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Article

Aging

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Neph1 and nephrin interaction in the slit diaphragm is an important determinant of glomerular permeability

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Neph1-deficient mice develop nephrotic syndrome at birth, indicating the importance of this protein in the development of a normal glomerular filtration barrier. While the precise subcellular localization of Neph1 remains unknown, its relationship with other components of the glomerular filtration barrier is of great interest in this field. In this paper, we localize the expression of Neph1 to the glomerular slit diaphragm by immunogold electron microscopy in rodents and describe its direct interaction with two other components of the slit diaphragm, nephrin and ZO-1. Both native and recombinant Neph1 associate with each other as dimers and multimers and interact with nephrin via their extracellular segments. Disruption of the Neph1-nephrin interaction in vivo by injecting combinations of individual subnephritogenic doses of anti-Neph1 and anti-nephrin results in complement- and leukocyte-independent proteinuria with preserved foot processes. This disruption modestly reduces Neph1 and nephrin protein expression in podocytes and dramatically reduces ZO-1 protein expression via the interaction of ZO-1 PDZ domains with the cytoplasmic tail of Neph1, independent of changes in mRNA expression of all three genes. The interaction between nephrin and Neph1 is specific and not shared by either protein with P-cadherin, another integral slit diaphragm protein. The interaction between nephrin and Neph1 therefore appears to be an important determinant of glomerular permeability.

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Introduction

A large number of genes with relevance to the biology of the podocyte and slit diaphragm have recently been characterized. Some encode transcriptional factors and are thought to regulate the expression of podocyte proteins (1–3), but the vast majority encode for structural components of the podocyte foot process or the slit diaphragm. Whereas the localization of ZO-1 (4) and the mAb 5-1-6 antigen (5) to the cytoplasmic and extracellular aspects, respectively, of the slit diaphragm were in themselves stellar observations, the discovery of nephrin (6) opened the field for the identification of other novel proteins. The rapid discovery of CD2 AP (7), podocin (8), α -actinin 4 (9), P-cadherin (10), and FAT (11) as components of the podocyte foot process/slit diaphragm complex, the recent identification of filtrin (12), and the recharacterization of mAb

5-1-6 as an anti-nephrin Ab (13) are just some of the important developments over the past 4 years.

This study addresses the characterization of *Neph1*, a gene recently mutated in mouse embryonic stem cells using a high-throughput mutagenesis method (14). Mutant mice generated from these stem cells develop nephrotic syndrome at birth, underscoring the importance of Neph1 in the development of a normal glomerular permeability barrier. Electron microscopy of the kidneys from the mutant mice revealed the absence of normal foot process development, and Lac-Z staining of these sections revealed the presence of Neph1- β -geo fusion transcripts in several cell types, including podocytes. A single 9-kb transcript was noted by Northern blot of several organs, with high expression in the kidney. Whereas the human Neph1 cDNA sequence published by Donoviel et al. (14) encoded for five Ig-like domains and a transmembrane domain, the mouse counterpart had only four Ig-like domains and lacked a transmembrane domain, suggesting that it was a partial clone. A more recent paper (15) included both full-length mouse and human sequences, and described the interaction of Neph1 with podocin. Whereas the expression of Neph1 in the podocyte is not in doubt, its localization in the slit diaphragm has been inferred from the nephrotic phenotype of *Neph1*^{-/-} mice and its protein-protein interactions (16).

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Nonstandard abbreviations used: rapid amplification of cDNA ends (RACE); APA, aminopeptidase A.

In this paper, we provide evidence for the subcellular localization of Neph1 in the glomerular slit diaphragm by immunogold electron microscopy. We present evidence that Neph1 interacts with nephrin *in vitro* and *in vivo*, and that this heterologous protein-protein interaction is an important factor in maintaining the normal permeability characteristics of the slit diaphragm. We also show the association of PDZ domains of ZO-1 with the cytoplasmic tail of Neph1 and characterize a novel model of the functional properties of the slit diaphragm.

Methods

Cloning of mouse Neph1 cDNA. Using primers designed from the published incomplete clone, we identified a full-length Neph1 cDNA by 3' rapid amplification of cDNA ends (RACE) and RT-PCR in the following manner. Total RNA was extracted from 4-week-old male C57BL/6 mouse kidney using TRIzol reagent (Invitrogen Corp., Carlsbad, California, USA), and cDNA templates were generated by reverse transcription using SuperScript II (Invitrogen Corp.). Using primers at either end of the published sequence, PCR fragments were cloned into pCRII (Invitrogen Corp.), sequenced, and compared with the published sequence. Using primers K7 (5'-GCAGAGGGGGCTGTGACTAGCAGCAGC-3') and K8 (nested primer 5'-GGGGCTGTGACTAGCAGCGAGCTGC-3') from this sequence, 3' RACE was performed using the GeneRacer kit (Invitrogen Corp.) per the manufacturer's instructions. A 2,673-bp RACE product was cloned into pCRII, and both sense and antisense strands were sequenced up to the polyA tail. Upon combining both sets of clones, a stop codon was noted 2,367 bp downstream of the open reading frame. Using primers overlapping the start and stop codons, several full-length cDNA clones were generated, sequenced, and compared for sequence identity. Protein translation, domain and motif analysis, and sequence comparisons were done using software on the BLAST (17), Scansite (18), and ExPASy (19) websites. Signal peptide prediction was done using SignalP version 1.1 software on the CBS World Wide Web Prediction server (20) and transmembrane domain prediction was done on the "DAS" Transmembrane Prediction server (21).

Since several experiments in rats were anticipated, we also cloned, sequenced, and assembled a partial rat Neph1 cDNA sequence using two primer pairs from the mouse sequence (K281, 5'-GTACAGTGGACCAAGGACGGGCTGG-3' and K283, 5'-CTGATGCGCTGCTGGAATCGTGGCC-3'; K282, 5'-GTCTGATGACGCTTCTATGAGTGCCAGG-3' and K284, 5'-CTCATAGTTCTCGTAGGACAGCTGGCTGG-3') and rat kidney cDNA. The rat cDNA sequence and its predicted protein sequence were used primarily to design real-time PCR primers and peptides for Ab generation, respectively.

Raising polyclonal Ab's. The extracellular segments of several mouse podocyte proteins were analyzed by hydrophobicity plots using Kyte-Doolittle curves on

the ProtScale web site (22); the most hydrophilic portions specific to the protein were selected for peptide synthesis. Peptide sequences that were chosen were homologous or nearly homologous (maximum of one AA difference) with corresponding rat sequences. The Neph1 peptide sequences that were synthesized had perfect homology with both mouse and rat Neph1 proteins. Prior to immunization, 15 ml of preimmune serum was obtained from each rabbit. Two KLH-conjugated peptides (of 15 AAs each) for each protein were used to immunize two rabbits each to raise polyclonal Ab's. Initial immunization was done with complete Freund's adjuvant, whereas boosters at days 20, 40, and 60 were given with incomplete Freund's adjuvant. ELISA titers for antigen-specific reactivity were checked after the first bleed, at day 70, using peptide coated wells. The following Ab's were raised: anti- α -dystroglycan (PNQRPELKNHIDRVD, STTTTTRRPTKKP-RTP), anti- β -dystroglycan (RRIADENGKPRPAFS, TEVPDRDPEKSSIEDD), anti-nephrin (DLQDPRTY-TEHKYHQG, FPRYSLEGDSAKGEF), anti-aminopeptidase A (YVQPNQKETAHEYAA, YTLEQYQKT-SLAQEK), and anti-Neph1 (EDAHESTRYETNVDYS, RSMNEAIPNGKETS). These Ab's were raised by Genemed Synthesis Inc. (South San Francisco, California, USA) on a fee-for-service basis.

Rabbit anti-ZO-1 and rat anti-P-cadherin with reactivity against the extracellular domain of P-cadherin (10) were purchased from Zymed Laboratories Inc. (South San Francisco, California, USA). Other Ab's purchased included mouse anti-rat CD45 (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada), goat anti-mouse CD2 AP (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), goat anti-rabbit IgG coupled with 10-nm gold particles (Electron Microscopy Services, Fort Washington, Pennsylvania, USA), rabbit anti-goat IgG coupled with 15-nm gold particles (Electron Microscopy Services), and goat anti-rat C3 (ICN Biomedical Inc., Aurora, Ohio, USA). Secondary Ab's for immunofluorescence and Western blotting were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Rabbit anti-rat albumin Ab was obtained from Research Diagnostics Inc. (Flanders, New Jersey, USA).

General laboratory reagents were obtained from Sigma-Aldrich. Reagents for electron microscopy were purchased from Electron Microscopy Services. Laboratory animals were purchased from Harlan Sprague Dawley Inc. (Indianapolis, Indiana, USA).

Animal studies. All animal studies were approved by the Animal Care and Use Committee of Northwestern University. In an attempt to study an *in vivo* association between various podocyte proteins as measured by the induction of proteinuria, the following groups of experiments were performed in male Sprague Dawley rats and female CD1 mice (four animals/group). Baseline 18-hour urinary protein excretion was performed for each animal used in the study. The choice of relatively young rats (approximately 100 g) whenever possible in these experiments is governed by the fact that they have

very little measurable baseline proteinuria. For the induction of proteinuria, all Ab's or antisera were heat-treated at 56°C for 30 minutes to inactivate complement prior to intravenous injection into rats or mice.

To study *in vivo* interactions between Neph1 and nephrin or P-cadherin, dose-response studies with anti-Neph1, anti-nephrin, and anti-P-cadherin were first performed by injecting rats via the tail vein with up to 500 µl of antisera (anti-nephrin, anti-Neph1, or preimmune serum) or up to 100 µg of anti-P-cadherin. The goal of this study was to define the optimum subnephritogenic dose of these Ab's beyond which a mild increase in slit diaphragm permeability would occur. The rats were placed in metabolic cages with access to water but no food for an 18-hour urine collection after injection. Proteinuria was measured using a protein assay kit from Bio-Rad Laboratories Inc. (Hercules, California, USA) based on the Bradford method; readings were multiplied by 1.33 to adjust for a 24-hour collection. Wherever proteinuria was noted, urine samples were appropriately diluted in PBS, resolved by SDS PAGE, and stained with Gel-Code Blue (Pierce Biotechnology Inc., Rockford, Illinois, USA). Samples were also processed for Western blot with an anti-rat albumin Ab. To test for an *in vivo* association between two proteins, the highest subnephritogenic dose of one of two Ab's or a combination of 50% of that dose of both Ab's was intravenously injected into three different groups of animals. In the anti-Neph1 and anti-nephrin study, two groups of rats were injected with 300 µl of either anti-Neph1 or anti-nephrin, while a third group received a combination of 150 µl of each Ab. A fourth group (control) received 300 µl of preimmune serum from the same rabbits. For the anti-Neph1 and anti-P-cadherin study, rats received either 300 µl of anti-Neph1, 75 µg of anti-P-cadherin, a combination of 50% of these doses, or 300 µl of preimmune serum. We also intravenously injected increasing doses up to 250 µl of preimmune serum, anti-nephrin, anti- α -dystroglycan, anti- β -dystroglycan, and anti-aminopeptidase A (anti-APA) separately into 8- to 12-week-old female CD1 mice, and analyzed the collected urine for proteinuria. Since rat glomeruli can be isolated by sieving with over 90% purity, most of the *in vivo* studies that required measurement of glomerular protein and mRNA expression were conducted in rats.

In all rodent studies, kidneys were removed after euthanasia and appropriate portions were used for fresh glomerular isolation for protein or total RNA extraction, or preserved for electron microscopy (with 2.5% glutaraldehyde), light microscopy (with 3.7% formaldehyde), or frozen sections (snap-frozen and preserved in 2-methylbutane).

Expression of FLAG-tagged proteins. To study their relative roles in protein-protein interactions, cDNA fragments corresponding to both extracellular and intracellular segments of Neph1 were cloned separately into pCAL-n-FLAG (Stratagene, La Jolla, California, USA) in the

following manner. A 1,424-bp fragment of mouse Neph1 cDNA corresponding to the extracellular segment was cloned into pCAL-n-FLAG between the EcoRI and HindII sites using primers K227 (GCGCGCAATTCCCGGGACTCAGACTCGTTCAGCC) and K230 (GCGCGCAAGCTTCTATAACACCTCTCGCTCTCCAGCTGG). A 717-bp fragment corresponding to the intracellular segment was cloned between the same restriction enzyme sites using primers K229 (GCGCGCAATTCTCTACCGACGTGCGAAAGGCAGTC) and K233 (GCGCGCAAGCTTCTACACATGAGTCTGCATGCGCTGCTGG). A segment of mouse ZO-1 containing the three PDZ domains was also cloned between the BamHI and HindIII sites of pCAL-n-FLAG using primers K238 (GCTATAGGATCCAAAGATGTCGCCAGGGCCGCGG) and K239 (GCGCTTAAAGCTTTCGCGGCGATAAACGTCCTTCTTC) and a cDNA template derived from mouse kidney total RNA by reverse transcription.

After verifying the accuracy of the plasmids by sequencing, protein expression was induced with isopropyl- β -D-thiogalactoside (IPTG) in BL21-Gold (DE3) *E. coli* (Stratagene) according to the product manual. Protein expression was verified by resolving preinduced and induced cultures by SDS PAGE under reducing and nonreducing conditions and staining with GelCode Blue, as well as transferring the proteins to nitrocellulose for Western blot with anti-FLAG (all expressions) or anti-Neph1 (extracellular segment only). The purified recombinant proteins were column-purified, dialyzed against PBS, and stored at -80°C. To generate non-FLAG-tagged recombinant protein, the FLAG domain and the calmodulin-binding peptide were cleaved from the recombinant protein using enterokinase as recommended in the product manual for pCAL-n-FLAG. Non-FLAG-tagged and FLAG-tagged recombinant proteins were used in competition experiments.

Western blot and coimmunoprecipitation studies. For glomerular studies involving the characterization of rat/mouse Neph1 and for coimmunoprecipitation studies, a standard protocol described previously was used (23). Autoradiographs were scanned into Adobe Photoshop 5.0, and band densitometry was measured using Gel-Pro Analyzer 3.1 software (Media Cybernetics Inc., Silver Spring, Maryland, USA).

For studies involving the injection of anti-Neph1, anti-nephrin, or both into rats, glomerular proteins were extracted by a different protocol (24) that also allowed for the concomitant extraction of ZO-1, which is resistant to detergent extraction by regular nonionic detergents like *n*-octyl- β -D-glucopyranoside or Triton X-100. Glomeruli were isolated by differential sieving and washed with PBS, protease inhibitors were added, and 100 µl of the pellet was solubilized by vortexing in 200 µl of SDS PAGE sample buffer without bromophenol blue. Insoluble material was removed by centrifugation at 15,000 *g* for 10 minutes. The protein concentration was measured by the BCA protein assay (Pierce Biotechnology Inc.) and 30 µg of protein was loaded into each lane and resolved by

performed in a 96-well format on the ABI Prism 7000 (Applied Biosystems) in multiplex mode (i.e., study primers/probe plus 18S control primers/probe) using preset cycling parameters specified by the manufacturer. Appropriate nontemplate controls were included in each experiment. Prior to the run, threshold settings were set at 0.2. After the run was concluded, the data was reanalyzed to obtain Ct values with a threshold setting of 0.75 in ΔRn versus Cycle mode for all groups. ΔCt values for each individual reading were calculated by subtracting the corresponding 18S Ct values from each study group reading, mean \pm SE was calculated for each group, and differences between combination and individual Ab groups were analyzed by Student *t* test. In addition, we subtracted the ΔCt of the individual Ab-injected groups from that of the combination injected group to obtain the $\Delta\Delta Ct$ and calculated the difference in mRNA expression between these groups using the formula $2^{-\Delta\Delta Ct}$ (see Applied Biosystems ABI 7000 user bulletin no. 2 for sequence detection at <http://docs.appliedbiosystems.com/search.taf>). A fourfold difference in mRNA expression, equivalent to a mean difference of two cycles by real-

time PCR, was used as a criterion to define a significant difference between the groups.

Imaging studies. For immunofluorescence studies, a standard protocol recently described in detail was used (23). The positive control for complement staining with anti-rat C3 Ab was day 5 kidney of a rat injected with sheep anti-rat Fx1A. Positive control for leukocyte staining with anti-CD45 was kidney from a rat injected with whole sheep anti-rat nephrotoxic serum 24 hours prior to euthanasia.

For immunogold electron microscopy of perfused rat kidney and for transmission electron microscopy, standard protocols described elsewhere were used (25, 26). The positive control for the procedure of immunogold localization of Neph1 was staining for CD2 AP in rat glomeruli. In addition, other rats were injected separately with 150 μ l of either anti-Neph1 or anti-nephrin 1 hour prior to perfusion fixation, followed by staining with 10 nm gold-coupled goat anti-rabbit IgG to assess for binding of injected Ab's to the slit diaphragm.

Statistical analysis to study the difference in protein expression between different experimental groups was done using the unpaired Student *t* test in Microsoft Excel 2000. A *P* value of < 0.05 was taken as significant.

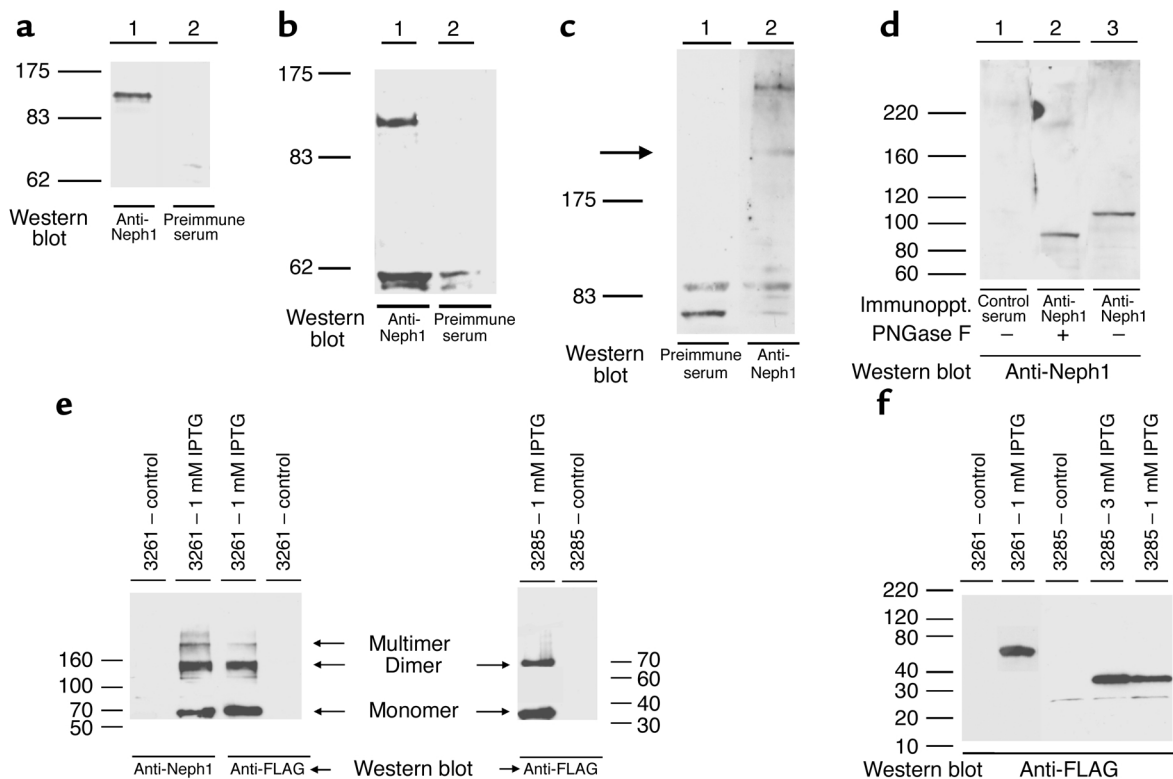


Figure 2

Characterization of native and recombinant mouse Neph1 by Western blot. (a and b) A distinct band is seen at approximately 110 kDa in rat and mouse glomerular extracts, respectively, under reducing conditions. (c) There is a band at approximately 220 kDa (arrow) and a higher band in mouse glomerular extracts under nonreducing conditions, suggesting dimer and multimer formation. (d) Migration of anti-Neph1 immunoprecipitates (Immunoppt.) from mouse glomerular extracts at approximately 90 kDa following deglycosylation with PNGase F. (e and f) Composite Western blots of the extracellular segment (clone 3,261) and intracellular segment (clone 3,285) expressed in *E. coli* by IPTG induction, run under nonreducing (e) and reducing (f) conditions, also demonstrating dimer formation under nonreducing conditions. IPTG, isopropyl- β -D-thiogalactoside.

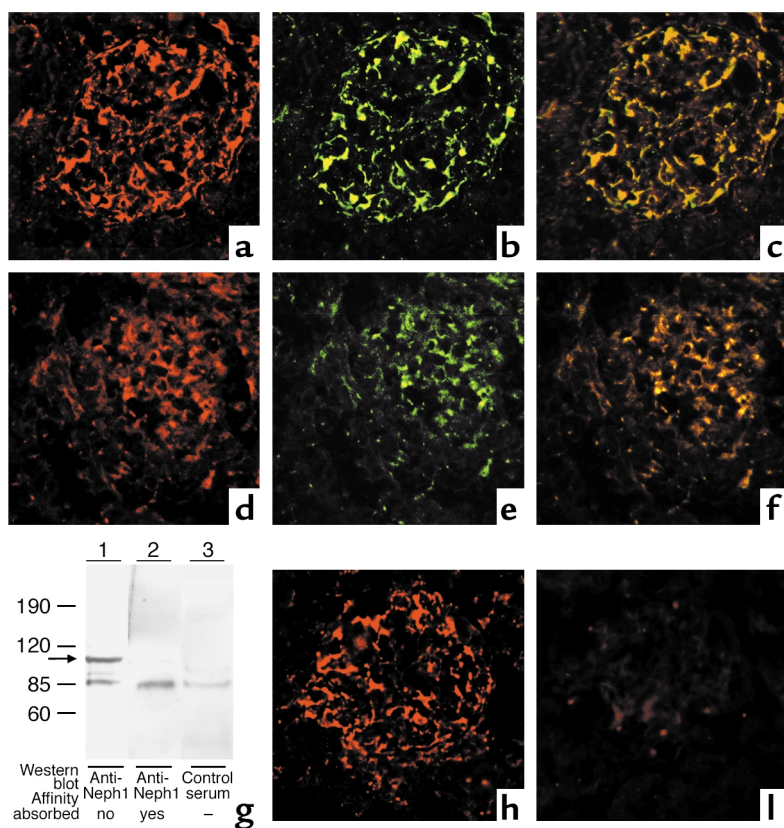


Figure 3 Localization of Neph1 in adult and developing rodent glomeruli. (a–c) Immunofluorescence images of adult rat glomeruli (3-µm sections; magnification, ×400) stained for Neph1 (a), CD2 AP (b), and merged images (c), localizing Neph1 to podocytes. (d–f) Immunofluorescence images of day 15 developing mouse glomeruli stained for Neph1 (d), CD2 AP (e), and merged images (f), localizing Neph1 to developing podocytes. (g) Western blot of rat glomerular extracts under reducing conditions with anti-Neph1 antibody prior to (lane 1) and following (lane 2) affinity absorption with a Neph1 peptide column, showing near-complete loss of immunoreactivity. (h and i) Immunofluorescence staining of 3-µm rat kidney sections prior to (h) and following (i) affinity absorption of anti-Neph1 with the Neph1 peptide affinity column, showing loss of immunoreactivity.

Results

Cloning of mouse *Neph1* cDNA. Cloning and sequencing by our group of the mouse cDNA fragment published by Donoviel et al. (14) revealed a single base pair disparity (absence of C after 1,204 in the Donoviel et al. sequence) which had led to a premature stop codon after 1,260 bp (Figure 1a). Combining our corresponding sequence with the 2,673-bp 3' RACE product revealed a 3,255-bp clone with a stop codon 2,367 bp downstream of the open reading frame, encoding for a 789-AA protein. This sequence has been submitted to GenBank (accession number AY243095). The 2,107-bp partial rat cDNA sequence also obtained had over 95% homology at the nucleotide level and 98% homology at the protein level with mouse Neph1 cDNA (GenBank accession number AY271309).

Structural analysis of the mouse *Neph1* gene. A BLAST search of the mouse genome with our cDNA sequence revealed the presence of 15 exons spanning a 57.4-kb region of chromosome 3, with intron 1 being disproportionately long (42.7 kb). Perfect identity between the predicted exons and our mouse cDNA sequence was noted. Our sequence, as well as the exons in the genomic sequence, differ from another recently submitted sequence by four base pairs (present study and Sellin et al., ref. 15) 1,064 C/T; 1,324 A/G, 2,008 A/G, 2,179 T/C).

Characterization of the mouse *Neph1* protein. Domain analysis of the predicted protein sequence revealed a signal peptide (AAs 1–47), five IgG-like domains with the middle domain overlapping with a PKD-like domain, an

RGD sequence (AAs 437–439), a transmembrane domain (AAs 529–551), and a cytoplasmic tail (552–789) (Figure 1b). The most significant motifs in the cytoplasmic tail include the Grb2 SH2 site at 637 and the PDZ1 motif at 787. Another motif on the cytoplasmic tail is the Itk SH2 at 672. However, the presence of Itk kinases in the podocyte has not yet been described, although this does not exclude potential binding by a member of the Tec kinase family at that site.

Western blot studies of native rodent glomerular protein suggest that Neph1 exists as a monomer of about 110 kDa under reducing conditions (Figure 2, a and b) and as a dimer and perhaps even as a multimer under nonreducing conditions (Figure 2c). These observations are confirmed by Western blot studies under nonreducing conditions with the recombinant protein that show dimeric bands of the extracellular and intracellular segments (Figure 2e), and a multimeric band of the extracellular segment. Association among Neph1 molecules is also favored by the coimmunoprecipitation of native Neph1 by an anti-FLAG Ab from a mixture of native mouse glomerular proteins and the FLAG-tagged recombinant extracellular domain (see Figure 5b, lane 11). The 110-kDa size of rodent Neph1 on reducing Western blot is identical to that noted by Sellin et al. (15), but differs from the 90-kDa protein noted by Barletta et al. (27). Since the predicted molecular weight of the protein, excluding the signal peptide, is 82 kDa, slower migration on Western blot may be related to posttranslational modification of the protein. Indeed,

there are four potential N-glycosylation sites (at AAs 78, 172, 329, and 503), seven potential O-glycosylation sites (at AAs 56, 256, 349, 597, 698, 700, and 703), and several potential phosphorylation sites in Neph1 (Figure 1c). Indeed, deglycosylation of immunoprecipitated mouse Neph1 with PNGase F causes the protein to migrate at approximately 90 kDa (Figure 2d).

Localization of Neph1 in adult and developing rodent glomeruli. Dual immunofluorescence staining of adult rat (Figure 3, a–c) and mouse glomeruli reveals an interrupted capillary loop pattern that is identical to, and colocalizes well with, the staining pattern for CD2 AP, suggesting that Neph1 is present in podocytes. Similar colocalization of Neph1 and CD2 AP was noted in the day 15 developing mouse kidney in the precapillary loop stage (Figure 3, d–f). In this immunofluorescence experiment, CD2 AP was used merely as a podocyte marker, since the limited resolution of immunofluorescence does not differentiate slit diaphragm proteins from other podocyte proteins present in the vicinity. Anti-Neph1 Ab depleted of immunoreactivity by absorption over a Neph1 peptide affinity column (Figure 3g) or recombinant Neph1 extracellular segment (blots not shown) failed to stain rodent glomeruli by immunofluorescence (Figure 3, h and i). Similar results were noted with anti-nephrin absorbed with a nephrin peptide column (data not shown). Immunogold electron microscopy, which is the technique of choice for precise subcellular localization, reveals most of the 10-nm gold particles to be present in the slit diaphragm, thereby establishing Neph1 as a slit diaphragm protein (Figure 4b). Some gold particles were also present on the foot processes in the vicinity of the slit diaphragm. The positive control for this study shows most of the 15-nm gold particles representing CD2 AP to be present on the cytoplasmic aspect of the slit diaphragm (Figure 4a). In a different experiment, both anti-Neph1 (Figure 4c) and anti-nephrin (Figure 4d) Ab's injected into separate rats 1 hour prior to perfusion fixation localized almost exclusively to the slit diaphragm by immunogold staining.

Overall, in a survey of ten glomeruli per study for binding of gold particles, more than 95% of the gold particles in the anti-Neph1- and anti-nephrin-injected rats were present in the slit diaphragm, compared with 75–80% in sections stained with the anti-Neph1 Ab in noninjected rats. The rest of the particles in both groups were equally divided between the foot process surface in close proximity to the slit diaphragm and the podocyte cytoplasm. In glomeruli stained with anti-CD2 AP, about 85% of the gold particles were present at the cytoplasmic aspect of the slit diaphragm, and the rest were present in the podocyte cytoplasm away from the slit diaphragm. Whereas Donoviel et al. had noted the presence of Neph1- β -geo fusion transcripts in podocytes, parietal epithelial cells, and mesangial cells, glomerular expression of native Neph1 as noted by our group appears to be restricted to podocytes.

Association of Neph1 with nephrin. We conducted coimmunoprecipitation experiments to study potential interactions between three slit diaphragm proteins: Neph1, nephrin, and P-cadherin. Coimmunoprecipitation studies under nonreducing conditions using anti-Neph1 and anti-nephrin Ab's, along with the corresponding preimmune antisera, revealed that nephrin coimmunoprecipitates with anti-Neph1 and vice versa (Figure 5a). Furthermore, we were able to coimmunoprecipitate the recombinant extracellular segment of mouse Neph1 with anti-nephrin, and vice versa (Figure 5b), but not the recombinant intracellular part (blots not shown), indicating that the Neph1-nephrin interaction occurs primarily at the level of the extracellular segments of these proteins. We were also able to demonstrate competition between FLAG-tagged and non-FLAG-tagged (unFLAG)

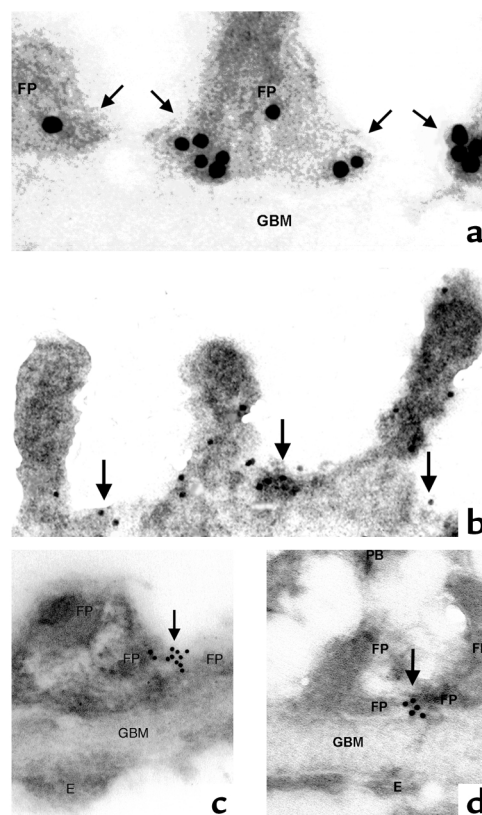


Figure 4 Immunogold localization of Neph1 to the slit diaphragm in adult rat glomeruli. (a) A control electron micrograph (magnification, $\times 50,000$) shows staining with goat anti-CD2 AP and rabbit anti-goat IgG coupled with 15-nm gold particles localizing CD2 AP predominantly to the cytoplasmic aspect of the slit diaphragm (arrows). (b) Staining with rabbit anti-Neph1 (magnification, $\times 27,000$) and goat anti-rabbit IgG coupled with 10-nm gold particles, localizing Neph1 mostly to the slit diaphragm (arrows), though some labeling of the foot process is also present (see text). (c and d) Staining of kidney sections with goat anti-rabbit IgG coupled with 10-nm gold particles (magnification, $\times 30,000$) from rats injected with either rabbit anti-Neph1 (c) or rabbit anti-nephrin (d). Both anti-Neph1 (c) and anti-nephrin (d) bind specifically to the slit diaphragm (arrows), as shown by the cluster of gold particles. FP, foot process; GBM, glomerular basement membrane; E, endothelial cell; PB, podocyte body.

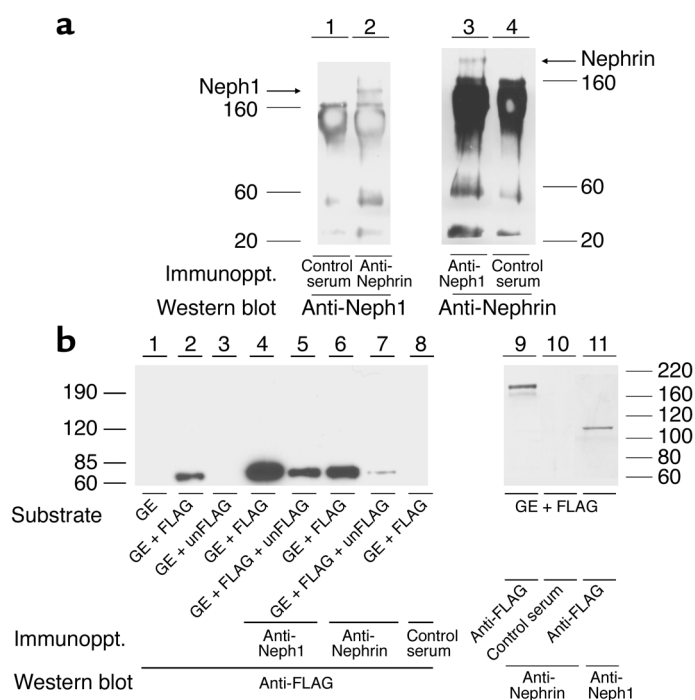


Figure 5

Composite Western blots showing the association of native (a) and recombinant (b) mouse Neph1 and nephrin. (a) Native Neph1 from mouse glomerular extracts coimmunoprecipitates with anti-nephrin (lane 2), and nephrin with anti-Neph1 (lane 3) under nonreducing conditions. All lanes, including control immunoprecipitations (lane 1 and lane 4), show the prominent band of nonreduced rabbit IgG at 150–160 kDa and traces of heavy chain (50 kDa) and light chain (25 kDa). (b) Lane 1 shows the absence of FLAG-reactive proteins in native mouse glomerular extracts. In lanes 2–11, native glomerular extracts were combined with FLAG-tagged recombinant extracellular Neph1 and also supplemented with an excess of recombinant extracellular Neph1 digested with enterokinase to remove the FLAG tag (labeled “unFLAG”) in lane 5 (unFLAG/FLAG ratio, 5:1) and lane 7 (unFLAG/FLAG ratio, 10:1). Coimmunoprecipitation of recombinant Neph1 by anti-Neph1 (lane 4) and anti-nephrin (lane 6); coimmunoprecipitation of nephrin (lane 9) and native Neph1 (lane 11) by anti-FLAG; and competition between FLAG-tagged and non-FLAG-tagged protein (lane 5 and lane 7) are demonstrated (see text for details). GE, glomerular extract.

proteins during coimmunoprecipitation studies with anti-Neph1 and anti-nephrin, as shown in Figure 5b. Lane 4 and lane 5 (unFLAG/FLAG-tagged ration, 5:1; $n = 3$ readings/group) show anti-Neph1 coimmunoprecipitates; densitometry readings were $2,235 \pm 29$ (lane 4) and 927 ± 26 (lane 5), densitometric units, $P < 0.01$. Lane 6 and lane 7 (unFLAG/FLAG ratio, 10:1) show anti-nephrin coimmunoprecipitates; densitometry readings were $1,272 \pm 20$ (lane 6) and 49 ± 3 (lane 7) densitometric units, $P < 0.01$. We were unable to coimmunoprecipitate P-cadherin with either anti-nephrin or anti-Neph1. As negative controls for the method of coimmunoprecipitation, we performed similar studies between slit diaphragm proteins (using anti-nephrin and anti-Neph1) and focal adhesion complex proteins (using anti- α - and anti- β -dystroglycan) or podocyte surface proteins (using anti-APA), all of which were negative (blots not shown).

In vivo study of the Neph1-nephrin association. We rationalized that disruption of slit diaphragm function would result in proteinuria and that targeting two interacting proteins critical to the barrier function

would cause this disruption more efficiently than targeting them alone. To study a functional association between slit diaphragm proteins, we first injected anti-nephrin, anti-Neph1 (Table 1 and Figure 6a), and anti-P-cadherin Ab's (Table 2) separately into rats in increasing amounts to find a threshold nephritogenic dose that would cause mild proteinuria compared with preimmune serum injection. Dose-response curves with individual Ab's helped us identify an optimum subnephritogenic dose in rats for anti-Neph1 (300 μ l, Figure 6a and Table 1), anti-nephrin (300 μ l, Figure 6a and Table 1), and anti-P-cadherin (75 μ g, Table 2). Similarly, in mouse experiments, the optimum subnephritogenic doses were 200 μ l for anti-APA and 150 μ l for anti-nephrin and anti-Neph1. We were unable to induce any proteinuria in mice beyond baseline with anti- α -dystroglycan and anti- β -dystroglycan. Of various subnephritogenic combinations tested, we were able to induce heterologous-phase proteinuria within the first 18 hours with anti-Neph1 and anti-nephrin; this proteinuria declined over the next 2 days (Table 3

Table 1

Urinary protein excretion in response to anti-Neph1 and anti-nephrin

Preimmune serum		Anti-Neph1		Anti-nephrin	
Dose injected	Proteinuria	Dose injected	Proteinuria	Dose injected	Proteinuria
Baseline	0.87 ± 0.19	Baseline	0.87 ± 0.19	Baseline	0.87 ± 0.19
100 μ l	0.65 ± 0.05	100 μ l	0.80 ± 0.05	100 μ l	0.70 ± 0.10
300 μ l	0.65 ± 0.02	300 μ l	0.80 ± 0.40	300 μ l	0.80 ± 0.09
500 μ l	1.30 ± 0.10	500 μ l	$2.36 \pm 0.38^{A,B}$	500 μ l	$1.84 \pm 0.04^{A,B}$

Protein excretion in urine over 24 hours, shown as mean \pm SE (in mg), in response to injection of increasing amounts of Ab or preimmune serum into Sprague Dawley rats ($n = 4$ male rats, 100 g each, per group). ^ASignificantly different from baseline, $P < 0.01$. ^BSignificantly different from corresponding preimmune serum value, $P < 0.05$.

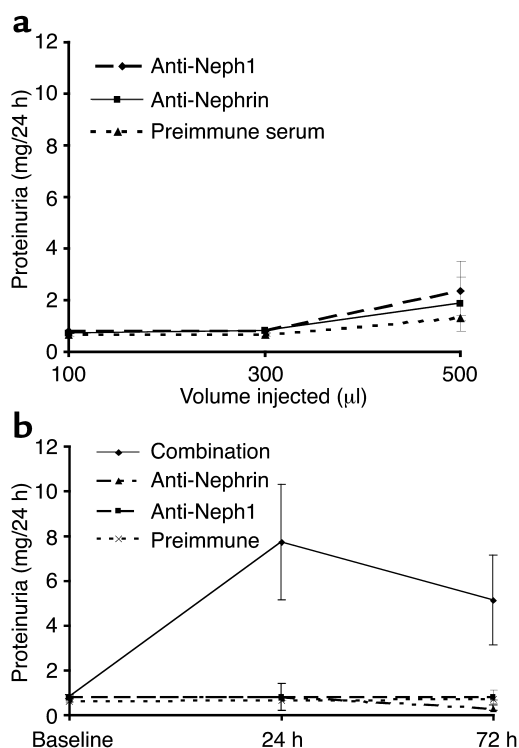


Figure 6 The induction of heterologous-phase proteinuria in rats by combinations of anti-Neph1 and anti-nephrin in individual subnephritogenic doses. (a) A dose-response plot of each individual Ab or preimmune serum to assess the optimum subnephritogenic dose. Some increase in permeability is noted at 500 μl for both study Ab's compared with preimmune serum ($P < 0.05$). (b) Induction of heterologous-phase proteinuria in rats by a combination of 150 μl each of anti-Neph1 and anti-nephrin, whereas 300 μl of each individual antibody and preimmune serum do not cause proteinuria.

and Figure 6b). This experiment was repeated with older rats and revealed similar results. The proteinuria was comprised primarily of albumin, as was obvious from the approximately 70-kDa band seen on GelCode Blue-stained gels in the combination-injected group (Figure 7b); this was confirmed to be albumin by Western blot (blots not shown). The following combinations did not result in significant proteinuria beyond baseline: anti-Neph1 and anti-P-cadherin; anti-nephrin and anti-P-cadherin; anti-nephrin and anti-APA; and anti-Neph1 and anti-APA.

Light microscopy of the kidney (hematoxylin and eosin stain) in all four groups of the anti-Neph1 and anti-nephrin experiment at 24 hours after injection showed the glomeruli to be normocellular, and staining for complement and leukocytes was negative by immunofluorescence with appropriate positive controls (images not shown). Transmission electron microscopy revealed preserved foot processes in all four groups, as exemplified by an electron micrograph from proteinuric rats at 24 hours (Figure 7a). Immunofluorescence for bound rabbit IgG (anti-Neph1, anti-nephrin, or combination) revealed a change from the normal

interrupted linear pattern to a cluster-like distribution in the three experimental groups starting 12 hours after intravenous injection of Ab (images not shown); this has been observed with several podocyte surface proteins by several investigators, including us, and appears to be unrelated to proteinuria (23). The induction of complement- and leukocyte-independent proteinuria by a combination of individually subproteinuric Ab doses indicates disruption of an in vivo association between nephrin and Neph1 in the slit diaphragm, without interference with foot process integrity.

As anticipated, we were unable to induce proteinuria in rats using a combination of 150 μl of anti-nephrin and 150 μl of the Neph1 peptide column unbound fraction, reiterating the specific role played by anti-Neph1 Ab's in this process: baseline proteinuria, 0.85 ± 0.15 mg/24 hours; Ab injected, 0.95 ± 0.17 mg/24 hours (mean \pm SE, $n = 4$ rats weighing 100 g, $P > 0.05$). Similar results were noted when 150 μl of anti-Neph1 was affinity-absorbed over a recombinant extracellular Neph1 column and injected in combination with 150 μl of anti-nephrin: baseline, 0.85 ± 0.15 mg/24 hours; antibody injected, 0.92 ± 0.15 mg/24 hours, mean \pm SE ($n = 4$ rats weighing 100 g each, $P > 0.05$). Similarly, when the anti-nephrin Ab used in the combination experiment was absorbed over a nephrin peptide affinity column, no significant proteinuria was induced by the combination: baseline, 0.85 ± 0.15 mg/24 hours; antibody injected, 0.90 ± 0.12 mg/24 hours ($n = 4$ rats weighing 100 g, mean \pm SE, $P > 0.05$).

Association of Neph1 with ZO-1. The presence of a PDZ-binding motif on the cytoplasmic tail of Neph1, and the absence in the literature of the direct interaction of any other slit diaphragm protein with ZO-1, prompted us to look for a potential association between Neph1 and ZO-1. As also recently shown by Huber et al. (28), we were able to coimmunoprecipitate the FLAG-tagged PDZ domains of ZO-1 with anti-Neph1, and vice versa, indicating that Neph1 and ZO-1 associate with each other on the cytoplasmic aspect of the slit diaphragm (Figure 8).

Protein and mRNA expression in anti-Neph1- and anti-nephrin-induced slit diaphragm dysfunction. Analysis of the glomerular content of nephrin, Neph1, and ZO-1

Table 2 Urinary protein excretion in response to anti-P-cadherin

Control serum		Anti-P-cadherin	
Dose injected	Proteinuria	Dose injected	Proteinuria
Baseline	1.61 ± 0.09	Baseline	1.61 ± 0.09
100 μl	1.56 ± 0.05	50 μg	1.60 ± 0.60
150 μl	1.63 ± 0.10	75 μg	1.65 ± 0.40
200 μl	1.66 ± 0.03	100 μg	2.40 ± 0.05^A

Urinary protein excretion over 24 hours, shown as mean \pm SE (in mg), in response to injection of increasing amounts of anti-P-cadherin Ab or control serum into 125-g male Sprague Dawley rats ($n = 4$ rats/group). ^ASignificantly different from baseline and corresponding control serum value, $P < 0.05$.

Table 3

Urinary protein excretion in response to subnephritogenic doses of Ab's

	Preimmune serum (300 μ l)	Anti-Neph1 (300 μ l)	Anti-nephrin (300 μ l)	Anti-Neph1 (150 μ l) + anti-nephrin (150 μ l)
Day 1	0.65 \pm 0.02	0.80 \pm 0.60	0.80 \pm 0.10	7.73 \pm 2.60 ^A
Day 3	0.67 \pm 0.04	0.90 \pm 0.30	0.38 \pm 0.20	5.10 \pm 2.07

Urinary protein excretion over 24 hours in response to injection of subnephritogenic doses of Ab's alone or in combination ($n = 4$ male rats/group, 100 g each, baseline proteinuria 0.87 \pm 0.19). Data are presented as mean \pm SE, in mg/24 hours. ^ASignificantly different from corresponding values of other three groups, $P < 0.05$.

proteins 24 hours after injection of the Ab's revealed modest reduction of nephrin and Neph1 in the combination group by densitometric analysis (Table 4 and Figure 9, a and b). In addition to being substantially reduced in the combination group, ZO-1 protein was also modestly reduced in the anti-Neph1 group.

The results of real-time PCR are illustrated in Figure 9, c-g, and the Δ Ct values and $2^{-\Delta\Delta$ Ct values are summarized in Table 5. The Δ Ct values of the combination versus individual Ab-injected groups were not significantly different by Student *t* test, and the $2^{-\Delta\Delta$ Ct values between the same groups were also less than 2, indicating that altered gene expression did not account for the pathogenesis of proteinuria.

Discussion

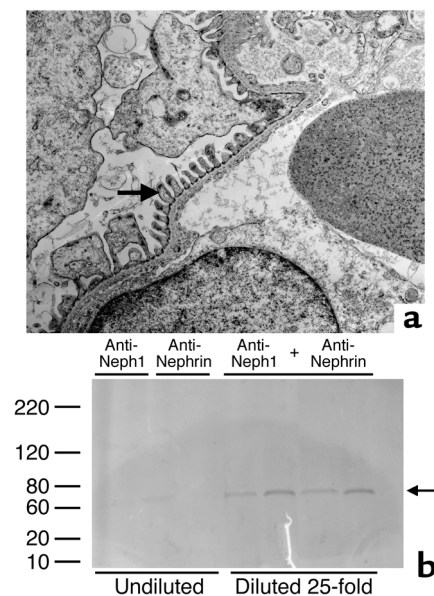
Over two decades of research has provided interesting insights into the structure and function of the glomerular capillary loop (29-31). More recent studies have addressed the relative role of podocyte versus basement membrane proteins in maintaining the permeability barrier (24). The paper on Neph1 by Donoviel et al. (32) describing nephrotic syndrome at birth in Neph1 mutant mice provides conclusive evidence that Neph1 plays a significant role in the normal development and function of the glomerular capillary loop. The paper by Sellin et al. (15) describes the interaction of Neph1 with podocin in vitro, the distribution of Neph1 in a glomerular capillary loop pattern, and the transactivation of AP-1 in the presence of Tec kinases. Our current study provides conclusive evidence that Neph1 is a slit diaphragm protein, as shown by immunogold localization in the slit diaphragm.

The coimmunoprecipitation studies using anti-Neph1, anti-nephrin, and anti-FLAG Ab's with glomerular extracts and recombinant proteins were highly suggestive of an interaction between Neph1 and nephrin at the level of their slit diaphragm spanning extracellular segments. We therefore decided to disrupt this interaction using an in vivo approach. The occurrence of complement- and leukocyte-independent proteinuria by a combination of anti-Neph1 and anti-nephrin Ab's in individually subnephritogenic doses is highly suggestive that this interaction exists in vivo, and is at least partially altered by this Ab combination approach. Moreover, the induction of heterologous-phase proteinuria by the combination of anti-Neph1

and anti-nephrin was not associated with foot process effacement or difference in the mRNA expression of ZO-1, nephrin, and Neph1 between proteinuric and nonproteinuric subgroups, indicating that it largely affected slit diaphragm permeability due to altered protein-protein interaction.

It is important to note that the dose-response curves performed as a prelude to these studies were intended to identify an optimum subnephritogenic dose of individual Ab's, and not to study the full extent of the nephritogenic potential of the individual Ab's. Indeed, both anti-Neph1 and anti-nephrin start increasing slit diaphragm permeability individually (compared with preimmune serum controls) at a dose of 500 μ l in rats. It is possible that future experiments directed toward investigating the nephritogenic potential of these individual Ab's may show more proteinuria at higher doses.

The choice of an in vivo approach to test the Neph1-nephrin association over an in vitro approach using monolayers of cultured glomerular epithelial cells was made for several reasons. The slit diaphragm is the final barrier that restricts passage of plasma proteins into the urine (29). We have now known for several years that

**Figure 7**

(a) Electron micrograph of rat kidney taken 24 hours after injection of the anti-Neph1 and anti-nephrin combination, showing that heterologous-phase proteinuria occurs in the absence of foot process effacement (arrow). Magnification, $\times 5,000$. (b) GelCode Blue-stained reducing SDS PAGE for urinary protein from rats injected with either anti-Neph1 or anti-nephrin (undiluted), or a combination of both (diluted 25-fold). Urine from only two of four rats injected with each individual antibody is shown. The rats in the combination group showed a prominent band at approximately 70 kDa (arrow) that was confirmed to be rat albumin by Western blot (see text).

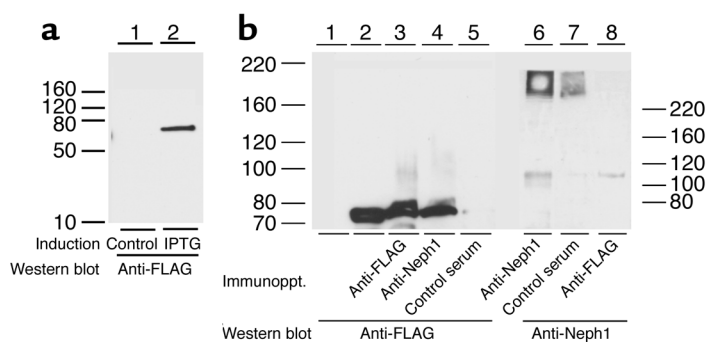


Figure 8 Composite Western blot analysis of (a) the expression of recombinant FLAG-tagged PDZ domains of mouse ZO-1 and (b) the association of Neph1 with FLAG-tagged ZO-1 PDZ domains by coimmunoprecipitation. (b) Lane 1 shows the absence of anti-FLAG reactivity in native mouse glomerular protein extracts. All other experiments shown were conducted with a combination of native mouse glomerular proteins and recombinant ZO-1 PDZ domains. Lane 4 shows the coimmunoprecipitation of ZO-1 PDZ domains by anti-Neph1; lane 8 shows the coimmunoprecipitation of Neph1 by anti-FLAG.

certain Ab's directed against podocyte proteins injected intravenously induce complement- and leukocyte-independent proteinuria (5, 33, 34). We therefore rationalized that Ab's directed against the extracellular segments of slit diaphragm proteins would bind their targets and induce slit diaphragm dysfunction that would be detectable as proteinuria. The classic example of this is mAb 5-1-6, which induces proteinuria in rats in certain doses after binding to an epitope on the extracellular segment of nephrin. An *in vitro* approach to this issue would be less than ideal, since there are no cultured glomerular epithelial cells that form foot processes and slit diaphragms with morphological similarity to their *in vivo* counterparts. In addition, cultured cells tend to form tight junctions, which are not seen in differentiated podocytes and would make the interpretation of monolayer permeability data more difficult.

The association of ZO-1 with the cytoplasmic tail of Neph1, and the interaction of nephrin with Neph1 in the slit diaphragm, also offer valuable insights into changes in podocyte proteins during proteinuria. The study complements the astute observation of Kawachi et al. (24) that the induction of proteinuria with mAb 5-1-6, an anti-nephrin Ab, results in reduced amounts of glomerular ZO-1 by immunostaining and Western blot. In the anti-Neph1/anti-nephrin model discussed above, glomerular ZO-1 was unchanged compared with controls when rats were injected with anti-nephrin at a dose that did not cause proteinuria. Some reduction of glomerular ZO-1 content was noted with the injection of anti-Neph1 alone, testifying further to the direct association of ZO-1 with Neph1. The most dramatic reduction, however, was observed when the Ab combination induced heterologous-phase proteinuria. This indicates that the interaction between nephrin and Neph1 helps to partially stabilize the association between Neph1 and ZO-1, and that disruption of this association results in a rapid reduction of glomerular ZO-1. This allows us to postulate that a nephrin/Neph1/ZO-1 macromolecular complex exists in the slit diaphragm and may be altered in some forms of proteinuria. Whether our anti-nephrin Ab given alone in higher doses reduces glomerular ZO-1 will be a subject of future investigations designed to examine the nephritogenic phase of this Ab. It would also be interesting to conduct similar studies with mAb 5-1-6.

A substantial amount of novel data presented in the Results section allows us to propose further refinements in the organization of Neph1 molecules in the slit diaphragm. The existence of Neph1 homodimers and multimers, the coimmunoprecipitation of nephrin with native Neph1, the selective coimmunoprecipitation of nephrin with the recombinant extracellular Neph1, and the induction of complement- and leukocyte-independent heterologous-phase proteinuria by the combination of anti-nephrin and anti-Neph1 Ab's in subnephritogenic doses all help in generating possible configurations. Neph1 homodimers/multimers may be arranged side by side in the slit diaphragm or adhere to each other from adjacent foot processes. It is also likely that Neph1 forms additional heterodimers with nephrin molecules either side by side or approaching each other from adjacent foot processes. Even though both the homodimeric association of Neph1 and a heterodimeric association with nephrin are both likely to be important in maintaining the permeability characteristics of the slit diaphragm, the occurrence of proteinuria after treatment with subnephritogenic combinations of Ab's rather than with individual Ab's would suggest that the latter may be more significant. In addition to the data presented above, the likelihood of a Neph1-nephrin interaction is also supported by the interaction in *Drosophila* between Hibris and Dumbfounded, two proteins with structural similarity to nephrin and Neph1, respectively, during myoblast fusion (35). Also, an association between nephrin and Neph1 has very recently also been shown by two other studies (27, 36).

Table 4 Densitometric analysis of protein expression

	<i>n</i>	Control	Anti-nephrin	Anti-Neph1	Anti-nephrin + anti-Neph1
Neph1	3	216 ± 12	194 ± 14	243 ± 34	98 ± 12 ^A
Nephrin	3	205 ± 3	197 ± 5	205 ± 41	92 ± 12 ^B
ZO-1	5	440 ± 40	436 ± 56	153 ± 36 ^B	33 ± 22 ^{C,D}

Expression of Neph1, nephrin, and ZO-1 in rat glomeruli extracted from experimental and control groups (mean ± SE densitometric units). ^ASignificantly different from corresponding control, anti-nephrin, and anti-Neph1 groups, *P* < 0.01. ^BSignificantly different from corresponding control and anti-nephrin groups, *P* < 0.01. ^CSignificantly different from corresponding anti-Neph1 group, *P* < 0.05. ^DSignificantly different from corresponding control and anti-nephrin groups, *P* < 0.001.

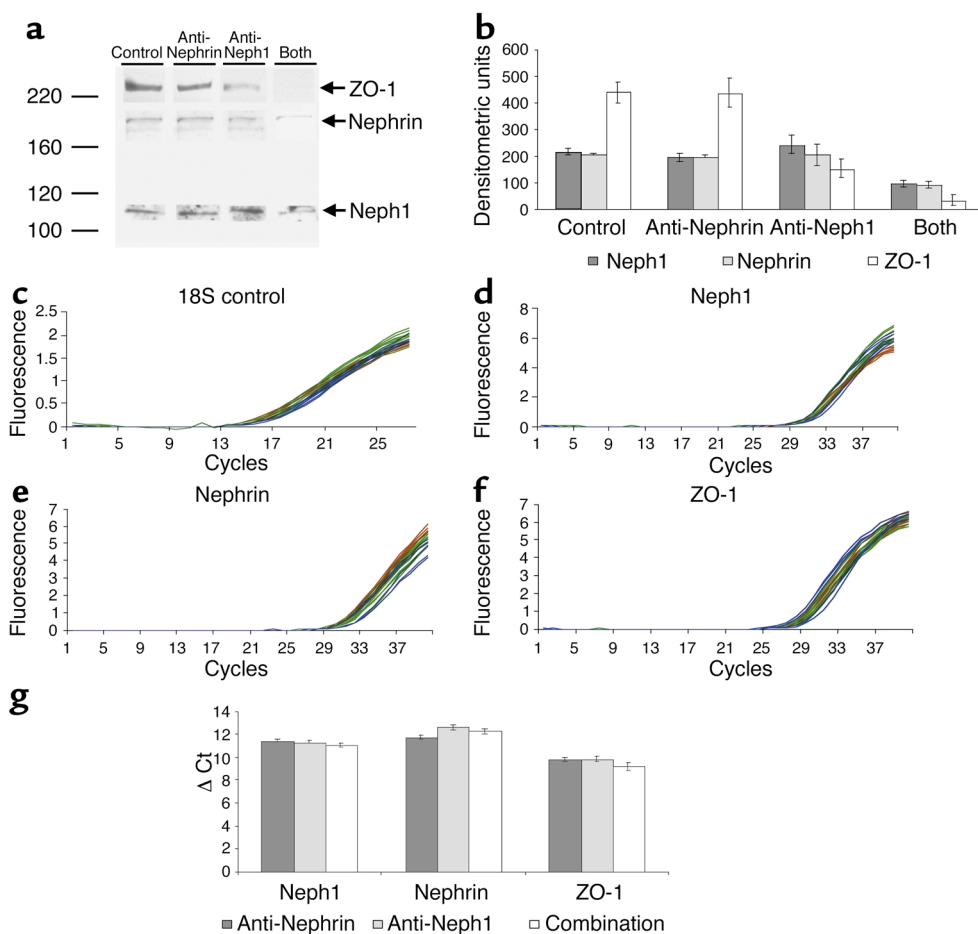


Figure 9

Composite image showing protein (a and b) and mRNA (c-g) expression in rat glomeruli of Neph1, nephrin, and ZO-1 24 hours after the injection into rats of anti-Neph1 or anti-nephrin alone, or a combination of both. (a) Composite Western blot of glomerular extracts for each protein in the three experimental subgroups or controls. (b) Densitometric analysis of bands shown in a (mean ± SE). Modest reduction of Neph1 and nephrin and dramatic reduction of ZO-1 protein expression were noted in the combination group. Modest reduction in ZO-1 expression was also noted in the anti-Neph1 group. (c-f) Real-time PCR tracings (n = 9 per group) for the three genes or 18S controls in the anti-nephrin (red), anti-Neph1 (green), and combination (blue) groups. (g) ΔCt (mean ± SE) values of each gene in the three groups. No significant difference in mRNA expression was noted for the individual genes between the combination and single-Ab groups (P > 0.05).

We have not as yet investigated the potential interactions of Neph1 or nephrin with FAT, a member of the cadherin superfamily that also spans the slit diaphragm (11). Interestingly, FAT also has a PDZ-binding motif that was not shown to bind ZO-1 in a recent paper also addressing the association between Neph1 and ZO-1 (28). Moreover, the data appears to suggest that very large cadherins, such as the FAT family members, may not be

involved in adhesion at all, but may have a sensing role (37). Also, two genes related to Neph1, named *Neph2* and *Neph3*, have now been shown to be expressed in human kidney cortex by RT PCR (15). Precise cellular and sub-cellular localization is still pending. Further studies are required to investigate other protein-protein associations in the slit diaphragm that may also be important in maintaining the permeability status of this important structure.

Table 5

ΔCt values for mRNA expression of individual groups and 2^{-ΔΔCt} values of difference between combination and individual groups by real-time PCR

	Anti-nephrin ΔCt	Anti-Neph1 ΔCt	Combination ΔCt	Combination minus anti-nephrin 2 ^{-ΔΔCt}	Combination minus anti-Neph1 2 ^{-ΔΔCt}
Neph1	11.46 ± 0.12	11.34 ± 0.13	11.07 ± 0.15	1.42 ± 0.24	1.22 ± 0.09
Nephrin	11.74 ± 0.18	12.62 ± 0.19	12.27 ± 0.22	0.83 ± 0.25 ^A	1.13 ± 0.05
ZO-1	9.85 ± 0.19	9.86 ± 0.20	9.20 ± 0.30	1.99 ± 0.59	1.67 ± 0.24

Data are presented as mean ± SE, n = 9 per measurement. ^AAnti-nephrin minus combination was 1.60 ± 0.45. P > 0.05 for ΔCt between all combination and corresponding individual Ab groups.

Acknowledgments

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