WT1 Activates a Glomerular-Specific Enhancer Identified from the Human Nephrin Gene

GORDON GUO,* DEBRA J. MORRISON,† JONATHAN D. LICHT,† and SUSAN E. QUAGGIN*‡

*Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, University of Toronto, Toronto, Ontario, Canada; †Division of Hematology/Oncology, Department of Medicine, Mount Sinai School of Medicine, New York, New York; ‡Division of Nephrology, St. Michael’s Hospital, Toronto, Ontario, Canada.

Abstract. The glomerular filtration barrier separates the blood from the urinary space. Nephrin is a transmembrane protein that belongs to the immunoglobulin superfamily and is localized to the slit diaphragms that are a critical component of this filtration barrier. Mutations in the nephrin gene (NPHS1) lead to congenital Finnish nephropathy, whereas alterations in the level of nephrin expression have been identified in a wide range of acquired glomerular diseases. A 186-bp fragment from the human NPHS1 promoter is capable of directing podocyte-specific expression of a β-galactosidase transgene when placed in front of a heterologous minimal promoter in transgenic mice. The Wilms tumor suppressor gene (WT1) is a zinc-finger–containing transcription factor that is coexpressed with NPHS1 in differentiated podocytes; gel shift binding assays demonstrate that a recombinant WT1 protein can bind and activate the 186-bp NPHS1 fragment in a sequence-specific manner. Taken together, these results suggest that WT1 may be required for regulation of the NPHS1 gene in vivo.

Kidney disease affects over 20 million individuals in the United States alone and is a major cause of morbidity and mortality worldwide. Although the causes of renal failure are diverse, the glomerulus and renal filtration barrier are often the target of injury (1,2). Despite the significant burden of disease, treatment options are limited. In the majority of cases, damage to the renal filtration barrier leads to progressive glomerular scarring and may result in end-stage kidney failure that requires dialysis or transplantation for survival. The glomerular filtration barrier consists of two individual cell types: glomerular visceral epithelial cells (podocytes), and fenestrated endothelial cells that are separated by a glomerular basement membrane. Approximately 180 L of blood per day passes through the glomerular capillaries in the average adult human kidney. Excess solutes and water are filtered into the urine, while critical blood proteins such as albumin and blood cells remain within the capillaries. Damage to this filtration barrier leads to the loss of protein into the urine (proteinuria).

Nephrin belongs to the immunoglobulin superfamily and is localized to the slit diaphragm, a specialized intercellular connection found between podocyte foot processes (3,4). Nephrin was originally identified as the gene that is mutated in congenital Finnish nephropathy (5), a rare autosomal recessive disease that results in severe proteinuria and death in infants unless they receive a renal transplant. Mutant mice that are missing the nephrin protein fail to form slit diaphragms and die at birth with massive proteinuria and kidney failure (6). Alterations in the level of nephrin expression have also been documented in a variety of acquired renal diseases that include diabetic nephropathy, focal segmental glomerulosclerosis, and minimal change nephropathy (7,8). Previous studies have shown that 8.3, 5.4, or 4.125 kb of the murine proximal nephrin promoter and 1.125 kb of the human proximal nephrin promoter are capable of directing expression of a β-galactosidase transgene to podocytes in vivo (9–11). Although the human fragment was exclusively expressed in podocytes, the larger fragments directed expression to the developing hindbrain as well. Recently, an alternate first exon that is required for neural expression of the nephrin gene has been identified (12).

By using the NCBI Pairwise-Blast sequence alignment program, we identified an 83-bp region of homology between the 4.125-kb murine and 1.125-kb human promoters. We found that a 186-bp fragment of the human promoter that contains this 83-bp region of homology is capable of directing podocyte-specific expression in transgenic mice. Furthermore, we demonstrate that the Wilms tumor suppressor protein can bind to a putative WT1 consensus sequence from this fragment in vitro and activates a nephrin-luciferase reporter transgene. By using this enhancer, it will be possible to identify additional trans-acting factors that are required for podocyte-specific gene regulation with the goal of finding new molecular targets for the treatment of renal patients.

Materials and Methods

Identification of Homology between the Human and Murine Promoters

We have previously shown that 4.125-kb of the murine nephrin promoter and 1.125-kb of the human nephrin promoter are capable of
directing expression of a reporter transgene (β-galactosidase) specifically to podocytes in vivo. To identify regions of homology within these DNA fragments, the sequences from these two promoters were aligned by the NCBI program (Pairwise BLAST; available at http://www.ncbi.nlm.nih.gov).

Generation of the Nephrin-LacZ Transgene

A total of 186 bp of the human nephrin promoter encoding the 83-bp core region of homology was amplified from the 1.125-kb human nephrin promoter fragment with the following primers: 5'-GGCCCTGGGTCAAGGAGCTGGGA-3' and 5'-CGGGTC-GACCTCAGATTTTAGGGGA-3' (Sigma); and under the following PCR conditions: 95°C × 30 s; 95°C × 1', 68°C × 4' for 30 cycles followed by 72°C for 4'. This 186-bp fragment was subcloned into the PCR 2.1 vector (Invitrogen) according to manufacturer’s directions. After digestion with EcoRI, either four copies or one copy of this fragment were subcloned into the hsp-lacZ vector that has been previously described (13). Both one- and four-copy transgenese were constructed to determine whether tandem repeats or a single copy of the enhancer alone were required for expression. This plasmid encodes the minimal promoter from the heat shock protein upstream of a β-galactosidase transgene (Figure 1). Both one-copy and four-copy transgenese were injected into one cell ICR (Charles River) murine embryos as described (14). To identify founder lines, genomic DNA was purified from tails as described (15), and Southern blot analysis of these tissues were prepared and stained as described previously (10,16). In addition, 10 μm cryosections of each of these tissues were prepared and stained as described previously (17).

Electrophoretic Mobility Shift Assays

A double-stranded DNA probe corresponding to the 186-bp human nephrin promoter fragment was released from pKS4 × 191 plasmid by EcoRI digestion and end-labeled with [α-32P] dATP by Superscript II (GibcoBRL). Cloning and expression of GST fusion proteins GST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion proteinsGST-WT1 (A isoform) and GST-ZF (WT1 lacking DNA-binding zinc fingers) (GibcoBRL). Cloning and expression of GST fusion protein...
nephrin gene and 444 bp upstream of the predicted initiation site for the murine nephrin gene (Figure 2B). The TF TRANSFAC database was used to identify putative consensus binding sites for transcription factors, an E-box site and retinoic acid receptor sites were identified, and a putative WT1-binding site was identified by visual inspection (Figure 3).

**Transgenic Founder Lines Exhibit Podocyte-Specific Expression**

Four independent founder mice that carried four copies of the nephrin "enhancer" and the hsp-lacZ transgene were identified. LacZ staining confirmed that 50% of these independent founders expressed β-galactosidase specifically in podocytes within the kidney from the late S-shape stage of glomerulogenesis onward (see Figure 4). The other two founder mice did not demonstrate any lacZ staining. In addition, two of four founder mice that carried one copy of the nephrin enhancer demonstrated podocyte-specific expression. One of these founder mice exhibited mosaic expression as only a subset of podocytes demonstrated β-galactosidase activity (Figure 4C). Transgene copy number was estimated by dot blot analysis. There was no correlation between copy number and presence or absence of lacZ staining (data not shown).

To determine whether there was extrarenal expression of the transgene, all tissues from the newborn founder pups were stained with lacZ. Notably, there was no neural expression in any of the mice. In one of the lines that carried four copies of the transgene, there was also staining in whisker follicles and in the epicardium of the heart.

**WT1 Binds to the Enhancer Region In Vitro**

To demonstrate a direct interaction between WT1 protein and the nephrin promoter, an electrophoretic mobility shift assay was performed (Figure 5). A DNA probe corresponding to the 186-bp nephrin "enhancer" was incubated with bacterial expressed and purified GST proteins, and subjected to electrophoresis. No complex was formed with GST alone or GSTAZF (lanes 1 and 3); however, a specific protein-DNA complex was formed when the DNA probe was incubated with GSTWT1 (lane 2). The complex was abolished by incubation with increasing amounts of excess cold competitor probe containing a canonical WT1 binding site (lanes 6 to 7) but was unaffected by a probe that did not contain WT1 binding sites (lanes 4 to 5). Formation of the Neph186-WT1 complex was specifically reduced by a WT1 antibody (lane 9) but unaffected by equal amounts of control IgG (lane 8).

**WT1 Activates a Nephrin-Luciferase Reporter Transgene**

To determine whether WT1 directly activates nephrin expression, four copies of the 186-bp nephrin enhancer were inserted into a promoter-less luciferase reporter (pGL2-neph-luc). The nephrin luciferase reporter was transfected into 293T cells along with expression vectors for wild-type and mutant WT1-A isoform (WTA) (Figure 6). WTA activated the promoter up to 30-fold compared with empty vector control. As expected, WTA-112, a tumor associated missense mutant that is deficient for transcriptional activation, did not activate the nephrin enhancer (18).
Glomerular injury is a leading cause of end-stage kidney failure worldwide. Unfortunately, few successful therapies exist to treat damaged glomeruli. Increased understanding of glomerular-specific gene regulation should provide insight into the molecular basis of renal disease. We demonstrate here that 186 bp of the human nephrin promoter is capable of directing gene expression specifically to the developing and mature podocyte in transgenic mice. We chose to examine this fragment because it is highly conserved between the human and mouse nephrin promoters, with over 80% identity at the nucleotide level. Although mutations have been reported in the nephrin promoter region in three patients with congenital Finnish nephropathy (19,20), they do not occur within this 186-bp region. After the identification of the nephrin gene, another podocyte-restricted gene named podocin was identified as the gene responsible for some cases of autosomal recessive focal segmental glomerulosclerosis (21). Moeller, et al. (22) have previously identified a 2.5-kb fragment of the podocin gene that is also capable of podocyte-specific expression. By using the human genome database and the pairwise BLAST program at NCBI, we also compared the 186-bp fragment to the podocin promoter but were unable to identify a homologous region and suggest that regulation of these two genes may differ. Accordingly, Miner, et al. (23) have shown that podocin expression disappears in lmx1b (a lim-homeodomain containing transcription factor) null mice but nephrin levels remain unaffected. Only 50% of the founder mice expressed the β-galactosidase transgene, and this did not correlate with transgene copy number. The most likely explanation for lack of expression in some founder mice is the site of integration of the transgene within the genome that is not controlled in these experiments. Of note, lacZ expression was detected as early as late S-shape stage glomeruli when podocytes are not fully differentiated. In contrast, both the 1.125-kb human and the 4.125-kb mouse promoter are incapable of driving expression in podocyte precursors (S-shape stage). Why this discrepancy in temporal expression exists is not clear, but endogenous nephrin is expressed in S-shape stage podocytes. Tryggvason and coworkers have reported that kidney regulatory elements reside within the fragment between -2.1 to -4 kb of the mouse nephrin gene, which does not contain the 186-bp fragment reported here (12,22). In a separate paper, the team of Moeller, et al. (22), by using 1.25 kb of the proximal murine nephrin promoter, were able to show podocyte-specific expression. It is likely that endogenous regulation of nephrin expression requires more than one regulatory element.

To date, a number of transcription factors have been identified that are expressed within the developing and mature podocyte (for review, see (24,25)). Null mutations in most of the...
these genes have been generated by using homologous recombination and gene targeting in embryonic stem cells in mice. However, no transcription factor has been shown to be essential for nephrin expression, although its expression is reduced in DNA-binding mutants of the Kreisler (Krn11/MafB) gene (26) and retinoic acid signaling leads to activation of the murine NPHS1 promoter in cultured podocytes (27). The Wilms tumor suppressor protein is a zinc-finger containing transcription factor that is required for early nephrogenesis; null mutant mice die during midgestation with multiple defects and fail to form kidneys or gonads, precluding the analysis of nephrin expression in glomeruli (28). In addition to its expression in the metanephric mesenchyme, WT1 becomes restricted to differentiating and mature podocytes later in nephrogenesis. Rescue experiments that used a human WT1-containing YAC in WT1 null embryos results in mesangial sclerosis or crescentic glomerulonephritis depending on the gene dose (29,30), suggesting that WT1 is required for normal podocyte differentiation and function. Furthermore, patients with point mutations in the WT1 gene develop Denys-Drash syndrome, which is characterized by glomerular defects and nephrotic syndrome, genital anomalies, and Wilms tumor. Also, mice carrying a common Denys-Drash syndrome mutation develop glomerulosclerosis (31). Finally, WT1 mutations have been identified in patients with nephrotic syndrome and sporadic glomerulosclerosis. Although WT1 transcriptional targets were identified in vitro and in vivo (32,33), downstream targets for WT1 in the podocyte have remained elusive. However, NPHS1 expression was dramatically decreased in mice with reduced levels of the WT1 protein (29), suggesting that nephrin is downstream of WT1 and in Drosophila, the WT1 and nephrin orthologues—Klumpfuss (34,35) and IrreC (36)—were shown to interact with differentiating and mature podocytes later in nephrogenesis. Rescue experiments that used a human WT1-containing YAC in WT1 null embryos results in mesangial sclerosis or crescentic glomerulonephritis depending on the gene dose (29,30), suggesting that WT1 is required for normal podocyte differentiation and function. Furthermore, patients with point mutations in the WT1 gene develop Denys-Drash syndrome, which is characterized by glomerular defects and nephrotic syndrome, genital anomalies, and Wilms tumor. Also, mice carrying a common Denys-Drash syndrome mutation develop glomerulosclerosis (31). Finally, WT1 mutations have been identified in patients with nephrotic syndrome and sporadic glomerulosclerosis. Although WT1 transcriptional targets were identified in vitro and in vivo (32,33), downstream targets for WT1 in the podocyte have remained elusive. However, NPHS1 expression was dramatically decreased in mice with reduced levels of the WT1 protein (29), suggesting that nephrin is downstream of WT1 and in Drosophila, the WT1 and nephrin orthologues—Klumpfuss (34,35) and IrreC (36)—were shown to interact genetically. By using the TRANSFAC database and visual inspection of the 83-bp core fragment of homology in the human and murine nephrin promoters, we identified a putative WT1 binding element. Gel shift assays demonstrated that a recombinant WT1 (-KTS) (A isoform) (37), does bind specifically to the nephrin enhancer because it can be competed by excess cold oligonucleotide and can be reduced with antisera to WT1. Furthermore, WT1 is able to transactivate a nephrin-enhancer luciferase reporter gene in vitro.

In summary, we report the first glomerular specific enhancer and demonstrate that WT1 can bind and activate this enhancer in vitro. Identification of this enhancer should facilitate the identification of other cis elements required for glomerular-specific expression through standard approaches such as footprinting and consensus sequence analysis. This element may also be used as the “bait” in yeast-one-hybrid assays or in DNA-Sepharose column purification experiments to pull out additional transacting factors (38) required for nephrin expression. Finally, future studies will determine whether NPHS1 is a direct target for WT1 in vivo.

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