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Cell Injury, Repair, Aging and Apoptosis

Podocyte Injury and Albuminuria in Mice with Podocyte-Specific Overexpression of the Ste20-Like Kinase, SLK

Andrey V. Cybulsky,* Tomoko Takano,* Joan Papillon,* Julie Guillemette,* Andrew M. Herzenberg,† and Chris R. J. Kennedy‡

From the Department of Medicine, McGill University Health Centre, McGill University, Montreal, Quebec; the Department of Pathology,† University of Toronto, Toronto, Ontario; and the Kidney Research Centre,‡ the Department of Medicine, The Ottawa Hospital, University of Ottawa, Ottawa, Ontario, Canada*

SLK expression and activity are increased during kidney development and recovery from renal ischemia-reperfusion injury. In cultured cells, SLK promotes F-actin destabilization as well as apoptosis, partially via the p38 kinase pathway. To better understand the effects of SLK *in vivo*, a transgenic mouse model was developed where SLK was expressed in a podocyte-specific manner using the mouse nephrin promoter. Offspring of four founder mice carried the SLK transgene. Among male transgenic mice, 66% developed albuminuria at approximately 3 months of age, and the albuminuric mice originated from three of four founders. Overall, the male transgenic mice demonstrated about fivefold greater urinary albumin/creatinine compared with male non-transgenic mice. Transgenic and non-transgenic female mice did not develop albuminuria, suggesting that females were less susceptible to glomerular filtration barrier damage than their male counterparts. In transgenic mice, electron microscopy revealed striking podocyte injury, including poorly formed or effaced foot processes, and edematous and vacuolated cell bodies. By immunoblotting, nephrin expression was decreased in glomeruli of the albuminuric transgenic mice. Activation-specific phosphorylation of p38 was increased in transgenic mice compared with non-transgenic animals. Glomeruli of SLK transgenic mice showed around 30% fewer podocytes, and a reduction in F-actin compared with control glomeruli. Thus, podocyte SLK overexpression *in vivo* results in injury and podocyte loss, consistent with the effects of SLK in cultured cells. ***(Am J Pathol 2010, 177:2290–2299; DOI: 10.2353/ajpath.2010.100263)***

Germinal center kinases (GCKs) comprise a family of protein kinases that are homologous to Ste20 of *S. cerevisiae*.[1–3] The GCK family has been subdivided into eight groups. The group I GCKs are situated upstream of mitogen-activated protein kinase kinase-1, and they activate the c-Jun N-terminal kinase (JNK) pathway.[1,2] GCKs in groups II-VIII are diverse kinases, and some may be activated *in vivo* by various stresses, eg, heat-shock, ischemic injury, or ATP depletion.[1–3] Most of these GCKs are expressed ubiquitously, and most do not fit into the well-defined mitogen-activated protein kinase pathways, although there are exceptions.[2–6] The pathophysiological roles of most GCKs are poorly understood. The Ste20-like kinase, SLK, is a group V GCK, expressed in kidney tubular epithelial cells, and to a lesser extent in glomerular visceral epithelial cells (GECs; podocytes). Previously, we demonstrated that expression and activity of SLK were increased in rat fetal kidneys, compared with adult control kidneys, and that renal ischemia-reperfusion injury enhanced SLK expression and activity in adult rat kidneys.[7] Thus, SLK is a renal epithelial protein kinase, whose expression and activity are increased during development, and recovery from acute renal failure, where injured tubular epithelial cells may regenerate by recapitulating developmental processes.[8] Moreover, expres-

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Address reprint requests to Andrey V. Cybulsky, M.D., Division of Nephrology, Royal Victoria Hospital, 687 Pine Avenue West, Montreal, Quebec, Canada H3A 1A1. E-mail: andrey.cybulsky@mcgill.ca.

sion of SLK in developing and mature podocytes suggests a possible role in glomerulogenesis or glomerular injury.

The functional and regulatory aspects of SLK have received considerable attention in recent years. Exposure of cultured kidney epithelial cells to chemical anoxia and re-exposure to glucose (which recapitulates ischemia-reperfusion *in vivo*) or to serum (a source of growth factors) stimulated SLK activity.[7] Overexpression of SLK in cultured GECs (which we have used to model the increased expression observed in the *in vivo* circumstances) resulted in a modest pro-apoptotic effect, whereas in the setting of ischemia-reperfusion, SLK overexpression markedly exacerbated cell death.[4,7] By analogy, transient overexpression of SLK also induced apoptosis in other cell lines.[5,6] In fibroblasts, SLK may regulate cytoskeletal remodeling. SLK was found to be associated with the microtubular network, and activation of SLK via focal adhesion kinase and ERK pathways destabilized the actin network. This process affected focal adhesion turnover, cell adhesion, spreading and motility.[9–11]

The level of expression, dimerization, and phosphorylation may be involved in the regulation of SLK activity. SLK mRNA has an extensive 3′-untranslated region, which may interact with kidney RNA-binding proteins to regulate expression.[12] Similar to other GCKs, the SLK protein possesses an N-terminal kinase domain, and an extensive C-terminal domain. The latter may be involved in regulation of kinase activity via dimerization.[4] Phosphorylation or dephosphorylation of SLK were associated with changes in SLK activity in some, but not all studies.[4–6,10,13,14] Downstream signaling by SLK may involve mitogen-activated protein kinase pathways. We demonstrated that in kidney epithelial cells, overexpression of SLK activated the p38 mitogen-activated protein kinase pathway.[4] In addition, SLK can lead to the activation of JNK[5,6,15] and an increase in the transactivation of p53.[15] In kidney epithelial cells, overexpression of SLK enhanced caspase activity and ischemia–reperfusion-induced apoptosis, and apoptosis was attenuated by inhibitors of p38, caspase-9, and p53.[4,15]

Podocytes are intrinsic components of the kidney glomerulus and play a key role in the maintenance of glomerular permselectivity.[16–19] Permselectivity is dependent on the maintenance of appropriate structure of podocytes and the filtration slit-diaphragms, including nephrin (a key component of the slit-diaphragm), and cytoskeletal proteins. Various forms of glomerulonephritis are associated with podocyte injury, which may lead to impaired glomerular function or permselectivity (proteinuria), apoptosis, and glomerulosclerosis. For example, based on studies in animal models, there is evidence that in focal segmental glomerulosclerosis, podocyte apoptosis may lead to "podocytopenia" and consequently glomerulosclerosis.[20–23] Alterations in expression of podocyte structural proteins and filtration slit diaphragm components have also been reported in glomerular diseases[16,18,19].

So far, studies have provided considerable insight into the functions of SLK in cultured cell lines, but information on the functional role of SLK *in vivo* is lacking. Transgenic (Tg) mice have been widely used as animal models of human disease. To gain a better understanding of potential effects of SLK *in vivo*, we overexpressed SLK in podocytes in mice. This approach was adopted since our previous studies have shown a functional role for SLK in GECs in culture, and because podocytes *in vivo* are terminally differentiated cells with low capacity for proliferation under normal circumstances and after injury.[19] Moreover, podocyte injury may be quantified as albuminuria. Our results show that overexpression of SLK resulted in striking podocyte injury, including poorly formed or effaced foot processes, as well as edematous and vacuolated cell bodies, in association with albuminuria.

Materials and Methods

Materials

Restriction enzymes and molecular biology reagents were purchased from New England Biolabs (Mississauga, ON), Invitrogen Life Technologies (Burlington, ON) and Fermentas (Burlington, ON). Electrophoresis and immunoblotting reagents were from Jackson ImmunoResearch (West Grove, PA), Pall Corporation (Pensacola, FL), and GE Health care (Baie d'Urfé, QC). Mouse anti-hemaglutinin antigen epitope tag (HA) and rabbit anti-Wilm's tumor (WT)−1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-HA was from Zymed Laboratories (San Francisco, CA). Rabbit antiserum to nephrin was described previously.[24] Rabbit anti-α-actinin-4 antiserum was produced by immunization with a synthetic peptide corresponding to the first 11 amino acids of the N-terminal region of mouse α-actinin-4, as described.[25] Rabbit anti-phospho-p38 and rabbit anti-p38 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-synaptopodin antibody was from Progen Biotechnik (Heidelberg, Germany). Rhodamine conjugated-phalloidin was obtained from Sigma-Aldrich Canada (Mississauga, ON).

Transgene Construction and Generation of Transgenic Mice

Full length human SLK open reading frame (3.7 kb) from the vector pEF-BOS-HA[4,7] was subcloned into the NotI restriction site in the plasmid pIND/Hygro (Invitrogen). A 255 bp PCR product containing a Kozak sequence, two HA tags and a 143 bp portion of the 5′ end of SLK was generated using the forward primer, 5′-GGACTAGTTA-ACGGCGCCACCATGTCGATGTACCCATACCCATACG-3′, and the reverse primer, 5′-GTGTACAAGGCCCA-GAATAA-3′. This product was subcloned between the SpeI restriction site in pIND/Hygro and the BsrG1 site in SLK (nucleotide 143). To remove most of the restriction sites 3′ to the NotI site in pIND/Hygro, a 742 bp fragment at the 3′ end of HA-SLK was amplified using the forward primer, 5′-AACAGCTCATGAGAGCTCGAGAA-3′ and the reverse primer, 5′-TTAATTAGGGCCCTTATGATCCGGT-GGAATGC-3′. This cDNA was subcloned into the XhoI

site of SLK (nucleotide 3008) and the ApaI site in pIND/Hygro. A 5.4 kb fragment of the mouse nephrin promoter[25] was subcloned into the vector pENTR. To introduce a SpeI restriction site at the 3′ end, a 558 bp PCR product was produced using the forward primer, 5′-GAGATGGCCCTGATCAGAAA-3′ and reverse primer 5′-ATAGTTTAGCGGCCGCGGCGCCGTTAACTAGTCACCAGCAGCTTGTTTGTT-3′. This product was subcloned between the BclI site of the nephrin promoter (548 nucleotides from the 3′ end) and NotI site of pENTR. The 5.4 kb nephrin promoter was subcloned into the KpnI and SpeI restriction sites in pIND/Hygro, just 5′ to HA-SLK. All PCR reactions were carried out with Pwo DNA polymerase, and the composition of all PCR products was verified with DNA sequencing.

The nephrin promoter-HA-SLK-bovine growth hormone polyA transgene was linearized with KpnI and RsrI and 1–2 pl at a concentration of 2 μg/ml was then microinjected into C3H/C57BL6 mouse embryos (Microinjection Laboratory Service, Montreal General Hospital). Embryos were surgically transferred to the oviduct of pseudopregnant C3H recipient female mice. Genotyping of the resultant pups was done by extracting tail DNA followed by PCR. The forward primer, 5′-AACAAACAAGCTGCTGGTGA-3′, is based on the DNA sequence 19 nucleotides from the 3′ end of the nephrin promoter. This primer lies downstream of the transcription start site of the mouse nephrin mRNA. The reverse primer, 5′-TGGGTGATCACAAGATGCTAA-3′, corresponds to amino acid 83 in human SLK (PCR product 395 bp). There is an intron in the SLK genomic DNA sequence between the two primer pairs. Four pups who were carriers of the transgene (founders) were crossed with C3H mice to generate the mice reported in this study. The studies were approved by the McGill University Animal Care Committee.

Urine Albumin Excretion

Mouse urine albumin concentration was quantified using an enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Montgomery, TX), according to the manufacturer. Urine creatinine concentration was measured using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Excretion of albumin is expressed as albumin to creatinine ratio. In addition, urine albumin concentration was confirmed in some mice using SDS-polyacrylamide gel electrophoresis.

Immunofluorescence, Light, and Electron Microscopy

For immunofluorescence microscopy, kidney fragments were snap-frozen in isopentane (−80°C). Four-μm sections were fixed in 1:1 ether/ethanol for 10 minutes, followed by ethanol for 20 minutes. After washing, sections were blocked with 5% serum. Sections were incubated with rabbit anti-HA antibody (4°C, overnight), washed, and incubated with rhodamine-conjugated goat anti-rabbit IgG (22°C, 30 minutes). Sections were examined with a Zeiss AxioObserver fluorescence microscope with visual output connected to an AxioCam digital camera. In some experiments, sections were incubated with rabbit anti-HA together with mouse anti-synaptopodin antibodies, followed by rhodamine-conjugated goat anti-rabbit IgG and fluorescein-conjugated rabbit anti-mouse IgG. These HA and synaptopodin images were collected from series of images derived from different focal planes (Z-stack). Merging of fluorescence images was carried out with Adobe Photoshop software.

Nephrin immunofluorescence staining was carried out similarly to HA. For rhodamine-phalloidin fluorescence, kidney sections were blocked as above, and were incubated with rhodamine-phalloidin, diluted 1 μg/ml in 3% bovine serum albumin in PBS (22°C, 30 minutes).[26,27] For WT-1 immunofluorescence, frozen sections were fixed with 4% paraformaldehyde for 5 minutes. Then, sections were immersed in 10 mmol/L sodium citrate, pH 6.0 for 8 minutes at 100°C. After cooling to 22°C, sections were blocked with 10% serum, and were incubated with rabbit anti-WT-1 antibody (22°C, 1 hour), followed by rhodamine-goat anti-rabbit IgG (22°C, 1 hour). Nephrin and phalloidin images (AxioCam digital camera) were collected from series of images derived from different focal planes (Z-stack). WT-1 positive cells were quantified in 4 to 5 glomeruli of Tg and control mice by visual counting. Glomerular rhodamine-phalloidin fluorescence intensity (4 to 5 glomeruli per mouse) was quantified using Adobe Photoshop software, and was normalized to the fluorescence intensity of Bowman's capsule.

Apoptosis was studied using the In Situ Cell Death Detection Kit (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay) from Roche Diagnostics (Laval, QC), according to the manufacturer's instructions. Briefly, kidney sections were fixed with 4% paraformaldehyde (22°C, 20 minutes), followed by 0.1% Triton X-100 and 0.1% sodium citrate (4°C, 2 minutes). After washing, sections were incubated with terminal transferase and fluorescein-dUTP (37°C, 60 minutes), to label free 3′-OH ends in genomic DNA. Positive control staining was produced by incubating sections of normal mouse kidney with DNase I (3 μl/ml, 22°C, 10 minutes) before the labeling reaction. Sections were examined by fluorescence microscopy, as above.

For light microscopy, kidney tissue was fixed in formalin, and was processed and embedded in paraffin according to conventional techniques. Sections were stained with periodic acid Schiff. Electron microscopy was carried out at the McGill University Facility for Electron Microscopy Research. Briefly, kidney tissue was fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer containing 0.1% $CaCl_2$, pH 7.4. Samples were washed and postfixed with 1% aqueous OsO_4 and 1.5% aqueous potassium ferrocyanide. Then, samples were dehydrated with serially-increasing concentrations of acetone (30 to 100%), and samples were infiltrated with epon/acetone. Sections of 90 to 100 nm were placed onto grids, and stained with uranyl acetate and Reynold's lead. Sections were viewed with a FEI Tecnai 12 transmission electron microscope operating at an accelerating voltage of 120 kV, and equipped with a Gatan Bioscan CCD camera, model 792.

Immunoblotting

Glomeruli were isolated from mouse kidneys by differential sieving.[28] The purity of glomerular isolation was ~65%. Following cells lysis, samples were adjusted to contain equal amounts of proteins, which were loaded into each lane of a gel. After SDS- polyacrylamide gel electrophoresis, proteins were electrophoretically transferred onto nitrocellulose paper, blocked, and incubated with primary antibody, and then with horseradish peroxidase-conjugated secondary antibody. The blots were developed using the enhanced chemiluminescence technique. Protein content was quantified by scanning densitometry, using NIH ImageJ software. Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

Reverse Transcriptase Real-Time PCR

Total RNA was prepared from mouse glomeruli using Trizol reagent (Invitrogen). cDNA synthesis was performed using the QuantiTect Reverse Transcription kit (Qiagen, Mississauga, ON). The PCR primer set for mouse nephrin was from Qiagen (catalog number QT01053276). Real-time PCR reactions were performed on an ABI 7300 Sequence Detection System (Applied Biosystems), and amplified DNA was detected by SYBR green incorporation. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels in the same sample.

Statistics

Data are presented as mean ± SEM. The t statistic was used to determine significant differences between two groups.

Results

Characterization of SLK Tg Mice

We generated mice carrying a HA-SLK transgene under the control of the nephrin promoter, which enables gene expression that in the kidney is specific to podocytes.[25,29,30] In total, 195 mice were born to four founder mice. Among these, 63 mice carried the SLK transgene. There were no apparent differences in weight between transgenic mice and normal littermate controls for up to 6 months of age (data not shown), and there was no excess mortality of transgenic mice. Kidney weight of transgenic mice at sacrifice was 0.89 ± 0.09 g, and was not significantly different from control 0.90 ± 06 g.

To assess expression of the SLK transgene in mouse glomeruli, kidney sections were incubated with anti-HA antibody, and were then examined by immunofluorescence microscopy. The SLK transgene was expressed in glomeruli of all male and female Tg mice studied (nine mice), At least one mouse from each founder was examined. Immunofluorescence staining was, however, relatively weak. Staining was focal and segmental in several animals, although occasional glomeruli showed diffuse staining of capillary loops (Figure 1, A–D). Staining with anti-HA-antibody together with antibody to synaptopodin (a protein expressed in podocytes[19]), showed that HA staining, although focal and segmental, colocalized with synaptopodin in capillary loops (Figure 1, E–G). We also confirmed expression of SLK transgene mRNA in glomeruli isolated from some Tg and control mice, using reverse transcription (RT)-PCR (Figure 1H). By immunoblotting,

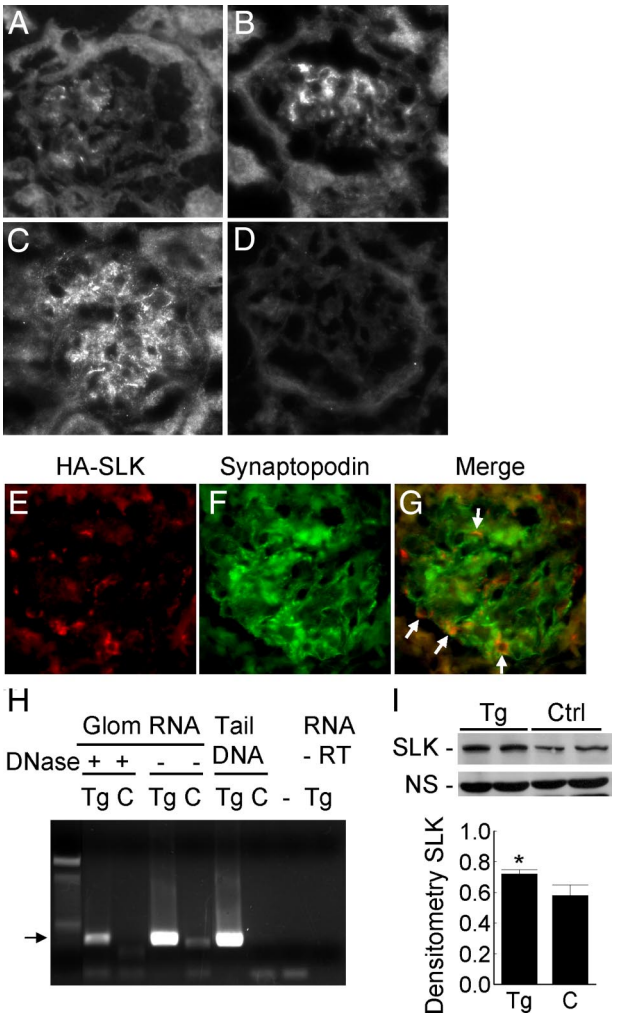

Figure 1. Expression of the HA-SLK transgene. **A–D:** Immunofluorescence microscopy. Kidney sections from Tg mice (3 months of age) were incubated with rabbit anti-HA antibody (**A–C**), or non-immune rabbit IgG (**D**), followed by rhodamine-conjugated anti-rabbit IgG. Transgene expression occurred frequently in a segmental pattern (**A** and **B**). Occasional glomeruli showed diffuse staining of capillary loops (**C**). Staining was negative in control incubations (**D**). **E–G:** HA (**E**) and synaptopodin (**F**) staining in Tg mouse glomerulus. Merged image (**G**); **arrows** point to some of the areas of HA and synaptopodin co-localization. **H:** RNA was extracted from isolated glomeruli of Tg mice or from non-Tg littermates (control; **C**), and was subjected to RT-PCR using the same primers as those used for genotyping of mice (*see Materials and Methods*). Some samples were first incubated with DNase (+). PCR of tail DNA is shown for comparison. Control reactions included all PCR components without RNA/DNA (−), and RNA without reverse transcriptase (−RT). The **arrow** points to the 395-bp PCR product, evident only in Tg mice. **I:** Proteins from isolated mouse glomeruli were immunoblotted with anti-SLK antibody. Immunoblot of two Tg and two control (Ctrl) mice (**upper panel**), and densitometric quantification (**lower panel**). $^*P < 0.05$ Tg ($N = 11$) vs. control ($N = 8$). NS, nonspecific band (loading control).

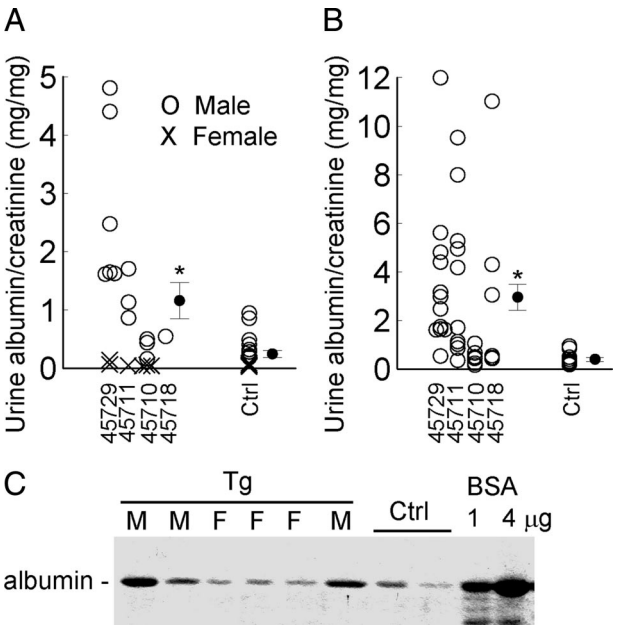

Figure 2. SLK overexpression increases urinary albumin excretion. **A** and **B:** Urinary albumin/creatinine ratios. Each point represents an average of two or three urine collections of a single mouse taken at monthly intervals between three and five months of age. Mean values for all Tg and control (Ctrl) mice are also presented. **A:** The first-born 44 male and female mice. The Tg mice (14 males and 6 females) were divided into groups according to founders. Littermate controls (13 males and 11 females) are presented as a single group (*$P < 0.025$ Tg versus control). **B:** Tg ($N = 35$) and control male mice ($N = 11$, *$P < 0.0001$ Tg versus control). **C:** Urinary albumin of male (M) and female (F) Tg and control mice (representative SDS-polyacrylamide gel electrophoresis and Coomassie blue stain, 15 μl urine per lane).

expression of total SLK in Tg mouse glomeruli was ~25% greater, as compared with non-Tg littermate controls (Figure 1I). This analysis provides an average level of expression in the entire glomerulus, which does not exclude significantly higher expression in individual podocytes, and lower levels in others.

Urinary Albumin Excretion

Mice up to 3 months of age showed low urine albumin excretion (monitored as albumin/creatinine), and there were no significant differences between Tg mice and littermate controls (data not shown). At three months, a subset of male Tg mice developed increased urinary albumin excretion. In the majority of these mice, albuminuria persisted at months 4 and 5, and there were no significant upward or downward trends in the magnitude of albuminuria. Therefore, urine albumin/creatinine measurements are presented as means of measurements during months 3, 4, and 5 (Figure 2, A–B). It should be noted that significantly increased urinary albumin excretion developed only in male Tg mice (Figure 2A; male Tg 1.6 ± 0.4 mg/mg versus female Tg 0.06 ± 0.02 mg/mg). Among control mice, males also showed greater urinary albumin/creatinine compared with female controls (0.3 ± 0.08 vs. 0.06 ± 0.01 mg/mg, $P < 0.002$). When analyzed together, the comparison of urinary albumin/creatinine of male and female Tg mice with male and female normal littermate controls showed that albuminuria was significantly greater in the Tg group (1.4 ± 0.3 mg/mg vs 0.3 ± 0.06 mg/mg; Figure 2A).

We then analyzed albuminuria in additional male offspring. Male Tg mice showed a greater mean urinary albumin/creatinine, compared with male controls (Figure 2B). Among all male Tg mice, 66% showed urinary albumin/creatinine above the mean plus 2 standard deviations of the normal littermate male controls. The albuminuric Tg mice were derived from 3 of the 4 founders (Figure 2, A and B). Examination of urinary albumin excretion using SDS-polyacrylamide gel electrophoresis and Coomassie blue staining confirmed that male Tg mice had the highest levels of urinary albumin excretion (Figure 2C).

Effects of SLK on Podocyte Structure

Glomeruli and tubules of Tg mice at age ~5 months ($N = 10$) appeared normal by light microscopy (Figure 3A). It should also be noted that male mice of this strain have large glomerular parietal epithelial cells, or proximal tubular epithelial cells which extend into Bowman's space (Figure 3A). Quantification of glomerular tuft circumference (excluding the parietal epithelial cells, or proximal tubular epithelial cells within Bowman's capsule) revealed a trend toward an increase in male Tg mice (3235 ± 268 μm^2/glomerulus; $N = 12$) vs. male controls (2712 ± 129 μm^2/glomerulus; $N = 5$), but the difference did not reach statistical significance.

Kidneys of three albuminuric male Tg mice and one male control mouse were examined by electron microscopy. The control mouse showed no glomerular abnormalities, and podocyte ultrastructure appeared entirely normal (Figure 3B). In contrast, all Tg mice showed focal abnormalities in podocyte ultrastructure. Podocyte cell bodies appeared vacuolated and swollen, and in some podocytes there was a paucity of intracellular organelles, consistent with "balloon degeneration" (Figure 3, C–G). Some podocyte nuclei showed condensation of chromatin. In some areas of the glomerulus, foot processes appeared normal, but in many other areas, there were marked abnormalities. These ranged from short, stubby, and widened foot processes to severely malformed foot processes, and areas of complete effacement (Figure 3, C–G).

Effect of SLK on Glomerular Expression of Nephrin and α-Actinin-4, and Phosphorylation of p38

Maintenance of glomerular permselectivity is dependent on an intact filtration slit diaphragm. Since overexpression of SLK increased albuminuria and induced abnormalities in podocyte architecture, we examined if there were linked abnormalities in slit diaphragm-associated proteins. By immunoblotting (Figure 4, A and B) and by immunofluorescence microscopy (Figure 4, F and G), expression of nephrin protein was reduced in male Tg

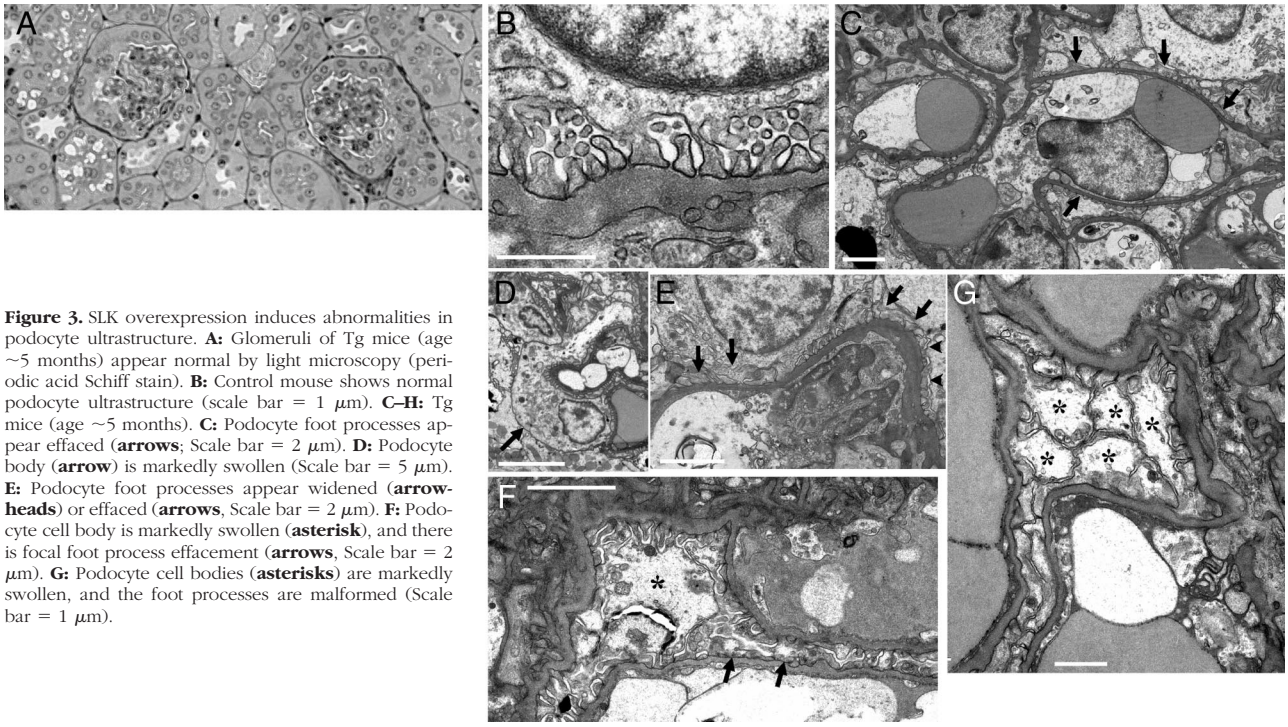

Figure 3. SLK overexpression induces abnormalities in podocyte ultrastructure. **A:** Glomeruli of Tg mice (age ~5 months) appear normal by light microscopy (periodic acid Schiff stain). **B:** Control mouse shows normal podocyte ultrastructure (scale bar = 1 μm). **C–H:** Tg mice (age ~5 months). **C:** Podocyte foot processes appear effaced (**arrows**; Scale bar = 2 μm). **D:** Podocyte body (**arrow**) is markedly swollen (Scale bar = 5 μm). **E:** Podocyte foot processes appear widened (**arrowheads**) or effaced (**arrows**, Scale bar = 2 μm). **F:** Podocyte cell body is markedly swollen (**asterisk**), and there is focal foot process effacement (**arrows**, Scale bar = 2 μm). **G:** Podocyte cell bodies (**asterisks**) are markedly swollen, and the foot processes are malformed (Scale bar = 1 μm).

mice, compared with male controls. However, by analogy to changes in albuminuria, nephrin protein expression was not significantly different in female Tg mice, compared with female controls. In contrast to nephrin protein, there was only a ~30% downward trend in nephrin mRNA expression (measured by real-time RT-PCR) in Tg mice, compared with control (Tg: 0.39 ± 0.07 units versus control: 0.60 ± 0.18 units; 3 male and 1 female mice per group). Moreover, if only the male mice were considered, differences between Tg (0.33 ± 0.05 units) and control (0.47 ± 0.16 units) remained nonsignificant. Glomerular α-actinin-4 protein tended to decrease in Tg mice, but the change did not reach statistical significance (Figure 4, A and C).

In cultured cells, overexpression of SLK induced activation of the p38 mitogen-activated protein kinase pathway.[4] To determine whether SLK overexpression activated the p38 pathway *in vivo*, we monitored p38 phosphorylation (which reflects activation) in glomerular lysates of mice derived from the four founders. Phosphorylation of p38 was enhanced 1.7-fold in Tg mice, compared with controls (Figure 4, A and D). Unlike albuminuria, there were no significant differences in phospho-p38 between Tg male and female mice, ie, increases in phospho-p38 were noted in both males and females. Expression of p38 was comparable in Tg and control mice (Figure 4, A and E). In certain cell lines, SLK activates the JNK pathway.[5,6,15] However, in contrast to p38, we did not detect phosphorylation of JNK in glomeruli of Tg mice (results not shown). Furthermore, there was no increase in phosphorylation of ERK in Tg mice (results not shown).

Effect of SLK on Podocyte Number and on the Cytoskeleton

Overexpression of SLK in cultured cells, including GECs induced apoptosis.[4–7] To determine whether overexpression of SLK in podocytes *in vivo* also induces apoptosis, we quantified the number of podocytes in glomeruli of Tg and non-Tg littermate control mice (age ~5 months), by using WT-1 immunostaining. In the glomerulus, WT-1 is expressed specifically in podocytes.[31] There was a ~30% decrease in the number of WT-1 positive cells in Tg glomeruli compared with control (Figure 5, A–E), suggesting that overexpression of SLK induced a loss of podocytes. This result does not, however, exclude the possibility that a reduction in WT-1 positive cells was related to injury instead of loss of cells. Examination of nine Tg and three control mice by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling did not, however, demonstrate the presence of apoptotic podocytes (Figure 5, F and G).

In cultured cells, SLK has been shown to affect cytoskeletal remodeling by destabilizing the F-actin network. To examine if SLK may affect the amount of F-actin *in vivo*, we incubated mouse kidney sections with rhodamine-phalloidin (Figure 5, H and I), and then quantified rhodamine-fluorescence intensity, which is proportional to the amount of F-actin. Fluorescence was ~35% lower in male Tg glomeruli, compared with male control (Figure 5J), implying that overexpression of SLK resulted in a reduction in F-actin content. This assay reflects F-actin content in the entire glomerulus, so we cannot conclude that the reduction in F-actin was due solely to loss in

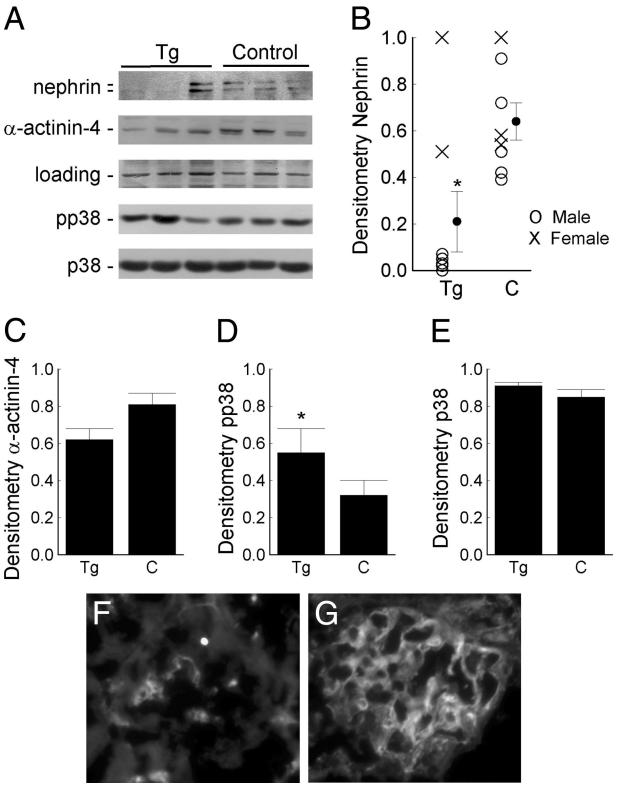

Figure 4. Effect of SLK on glomerular expression of nephrin and α-actinin-4, and phosphorylation of p38. Glomeruli were isolated from Tg and control (C) mice (age 5 to 6 months). Lysates were immunoblotted with antibodies to nephrin, α-actinin-4, phospho-p38 (pp38), and p38. A band from the membrane stained with amido black is presented to demonstrate protein loading. **A:** Representative immunoblot. **B–E:** densitometric quantification. B: $*P <$ 0.02 Tg versus control (8 Tg mice and 8 control mice). **D:** $*P = 0.05$ Tg versus control (9 Tg mice and 8 control mice). **C** and **E:** There are no significant differences between groups (9 Tg mice and 8 control mice). **F** and **G:** Kidney sections of Tg (**F**) and control male mice (**G**) were immunostained with anti-nephrin antibody. There is reduction in staining in the Tg mouse.

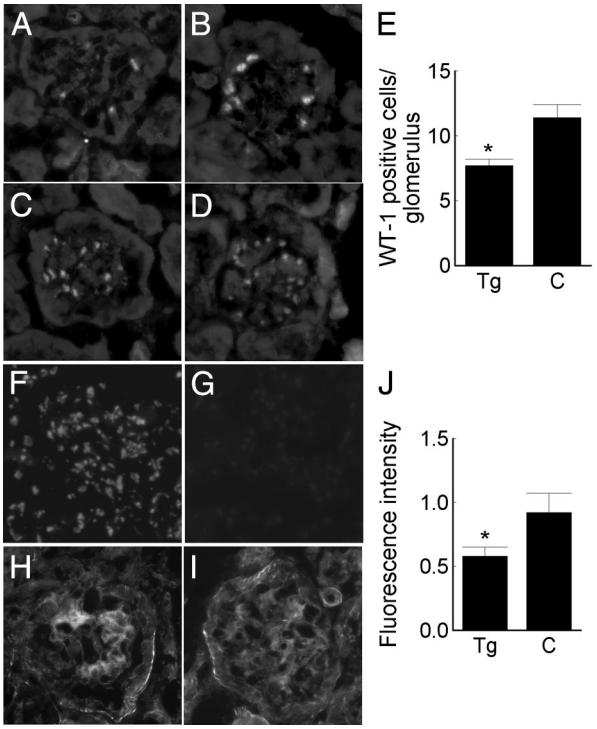

Figure 5. Effects of SLK on podocyte number and actin cytoskeleton. WT-1 immunofluorescence in two Tg mice (**A** and **B**) and two control mice (**C** and **D**). **E:** Quantification of WT-1-positive cells per glomerulus. $*P = 0.01$ Tg ($N = 11$) vs. control (**C**; $N = 8$). **F** and **G:** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end staining of mouse kidney sections. **F:** Section of normal mouse kidney treated with DNase I (positive control). **G:** Section of Tg mouse kidney shows absence of staining. **H–J:** Rhodamine-phallodin fluorescence in Tg (**H**) and control (**I**) mouse kidney sections. **J:** Quantification of rhodamine-phallodin fluorescence (arbitrary units). $*P <$ 0.035 Tg ($N = 11$) vs. control (C; $N = 4$).

podocytes and not in other glomerular cells. However, in non-Tg mice, there was substantial F-actin staining of the capillary loops, suggesting that there was abundant F-actin in the podocytes (Figure 5I).

Discussion

In this study, we demonstrate that overexpression of SLK in podocytes leads to albuminuria, podocyte injury, and a decrease in podocytes. We generated mice carrying a HA-SLK transgene under the control of the nephrin promoter, which enables gene expression that in the kidney is exclusive in podocytes.[25,29,30] SLK Tg mice were born to four founders, and male and female mice derived from the four founders expressed the SLK transgene in glomeruli. By immunofluorescence microscopy, HA-SLK was detected in a focal and segmental pattern in most animals, although occasional glomeruli showed diffuse capillary loop localization (Figure 1). The HA-SLK staining intensity was relatively weak when compared with staining for endogenous nephrin in control mice (Figure 4), or when compared, in parallel, with the expression of HA-α-actinin K256E transgene under the control of the nephrin promoter in another Tg mouse line.[25] Nevertheless, the HA-SLK staining pattern suggests that the transgene was expressed more robustly in a subset of podocytes, even though global expression was relatively low (Figure 1). In rodents, nephrin promoter activity has been detected in some parts of the central nervous system, β-cells of the pancreas, testis, spleen, and thymus, while in humans, nephrin has been detected in pancreatic and lymphoid tissue. The significance of nephrin expression in these other organs has not been established conclusively.[32] We did not examine extrarenal SLK transgene expression in the Tg mice, but the mice did not manifest any obvious extrarenal abnormalities.

In cultured cells, overexpression of SLK induced activation of apoptosis signal-regulating kinase-1, and the p38 pathway.[4] By analogy, activation-specific phosphorylation of p38 was enhanced in glomeruli of Tg mice, compared with controls, and increases in phospho-p38 were noted in both male and female mice (Figure 4). Based on this result, it is reasonable to conclude that SLK overexpression activated downstream signaling in podocytes. Although SLK is also reported to activate the JNK pathway in certain cell lines,[5,6,15] in contrast to p38, we did not detect glomerular phosphorylation of JNK in Tg mice. SLK does not induce ERK activation in cell culture models,[4] and consistent with this finding, we did not detect ERK phosphorylation in Tg mice.

The principal phenotypic finding in Tg mice became evident at three months of age, when 66% of male Tg mice developed albuminuria. This increase in urinary albumin excretion persisted at months four and five (Figure 2). The albuminuric male Tg mice were derived from three separate founder mice, whereas there were no proteinuric mice born to one of the founders (Figure 2). This result implies that the presence of albuminuria was most likely due to an effect of the SLK transgene, rather than disruption of the function of another gene after incorporation of the transgene into chromosomal DNA. In association with albuminuria, the Tg mice showed focal abnormalities in podocyte ultrastructure. In particular, certain podocyte cell bodies were vacuolated and swollen, with a paucity of intracellular organelles (Figure 3). There were focal abnormalities of the foot processes, ranging from short, stubby, and widened foot processes to severely malformed foot processes, and areas of complete effacement (Figure 3). The focal nature of podocyte ultrastructural injury is in keeping with the focal pattern of SLK transgene expression (Figure 1).

The podocyte plays an important role in maintaining glomerular permselectivity. Actin filaments are the core structural component of podocyte foot processes. Condensation of the actin cytoskeleton at the base of effaced podocyte foot processes together with alterations in filtration slits, and displacement and disruption of slit diaphragms are features of proteinuric glomerular diseases.[33] Heritable mutations of slit diaphragm-associated proteins or proteins associated with the actin cytoskeleton (eg, nephrin, podocin, α-actinin-4) may result in proteinuria,[16,18,19] implying that these proteins play key roles in maintaining podocyte ultrastructure. Changes in nephrin expression are also observed in acquired proteinuric kidney disease.[33] Since overexpression of SLK induced albuminuria and abnormalities in podocyte architecture, we examined if there were associated abnormalities in nephrin expression. By analogy to changes in albuminuria, nephrin protein expression was reduced significantly in male Tg mice, but was not significantly different in female Tg mice, compared with controls. Glomerular α-actinin-4 tended to decrease in Tg mice, but the change did not reach statistical significance (Figure 4). The decrease in nephrin protein expression measured at 5 to 6 months of age was not paralleled by an analogous reduction in nephrin mRNA, suggesting that the nephrin promoter was active. Thus, the decrease in nephrin protein expression was most likely due to a post-transcriptional change,[34] including a reduction in translation or increased degradation.

Interestingly, increased urinary albumin excretion was found only in male, but not female Tg mice, whereas SLK transgene expression and p38 activation were not significantly different between males and females (Figure 2). Also female control mice had lower basal levels of urine albumin excretion, compared with male controls. These results suggest that sex hormones influenced the mechanism for the pathogenesis of podocyte injury and proteinuria. A link between sex and progression of proteinuric kidney disease has been described in humans and animal models, and may involve cell response systems and glomerular hemodynamic factors.[35] Aging male rats develop proteinuria and glomerulosclerosis, whereas females and orchidectomized males appear to be resistant.[36] Male animals have larger glomeruli than females,[37,38] and glomerular hypertrophy may be a risk factor for glomerulosclerosis. Extracellular matrix production may be modified by the presence of estrogen.[39] Experimental work also supports an interaction between the renin-angiotensin system and intraglomerular pressure that is at least partially estrogen dependent.[40]

Overexpression of SLK in mice resulted in a ~30% decrease in podocytes, measured by WT-1 staining (Figure 5). In view of the ultrastructural evidence of podocyte injury, it is likely that the reduction in podocytes in Tg mice was associated with apoptosis. Although we could not demonstrate the presence of apoptotic cells in glomeruli of Tg mice by terminal deoxynucleotidyl transferase-mediated dUTP nick-end staining, it is possible that the technique was not sufficiently sensitive to detect a low-grade apoptotic process. For example, in other studies, rats with extensive podocyte injury and nephrotic syndrome induced by the podocyte toxin, puromycin aminonucleoside, showed few apoptotic podocytes.[41,42] In addition, it has been suggested that apoptotic podocytes are flushed out in the urine, making it technically difficult to detect these cells.[43] Reduction in podocytes *in vivo* due to apoptosis is in keeping with the effect of SLK overexpression in cultured GECs, and other renal and non-renal cells in culture.[4–7] Loss of podocytes *in vivo* may lead to glomerulosclerosis.[20–23] However, the decrease in podocytes in the SLK Tg mice was not associated with glomerular sclerotic lesions, at least up to 5 to 6 months of age, although there was a tendency toward a greater glomerular circumference in Tg mice, suggesting early glomerular hypertrophy, which may be a risk factor for glomerulosclerosis. Probably, a greater amount of podocyte loss may be necessary for sclerotic lesions to appear, or alternatively other factors, such as hypertension, may also be required. It a rat model of podocyte loss, it was demonstrated that loss of ~70% of podocytes resulted in significant glomerular capsular adhesions, while glomeruli remained relatively free of lesions if podocyte loss was <25%.[22] In two mouse models of diabetic nephropathy, there was a 27 to 37% decrease in podocytes. These changes in podocyte numbers were associated with some mesangial expansion, but glomerular sclerotic lesions were absent.[44]

In cultured fibroblasts, SLK was shown to regulate cytoskeletal remodeling. SLK was associated with the microtubular network, and activation of SLK via focal adhesion kinase and ERK pathways destabilized the actin network, thereby affecting focal adhesion turnover, cell adhesion, spreading, and motility.[9–11] By analogy, we observed that in the SLK Tg mice, there was a reduction in glomerular F-actin content (Figure 5). Disassembly of podocyte F-actin could contribute to changes in ultrastructure and proteinuria. Podocyte foot processes harbor an actin-based contractile apparatus, and defects in proteins which interact with the actin cytoskeleton (eg, α-actinin-4) impair glomerular permselectivity.[16,18,19] A reduction in podocyte F-actin may be associated with foot process effacement, and po-

tentially, detachment from the glomerular basement membrane.[33] Thus, podocyte detachment is another potential explanation for the reduction in podocyte number.

Tg mice were born at a frequency of ~33%, which is somewhat less than expected. We did not observe any apparent increase in mortality of adult Tg mice up to 6 months of age, compared with normal littermate controls, and there were no significant differences in kidney weights. In addition, the abnormal phenotype of Tg mice (albuminuria) was not detected until 3 months of age. These results would suggest that overexpression of SLK was not detrimental to normal podocyte development, but instead exerted an injurious effect on mature cells. Furthermore, expression of genes under control of the nephrin promoter is highly restricted, further arguing against toxicity of the transgene. Thus, the reduced frequency of Tg mice may have been due to chance. Nevertheless, we cannot entirely exclude the possibility of excess intrauterine or perinatal mortality in mice with very high levels of SLK overexpression.

There is only limited information on the functional effects of GCKs *in vivo*. The Ste20-like kinase, MstI, is activated by stress and stimulates apoptosis in some cultured cell types. *In vivo*, cardiac-specific overexpression of MstI in transgenic mice induced caspase activation, apoptosis, and a dilated cardiomyopathy.[45] In addition, MstI inhibited cardiac muscle hypertrophy. By analogy to SLK in the kidney, MstI was activated by ischemia-reperfusion in the mouse heart *in vivo*. Suppression of endogenous MstI by cardiac-specific overexpression of a dominant-negative MstI mutant in transgenic mice prevented myocyte death by pathological insults.[45] Thus, MstI is an initiator of apoptosis and an inhibitor of hypertrophy in cardiac myocytes.

The Ste20-related proline/alanine-rich kinase (SPAK) plays important roles in cell differentiation and regulation of chloride transport.[46] Cultured intestinal epithelial cells stably transfected with SPAK showed increased permeability. Transgenic mice that expressed a constitutively active form of SPAK under the control of the villin gene locus control region, which can target SPAK expression only in intestinal epithelial cells were generated to study intestinal barrier function *in vivo*. The mice showed a decreased intestinal barrier function.[46]

We have previously demonstrated that SLK expression and activity increase in the developing kidney and in kidneys subjected to ischemia-reperfusion injury.[7] The upregulation of expression may then favor homodimerization and phosphorylation, and possibly reversal of auto-inhibition, together leading to an increase in kinase activity.[4,6] Decisive proof of this mechanism will require further study. It will also be of interest to determine whether increased SLK expression and/or activity are evident in experimental models of glomerular diseases associated with podocyte injury.

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