Podocan, a Novel Small Leucine-rich Repeat Protein Expressed in the Sclerotic Glomerular Lesion of Experimental HIV-associated Nephropathy*

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Michael D. Ross‡‡, Leslie A. Bruggeman†, Basil Hanss‡, Masaaki Sunamoto‡, Daniele Marras‡, Mary E. Klotman‡, and Paul E. Klotman‡

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† To whom correspondence should be addressed: Mount Sinai School of Medicine, Annenberg Bldg., Rm. 23-44, Box 1249, One Gustave L. Levy Pl., New York, NY 10029. Tel.: 212-241-7088; Fax: 212-987-0389; E-mail: michael.d.ross@mssm.edu.

‡‡ To whom correspondence should be addressed: Mount Sinai School of Medicine, Divisions of Nephrology and Infectious Diseases, Mount Sinai School of Medicine, New York, New York 10029, Rammelkamp Center for Education and Research, Case Western Reserve University, Cleveland, Ohio 44109, and Laboratorio di Immunologia, Istituto Scientifico Tumori, 16132 Genova, Italy

Growing evidence suggests that human immunodeficiency virus (HIV)-1 infection of podocytes plays a central role in the glomerular disease of HIV-associated nephropathy (HIVAN). As an approach to identify host genes involved in the pathogenesis of the sclerotic glomerular lesion in HIVAN, representational difference analysis of cDNA was used to identify differentially expressed genes in HIV-1 transgenic and nontransgenic podocytes. We isolated a novel member of the small leucine-rich repeat (SLR) protein family, podocan, that is expressed at high levels in the HIV-1 transgenic podocytes. In normal embryonic kidney, a 3.2-kb podocan transcript was detected at low levels, and expression increased dramatically within 24 h following birth. Expression of a 2.3-kb transcript became evident after birth and gradually increased to 50% of the total podocan RNA in the mature kidney. Phylogenetically, podocan represents a new class in the SLR protein family, an expanding protein family sharing homology with the small leucine-rich repeat proteoglycans. The 3.2-kb transcript encodes a predicted 611-amino acid secretory protein with 20 leucine-rich repeats, a unique N-terminal cysteine-rich cluster pattern and a highly acidic C-terminal domain. In situ hybridization of normal kidney revealed podocan mRNA expression in podocytes and likely vascular endothelial cells within the glomerulus. The immunohistochemical staining pattern of podocan protein in normal kidney glomeruli was consistent with that of the glomerular basement membrane, and staining was markedly increased in sclerotic glomerular lesions in the transgenic HIVAN model. Thus, podocan defines a new class within the SLR protein family and is a previously unrecognized component of the sclerotic glomerular lesion that develops in the course of experimental HIVAN.

HIV-associated nephropathy (HIVAN) is now the third leading cause of end-stage renal disease in African Americans between the ages of 20 and 64 (1, 2). HIVAN occurs almost exclusively in Blacks and Hispanics, suggesting that genetic factors play an important role in susceptibility to disease (1, 3–5). The characteristic clinical and histopathologic findings of HIVAN include proteinuria and focal segmental glomerulosclerosis (FSGS), often of the collapsing variant, combined with microcystic tubulointerstitial disease (6–8). The disease untreated can be rapidly progressive, resulting in renal failure often within only weeks or months of the initial diagnosis.

Evidence now indicates that HIV-1 infection of renal epithelial cells plays a direct role in the pathogenesis of HIVAN. Transgenic mice expressing the HIV-1 envelope and regulatory genes under the control of an HIV-1 long terminal repeat promoter develop renal disease that is virtually identical to that seen in humans (9, 10). Reciprocal renal transplantation studies between HIV-1 transgenic mice and nontransgenic littermates reveal that the development of renal disease is intrinsic to the kidney expressing the HIV-1 transgene (11). In human kidney, we demonstrated the presence of HIV-1 mRNA and DNA in renal tubular epithelial cells and parietal and visceral epithelial cells of the glomerulus in HIVAN renal biopsy samples by in situ hybridization and in situ DNA PCR (12). The distribution of HIV-1 RNA expression in patients is remarkably similar to that observed in the transgenic HIVAN model. More recently, PCR was used to amplify HIV proviral sequences from individual renal tubules obtained by laser capture microdissection and from peripheral blood mononuclear cells from the same patients (13). Quasispecies analysis of the HIV-1 gp120 sequences obtained revealed that HIV-1 replication is supported by tubular epithelial cells and that replication in the renal epithelial compartment is distinct from the peripheral blood.

One of the most distinctive pathological features of HIVAN is collapsing FSGS. This morphological feature is accompanied by evidence of HIV-1 infection of podocytes and consequent loss of normal function. The development of the FSGS lesion in HIVAN correlates with the loss or reduction of critical podocyte differentiation markers, most notably in collapsed glomeruli (14, 15). Decreased expression of the cyclin-dependent kinase inhibitors p27 and p57, as well as an increase in the expression of cyclin A and the proliferative marker Ki-67, is also observed.

The abbreviations used are: HIVAN, HIV-associated nephropathy; FSGS, focal and segmental glomerular sclerosis; RDA, representational difference analysis; SLR, small leucine-rich-repeat; SLRP, small leucine-rich-repeat proteoglycan; LRR, leucine-rich repeat; GBM, glomerular basement membrane.
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14–16). Collectively, these changes represent a dysregulated podocyte phenotype characteristic of collapsing FSGS.

In this study, we begin to explore the relationship between HIV-1 gene expression in podocytes and the pathogenic host response that results in the FSGS lesion of HIVAN. To approach this, we used representational difference analysis (RDA) of cDNA to identify host genes that are differentially expressed in HIV-1-transgenic vs. nontransgenic podocytes. We describe here the isolation and characterization of a novel small leucine-rich repeat (SLR) protein that is overexpressed in the sclerotic glomerular lesion of HIVAN. Phylogenetically, podocan represents a new class within this family of proteins that bear in common their homology to the small leucine-rich repeat proteoglycans (SLRPs) (17). We further demonstrate that podocan is expressed in normal glomeruli in a pattern consistent with that of the glomerular basement membrane (GBM) and accumulates at high levels as a previously unrecognized component of the FSGS lesion of HIVAN.

EXPERIMENTAL PROCEDURES

RDA of cDNA—Propagation and differentiation of the conditionally immortalized HIV-1 transgenic and wild-type podocytes were performed as previously reported (18). Prior to all experiments, the podocytes were cultured for 14 days under “nonpermissive” conditions to induce maximal differentiation. RDA of cDNA was performed as described by Hubank and Schatz (19) and carried out through the generation of the second difference product. The nontransgenic driver was supplemented with 4 µg of the plasmid DNA used to generate the HIV-1 transgenic line (pNL4-3-d1443) digested with DpnI.

Cloning the Mouse and Human Podocan cDNAs—The mouse podocan cDNA was isolated by screening a λZapII (Stratagene) phage library, generated from size-selected poly(A)+ RNA from the HIV-1 transgenic podocytes, with a 0.7-kb novel difference product obtained by RDA. The mouse podocan cDNA sequence was used to identify homologous human genomic and expressed sequence tag entries in the GenBankTM database. Oligonucleotide primers were designed from the homologous human genomic and expressed sequence tag entries in the GenBankTM database. Oligonucleotide primers were designed from the mouse podocan cDNA coding sequence 2; sense (5’-gacttgaatggaaggagc-3’), antisense (5’-catctgtttctcttttgct-3’), and sense (5’-cattgccatatcacttcatct-3’) human podocan amplification was achieved from adult human kidney RNA (Stratagene) by reverse transcriptase-PCR using Superscript II reverse transcriptase (Invitrogen) and Pfu turbo DNA polymerase (Stratagene) with the following PCR parameters: 94 °C for 1 min, 59 °C for 45 s, and 72 °C for 2 min for 35 cycles.

Analysis of Podocan RNA Expression—Whole cell RNA was isolated from the cultured podocytes using TRIzol (Invitrogen), and 10 µg of each sample was separated by electrophoresis through a 1.2% agarose gel supplemented with 4 µg of the plasmid DNA used to generate the HIV-1 transgenic line (pNL4-3-d1443) digested with DpnI.

Expression and Detection of Secreted Podocan in Cultured Cells—The HindIII-XhoI fragment of the mouse podocan cDNA was cloned into the pCDNA3.1 (+) vector (Invitrogen) following Klenow blunting of the Ndel and XhoI sites. The resulting expression vector encoding the full-length mouse podocan protein (p-mPCAN) and control vector (pCDNA3.1) were transfected into HEK-293T cells using LipofectAMINE 2000 (Invitrogen). The cells were washed three times in 1× PBS 24 h post-transfection and cultured an additional 24 h in Dulbecco’s modified Eagle’s medium in the absence of fetal bovine serum. Similarly, HIV-1 transgenic and nontransgenic podocytes were washed three times with 1× PBS and cultured 24 h in Dulbecco’s modified Eagle’s medium in the absence of phenol red and fetal bovine serum. Supernatants were harvested, passed through a 0.45-µm syringe filter, and concentrated 10-fold using Microcon YM-10 centrifugal filters (Amicon). The samples (15 µl each) were separated by SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore Corp.). Western blot was performed using a 1:5000 dilution of the affinity-purified anti-podocan antibody and a 1:7500 dilution of horse immunoglobulin G (IgG) (Kirkegaard and Perry). Chemiluminescence for Western blot detection was performed using ECL Western blotting detection reagent (Amerham Biosciences). Immunolocalization of Podocan in Kidney—Kidneys were isolated from adult HIV-1 transgenic and nontransgenic age-matched littermates and frozen in Cryo-Gel embedding medium (Intramedics). Thin sections (5 µm) prepared using the CryoJande Tape-Transfer System (Intramedics) were fixed for 7 min in ice-cold acetone and washed three times in 1× PBS, and then incubated overnight at 4 °C with 1:50 dilution of the antibody followed by incubation for 15 min in 7.5% H2O2. Tissue staining was performed using the TSA Plus DNP (horse-radish peroxidase) and (Cy3) Systems (PerkinElmer Life Sciences). The anti-podocan and preimmune sera were used at a dilution of 1:500 with overnight incubations at 4 °C. The biotinylated goat anti-rabbit IgG (Kirkegaard and Perry) secondary antibody was used at a dilution of 1:400, and the streptavidin/biotinylated goat anti-rabbit IgG (Kirkegaard and Perry) secondary antibody was used at a dilution of 1:500 with overnight incubations at 4 °C. The anti-podocan polyclonal antibody was affinity-puriﬁed using a modification of a procedure described by Hale et al. (20). The Klenow-blunted NeoI-Nhel fragment from the mouse podocan cDNA was cloned into the XhoI site of the pET-15b bacterial expression vector (Novagen) following treatment with the DNA polymerase I Klenow fragment to introduce HpaI and NheI restriction sites. The resulting His-tagged protein was expressed at about 500-ml culture volume, and the bacteria were lysed in 20 ml of BugBuster reagent as per the manufacturer’s protocol supplemented with 1× Complete protease inhibitor mixture (Roche Applied Science) and 100 µg/ml lysozyme (Sigma). The soluble fraction was clarified through a 0.45-µm syringe filter (Millex-HV, Millipore Corp.), imidazole was added to a ﬁnal concentration of 10 mM, and 10 ml was used to hydrate 5 ml of 1× PBS, incubated with the resin for 15 min, and drained by gravity flow. The column was washed with 10 ml of IgG elution buffer (Pierce) followed by 30 ml of 1× PBS. The anti-podocan polyclonal serum (10 µl) was eluted with 30 ml of 1× PBS. To block any unbound cobalt, 1 ml of normal rabbit serum (obtained from Research Genetic for animal prescreening) was diluted with 4 ml of 1× PBS, incubated with the resin for 15 min, and drained by gravity flow. The column was washed with 10 ml of IgG elution buffer (Pierce) followed by 30 ml of 1× PBS. The anti-podocan polyclonal serum (20 ml) was passed three times over the affinity resin followed by a 30-ml wash with 1× PBS. The antibodies were eluted with 10 ml of IgG elution buffer, collected in 1 ml fractions, and adjusted to pH 7.0 with 1 N NaOH. A 1-µl volume from each fraction was then separated by SDS-PAGE. Fractions in which IgG could be visualized by Coomassie staining were then pooled for use in subsequent experiments.

The nucleotide sequences used to design the PCR primers for the amplification of the human podocan coding sequence can be obtained from the GenBankTM data base under the following accession numbers: 5′ sequences, GenBankTM accession numbers AL445183, BG721399, and AC022728; 3′ sequence, GenBankTM accession number AW361218.
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RESULTS

Identification of Candidate Genes in HIVAN Pathogenesis—RDA of cDNA was used to identify host genes that were differentially regulated in podocytes expressing an HIV-1 transgene. More than 250 difference products were analyzed corresponding to 29 differentially expressed genes between the cell populations. Included in the differentially expressed genes were several adhesion molecules, cell surface receptors, transcription factors, known extracellular matrix components, and pre-senescence markers. Included in the differentially expressed genes were several adhesion molecules, cell surface receptors, transcription factors, known extracellular matrix components, and pre-senescence markers.

Northern blot analysis of podocan RNA from the conditionally immortalized podocytes was performed using the 0.7-kb difference product isolated from the RDA procedure as a probe. Hybridization of a 3.2-kb mRNA transcript was seen only in the HIV-1 transgenic podocytes (top panel). Ethidium bromide staining of the RNA prior to transfer is shown to demonstrate that an equivalent amount of RNA from both podocyte cultures was used (bottom panel).

Podocan Defines a New Fifth Class in the SLR Protein Gene Family—Phylogenetic analysis of podocan with the known mouse SLR proteins indicates that podocan defines a new class within this protein family (Fig. 2B). Although podocan shares much of its structural organization with the well characterized proteoglycans within this family, it is unique in many respects. Each SLRP family member contains a class-specific N-terminal cysteine-rich cluster preceding its leucine-rich repeat (LRR) domain. Podocan exhibits a unique cysteine-rich cluster pattern of C\textsubscript{X\textsubscript{C}}\textsubscript{X\textsubscript{C}}\textsubscript{X\textsubscript{C}}\textsubscript{C}, where X denotes any amino acid and the subscript indicates the number of intervening residues (Fig. 2A). Based on studies by Matsushima et al. (21) of tandem LRR evolution within the SLRP family, the central domain of SLRPs can be characterized by a series of type S and/or type T LRRs. Class I and II proteins contain 12 LRRs composed of four tandem STT superrepeat units. Class III proteins have seven LRRs organized as four ST superrepeats, while the second superrepeat lacks the type S LRR. Chondroadherin, the only identified member of class IV, is composed of 10 type T LRRs. Although podocan shares the STT superrepeat structure of the class I and II proteins, the LRR domain of podocan is much larger, containing seven tandem STT superrepeats (Fig. 2A). Due to a nonconserved substitution of the terminal leucine with glutamic acid in the C-terminal type T LRR, however, we have determined that podocan contains 20 and not 21 complete LRRs. In addition, podocan lacks the C-terminal cysteine residues present in other SLRP family members and has a distinct acidic C-terminal domain.

In addition to differences in protein primary structure, podocan also differs from the class I–IV SLRPs with respect to genomic organization. Alignment of the mouse podocan cDNA with the newly available mouse genomic data base at the National Center for Biotechnology Information indicates that the gene is composed of 13 exons, including nine coding exons, residing on chromosome 4 (data not shown). Human podocan was found to share all of the features described for the mouse protein and also conserves the genomic organization of its coding exons (Fig. 2A and data not shown). This analysis leads us to conclude that podocan is the first identified member of a new class V SLR protein.

Podocan Expression in the Kidney Is Developmentally Regulated—Analysis of podocan expression by Northern blot of normal mouse kidney RNA indicated that two podocan transcripts were expressed with distinct temporal regulation (Fig. 3). Expression of the cloned 3.2-kb podocan transcript was evident in the kidney at low levels from embryonic day 14 through birth (Fig. 3). A dramatic increase in the expression of the 3.2-kb transcript occurred within the first 24 h of life, and maximal levels coincided with the completion of nephrogenesis at ~2 weeks of age. Expression of an additional podocan transcript 2.3 kb in size was evident in the second week of life. In the adult kidney, expression levels of the 3.2- and 2.3-kb transcripts were approximately equivalent. Efforts to isolate and characterize the 2.3-kb transcript are ongoing.

Glomerular Distribution of Podocan mRNA Expression in the Kidney—RNA in situ hybridization was performed to localize podocan expression in the normal adult mouse kidney. Staining of the hybridized riboprobe appeared restricted to the glomeruli of the adult kidney (Fig. 4). Under high magnification, staining of podocan RNA was clearly evident in the cytoplasm of podocytes within the glomerulus. Staining also appeared to be present in the cytoplasm of vascular endothelial cells within the glomerulus. The observed expression pattern of podocan within the glomerulus suggested that it may be a previously unrecognized component of the mature glomerular basement membrane.
Fig. 2. Alignment of the mouse and human podocan protein sequences (A) and phylogenetic analysis of podocan with the known mouse SLR proteins (B). A, identical amino acids in the mouse and human podocan proteins are shown in blue lettering. Nonidentical amino acids are shown in black lettering with conserved substitutions shaded in gray. The signal sequences and cleavage sites (arrow) were predicted using SignalP version 1.1 available from the Center for Biological Sequence Analysis. Characterization of the LRRs was based on the general consensus sequences for type S repeats (xxaPzxLPxxLxxLxxNxL) shaded in red and type T repeats (zzxxaxxxxFxxaxxLxxLxxNxL) shaded in blue. In both sequences “x” indicates variable residues; “z” is frequently a gap, a is Val, Leu, or Ile; and “I” is Ile or Leu. In LRRs that deviate from the consensus sequence, the consensus amino acid appears above the aligned sequences. All of the conserved substitutions can be found in the SLRP proteins from which the consensus sequences were derived with the exception of the substitution of Phe with Trp in the 12th type T repeat. Due to the nonconservative substitution of Lys with Glu in the final position of the 14th type T repeat shown, we do not consider it a true LRR and therefore conclude that podocan has a total of 20 and not 21 LRRs. Potential sites for N-linked glycosylation are circled. B, horizontal distances of the bars are proportional to the predicted evolutionary distance of the available mouse SLR protein sequences. Subdivision by class and the class-specific C-terminal cysteine-rich cluster patterns are also shown. This analysis was performed using ClustalW, and the resulting dendrogram was generated using DRAWGRAM on Biology Workbench 3.2 available from the San Diego Supercomputing Center.
Detection of Secreted Podocan in Cultured Cell Supernatants—To characterize podocan expression at the protein level, polyclonal antibodies were raised against a glutathione S-transferase fusion protein containing the N-terminal 203 amino acids of the mouse podocan protein. The antiserum was subsequently affinity-purified and used for Western blot analysis of serum-free cell culture supernatants. In the supernatant of both HIV-1 transgenic podocytes and HEK-293T cells transfected with a mouse podocan expression vector, a single protein with an apparent molecular mass of 95 kDa was detected (Fig. 5). No reactivity was seen with supernatants from the non-transgenic podocytes or vector control-transfected HEK-293T cells. The disparity between the predicted molecular mass of the secreted core protein of 66 kDa and the observed molecular mass of 95 kDa indicates that podocan is probably subject to post-translational modification. Furthermore, these results confirm that podocan is secreted from the podocyte.

Distribution of Podocan Protein in the Normal Kidney and in the Transgenic HIVAN Model—Immunodetection was performed to localize podocan protein in frozen kidney sections of normal adult and HIV-1 transgenic adult mice. In normal kidney, podocan was seen in the glomerulus in a pattern consistent with GBM staining (Fig. 6). Podocan staining was also observed in the afferent arterioles and interlobular arteries in sections where these structures were seen. This latter finding suggests that the vascular endothelial cells of the glomerulus and proximal vessels probably contribute to the deposition of podocan in their associated basement membranes.
In the HIV-1 transgenic kidney, podocan staining in normal appearing glomeruli was comparable with the nontransgenic kidney (Fig. 6). In contrast, affected glomeruli showed a marked accumulation of podocan protein in sclerotic lesions. In the HIV-1 transgenic kidney sections evaluated, few glomeruli could be identified that did not have some degree of focal podocan accumulation. No staining was observed in normal or HIV-1 transgenic kidney using preimmune serum from the animal in which the anti-podocan antibodies were raised. Our results indicate that podocan is dysregulated in diseased glomeruli in the HIV-1 transgenic mouse and is an integral component of the FSGS lesion.

**DISCUSSION**

The objective of our study was to better understand how HIV-1 gene expression in podocytes leads to the FSGS lesion of HIVAN. Using RDA of cDNA, we identified a novel SLR protein that is expressed at high levels in HIV-1 transgenic podocytes. Immunohistochemical localization of podocan in normal kidney demonstrates that it is expressed in glomeruli in a pattern consistent with that of the GBM. Staining of afferent arterioles (black arrows) and interlobular arteries (black arrowhead) was also seen (B). In the HIV-1 transgenic kidney (D–F), the distribution of podocan in nondiseased glomeruli was comparable with the nontransgenic kidney (E, arrowhead). In affected glomeruli, a marked increase in podocan deposition was clearly evident in the sclerotic glomerular lesions (E and F, arrows). To illustrate the increased level of podocan deposition in the FSGS lesions of HIV-1 transgenic kidneys (red fluorescence, G–I), confocal images of a nondiseased glomerulus (G) and two sclerotic glomeruli (H and I) are shown. To allow a direct comparison, all three images were taken from the same section using identical settings on the confocal microscope. The sections were co-labeled for laminin A chain (green fluorescence) to delineate Bowman’s capsule. No staining was detected in glomeruli (asterisks), afferent arterioles, or interlobular arteries with the preimmune serum from the animal in which the anti-podocan antibodies were raised (A and D).

**Fig. 6. Immunolocalization of podocan protein in normal mouse kidney and in diseased kidney from the HIV-1 transgenic HIVAN model.** In the normal mouse kidney (A–C) podocan staining was observed surrounding capillary loops in the glomerulus in a pattern consistent with that of the GBM (B and C, white arrowheads). Staining of afferent arterioles (black arrows) and interlobular arteries (black arrowhead) was also seen (B). In the HIV-1 transgenic kidney (D–F), the distribution of podocan in nondiseased glomeruli was comparable with the nontransgenic kidney (E, arrowhead). In affected glomeruli, a marked increase in podocan deposition was clearly evident in the sclerotic glomerular lesions (E and F, arrows). To illustrate the increased level of podocan deposition in the FSGS lesions of HIV-1 transgenic kidneys (red fluorescence, G–I), confocal images of a nondiseased glomerulus (G) and two sclerotic glomeruli (H and I) are shown. To allow a direct comparison, all three images were taken from the same section using identical settings on the confocal microscope. The sections were co-labeled for laminin A chain (green fluorescence) to delineate Bowman’s capsule. No staining was detected in glomeruli (asterisks), afferent arterioles, or interlobular arteries with the preimmune serum from the animal in which the anti-podocan antibodies were raised (A and D).

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development of the sclerotic lesion in HIVAN (28–32). It has been shown that decorin, administered as either purified protein or using a gene therapy approach, can reduce the development of glomerular sclerosis in an anti-Thy-1 model of glomerulonephritis (33, 34). Furthermore, increased glomerular expression of decorin, biglycan, fibromodulin, and lumican has been demonstrated in several forms of renal disease (35–37). Thus, increased expression of decorin and other SLRP's capable of binding transforming growth factor-β may be a protective mechanism in renal disease.

The role of SLRPs in modulating collagen fibrillogenesis is perhaps the most well established function of SLRP proteins. Whereas several family members have been shown to directly interact with collagen fibrils in vitro (38, 39) and alter fibril formation (40–43), the most striking evidence comes from the study of transgenic animals with targeted disruption of specific SLRP genes. Individual targeted disruptions of decorin, fibromodulin, lumican, or biglycan all result in ultrastructural abnormalities of the collagen fibrils with which they are associated (44–47). Although further studies will be necessary to confirm the localization of podocan within the glomerulus, we have postulated that it is a component of the GBM. We have demonstrated that podocan is secreted from podocytes cultured in vitro and is present in the glomerulus in a pattern consistent with that of the GBM. Although a similar staining pattern would be observed by confocal microscopy if podocan were localized to podocyte foot processes or the slit diaphragm, staining of podocan in the proximal vasculature outside of the glomerulus suggests that this is unlikely. As such, podocan may in part serve to modulate fibrillogenesis in the mature GBM and help to maintain the integrity of the glomerular filtration barrier.

Studies to localize podocan in the kidney by electron microscopy and to determine the ability of podocan to interact with type IV collagen are ongoing. As can be seen with proline/arginine-rich end leucine-rich repeat protein (48, 49) and as is probably true of asporin (50, 51), podocan is probably secreted as a glycoprotein and not a proteoglycan. The mouse podocan protein does not contain a serine/glycine dipeptide consensus sequence for glycosaminoglycan attachment as is typical of SLRPs in the N-terminal region preceding the LRR domain. Furthermore, Western blot analysis of the secreted mouse podocan protein reveals a sharp band migrating with an apparent molecular mass only 30 kDa greater than the predicted molecular mass of the core protein (Fig. 4). This disparity may be the result of that of the GBM proteoglycans are critical to maintaining charge selection of the glomerular filtration barrier (52). Determining the molecular interactions of podocan in the GBM with collagen and the highly charged proteoglycans may prove insightful to understanding the architecture necessary for maintaining both size and charge selection of the glomerular filter. Furthermore, it may help elucidate how the dysregulation of podocan expression in HIVAN may contribute to the pathogenesis of FSGS.

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