WT1-interacting protein and ZO-1 translocate into podocyte nuclei after puromycin aminonucleoside treatment

Maribel Rico,1 Amitava Mukherjee,2 Martha Konieczkowski,2 Leslie A. Bruggeman,2 R. Tyler Miller,1,2,3 Shenaz Khan,2 Jeffrey R. Schelling,2 and John R. Sedor1,2

Departments of 1Physiology and Biophysics and 2Medicine, School of Medicine, Case Western Reserve University, and 3Louis Stokes Veterans Administration Medical Center and Kidney Disease Research Center, Rammelkamp Center for Research and Education, MetroHealth System Campus, Cleveland, Ohio

Submitted 21 October 2004; accepted in final form 25 March 2005

Rico, Maribel, Amitava Mukherjee, Martha Konieczkowski, Leslie A. Bruggeman, R. Tyler Miller, Shenaz Khan, Jeffrey R. Schelling, and John R. Sedor. WT1-interacting protein and ZO-1 translocate into podocyte nuclei after puromycin aminonucleoside treatment. Am J Physiol Renal Physiol 289: F431–F441, 2005. First published March 29, 2005; doi:10.1152/ajprenal.00389.2004.—Podocyte differentiation is required for normal glomerular filtration barrier function and is regulated by the transcription factor WT1. We identified WT1-interacting protein (WTIP) and hypothesized that it functions as both a scaffold for slit diaphragm proteins and a corepressor of WT1 transcriptional activity by shuttling from cell-cell junctions to the nucleus after injury. Endogenous WTIP colocalizes with zonula occludens-1 (ZO-1) in cultured mouse podocyte adherens junctions. To model podocyte injury in vitro, we incubated differentiated podocytes with puromycin aminonucleoside (PAN; 100 μg/ml) for 24 h, which disassembled cell-cell contacts, rearranged actin cytoskeleton, and caused process retraction. Podocyte synaptic pododin expression diminished after PAN treatment, consistent with podocyte dedifferentiation in some human glomerular diseases. To assess podocyte function, we measured albumin flux across differentiated podocytes cultured on collagen-coated Transwell filters. Albumin transit across PAN-treated cells increased to levels observed with undifferentiated podocytes. Consistent with our hypothesis, WTIP, as well as ZO-1, translocated from podocyte adherens to nuclei in PAN-treated cells. Because WTIP is a transcriptional corepressor for WT1, we examined the effect of PAN on expression of retinoblastoma binding protein RbAp7 (also known as Rhap46), a WT1 target gene expressed in S-shaped bodies during nephrogenesis. RbAp7 expression in PAN-treated podocytes was reduced compared with untreated cells. In conclusion, WTIP translocates from cell-cell junctions to the nucleus in PAN-treated podocytes. We suggest that WTIP monitors slit diaphragm protein assembly and shuttles into the nucleus after podocyte injury, translating changes in slit diaphragm structure into altered gene expression and a less differentiated phenotype.

nuclerocyttoplasmic translocation; glomerulosclerosis; LIM domain; slit diaphragm; cell-cell contacts

The glomerular filtration barrier is composed of a highly fenestrated endothelium, the glomerular basement membrane, and the podocyte. After a tightly orchestrated differentiation program, podocytes develop foot processes and assemble a specialized adherens junction, the slit diaphragm, which mediates contact between adjacent cells (44). In proteinuric diseases, regardless of the etiology, podocytes undergo marked morphological change. The actin cytoskeleton rearranges into a mat below the plasma membrane opposed to the glomerular basement membrane, slit diaphragm structures are lost, and the podocyte assumes a cuboidal shape. At a molecular level in both human biopsies and experimental models, this stereotypical morphological response is associated with changes in cytoplasmic, plasma membrane, and nuclear podocyte differentiation marker expression (5, 6). In addition, podocytes in some glomerular diseases also revert to a less differentiated phenotype more characteristic of the developing, rather than the fully differentiated, glomerulus (5). Appropriate treatment can restore normal podocyte structure and filtration barrier function, suggesting that regulation of foot process architecture is plastic and dynamic. Given its unique microenvironment with exposure to hemodynamic forces and high ultrafiltrate flow, the podocyte must rapidly respond to changes in physical forces or soluble signals that occur with injury. We have hypothesized that slit diaphragm-associated proteins monitor the podocyte microenvironment and may trigger signaling cascades that alter the podocyte differentiation state (52).

Cell-cell junction molecules can transmit extracellular cues by shutting into the nucleus to regulate gene expression (9, 12, 14). Two podocyte molecules, WT1-interacting protein (WTIP) and zonula occludens-1 (ZO-1), are candidates for this important adaptive role. The Wilms' tumor gene 1 (WT1) is a zinc finger transcription factor whose function is required for normal nephrogenesis and podocyte differentiation (18, 28). Investigators in our laboratory (52) previously reported that a WT1 coregulator, the LIM domain protein WTIP, localized to nascent cell-cell contacts and interacted with the cell junction scaffold proteins Mena and CD2AP. A truncated WTIP, containing only its LIM domains, colocalized with WT1 in nuclei, coprecipitated with WT1, and inhibited WT1-dependent transcriptional activation of the amphiuregulin promoter. Although full-length WTIP was excluded from cell nuclei, it accumulated in the nucleus and coprecipitated with WT1 after the addition of an inhibitor of Crm1-mediated nuclear export, leptomycin B. These data suggest that WTIP may function to transfer information from the slit diaphragm microenvironment into the nucleus. After wounding, the MAGUK (membrane-associated guanylate kinase) family member ZO-1 translocates into epithelial cell nuclei from tight junctions (15) with its cognate Y-box transcription factor ZONAB (ZO-1-associated nucleic acid binding protein) to regulate proliferation and to promote epithelial cell dedifferentiation (4, 16, 29). An analogous ZO-1 function has not been described in the podocyte.
where ZO-1 is normally colocalized with P-cadherin and Nephrin (20). In this study, we test the hypothesis that WTIP and ZO-1, components of podocyte cell-cell junctions, translocate into the nucleus after injury. As an in vitro model of damage, podocytes were treated with puromycin aminonucleoside (PAN), and injury was assessed by morphological characteristics and functional response by an albumin diffusion assay. Both WTIP and ZO-1 translocate to the nucleus after PAN treatment, a finding associated with deregulated expression of a WT1 target gene, retinoblastoma binding protein Rbpb7 (also known as RbAp46).

MATERIALS AND METHODS

Cell lines. The conditionally immortalized podocyte cell line MPC was a generous gift of Dr. Peter Mundel (Albert Einstein Medical College, New York, NY) (35). Cells were maintained in RPMI 1640 medium (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. As described previously (35, 52), podocytes were cultivated at 5% CO₂ and 37°C (permissive conditions), and culture medium was supplemented with 10 U/ml mouse recombinant γ-interferon (Sigma Chemical, St. Louis, MO) to enhance expression of the SV40 Large T-antigen. To induce differentiation, we maintained podocytes on type I collagen at 5% CO₂ and 37°C without γ-interferon (nonpermissive conditions) for at least 10 days. A detailed characterization of these cells was published previously (35). All batches of podocytes (between passages 10 and 25) used in these studies expressed the podocyte-selective transcription factor WT1. Before experiments, expression of the podocyte differentiation marker synaptopodin was confirmed by immunofluorescence analysis in a parallel well of podocytes. Madin-Darby canine kidney (MDCK) cells (clone 8) were a generous gift of Dr. Bingcheng Wang (Case School of Medicine) and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin at 5% CO₂ and 37°C. COS-7 cells (American Type Culture Collection, Manassas, VA) were cultured at 5% CO₂ and 37°C in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Phylogenetic analysis of zyxin family LIM domain proteins. The ClustalW (slow, accurate) algorithm of the MegAlign module of Lasergene (DNASTAR) was used to determine unrooted phylogenetic relationships between LIM domain family proteins. For generation of the multiple alignment analysis, the gap penalty was 10.0, the gap length penalty was 0.2, and the Gonnet protein weight matrix was used. The length of each pair of branches represents the distance between sequence pairs, and the units at the bottom of the tree indicate the number of substitution events. Protein sequence sources are provided (see Fig. 1).

Generation of expression vectors and transient transfections. Expression vectors for full-length WTIP, WTIP with deletion of the LIM domain region of WTIP (NΔWTIP, amino acids 186–430). The crude antiserum was purified against GST-NΔWTIP after preclearing on a GST affinity column. To eliminate residual anti-GST antibodies, we further affinity-purified the antibody using a 6× His-WTIP fusion protein conjugated to cyanogen bromide (CNBr)-activated beads (Amersham Biosciences, Piscataway, NJ). Antibody was eluted from the CNBr column by using 0.1 M glycine (pH 2.4), immediately followed by neutralization with 1 M Tris base (pH 9) and subsequent dialysis against PBS. In some immunoblots, we assessed anti-WTIP specificity by incubating the affinity-purified anti-WTIP antibody with the His-WTIP fusion protein.

Immunofluorescence microscopy. Differentiated podocytes grown on collagen-coated slides were analyzed using immunofluorescence, as described previously (52). Briefly, cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then permeabilized with PBS containing 0.2% Triton X-100 and 2% BSA (permeabilization buffer) for an additional 30 min at room temperature. After blocking, cells were incubated with primary antibodies at 1:50 dilution in permeabilization buffer for 1 h at 37°C or overnight at 4°C. Primary antibodies included mouse monoclonal anti-myc (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-P-cadherin (Zymed Laboratories, San Francisco, CA), rat monoclonal anti-ZO-1 (Chemicon International, Temecula, CA), mouse monoclonal anti-synaptopodin (Maine Biotechnology, Portland, MA), mouse monoclonal anti-TFIIH, both rabbit polyclonal and mouse monoclonal anti-WT1 (Santa Cruz Biotechnology), and anti-WTIP (0.2 mg/ml, see WTIP antibody generation). Antibody binding was detected with secondary anti-rabbit (Molecular Probes, Eugene, OR), anti-mouse (Molecular Probes), or anti-rat (Jackson ImmunoResearch Laboratories, West Grove, PA) antibodies conjugated with either FITC or rhodamine at a concentration of 1:200. Antibody staining was visualized using a Nikon epifluorescence E600 microscope, and photographs were taken with a SPOT digital system camera model 2.3.0. Confocal images were obtained with a Leica TCS SP2 confocal system (Leica Microsystems, Wetzlar, Germany). Digital images were processed and grouped using Adobe Photoshop version 7.0.1 (Adobe Systems, San Jose, CA).

Podocyte BSA filter assay. Transwell-Col PTFE filters (3-μm pore; Corning, New York, NY) were seeded with 5 × 10⁵ podocytes/filter and cultured under differentiating or permissive conditions. Cell density after plating was monitored using fluorescence microscopy of parallel filters stained with Cell Tracker (Molecular Probes). After 10–18 days, podocyte differentiation was confirmed by synaptopodin expression using immunofluorescence analysis of cells on parallel filters. Upon confirmation of differentiation, cells were washed twice with PBS supplemented with 1 mM MgCl₂ and 1 mM CaCl₂ to preserve the cadherin-based junctions. The upper compartment was then refilled with 0.5 ml RPMI 1640 and the lower compartment with 1 ml BSA medium (RPMI 1640 supplemented with 40 mg/ml BSA) and incubated for 2 h or as indicated at 37°C. Total protein concentration in the upper compartment was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Coomassie-blue-stained gels demonstrated that virtually all of the protein in the upper chamber was derived from the DMEM containing BSA and that the contribution of cellular proteins was negligible (not shown). In some experiments, podocytes were treated with PAN.

Induction of podocyte injury by PAN treatment. Podocytes differentiated for 10–18 days on coverslips or Transwell filters were incubated with 100 μg/ml PAN (Sigma). After 24 h at 37°C, cells were analyzed using immunofluorescence microscopy or the BSA filter assay described above.

Apoptosis assays. Podocytes were cultured to confluence, treated with PAN or vehicle as described above, fixed in paraformaldehyde (4%, 30 min, room temperature) and mounted in 4–6-diamidino-2-phenylindole (DAPI)-containing medium. Cells were assayed for apoptosis by examiners blinded to experimental conditions; apoptosis...
was indicated by DAPI-labeled pyknotic nuclei or apoptotic bodies from >300 cells, as previously described (23).

Quantification of nuclear fluorescence. Differentiated podocytes were fixed on coverslips and stained with the nuclear dye TOPO (Molecular Probes) and anti-WTIP, anti-ZO-1, or anti-TIFIIß before and after treatment with PAN. Stacks of nonoverlapping 3-µm-thick sections of 20–50 cells were generated and projected into a single confocal image with the Leica TCS SP2 confocal system. The fluorescence intensity of the nuclear area, as determined by a region of interest that corresponded to the TOPO nuclear dye, was quantified for the fluorescence intensity of the nuclear area, as determined by a region of interest that corresponded to the TOPO nuclear dye, was quantified for localization of endogenous WTIP.

RESULTS

WTIP phylogeny. Sequence alignments suggest that WTIP is a member of the zyxin family of LIM domain-containing proteins (Ref. 52 and Fig. 1A). As indicated by analysis of nucleotide substitutions using an unrooted method (ClustalW, see MATERIALS AND METHODS), WTIP appears to belong to a zyxin subfamily, which is related to the Drosophila zyxin paralog CG11063 (45), and is most similar to ajuba, which is contained in cadherin-based cell-cell contacts and translocates into the nucleus to regulate mitotic commitment. WTIP LIM domains share significant sequence homology with other zyxin family paralogs (52), although ajuba and LIMD1 mRNA transcripts are not detected by RT-PCR in isolated glomeruli (Padiyar A and Sedor JR, unpublished data).

WTIP antibody specificity. We generated a rabbit anti-WTIP antibody against a GST-N∆WTIP fusion protein. To test specificity of the affinity-purified WTIP antibody, we immunoblotted lysates from COS-7 cells transiently expressing either the immunogen (myc epitope-tagged N∆WTIP), a myc epitope-tagged full-length WTIP, FLAG epitope-tagged ajuba, or myc epitope-tagged zyxin (Fig. 1B). The WTIP antibody detected a single band of the appropriate size for N∆WTIP and full-length WTIP and did not cross-react with ajuba or zyxin, other LIM domain proteins that also translocate from sites of cell adhesion into the cell nucleus (22, 37, 53). No proteins were recognized in control COS-7 cells transfected with empty vector (pCMV-Tag). Western blot analysis of podocyte cell lysates revealed that both differentiated and undifferentiated podocytes expressed equivalent amounts of WTIP (Fig. 1C), suggesting that WTIP function would be regulated by its subcellular localization rather than by differences in abundance. Antibody specificity was verified by competition of anti-WTIP binding with the 6X-His-WTIP fusion protein (Fig. 1C). The same single band was detected in rat glomerular lysates (not shown). To further characterize the antibody for immunocytochemistry, we transiently transfected COS-7 cells with myc-WTIP. Both the confocal image (Fig. 1D, middle) and quantification of signal intensity (Fig. 1D, right) demonstrate complete overlap of the signal from affinity-purified anti-WTIP (green channel) and anti-myc antibodies (red channel). Untransfected COS-7 cells were also fixed and incubated with both commercial anti-myc and affinity-purified anti-WTIP antibodies. A low-power magnification shows only DAPI-stained nuclei (Fig. 1D, left). No signal could be detected from either channel in untransfected cells, indicating that the affinity-purified anti-WTIP antibody specifically recognizes WTIP and does not cross-react with other cellular proteins.

Localization of endogenous WTIP. Because the anti-WTIP antibody detected endogenous podocyte WTIP, as indicated by...
Western blotting (Fig. 1C), we next localized WTIP in cultured podocytes. In undifferentiated podocytes, WTIP was detected in the nucleus as well as at the plasma membranes (Fig. 1E). In differentiated podocytes, nuclear localization of WTIP was reduced and WTIP localization was relatively more prominent in cytoplasm and plasma membrane (Fig. 1E). Significant perinuclear WTIP expression was observed in both differentiated and undifferentiated podocytes.
Colocalization of ZO-1 and WTIP at podocyte adherens junctions. Because endogenous WTIP is localized in the cell periphery and we had hypothesized that it would be localized to the specialized cell junction of the differentiated podocytes (52), we next characterized the cell-cell contacts of cultured differentiated podocytes. Differentiated podocytes form cell-cell contacts, which can be visualized using Cell Tracker (Fig. 2A), contain both ZO-1 and a cadherin identified using an anti-pan-cadherin antibody. As previously reported by Reiser et al. (44), P-cadherin is the major cadherin isoform expressed in cultured, immortalized podocytes. E-cadherin does not localize at cell-cell junctions but is distributed diffusely throughout cytoplasm (44). Use of a specific anti-P-cadherin antibody confirmed that the cadherin expressed at cell-cell contacts of cultured podocytes was P-cadherin (not shown). High-resolution immunofluorescence micrographs revealed that P-cadherin and ZO-1 are juxtaposed at most cell-cell junctions in differentiated podocytes (Fig. 2B). ZO-1 localizes to the cytoplasmic side of filtration slits, where it has been shown to colocalize with P-cadherin (21) and to interact with Neph1 (20), a slit diaphragm protein of the immunoglobulin superfamily with a PDZ binding site.

As reported, WTIP contains a PDZ binding domain, suggesting that it may associate with ZO-1 at sites of cell-cell contact. To test this hypothesis, we seeded cells on collagen-coated permeable Transwell filters to maximize epithelial polarity. Cells were differentiated for 10–18 days until synaptopodin was expressed, and they were then assessed for colocalization of WTIP with ZO-1 using confocal microscopy. WTIP and ZO-1 colocalized precisely (Fig. 2C), suggesting that ZO-1 and WTIP may be components of a multiprotein complex of slit diaphragm proteins. MDCK cells abundantly express ZO-1, and immunoprecipitation assays performed using a 6× His-WTIP fusion protein confirmed that WTIP physically associated with ZO-1 (Fig. 2D).

Podocyte injury with PAN causes cytoskeletal reorganization and loss of synaptopodin expression. In vivo, podocyte differentiation state is critical for establishment and maintenance of the slit diaphragm, which functions to exclude proteins from the ultrafiltrate. To have a functional correlate to assess podocyte injury, we first developed a method to measure vectorial BSA diffusion across podocytes, maintained on permeable Transwell supports, as an assay of filtration barrier function. With this system, confluent but undifferentiated podocytes permitted much greater albumin flux over time compared with confluent and differentiated podocytes (Fig. 2E, left). At 2 h, albumin diffusion across filters on which undifferentiated podocytes were cultured was not different compared with that across Transwell filters on which no cells were cultured (Fig. 2E, right). In contrast, albumin transit was significantly lower across Transwell filters that contained differentiated podocytes. Although this in vitro assay does not fully model the complexity of the podocyte slit diaphragm, it does provide information about barrier function of cell-cell contacts. Consistent with this premise, MDCK cells, which develop tight junctions, do not permit significant albumin diffusion, as expected (not shown).

We next treated cultured podocytes with PAN, which causes podocyte injury and proteinuria in animal models (25, 54), and determined its effects on podocyte morphology, cytoskeleton, and cell-cell junction assembly. We detected podocyte morphology changes with 100 µg/ml PAN, which has been used before to stimulate injury in cultured mouse podocytes (13, 47). Figure 3A shows that differentiated podocytes robustly expressed filamentous actin, whereas podocytes treated with PAN displayed remarkable rearrangement of actin filaments. These data are consistent with a prior report that PAN promotes cytoskeletal changes in podocytes (47). Upon closer examination of adherens junctions, we found that actin filaments were highly organized in control, differentiated podocytes (Fig. 3, A and B, top left). Conversely, after treatment with PAN, actin filaments were distributed in a less organized actin mat (Fig. 3, A and B, bottom left), consistent with observations of podocytes in situ from animal models of glomerular injury (51). Tubulin arrangement paralleled actin patterns in control cells but was not as dramatically affected by treatment with PAN (Fig. 3B, right). Withdrawal of PAN permitted podocytes to revert to a normal morphology (not shown), suggesting that PAN did not stimulate irreversible cell death after the 24-h treatment. Moreover, morphological analysis of DAPI-stained nuclei revealed no significant difference in frequency of apoptosis between PAN-treated (0.3%) and untreated (0%) podocytes [n > 300 cells for each treatment (not shown)].

Differentiated podocytes assembled extensive P-cadherin-based adhesive contacts between cells (Fig. 3C, left), which disassembled after PAN treatment and permitted cells to retract from one another (Fig. 3C, right). Consistent with this mor-
Fig. 2. A: confocal microscopy images showing 2 podocytes differentiated for 15 days and stained with Cell Tracker to show cell morphology (top), anti-ZO-1 antibody (middle), and anti-cadherin antibody (bottom), which recognizes E- and P-cadherin. Whole cells are shown at left (bar, 50 μm) and magnified images of cell-cell contacts at right (bar, 10 μm) of each panel. B: confocal zoom image of cell-cell contact between 2 podocytes differentiated for 15 days demonstrating that ZO-1 and P-cadherin are in close spatial association but do not necessarily overlap. Bar, 10 μm. C: podocytes were seeded on collagen-coated Transwell filters and then analyzed by confocal microscopy for localization of WTIP (green, left) and ZO-1 (red, middle). Merged image (right) demonstrates close association between both molecules (bar, 50 μm). D: His-WTIP and endogenous Madin-Darby canine kidney cell ZO-1 coprecipitate (see MATERIALS AND METHODS). IP, immunoprecipitate; WB, Western blot. E: representative graph of time course of BSA diffusion across collagen-coated Transwell filters alone or seeded with podocytes (f, collagen-coated filter only; undiff, collagen-coated filter seeded with 5 × 10^3 undifferentiated podocytes; dif, collagen-coated filter seeded with 5 × 10^3 podocytes differentiated for 8–12 days). F: after 2 h, BSA diffusion was quantified in Transwell assays by using collagen-coated filters (F), collagen-coated filters seeded with 5 × 10^3 undifferentiated podocytes (UND), collagens filter seeded with 5 × 10^3 podocytes differentiated for 8–12 days. Data are presented as means ± SE; n = 5 independent experiments. *P < 0.01 (by Kruskal-Wallis ANOVA followed by individual comparisons of medians using Dunn’s method).
that is disrupted by treatment with the nuclear export inhibitor leptomycin B. Consistent with data from Figs. 1 and 2, WTIP localized in perinuclear regions and at cell-cell contacts in differentiated podocytes. However, upon PAN treatment, WTIP moved to the nucleus (Fig. 4C). Consistent with previous reports (33), nuclear translocation of WTIP was confirmed by Western blot analysis of nuclear lysates that showed increased levels of endogenous WT1 mRNA (Fig. 4D). Immunochemistry and confocal microscopy revealed that nuclear WTIP content increased significantly after PAN treatment, as did ZO-1. In contrast, nuclear content of the general transcription factor TFIIHβ was unchanged with PAN treatment (Fig. 4B). Immunohistochemistry and confocal microscopy revealed that nuclear WTIP content increased significantly after PAN treatment, as did ZO-1. In contrast, nuclear content of the general transcription factor TFIIHβ was unchanged with PAN treatment (Fig. 4B). Immunohistochemistry and confocal microscopy revealed that nuclear WTIP content increased significantly after PAN treatment, as did ZO-1.

Investigators in our laboratory (52) using an amphieregin promoter-luciferase reporter assay in WTIP-expressing HEK293 cells showed that WTIP inhibited WT1-dependent transcription. In the current studies, we examined the effect of PAN on expression of the retinoic acid binding protein Rbbp7 (also called RbAp46), an endogenous kidney protein
expressed during nephrogenesis and a known WT1 target gene (17). Translocation of WTIP in PAN-treated cells was associated with reduced expression of Rbbp7 protein (Fig. 4D), a finding consistent with our previous work but that does not exclude WTIP effects on posttranscriptional regulatory mechanisms. The data suggest that WTIP may play a pathophysiological role in the podocyte by translocating from cell junctions to the nucleus, where it regulates WT1-dependent podocyte differentiation.

**DISCUSSION**

Podocyte differentiation is critical for the filtration function of the glomerulus (44). The tumor suppressor gene WT1 regulates podocyte differentiation (34, 43) and is mutated in syndromes of familial glomerulosclerosis (5, 6, 18). We have demonstrated that WT1 single nucleotide polymorphisms are associated with focal segmental glomerulosclerosis in African Americans, suggesting that dysfunction of WT1 pathways may contribute to more common causes of nephropathy (39). We have identified WTIP and hypothesized that it functions as both a scaffold for slit diaphragm proteins and a corepressor of WT1 transcriptional activity by shuttling from cell-cell adhesions to the nucleus after injury (52). Consistent with our hypothesis, WTIP as well as ZO-1 translocated from podocyte adherens junctions to nuclei in PAN-treated cells, findings associated with loss of the podocyte differentiation marker synaptopodin and increased albumin transit across podocytes cultured on Transwell filters. Taken together, data in this study and our previously published data suggest that WTIP monitors podocyte cell-cell junction assembly and shuttles into the nucleus after podocyte injury, translating changes in junctional structure into altered gene expression and a less differentiated phenotype.

In vivo, podocyte differentiation is required for normal filtration barrier function, and podocyte injury results in proteinuria (5, 6). Differentiated, cultured podocytes develop cell-cell junctions that are modified adherens junctions, containing P-cadherin and ZO-1 (44). WTIP colocalizes and physically interacts with ZO-1 at these cell junctions. PAN, an agent that causes proteinuria in animal models (31), has been proposed to cause changes in podocyte morphology by the same mechanisms fibroblast growth factor-2 (48). In our studies, PAN caused podocyte actin filaments at cell-cell contacts to reorganize into a subcortical ring of F-actin, similar to the diffuse
cytoplasmic sheet along the glomerular basement membrane described in experimental glomerulopathies (51). We used an albumin diffusion assay as a functional correlate to corroborate our immunocytochemical findings in vehicle- and PAN-treated podocytes. PAN-treated, differentiated podocytes permitted greater albumin diffusion compared with untreated, differenti-
ated podocytes. Total protein levels in upper compartments from filters containing PAN-treated, differentiated podocytes were similar to those measured in filters with undifferentiated podocytes and significantly greater than those in filters seeded with differentiated but untreated podocytes. Of course, this in vitro assay incompletely models the barrier of slit diaphragm, because cultured podocytes only express some of the protein components (35, 44). We believe that loss of mature cell-cell contacts after PAN treatment is the proximate cause of increased albumin diffusion, because undifferentiated but con-
fluent podocytes without mature cell-cell contacts permit albumin diffusion similar to that observed across filters without cells. Coupled with the immunocytochemical changes that demonstrate disassembly of cadherin-based cell-cell contacts and nuclear translocation of both WTIP and ZO-1 with PAN treatment, the data suggest that injured podocytes use these molecules to signal changes in the nucleus concomitant with albumin leak. Of course, these data do not directly demonstrate that WTIP is necessary for either cell-cell contact assembly in vitro or filtration barrier function in vivo. Further experiments are needed to demonstrate the functional importance of WTIP and its translocation into nuclei after podocyte injury, experiments that are ongoing in our laboratory.

The correct temporal and spatial expression of the Wilms’ tumor suppressor gene (WT1) is critical for the induction and maintenance of a differentiated podocyte phenotype. Although persistent expression of WT1 protein in podocyte nuclei sug-
gests that podocyte differentiation requires ongoing transcription of WT1-dependent genes, none of the previously known WT1 protein partners appear to regulate podocyte WT1. An-
other WT1-interacting protein (WTAP) was identified through yeast two-hybrid screening and localizes in nuclear splices-
somes, but its function in kidney is unknown (28). Given the increasing number of molecules known to shuttle between cytoplasm and nucleus to regulate gene expression, we speculated that specific molecules may transmit information from the filtration barrier to the nucleus to allow the podocyte to regulate its differentiation state in response to environmental signals. A yeast two-hybrid assay screen identified WTIP as a candidate molecule for this function (52).

WTIP LIM domains are similar to LIM domains in zyxin, the prototype protein for a family of molecules that localize to cell adhesion junctions (37, 38). The LIM domain is a con-
served zinc finger protein-interaction motif, and proteins contain-
ing LIM domains mediate cytoskeletal organization, cell lineage specification, organogenesis, and oncogenesis (1, 2, 10, 24, 27). A number of LIM domain-containing zyxin paralogs shuttle from sites of cell-cell contacts to the nucleus and can regulate cell differentiation state (22, 41, 42, 50, 53). Nuclear accumulation of ajuba, which is phylogenetically the closest of these junctional complexes to influence cellular proliferation and expression of the EGF receptor Erb2b and E-cadherin (3, 4, 15). In this study, we demonstrated that PAN-induced podocyte injury stimulates ZO-1 shuttling to the nucleus. These data suggest that cell junctions, including the highly specialized podocyte adherens junction, contain proteins that transmit information to the nucleus, regulate cell responses to extracellular stresses, and function as molecular switching stations. We suggest that molecules such as WTIP and ZO-1, either independently or in concert, signal the podocyte to simplify its differentiation state in response to environmental stress, a potentially adaptive response that would promote cell survival until the resolution of the injury. The response of human and experimental glomerular diseases to therapy sug-
gests that podocytes can reestablish their differentiated pheno-
type. Altered subcellular compartmentalization of signaling molecules has been implicated in human disease pathogenesis. IGF-I-activated Akt phosphorylated p27Kip1 in human breast cancers and prevented its import into the nucleus (30), showing that an extracellular stimulus can change cell phenotype by redirecting an intracellular signaling molecule to a different subcellular location. Although the in vitro data in this study support our hypothesis, translocation of junctional molecules like WTIP and ZO-1 from sites of cell-cell contact into the nucleus needs to be demonstrated in animal models of podo-
cyte injury.

When WTIP is retained in the nucleus, prior data suggest that it functions as a transcriptional repressor of WT1 activity (52). In the current study, we determined that translocation of WTIP into nuclei of WT1-treated podocytes was associated with decreased expression of the WT1 target gene Rb atp7 (also known as RbAp46). Although these data are consistent with WTIP function as a transcriptional repressor, our studies do not exclude regulation at the level of RNA processing or transla-
tion. WT1 has been shown to bind to RNA and associate with proteins comprising the splicing machinery (11). WT1 also can shuttle into cytoplasm, where it is associated with actively processing polysomes, suggesting a role in regulation of trans-
lation (36). Further experiments are needed to specifically determine whether WTIP interferes with both WT-1-dependent transcriptional and posttranscriptional regulation of gene ex-
pression.

Rb atp7 is a retinoblastoma (Rb)-associated protein that was discovered to be upregulated by WT1 with suppression subtractive hybridization PCR and coexpressed with WT1 in E13 developing kidney (17). Reduction in Rb atp7 protein expres-
sion might have important consequences for podocyte develop-
ment, because Rb atp7 is a member of the NuRD and Sin3 transcriptional corepressor complexes and has been shown to repress the c-Fos transactivation domain (55). In addition, its Caenorhabditis elegans orthologs have been shown to inhibit Ras-dependent signaling during worm development (56). Al-
though podocytes do not express Ras isoforms in normal glomeruli, expression of some isoforms is observed in biopsies from a variety of glomerular diseases (26). Downregulation of Rb atp7 promotes Ras signaling either directly, by increasing Ras expression, or indirectly, by disinhibiting Ras-dependent signaling pathways. The evidence presented in this study for
WTIP-dependent repression of WT1 transcriptional activity is indirect. Chromatin immunoprecipitation assays are necessary to prove that WTIP functions as a transcriptional repressor but require that actual WT1 target genes are known. WT1 has been shown to both transcriptionally activate and repress a number of target genes in vitro with promoter-reporter assays, but none of these target genes have been confirmed in vivo in either cells or animals.

Based on the data in this report, our model of WTIP function in the podocyte continues to evolve. In normal glomeruli WTIP is part of a multiprotein complex in the podocyte foot process at cell-cell contacts. After injury, WTIP translocates into the nucleus, where it represses WT1-dependent gene expression and dysregulates podocyte phenotype. Loss of WTIP from its location as cell-cell junctions may promote concomitant redistribution of slit diaphragm proteins, which leads to filtration barrier dysfunction. We suggest that WTIP regulates podocyte phenotype by monitoring slit diaphragm protein integrity, ultimately translating changes in slit diaphragm structure or function into altered expression of podocyte differentiation genes.

GRANTS
Support for this project was provided by National Institute of Diabetes and Digestive and Kidney Diseases. Grants DK-07470, F50 DK-054178, and DK-064719 and by the Kidney Foundation of Ohio.

REFERENCES


