Nephrin and Neph1 Co-localize at the Podocyte Foot Process Intercellular Junction and Form cis Hetero-oligomers*

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Glomerular visceral epithelial cells (podocytes) appear to play a central role in maintaining the selective filtration barrier of the renal glomerulus. While the immunoglobulin superfamily member Nephrin was proposed to act as a cell adhesion molecule at the podocyte intercellular junction necessary for maintaining glomerular perm selectivity, the Nephrin ligand has not been identified. The existence of a new subfamily of Nephrin-like molecules including Neph1 was recently described. Genetic deletion of Nephrin or Neph1 resulted in similar phenotypes of podocyte foot process effacement and proteinuria. The subcellular localization of Neph1 and the possibility that Nephrin and Neph1 interact was investigated. Polyclonal antiserum for Neph1 was raised and characterized. Neph1 migrated as a 90-kDa protein on SDS-PAGE under reducing conditions. Neph1 was identified in a glomerular and podocyte-specific distribution in adult rat kidney. Like Nephrin and Podocin, Neph1 was enriched in Triton X-100 detergent-resistant membrane fractions. Consistent with this observation, immunogold electron microscopy demonstrated that Neph1 localized exclusively to lateral margins of podocyte foot processes at the insertion of the slit diaphragm. Neph1 and Nephrin participate in a direct cis-interaction involving their cytoplasmic domains. In addition, interactions between the extracellular domain of Nephrin and itself and between the extracellular domain of Neph1 and that of Nephrin were detected. Neph1 did not interact via a homophilic interaction. These observations suggest that Nephrin and Neph1 form a hetero-oligomeric receptor complex in the plane of the membrane that might interact across the foot process intercellular junction through interactions between Nephrin with itself and Neph1.

Glomerular visceral epithelial cells or podocytes are responsible for maintaining the filtration barrier of the renal glomerulus. These cells attach to the basement membrane of glomerular capillaries via their octopus-like, interdigitated “foot processes” that arise from a central cell body. Normally, glomerular filtrate passes between these foot processes and across a specialized intercellular junction known as the slit diaphragm. When podocytes are injured, the intercellular junctions and cytoskeletal structure of the foot processes is altered and the cell takes on an “effaced” phenotype that is associated with the development of proteinuria (1). For this reason, it is thought that the slit diaphragm is an important component of the glomerular filter.

The molecular composition and function of the foot process intercellular junction remains incompletely characterized. Emerging evidence suggests that this specialized junction is composed of a large complex of proteins associated in part within a specialized lipid microdomain. Presently, at least three transmembrane proteins have been identified at this site, including Nephrin (2), FAT1 (3), and P-cadherin (4). Significant attention has focused on Nephrin, the protein product of NPHS1. In humans, mutation of NPHS1 results in congenital nephrotic syndrome of the Finnish-type that is manifest by foot process effacement and proteinuria (5). That Nephrin participates in a protein complex important for maintenance of glomerular filter integrity has been suggested by observations that deletion or mutation of proteins that are physically associated with Nephrin, such as Podocin and CD2ap, also result in foot process effacement and proteinuria (6–8). Nephrin is a member of the immunoglobulin (Ig) superfamily characterized by eight C2 type Ig-like domains and a fibronectin type III repeat in its extracellular region. For this reason, it has been proposed that Nephrin is a cell adhesion molecule (CAM)1 that participates in forming the glomerular filter via homophilic or heterophilic interactions involving its extracellular domain (9, 10). However, the Nephrin ligand has not been identified. Recent progress in genome and expressed sequence tag sequencing and work by mouse and Drosophila geneticists has provided important new clues regarding the true Nephrin ligand. Search of the genetic data base demonstrated the existence of three mammalian Nephrin-like Ig superfamily CAMs including Neph1 and two additional structurally similar proteins (11). While little characterization of Nephrin has been published, deletion of mouse Nephrin results in a podocyte effacement phenotype (12). A family of four CAMs was also identified in Drosophila that are structurally related to Nephrin (13).

These proteins form two subfamilies: Hibris and Sticks and Stones that are most closely related to Nephrin, and Dumbfounded and Irregular Chiasm that are structurally similar to mammalian Neph1, Neph2, and Neph3. Importantly, Dumbfounded (the Neph1 homologue) interacts with Hibris.
and Sticks and Stones, but Hibris (the Nephrin homologue) does not interact with itself (13). These results predict that Nephrin interacts with Nephi1.

In the present study we sought to investigate whether Nephi1 and Nephrin interact via heterophilic and/or homophilic interactions. It was observed that both Nephrin and Nephi1 are expressed in a glomerular epithelial cell-specific fashion in kidney and co-localize at the insertion of the glomerular slit diaphragm. Nephrin and Nephi1 co-fractionate with a detergent-resistant lipid micro-domain and participate in a direct cis-interaction involving their cytoplasmic domains. In addition, interactions between the extracellular domain of Nephrin and itself and between the extracellular domain of Nephrin and that of Nephi1 were detected. Nephi1 did not interact via a homophilic interaction.

EXPERIMENTAL PROCEDURES

Eukaryotic Expression Constructs—A mammalian expression plasmid encoding mouse Mcp-Nephrin has been described previously (2). Full-length mouse Nephi1 cDNA was a gift of Dr. G. Walz (University of Freiburg, Freiburg, Germany). The cDNA encoding mouse Nephrin open reading frame was subcloned into a mammalian expression vector pCDNAS1 (Invitrogen) and a FLAG epitope or V5 epitope tag was placed at the 3’ end of the open reading frame using standard a PCR-based cloning technique (16). A mammalian construct expressing a FLAG-tagged mouse Nephrin cytoplasmic domain (Nephrin-CD) fragment encoding the COOH-terminal 155 amino acid residues was prepared in a similar fashion (2). The extracellular domain (ECD) of Nephrin (Fc-mNephrin-ECD) or Nephi1 (Fc-FLAG-Neph1-ECD) was fused to a human immunoglobulin Fc segment by placing it in a modified pCDM7 vector (Invitrogen). This vector also contained a signal peptide sequence, a CD5:Hinge:CH2:CH3 region, and a Myc tag at the 3’ end of the expressed Nephrin or a FLAG tag at the 3’ end of the expressed Nephi1 ECD open reading frame (14, 15). The ECD of Nephrin included amino acid residues 55–521 of the previously published mouse Nephrin sequence (2), and the ECD of Nephi1 included amino acid residues 55–521 of the previously published mouse Nephi1 sequence (11). The Fc-mNephrin-ECD was expressed as a 180-kDa protein and the Fc-FLAG-Nephi1-ECD expressed a 90-kDa protein when reduced on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Bacterial Fusion Proteins—Plasmid encoding GST-Nephi1-CD (amino acids residues 554–789 (11) (Invitrogen) fusion protein was prepared using standard techniques. Briefly, PCR was performed using 5’ primer (GGA ATC CCG ACG TCG CAA AGG CAG T-3’) and 3’ primer (CCG TCG AGC ACA TGA TGC TGG ATG CG-5’) and using the full-length mouse Nephrin open reading frame residues 55–1059 of the previously published mouse Nephrin sequence (2), and the ECD of Nephi1 expressed in Echerichia coli, then purified by affinity chromatography on glutathione-Sepharose (Sigma, G-4510). The eluted GST fusion protein was covalently linked to cyanogen bromide activated Sepharose C-4B beads (Sigma, CL-4B-200) and used for pull-down experiments as well as affinity purification of Nephi1 antiserum. A plasmid (pBSET-A, Invitrogen) encoding hexahistidine-tagged Nephi1-CD was prepared as described above. A hexahistidine-tagged Nephi1-CD construct was described previously (2). Both proteins were prepared from bacterial lysate and purified using a commercially prepared nickel-agarose column according to the directions of the manufacturer (Invitrogen).

Antibodies—Rabbit anti-Nephrin cytoplasmic domain (CD) polyclonal antibody was prepared as described previously (2). Anti-FLAG (M2, Sigma), anti-Myc (9E10, Oncogene Science), anti-V5 (Invitrogen), and anti-human IgG (Fc specific) (Sigma, A-0170) antibodies were obtained commercially. Mouse anti-rat GLEPP1 monoclonal antibody was a gift of Dr. Roger Wiggins (University of Michigan). Nephi1 antiserum was produced by immunization of rabbits with hexahistidine-tagged Nephi1 cytoplasmic domain antigen. For antibody preparation, hexahistidine-Nephi1 CD was solubilized in 6 M urea, purified by nickel affinity chromatography, separated on SDS-PAGE, and gel slices were used for rabbit inoculation. Antiserum was affinity purified on a GST-Nephi1-CD affinity column after ammonium sulfate precipitation as described previously (2).

Cell Culture—COS7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen) and 200 units/ml of penicillin and streptomycin (Roche Molecular Biochemicals). Transient transfections were performed with FuGENE 6 (Roche Molecular Biochemicals) as described previously (16). Cell lysates were prepared 24 h after transfection using RIPA buffer (0.1 M HEPES, 0.15 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% glycerol, 0.1 M sodium fluoride, 10 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 1% Nonidet P-40, and protease inhibitor mixture tablet (Roche Molecular Biochemicals)).

Immunoblotting and Immunoprecipitations—Immunoblotting was performed as described previously (17, 18). Blots were developed using the ECL chemiluminescent reagent (Amersham Biosciences) and were autoradiographed. Immunoprecipitations were performed as described previously (16). For affinity chromatography experiments using Fc-chimeric proteins, lysates were incubated with protein A-Sepharose (Sigma, P-5906). Beads were washed six times with 1% phosphate-buffered saline containing 1% BSA and 0.1% Tween 20 and the eluted immune complexes were separated under reducing conditions by SDS-PAGE, transferred to nitrocellulose, then immunoblotted with indicated antibodies.

Glomerular Isolation and Flotation Gradient Preparation—Adult rat kidney glomeruli were isolated by sieving as described elsewhere (2, 19). The average purity of glomerular preparations was >90%. Glomeruli were lysed using RIPA buffer containing protease inhibitors at a concentration of ~10,000 glomeruli/ml of extraction buffer.

For preparation of the flotation gradient, glomeruli were isolated and homogenized from rat kidneys at 4 °C with 14 strokes with a Dounce homogenizer in a buffer containing 250 mM sodium chloride, 5 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and protease inhibitor mixture tablet (Roche Molecular Biochemicals). Triton X-100 was added to the homogenate to give a final concentration of 1%. The homogenate was mixed with 60% Optiprep solution (Nycomed Pharma AS) to obtain a final density of 40%. Five ml of this mixture was added to precooled centrifuge tubes and layered with 3 ml of 30% Optiprep and 4 ml of 5% Optiprep solution. Optiprep dilutions were obtained by diluting 60% stock Optiprep with Tricine buffer containing 20 m M Tricine, 0.25 mM sucrose, and 1 mM EDTA. The gradient was centrifuged at 37,000 rpm using a Beckman SW 41Ti rotor (180,000 g × g) for 3 h at 4 °C. The Triton X-100-insoluble lipid layer was identifiable at the interface between the 50% and 30% Optiprep solutions. Top to bottom 1-ml fractions were sequentially collected.

Immunofluorescence—Six-μm adult rat kidney cryosections were fixed with cold acetone. Affinity-purified rabbit anti-mouse Nephi1 polyclonal antibody (1:50) or mouse anti-rat GLEPP1 monoclonal antibody (1:200) were used as a primary antibody. Species-appropriate secondary antibodies conjugated to fluorescein isothyocyanate or Cy3 (Jackson Laboratories, West Grove, PA) were employed.

Immunogold Electron Microscopy—Kidneys from adult rats were fixed in 4% freshly prepared paraformaldehyde, 0.1% distilled glutaraldehyde in 100 mM phosphate buffer, pH 7.2, for 6–12 h at 4 °C, soaked in sucrose, and stored in liquid nitrogen. Ultra-thin frozen sections of rat kidney were processed for indirect immunogold labeling as described elsewhere (20, 21). In all experiments, control sections were prepared with affinity-purified rabbit anti-podocalyxin IgG to assess background and specificity.

RESULTS

A polyclonal antiserum directed against the cytoplasmic domain of mouse Nephi1 was raised in rabbits and was affinity purified. To assess the specificity of the affinity-purified antibody and to test the structural predictions made by the mouse Nephi1 cDNA sequence, full-length mouse Nephi1 was expressed by transient transfection in COS7 cells. Fig. 1 demonstrates that anti-Nephi1 antibody specifically recognized a protein with an apparent molecular mass of ~90 kDa in Nephi1 transfected cells but not in vector control transfectants or in untransfected COS7 cell lysates. A protein of similar mobility was identified using this antibody in lysates obtained from isolated rat glomeruli.

Nephi1 Is Expressed in a Podocyte-specific Fashion in Kidney—Indirect immunofluorescence was used to examine the expression pattern of Nephi1 in adult rat kidney. Nephi1 immunoreactivity was identified in a glomerular-specific pattern (Fig. 2, A–C). To determine whether the immunoreactivity observed was podocyte-specific, cryosections of adult rat kidney were double labeled with a mouse monoclonal antibody di-
rected against GLEPP1 and with rabbit anti-Neph1 antibody (Fig. 2, D–F). GLEPP1 is a transmembrane protein-tyrosine phosphatase expressed only on podocytes in the kidney (22). Merged photomicrographs demonstrated that immunoreactivity for GLEPP1 and Neph1 produce superimposable signals and suggested that Neph1 is expressed in a podocyte-specific pattern in kidney.

Neph1 Fractionates with the Triton X-100-insoluble Lipid Microdomain of Isolated Rat Glomeruli—Nephrin was found previously to associate in part with a Triton X-100-insoluble membrane fraction obtained from isolated glomeruli (23, 24). For this reason, the possibility that Neph1 also fractionates with this membrane fraction was investigated. Rat glomeruli were isolated and lysed at 4 °C in buffer containing 1% Triton X-100. Lysate was fractionated by floatation gradient centrifugation. The lipid fraction was identified in fraction four at the interface between 5 and 30% Optiprep (Fig. 3). One-ml fractions were collected from top to bottom, and each fraction was analyzed by immunoblotting for Neph1, Nephrin, Podocin, and CD2ap (Fig. 3). Like Podocin, another podocyte-specific detergent-resistant membrane fraction associated-protein (27), Neph1 was isolated only in the Triton X-100-insoluble membrane fraction (fraction 4). As described previously, Nephrin, in part, fractionated in the same lipid membrane fraction.

Neph1 Subcellular Localization in Glomeruli—To examine the distribution of Neph1 within glomeruli in more detail, immunoelectron microscopy was carried out using post-embedding colloidal gold immunolabeling of rat kidney sections (Fig. 4). Gold particles were identified exclusively on lateral pedicel surfaces of podocytes in a distribution consistent with that of the insertion of the slit diaphragm. As anticipated, because the polyclonal antibody utilized in these experiments was raised against the cytoplasmic domain of Neph1, gold particles were not observed within the slit pore itself. There was no labeling for Neph1 detected elsewhere within membrane or cell body of the podocyte or within glomerular basement membrane or endothelial cells (Fig. 4). Importantly, this subcellular distribution of Neph1 within the podocyte was similar to that previously reported for Nephrin (2, 9).

Neph1 and Nephrin Interactions—Given the co-localization and structural similarity of Neph1 and Nephrin, it was hypothesized that Neph1 and Nephrin might engage in heterophilic and/or homophilic interactions occurring either in cis or trans at the podocyte foot process intercellular junction. To investigate this hypothesis, an initial series of co-immunoprecipitation experiments were performed after co-expressing Nephrin and/or Neph1, or their truncated mutants, by transient transfection of COS7 cells.

In preliminary experiments, full-length Neph1 co-immunoprecipitated with full-length Nephrin (Fig. 5A). However, full-length FLAG-tagged Neph1 did not co-immunoprecipitate with full-length V5-tagged Nephrin (Fig. 5B). A potential Nephin-Nephrin interaction was examined in experiments described below. To assess whether endogenous Nephrin interacts with endogenous Neph1, Nephrin was immunoprecipitated from isolated glomerular lysate prepared by extraction with RIPA buffer (that had been shown in preliminary experiments to extract both Nephrin and Neph1 from membrane fractions). In
To define the domains that mediate the observed interaction between Neph1 and Nephrin, additional co-immunoprecipitation experiments were performed. ECD interactions were examined by preparing plasmids encoding chimeric proteins of the ECD of Nephrin (Fc-mNephrin-ECD) and the ECD of Neph1 (Fc-FLAG-Neph1-ECD) fused to the Fc domain of human Ig (Fig. 6). In these experiments, the ECD of Nephrin co-immunoprecipitated with full-length FLAG-tagged Neph1 (Fig. 6A). Reciprocally, the ECD of Neph1 co-immunoprecipitated with full-length Nephrin (Fig. 6B). In similar experiments, Fc-mNephrin-ECD co-immunoprecipitated with full-length Neph1, while Fc-FLAG-Neph1-ECD did not interact with full-length Nephrin (Fig. 6D and C, respectively). In a control experiment, Nephin ECD did not co-immunoprecipitate with the Nephin CD (Fig. 6E). In summary, the extracellular domain of Nephin interacts both with itself and with that of Nephrin, while the extracellular domain of Nephrin does not interact with itself.

Intracellular domain interactions were examined by preparing plasmids encoding the cytoplasmic domain of Nephrin. COS7 cells were co-transfected with indicated combinations of expression plasmids. Cell lysates were immunoprecipitated with specified antibody. Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, then immunoblotted with indicated antibody and visualized by autoradiography. Western blots were also performed using 30 μl of whole cell lysate obtained from each group of transfected cells and immunoblotted with the indicated antibodies that served as mobility markers and confirmed the presence of each protein in the corresponding lysates. C, endogenous Nephin co-immunoprecipitates with endogenous Neph1. Isolated glomeruli were extracted with RIPA buffer, and lysates were immunoprecipitated with indicated antibodies. Immune complexes obtained were resolved by SDS-PAGE and immunoblotted with affinity-purified Nephrin antibody. Apparent molecular mass (in kilodaltons) is indicated. All experiments were repeated at least three times with similar results.
neither Nephrin nor Neph1 engage in homophilic interactions via their respective cytoplasmic domains.

DISCUSSION

The composition and function of the Nephrin-associated protein complex remains incompletely characterized. Given its structure and localization, it has been hypothesized that Nephrin mediates homophilic cell-cell interactions between podocyte foot processes that may account for the electron microscopic appearance and theoretical filtering function of the slit diaphragm (9, 25, 26). However, this model has not been validated experimentally.

Nephrin and Neph1 share a number of important similarities. Deletion of either Neph1 (12) or Nephrin (5) results in similar phenotypes of foot process effacement with proteinuria and early postnatal death in mice. Both proteins are expressed in a glomerular epithelial cell-specific fashion in kidney and are localized to the podocyte slit diaphragm as demonstrated by immunoelectron microscopy of glomerular sections (2). Neph1 and Nephrin also have been shown to interact with Podocin, a lipid raft-associated component of the glomerular slit diaphragm (11, 27, 28). As suggested by recent work in Drosophila, a new family of structurally related Nephrin-like molecules has been described (13). Within this family is Hibris, a protein member of the Ig superfamily. Although their location and activity differ, Hibris bears strong structural similarity to mammalian Nephrin. In a study by Dworak et al. (13), Hibris mediated cell aggregation via a heterophilic interaction with Dumbfounded, a protein that bears structural similarity to Neph1. Therefore, these results predict that Nephrin interacts with Neph1.

The present results identify Neph1 as a newly recognized component of the Nephrin-associated protein complex existent at the intercellular junction between foot processes. Importantly, Nephrin and Neph1 appear to form a cis-interacting hetero-oligomeric complex associated with a detergent resistant lipid membrane microdomain. Nephrin and Neph1 directly

![Fig. 6. Co-immunoprecipitation experiments to examine interactions of extracellular domains of Nephrin and Neph1. A–E, COS7 cells were co-transfected with indicated combinations of expression plasmids. Affinity chromatography (AC) was performed by incubating cell lysates with protein A-Sepharose that bound the human Fc portion of indicated ECD constructs. Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with indicated antibody and visualized by autoradiography. Western blots were also performed using 30 μl of whole cell lysate obtained from each group of transfected cells and immunoblotted with the indicated antibodies that served as mobility markers and confirmed the presence of each protein in the corresponding lysates. Apparent molecular mass (in kilodaltons) is indicated. All experiments were repeated at least three times with similar results.](http://www.jbc.org/)

![Fig. 7. Demonstration of a direct interaction between the cytoplasmic domain of Nephrin and the cytoplasmic domain of Neph1. A and B, COS7 cells were co-transfected with indicated combinations of expression plasmids. Affinity chromatography (AC) was performed by incubating cell lysates with protein A-Sepharose that bound the human Fc portion of indicated ECD constructs. Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and then immunoblotted with indicated antibody and visualized by autoradiography. Western blots were also performed using 30 μl of whole cell lysate obtained from each group of transfected cells and immunoblotted with the indicated antibodies that served as mobility markers and confirmed the presence of each protein in the corresponding lysates. C, rat glomerular lysate was incubated with either GST alone or GST-Neph1-CD covalently linked to Sepharose beads. Pelleted beads were subjected to Western blot analysis using an anti-Nephrin antibody. D, GST alone and GST-Neph1-CD covalently linked to Sepharose beads were each incubated with 1 μg of hexahistidine-tagged purified recombinant Nephrin-CD. Pelleted beads were subjected to Western blot analysis using an anti-Nephrin antibody. Apparent molecular mass (in kilodaltons) is indicated in figures. All experiments were repeated at least three times with similar results.](http://www.jbc.org/)
interact via their cytoplasmic domains. In addition, Podocin, a stomatin-like protein, binds both Nephrin and Neph1 via their cytoplasmic domains (11, 27), and all three fractionate with the detergent-resistant membrane fraction. While Nephrin and Neph1 interact directly via their cytoplasmic domains, Podocin interaction might serve to stabilize their interaction and target the entire complex to the detergent-resistant membrane at the intercellular junction.

In the present study both homophilic and heterophilic interactions were also identified between the ECD of Nephrin and itself and between Nephrin and Neph1. Because multiple members of the Ig superfamily mediate cell-cell interaction via either homophilic or heterophilic associations, it is tempting to speculate that the Nephrin-Neph1 complex functions in a similar fashion. Several models have been proposed for cell adhesion mediated by classical CAMs (29–34). In general, these models suggest that CAM-mediated cell-cell adhesion may be initiated by parallel cis-dimerization followed by an anti-parallel adhesive trans-interaction of the cis-dimers on opposing cells (31, 33, 34). Therefore, it is possible that Neph1 and Nephrin participate in foot process-to-foot process interactions only after formation of a cis-hetero-oligomeric complex. This model would be consistent with our previous failure to demonstrate Nephrin-only mediated homophilic cell-cell interactions2 and suggests a new experimental direction.

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