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Article

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Nephritogenic mAb 5-1-6 is directed at the extracellular domain of rat nephrin

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Introduction

This report represents the confluence of two independent lines of investigation of proteinuric disorders: one an inherited form of the nephrotic syndrome in humans, and the other an antibody-induced model of nephrotic syndrome in rats. After the advent of hybridoma technology, several mAb's were developed to identify potential nephritogenic glomerular antigens. Arguably, the most interesting of these antibodies is mAb 5-1-6 because of its unique ability to produce massive proteinuria when injected into rats (1). It was produced by immunizing a mouse with isolated rat glomeruli and was selected because of its reactivity with rat glomeruli on immunofluorescence. Proteinuria develops immediately, without complement activation or leukocyte recruitment, and occurs without ultrastructural alterations in glomerular morphology apart from mild, focal foot process effacement (1). Immunohistological analysis has demonstrated redistribution of mAb 5-1-6 staining coincident with the development of proteinuria, which suggests that its antigen is critically involved in the maintenance of the permselective barrier function of the glomerulus (1). Immunoelectron microscopy has localized the target antigen to the podocyte slit-diaphragm and outer surface of the adjacent plasma membrane (2, 3), but its identity has remained elusive for 10 years.

In 1998, the gene mutated in congenital nephrotic syndrome of the Finnish type (NSF1) was cloned (4). The product of this gene, nephrin, is a 1,241–amino acid transmembrane protein of the immunoglobulin superfamily. Published in situ hybridization data suggested that gene expression is limited to glomerular epithelial cells. Subsequent immunoelectron microscopy studies have localized nephrin to the slit-diaphragm (5), exactly akin to our observations with mAb 5-1-6 (2).

The slit-diaphragm is a continuous membranelike structure that spans the filtration slits between adjacent foot processes of mature glomerular epithelial cells (GECs). Until the discovery that the extracellular domain of nephrin is a component of the slit-diaphragm, little was known about its composition. The only other protein known to be associated with the slit-diaphragm was the tight junction protein, zonula occludens-1 (ZO-1), which resides on the cytoplasmic face (6) and redistributes in response to mAb 5-1-6 injection and other agents that alter the slit-diaphragm (2, 7). Despite uncertainty about its structure and composition, there is general agreement that the slit-diaphragm lies in the pathway of solute and water filtration. More contentious has been the question as to what extent it forms the final barrier to filtration of plasma proteins (8). Ultrastructural studies with variously charged ferritin tracers showed a charge-depend-

ent penetration of the glomerular capillary wall, but even the most cationic of these macromolecules failed to cross the slit-diaphragm (9). In addition, it is quite evident that IgG antibodies are able to reach target antigens on the podocyte, mAb 5-1-6 being a prime example. Thus, although these observations suggest that the podocyte slit-diaphragm forms the final barrier to macromolecular permeability, and mutations of nephrin are associated with massive nephrotic syndrome at birth (3, 10), they do not provide conclusive evidence that alterations in the slit-diaphragm itself are responsible for the development of proteinuria. In the studies reported here, we demonstrate that the slit-diaphragm–reactive nephritogenic mAb 5-1-6 identifies the extracellular domain of nephrin, thereby documenting the importance of the slit-diaphragm and its component, nephrin, in the regulation of glomerular permselectivity.

Methods

Antibodies. Ascitic fluid containing mAb 5-1-6 was produced in mice primed with 2,6,10,14-tetramethylpentadecane (Sigma Chemical Co., St. Louis, Missouri, USA) and injected intraperitoneally with a mouse IgG1 hybridoma prepared as described previously (1). This fluid was subjected to 50% ammonium sulfate precipitation, and the immunoglobulin-rich fraction was dialyzed against PBS (0.9% NaCl in 10 mM sodium phosphate buffer [pH 7.4]) for 2 days and stored at –80°C. An irrelevant mouse monoclonal IgG1 antibody, RVG-1, was treated in the same way and used as a control. Rabbit antibody to the complete cytoplasmic domain of mouse nephrin was raised by immunizing rabbits with a hexahistidine-tagged peptide expressed in transformed *Escherichia coli* (11). A mouse nephrin cDNA fragment encoding the COOH-terminal 155 amino acids was amplified by PCR, directionally cloned into the bacterial expression vector, pRSETA (Invitrogen Corp., San Diego, California, USA), and transfected into *E. coli.* The expressed fusion protein was purified from the lysed bacteria on a nickel-agarose column and used to immunize rabbits by intramuscular injection. A 50% ammonium sulfate precipitate was prepared from the antiserum after the third immunization and dialyzed against PBS before use in these studies. Western blotting studies have shown that this antiserum specifically identifies a 185-kDa protein in mouse, rat, and human glomerular extracts, and, on immunoelectron microscopy, it localizes to the lateral surfaces of the podocyte foot processes in the filtration slits (11). Normal rabbit serum was used as a control. CY3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) and FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) were used for immunofluorescence microscopy, and horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, Alabama, USA) and HRP-conjugated goat anti-rabbit IgG (Sigma Chemical Co.) were used for Western blotting.

Animals. Normal adult Wistar rats weighing 150–175 g were purchased from Charles River Laboratories (Wilmington, Massachusetts, USA). For large-scale glomerular isolation, frozen Sprague-Dawley rat kidneys were purchased from Pel Freez (Rogers, Arkansas, USA).

Materials. All materials were purchased from Sigma Chemical Co. unless otherwise stated.

Preparation of material for protein sequencing and 2-dimensional gel electrophoresis. The identification of the mAb 5-1-6 antigen has been hampered by the absence of reactivity on Western blotting. In addition, preliminary studies had shown that conventional immunoprecipitation of glomerular extracts with mAb 5-1-6 would not yield sufficient material for peptide sequence analysis. To overcome these problems, we adopted the following procedure. The idea was based on our repeated observation that mAb 5-1-6 binds to its antigen in situ in isolated glomeruli, where it can be readily detected by immunohistological techniques (2). Therefore, we reasoned that if we stabilized the antigen/antibody complex with an impermeant, cleavable chemical cross-linker, we should be able to detect an upshifted band of mouse IgG bound to the antigen when the complex is resolved on SDS-PAGE under nonreducing conditions. In addition, by submitting the complex to 2-dimensional (2D) gel electrophoresis under reducing conditions, the cross-link would be disrupted, releasing of

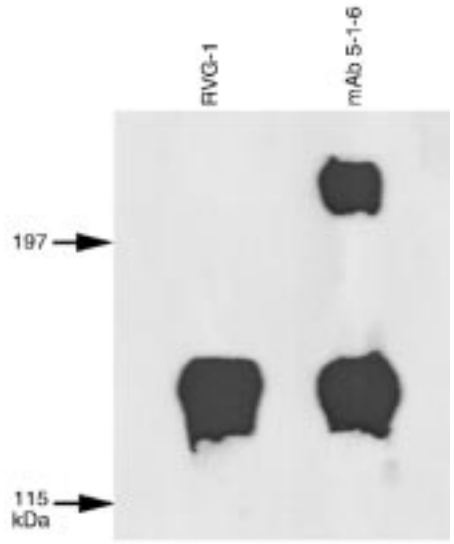

Figure 1
Representative Western blot analysis for mouse IgG in cross-linked immunoprecipitates. Isolated Sprague-Dawley rat glomeruli were incubated with either mAb 5-1-6 or RVG-1. After washing, the antibody/antigen complex was cross-linked with DTSSP followed by detergent solubilization and immunoprecipitation with agarose bound goat anti-mouse IgG. The precipitates were resolved by nonreducing 5% SDS-PAGE, electroblotted to nitrocellulose, immunoblotted for mouse IgG, and developed using a chemiluminescent technique. A mouse IgG–containing band is visible at 150 kDa in both lanes, consistent with uncomplexed mouse mAb 5-1-6 or RVG-1. In addition, a band is specifically present in the mAb 5-1-6 IP lane at approximately 300 kDa, which represents the cross-linked complex of mAb 5-1-6 and its antigen.

the antigen from the heavy and light chains of IgG. An impermeant cross-linker was used to avoid the risk of coprecipitating putative cytoplasmic binding proteins.

Glomeruli were isolated by differential sieving from 20 frozen Sprague-Dawley rat kidneys. After blocking with 1% BSA in PBS with protease inhibitors (PI) (1 mM phenylmethylsulfonyl fluoride, 5 μg/mL soybean trypsin inhibitor, 4 mM *N*-ethylmaleimide, and 5 mM benzamidine hydrochloride) for 1 hour at 4°C, the glomeruli were incubated with 2 mg/mL of either mAb 5-1-6 or RVG-1 in blocking buffer for 1 hour at 4°C. After 5 washes in PBS/PI, the glomeruli were incubated with the chemical cross-linker dithiobis(sulfosuccinimidylpropionate) (1 mM DTSSP; Pierce Chemical Company, Rockford, Illinois, USA) for 30 minutes at room temperature. Reactive sites were quenched with the addition of 1 M Tris (pH 8.0) to a final concentration of 50 mM for a further 15 minutes. After 3 washes with PBS, the glomeruli were solubilized in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in 50 mM Tris, and 150 mM NaCl [pH 8.0]) with PI. The detergent extract was centrifuged at 16,000 *g* in a desktop microfuge for 30 minutes to remove insoluble material, and the supernatant was incubated with agarose-conjugated rabbit anti-mouse IgG to precipitate the stabilized antigen/antibody complex. The pellet was washed twice with each of the following buffers: high-stringency buffer (HSB; 0.1% SDS, 1% deoxycholate, 0.5% Triton-X 100, 20 mM Tris-HCl [pH 7.5], 120 mM NaCl, 25 mM KCl, 5 mM EDTA, 5 mM EGTA, and 0.1 mM DTT), HSB with 1 M NaCl, and low-salt buffer (2 mM EDTA, 10 mM Tris-HCl [pH 7.5], and 0.5 mM DTT). After solubilization in 100 μL of nonreducing SDS-PAGE sample buffer and centrifugation, 10 μL of the supernatant was subjected to SDS-PAGE. The resolved proteins were electroblotted to nitrocellulose membranes (Micron Separations Inc., Westborough, Massachusetts, USA) or stained in the gel with Coomassie Brilliant Blue. The location of mouse IgG was determined by Western blotting. The nitrocellulose membranes were blocked with 5% nonfat milk in Tris-buffered saline/Tween 20 (TBST; 50 mM Tris, 150 mM NaCl, 0.2% Tween 20 [pH 7.6]) for 1 hour followed by incubation with HRP-conjugated goat anti-mouse IgG (1:2,000) for 1 hour. After the membranes were washed 3 times with TBST, immunoreactive proteins were identified using a chemiluminescent technique (Supersignal Chemiluminescent Substrate; Pierce Chemical Co.). Reactive bands were used to identify the relevant bands on the Coomassie-stained gel. The same protocol was used to prepare the samples for 2D-gel analysis, except that glomeruli were isolated from 100 frozen kidneys and the final immunoprecipitate was solubilized in 50 μL of reducing sample buffer. The residual pellet was washed twice with water, and the supernatants were pooled with the solubilized sample, lyophilized, and resuspended in 50 μL of water.

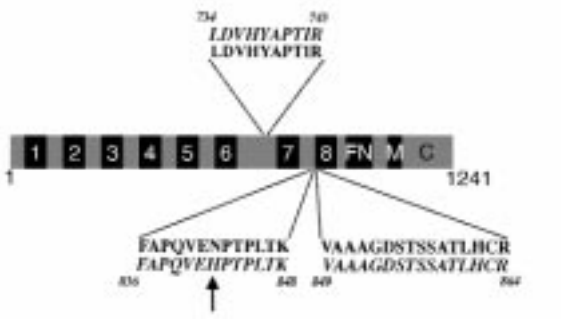

Figure 2
Schematic representation of nephrin demonstrating the origin of the peptide sequences identified by MS/MS in immunoprecipitates of rat glomeruli. The sequence above nephrin was obtained from the cross-linked immunoprecipitate, and the 2 below were identified from the 185-kDa 2D-gel spot. The corresponding human sequence is shown in italics. The arrow highlights the single amino acid mismatch between the MS/MS–derived sequence and human sequence. In the nephrin schematic, numbered black regions indicate the Ig-like domains; FN, the fibronectin type III–like domain; M, the transmembrane domain; and C, the cytoplasmic domain.

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell by Kendrick Labs Inc. (Madison, Wisconsin, USA) (12). Five microliters of the solubilized immunoprecipitate was subjected to isoelectric focusing in glass tubes of 2-mm inner diameter using 2% (pH 4–8) ampholines (BDH; Hoefer Scientific Instruments, San Francisco, California, USA) for 9,600 volt-hours. An internal isoelectric focusing standard, tropomyosin (50 ng, pI 5.2, molecular weight [MW] 33,000) was added to each sample. The tube gel pH gradient was determined with a surface pH electrode. After equilibration for 10 minutes in buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, 62.5 mM Tris [pH 6.8]), the tube gel was sealed to the top of a stacking gel, which in turn was on top of a 10% acrylamide slab gel (0.75-mm thick). Molecular weight standards were added to the agarose that sealed the tube gel in place: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000) (Sigma Chemical Co.). SDS slab gel electrophoresis was carried out for 4 hours at 12.5 mA/gel. The slab gels were either stained with Coomassie Brilliant Blue R-250 or fixed overnight in 10% acetic acid/50% methanol and silver stained.

Protein identification from 1-dimensional and 2D gel bands by mass spectrometry. Protein bands of interest were excised from the SDS-PAGE and 2D slab gels and subjected to in-gel proteolytic digestion with trypsin according to the procedure by Hellman et al. (13).

Liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) spectra were collected on a Finnigan MAT (San Jose, California, USA) LCQ mass spectrometer fitted with a custom design LC source for delivery of ultra low flow rates (120–180 nL/min). Peptides resulting from the in-gel digests were separated using a 75-μm ID × 360-μm OD fused silica column packed in-

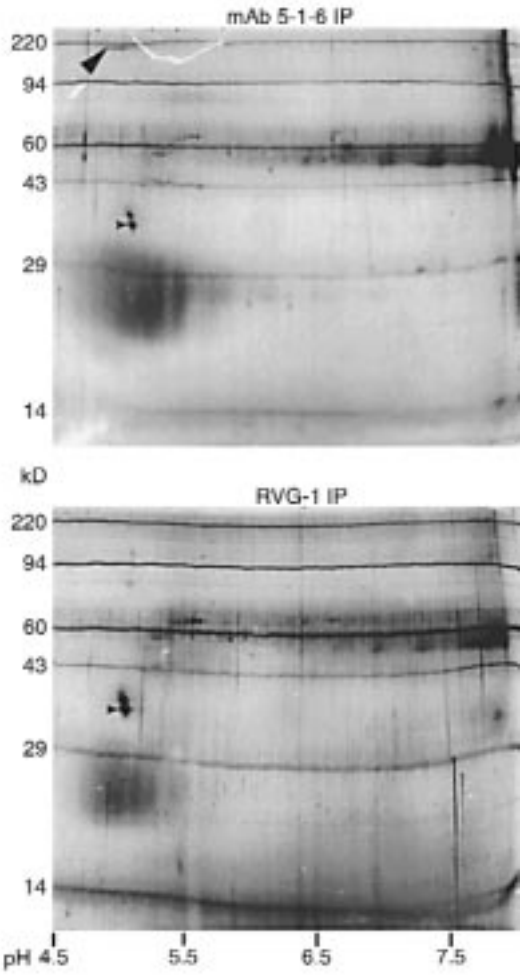

Figure 3
Silver-stained 2-dimensional gels. Isolated Sprague-Dawley rat glomeruli were incubated with either mAb 5-1-6 or RVG-1. After washing, the antibody/antigen complex was cross-linked with DTSSP followed by detergent solubilization and immunoprecipitation with agarose bound goat anti-mouse IgG. The precipitates were then resolved by 2D gel electrophoresis under reducing conditions, and the gels were stained with silver. The pH of the electrofocusing gel was recorded, and the values are shown below the lower gel. The smaller arrowheads indicate the tropomyosin internal control (pI 5.2; 33,000 kDa). The large arrowhead indicates a spot at 185 kDa, pI 4.95–5.2 that is specifically immunoprecipitated by mAb 5-1-6. The large anionic silver-stained spot below the 29-kDa marker represents IgG light chain and the series of cationic spots below the 60-kDa marker are IgG heavy chain in both the mAb 5-1-6 and control gels.

house at Millennium Pharmaceuticals with POROS 10R2 reverse-phase material (Perceptive Biosystems, Framingham, Massachusetts, USA), and a gradient of 5–65% acetonitrile in 30 minutes, 65–80% acetonitrile in 5 minutes, in 0.1 M acetic acid. The LCQ was operated in a data-dependent mode whereby MS scans were alternated with automated collection of MS/MS for the top most intense precursor. Maximum inject time was set to 400 ms. All MS/MS spectra were searched against a nonredundant, protein database from the National Center for Biotechnology Information (NCBI) using MS-Tag (14).

Conventional immunoprecipitation for antinephrin immunoblotting. Although conventional immunoprecipitation techniques did not isolate sufficient material for sequencing, we suspected that mAb 5-1-6 was able to precipitate some antigen after extraction. With the availability of a specific antinephrin antibody, we sought the presence of nephrin in the precipitate by Western blotting. Glomeruli were isolated by differential sieving from 10 frozen kidneys and solubilized with 0.5% Triton X-100 in PBS (pH 7.4) containing 0.1% BSA and PI for 30 minutes on ice. After centrifugation, the supernatant was precleared with 50 μL protein G-agarose (GIBCO BRL, Gaithersburg, Maryland, USA) for 1 hour at 4°C. The precleared supernatant was incubated with 2 mg/mL of either mAb 5-1-6 or RVG-1 overnight at 4°C followed by 50 μL protein G-agarose for 1 hour. After centrifugation, the pellet was washed 5 times with PBS. The washed pellet and pre- and postprecipitation samples of the glomerular extract were boiled in SDS sample buffer containing dithiothreitol for 5 minutes and centrifuged, and 10 μL of each supernatant was subjected to SDS-PAGE using 7.5% or 5% gels. After electroblotting to nitrocellulose and blocking in 4% nonfat milk in PBS for 1 hour, the membrane was incubated with the polyclonal antinephrin antibody (1:1,000 dilution in blocking reagent) for 1 hour at room temperature and then washed 3 times with TBST. Rabbit IgG was detected using HRP-conjugated goat anti-rabbit IgG (1:5,000 dilution) for 1 hour. After a further series of washes in TBST immunoreactive proteins were detected using the chemiluminescent technique. Autoradiographs were scanned into Adobe Photoshop 4.01 (Adobe Systems Inc., Mountainview, California, USA) and densitometry was measured with NIH Image software (version 1.61; National Institutes of Health, Bethesda, Maryland, USA).

Immunofluorescence. Snap-frozen kidney tissue from normal Wistar rats was examined. For double-labeling indirect immunofluorescence, 5-μm cryostat sections were blocked with 1% BSA in PBS for 30 minutes. Normal kidney cryosections were incubated sequentially with mAb 5-1-6 (1:50) and rabbit anti-nephrin (1:200) for 1 hour each. After several washes with PBS, CY3-conjugated donkey anti-mouse IgG (1:800) and FITC-conjugated goat anti-rabbit IgG (1:100) were added sequentially for 1 hour each. Lack of spectral overlap and cross-reactivity of the secondary antibodies was verified by exclusion of the respective primary antibody. The sections were examined by epifluorescent microscopy using a Nikon 40× Plan Apo oil-immersion lens (Nikon Inc., Melville, New York, USA). The images were captured with a Spot CCD camera (Diagnostic Instruments, Inc., Sterling Heights, Michigan, USA) and exported into Adobe Photoshop. After construction of a composite plate, color output was generated using a Kodak ColorEase thermal dye sublimation printer.

Results

Cross-linked immunoprecipitation of mAb 5-1-6/antigen complex. Figure 1 shows a representative Western blot for mouse IgG of the DTSSP cross-linked immunoprecip-

itate following SDS-PAGE under nonreducing conditions. In the mAb 5-1-6 lane, a mouse IgG band is identified at 150 kDa, representing uncomplexed mAb 5-1-6. A second, upshifted band is also present at approximately 300 kDa in this lane, which represents the complex of mAb 5-1-6 with its antigen. A 150-kDa band is also identified in the RVG-1 control lane, presumably as a result of nonspecific glomerular binding. In contrast to the mAb 5-1-6 lane, however, there is no upshifted mouse IgG–containing complex, which indicates that the 300-kDa complex is the result of specific interaction between mAb 5-1-6 and its antigen.

MS analysis of mAb 5-1-6 immunoprecipitates. The same immunoprecipitates as shown in Figure 1 were also resolved by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. After destaining with 30% methanol, the upshifted band was excised and subjected to in-gel proteolytic digestion with trypsin. As a control, the 150-kDa band corresponding to uncomplexed mAb 5-1-6 was excised and processed similarly. Both the control and upshifted bands were analyzed by MS (by T.A. Addona and K.B. Charron). As expected, several peptides of IgG were identified in the 300-kDa complex. In addition, a single peptide, 10 amino acids in length (LDVHYAPTIR) with complete identity to a sequence in the extracellular domain of the published human nephrin sequence, was identified (Figure 2). Finally, a single peptide with homology to rat actin was also identified in the 300-kDa complex. All MS/MS spectra were analyzed, and no other relevant protein sequences were identified. In particular, the nephrin sequence was the only transmembrane protein present. Only IgG sequences were obtained from the 150-kDa band of mAb 5-1-6.

To specifically isolate the protein of interest and simplify the MS/MS analysis, we resolved the mAb 5-1-6 immunoprecipitate by 2D gel electrophoresis. Under reducing conditions, the DTSSP is cleaved and the complex dissociates into its individual components. Figure 3 shows a silver-stained 2D-gel of the mAb 5-1-6 immunoprecipitate with its control for comparison. A spot is clearly visible just below the 220-kDa marker with a pI of 4.95–5.2 in the mAb 5-1-6 gel. No other specifically stained areas were identified in the mAb 5-1-6 gel, and no equivalent spot was present in the control gel. An identical, but fainter, spot was viewed at 185 kDa in a Coomassie blue-stained gel from a second experiment (not shown). The 185-kDa spot was excised from the silver-stained gel, together with the corresponding area from the control gel and analyzed by LC-MS/MS following in-gel proteolytic digestion with trypsin. Two peptides were identified through database matching from the 185-kDa gel band. One peptide had 100% sequence identity to human nephrin (VAAAGDSTSSATLHCR). The second peptide was observed to have strong homology to another sequence from human nephrin (H7toN: FAPQVENPTPLTK). Both peptides were identified through database searching of the MS/MS spectra with MS-Tag in identity and homology modes (Figure 2).

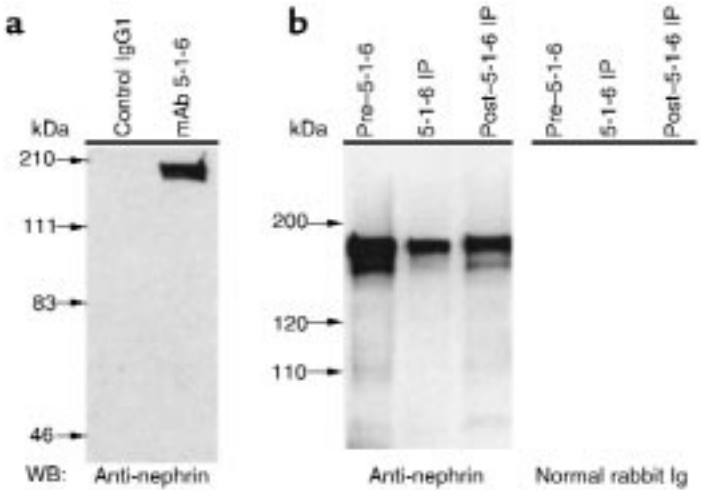

Figure 4

Representative Western blot analysis of nephrin in non–cross-linked mAb 5-1-6 and RVG-1 immunoprecipitations. (**a**) A Triton X-100 glomerular extract was incubated with either mAb 5-1-6 or RVG-1 followed by Protein G-agarose. The washed pellets were solubilized in reducing sample buffer and resolved by 7.5% SDS-PAGE. After electroblotting to nitrocellulose, the membrane was incubated with rabbit antinephrin (1:1,000) followed by HRP-conjugated goat anti-rabbit IgG (1:2,000). Reactive proteins were detected using a chemiluminescent technique. A specific antinephrin reactive protein of 185 kDa is present in the mAb 5-1-6 precipitate lane and is absent from the control lane. (**b**) A Triton X-100 glomerular extract was incubated with mAb 5-1-6 followed by Protein G-agarose. The washed pellet was solubilized in reducing sample buffer and resolved by 5% SDS-PAGE together with equal samples (10 μL/lane) from the glomerular extract before and after the mAb 5-1-6 IP. After electroblotting to nitrocellulose, the membrane was incubated with rabbit anti-nephrin (1:1,000) or normal rabbit Ig (1:1,000) followed by HRP-conjugated goat anti-rabbit IgG (1:5000). Reactive proteins were detected by chemiluminescence (antinephrin: 1- to 2-second exposure; normal rabbit Ig: 30-second exposure). In addition to the 185-kDa band in the mAb 5-1-6 precipitate lane, there is a double band in the glomerular extract lanes with partial depletion of nephrin from the postprecipitation lane (densitometry of the upper band pre- versus postprecipitation: 174 ± 20.9 versus 137 ± 24.0; $n = 5$, mean ± SEM, $P < 0.02$, paired t test).

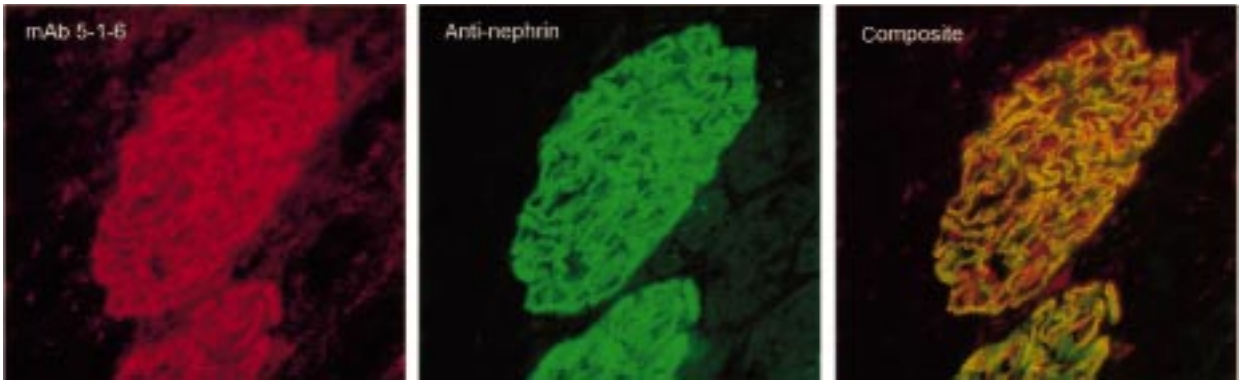

Figure 5
Colocalization of nephrin and mAb 5-1-6 antigen. Dual-label immunofluorescence with mAb 5-1-6 (red) and rabbit anti-nephrin antibody (green) in 4-μm cryosections from normal Wistar rat kidney. Identical localization of nephrin and the mAb 5-1-6 antigen in normal kidney is apparent from the yellow staining in the composite image representing superimposition of the 2 fluoroprobes. Magnification, ×400.

Western blotting of mAb 5-1-6 immunoprecipitate with rabbit antinephrin antibody. A Triton X-100 glomerular extract was immunoprecipitated with either mAb 5-1-6 or RVG-1, and the resulting pellet was resolved by SDS-PAGE under reducing conditions. Figure 4 shows the results of a Western blot of this preparation with an antinephrin polyclonal antibody. This antibody was raised against the cytoplasmic domain of mouse nephrin but also identifies human and rat nephrin. A specific band was present at 185 kDa in the mAb 5-1-6 lane (Figure 4a). Immunoprecipitation with control mAb RVG-1 was negative (Figure 4a). Furthermore, Western blot analysis of the glomerular extract revealed a doublet of nephrin bands that were partially depleted after immunoprecipitation with mAb 5-1-6 (Figure 4b). Immunoblotting with nonimmune rabbit serum was negative.

Immunohistological comparison of glomerular distribution of nephrin and mAb 5-1-6 antigen. As previously reported, mAb 5-1-6 staining of normal kidney produced a confluent granular staining pattern of the peripheral glomerular capillary loops (Figure 5). Antinephrin gave an identical pattern of staining. Superimposition of the red and green fluorescence images of dual-stained sections confirmed this impression.

Discussion

These results document that the antigen identified by mAb 5-1-6 is rat nephrin. They also establish nephrin as an integral component of the podocyte slit-diaphragm and a potential target of injury in acquired, as well as in inherited, forms of proteinuric renal diseases. Furthermore, the results provide new information on the nature of nephrin and possible insights into its role in regulating glomerular permselectivity.

The slit-diaphragm is considered by many to be the major structural barrier to macromolecular leak in the glomerulus. Its structure and molecular composition remain incompletely understood, however. Early electron microscopic analysis of tannic acid-gluteraldehyde-fixed glomeruli revealed a highly ordered, isoporous substructure of the slit-diaphragm, with either a ladder- or zipperlike configuration (15, 16). More recently, quick-freeze preparation of unfixed tissue has revealed a nonporous substructure with narrower filtration slits than those seen with fixed tissue, suggesting that the previously identified pores in the slit-diaphragm were simply a fixation artifact resulting from shrinkage and retraction of the foot processes (17, 18). Until recently, the only confirmed slit-diaphragm associated protein was ZO-1 (6). This is a 210-kDa protein that resides on the cytoplasmic face of the slit-diaphragm. Last year, through the investigation of patients with congenital nephrotic syndrome of the Finnish type, the gene for nephrin was cloned (4). Nephrin is a 1,241–amino acid protein with a large putative extracellular domain that contains 8 immunoglobulin-like modules and a fibronectin type III–like module. The short intracellular domain has no homology with other known proteins but contains 9 tyrosine residues that could potentially be phosphorylated, suggesting a possible role in cell signaling. From this data, it has been suggested that nephrin functions as a cell adhesion receptor, although no data to support this have yet been produced.

Regarding the location of nephrin, Northern blot analysis revealed a kidney-specific distribution, and in situ hybridization demonstrated that nephrin is exclusively expressed in visceral glomerular epithelial cells (4). More recently, an antibody to the extracellular NH_2-terminus of human nephrin localized on the slit-diaphragm of human kidney on immunogold electron microscopy in exactly the same distribution as previously reported for mAb 5-1-6 (1–3, 5). Moreover, the antibody to the intracellular COOH-terminus of nephrin used in this study localizes to the lateral membranes of mouse podocyte foot processes as would be expected for a transmembrane protein (11). The present studies confirm the colocalization by dual-staining immunofluorescence in normal glomeruli. In addition, dual staining for mouse IgG and nephrin in rat kidneys 5 days after mAb 5-1-6 injection showed an identical and superimposable redistribution in staining from a pseudolinear

to a punctate granular pattern, indicating that the antibody and antigen (nephrin) shift as a complex during the development of proteinuria (data not shown; and H. Kawachi et al., manuscript submitted for publication).

The identity of the mAb 5-1-6 antigen as nephrin was established by immunoprecipitation and MS. Analysis of the entire immune complex revealed a 10–amino acid peptide with identical sequence homology to a region of the extracellular domain of human and mouse nephrin. This was the only peptide in the complex with homology to a cell-surface protein. Two-dimensional gel electrophoresis of the mAb 5-1-6 immune precipitate of rat glomeruli under reducing conditions released a protein that migrated to 185 kDa in the highly anionic region (pI 4.95–5.2), which is very close to the theoretical pI of nephrin (pI 5.42) predicted from its primary structure (19). Mass spectrometry of this spot revealed that this too contained peptides with identity to nephrin. Given that the size of nephrin is predicted to be about 130 kDa from its amino acid sequence, the size of 185 kDa indicates substantial post-translational modification. Considering that nephrin contains 10 putative *N*-glycosylation sites (4, 11), this is not surprising. Thus, it is clear that mAb 5-1-6 precipitates nephrin from glomerular extracts, but, because of the need to use a cross-linking agent, it might be argued that it could have been coprecipitated with an associated or neighboring protein. This seems very unlikely, however, given the size of the upshifted band (~300 kDa) and its composition (IgG: 150 kDa; nephrin: 185 kDa), as well as the lack of any other detectable cell-surface protein in either the immune complex or on 2D-gel electrophoresis. Final proof that nephrin is immunoprecipitated by mAb 5-1-6 is given by the positive Western blot of the resolved non–cross-linked precipitate with a specific antinephrin antibody, which showed a specific band at 185 kDa. Western blot of the glomerular extract showed this band to be a doublet, suggesting either alternative splicing or variable glycosylation or phosphorylation. In addition, it may be relevant that mAb 5-1-6 appeared to preferentially precipitate the upper band.

These results also provide new information on nephrin, indicating that, in addition to being heavily glycosylated, it is highly anionic. Previous studies showed no alteration in immunofluorescent staining with mAb 5-1-6 after rat tissue sections were treated with neuraminadase (1), which suggests that the epitope does not depend on the presence of sialic acid. This is substantiated by recent observations, showing that nephrin is not sialated (11). This raises the question of what accounts for the anionic charge. One possibility is that the sugar side chains are sulfated glycosaminoglycans such as heparan sulfate. Others include a high degree of sulfation or phosphorylation. Whatever the explanation, this property suggests that the slit-diaphragm may regulate glomerular permeability to plasma proteins by serving as a charge- and size-restrictive barrier.

The mechanism by which mAb 5-1-6 causes proteinuria, and the specific nephrin epitope identified by the antibody, have yet to be established. Given that the morphology of podocytes remains essentially normal during the development of proteinuria after mAb 5-1-6 injection (1, 2), it seems that a molecular rearrangement of the slit-diaphragm is the most likely explanation. There is reason to believe that the epitope of mAb 5-1-6 may be a sugar moiety or at least depend on glycosylation of the protein. This inference is made because of our inability to detect nephrin protein or cDNA with mAb 5-1-6 on direct Western blotting of glomerular lysates or by screening of either bacterial or eukaryotic expression cDNA libraries (data not shown). If this is so, it is conceivable that mAb 5-1-6 changes the conformation of nephrin without visibly altering the appearance of the slit-diaphragm or podocyte foot processes. But this can not be the whole explanation because mAb 5-1-6 and nephrin shift their location during the development of proteinuria, as seen on immunofluorescence, and at least some of the antibody (and possibly the antigen) is endocytosed by podocytes into a lysosomal compartment (1, 2). Simultaneously, there is a loss of podocyte ZO-1 (2). Although nephrin has no recognizable protein interaction motifs in the cytoplasmic tail and, in particular, lacks a PDZ domain for interaction with ZO-1, it does have several tyrosine residues that might be phosphorylated and serve a signaling function. It may also be significant that the only other cellular protein detected in the mAb 5-1-6 immunoprecipitate was actin. Although the specificity of this finding has yet to be established, it raises the possibility of a nephrin-cytoskeletal interaction. Thus it is conceivable that mAb 5-1-6 induces a transmembrane signaling process that may produce subtle alterations in podocyte function or cytoskeletal organization.

Whatever the mechanism of injury, these data have profound implications for our understanding of glomerular function and the nature of the permselective barrier. This is, to our knowledge, the first demonstration that interaction of an antibody with the extracellular domain of nephrin results in massive proteinuria. Antibody binding leads to the redistribution of nephrin, disruption of the molecular structure of the slit-diaphragm, compromise of its permselective integrity, and massive leakage of macromolecules. Thus, nephrin is critical role for maintaining the functional integrity of the slit-diaphragm. In addition to its role in congenital nephrotic syndrome of the Finnish type, it should be considered a potential target of injury in acquired proteinuric states such as minimal change disease in humans.

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