). Wnt signaling induces the expression of Snail, a transcription factor that is sufficient to initiate EMT through the downregulation of E-cadherin (7, 53). β-Catenin, when transported to the nucleus, interacts with lymphoid enhancer-binding factor-T cell factor to induce gene transcription required for EMT (26). This shows the importance of β-catenin signaling through the canonical Wnt pathway in renal interstitial fibrosis. A number of studies have shown an increase in TDAG51 (PHLD1) expression when Wnt signaling is activated (23, 50); however, it has been demonstrated that TDAG51 is not a direct downstream target of canonical Wnt signaling (48). Our results suggest that increased TDAG51 expression activates Wnt signaling, priming the cells for an EMT response. We have shown that ER stress inducers that alter cytosolic [Ca\textsuperscript{2+}] cause TDAG51 overexpression, disrupt epithelial junctions, and cause β-catenin cytoplasmic-to-nuclear translocation, leading to proximal tubular cell EMT. This appears to be the first hit in our ER stress-induced model of EMT, sensitizing the cells for the action of TGF-β1. Our results demonstrate that TDAG51 is required for the progression of peritoneal fibrosis in vivo, indicating it plays an important role in this process. The model utilized an adenovirus-mediated TGF-β1-induced mesothelial cell EMT and peritoneal fibrosis model (38), allowed the direct
testing of our hypothesis since it does not rely on a nonspecific induction of EMT through inflammation or injury. This model is dependent solely on the direct action of TGF-β1 to induce EMT. TDAG51 is a potential molecular mediator of ER stress-induced Wnt/β-catenin signaling, since overexpression of the TDAG51 protein causes proximal tubular cell shape change, leading to junctional breakage and β-catenin cytoplasmic and nuclear translocation, and TDAG51 deficiency prevents the development of TGF-β1-induced peritoneal fibrosis.

In proximal tubular epithelial cells, a decrease in ER Ca^{2+}, which leads to the disruption of ER Ca^{2+} homeostasis, causes ER stress. Unresolved Ca^{2+} dysregulation-mediated ER stress appears to lead to an increase in TDAG51 protein expression. Increased TDAG51 expression causes cytoskeletal reorganization, characterized by rounding up or elongation of the cells. Cytoskeletal reorganization results in loss of cell-cell adhesions, thereby causing disruption of the monolayer. After loss of epithelial cell adhesion and monolayer disruption, β-catenin is translocated to the nucleus, where it upregulates transcription of EMT-related genes. This is one effector in the two-hit model, the other being TGF-β1 signaling. Together, β-catenin nuclear translocation and TGF-β1 signaling lead to the final stage of EMT: an increase in EMT-related gene expression and protein synthesis, including α-SMA and vinculin, resulting in a mesenchymal cell (Fig. 9).

In summary, our data indicate that TDAG51 is a novel mediator of EMT in hRPTECs. As stated, TDAG51 expression is associated with ER stress caused by Ca^{2+}-mediated mechanisms. We demonstrated that Ca^{2+} dysregulation, as induced by select ER stressors, upregulates TDAG51 expression, although this upregulation is not seen in ER stress caused via other mechanisms. It has previously been shown that intracellular Ca^{2+} fluctuations can cause EMT (22); however, Ca^{2+}-mediated ER stress-induced TDAG51 expression leading to EMT is a novel finding. Our results indicate that TDAG51 leads to cytoskeletal rearrangement, priming the cells for TGF-β1-induced EMT, and that TDAG51 is essential for the development of TGF-β1-induced peritoneal fibrosis in vivo. The elucidation of this novel molecular mediator of hRPTEC EMT holds promise for the development of therapeutic compounds that can reduce the ER stress response and specifically downregulate the expression of TDAG51, preventing the progression of CKD through the inhibition of ER stress-induced renal interstitial fibrosis.

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