Disease-causing missense mutations in NPHS2 gene alter normal nephrin trafficking to the plasma membrane

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Background. Podocin is a membrane-integrated protein that is located at the glomerular slit diaphragm and directly interacts with nephrin. The gene encoding podocin, NPHS2, is mutated in patients with autosomal-recessive steroid-resistant nephrotic syndrome (SRN). In order to study a potential pathomechanism of massive proteinuria in patients with SRN, we have investigated the trafficking and subcellular localization of five common disease-causing missense mutants of human podocin.

Methods. Site-directed mutagenesis was applied to generate cDNA constructs encoding five different missense mutations of human podocin (P20L, G92C, R138Q, V180M, and R291W). To identify the subcellular localization of each mutant in transfected human embryonic kidney (HEK)293 cells, we have generated and characterized a rabbit polyclonal antibody against human podocin. Specificity of the antibody was determined by light and immunoelectron microscopy, as well as immunoblot analysis using human glomeruli. Confocal microscopy was applied to determine subcellular localization of the wild-type and the mutated podocin molecules, as well as wild-type nephrin in transfected cells. Immunoprecipitation and pull-down studies were carried out to investigate the molecular interaction of podocin mutants and wild-type nephrin.

Results. Immunofluorescence and confocal microscopy showed that wild-type podocin located to the plasma membrane when expressed in HEK293 cells. Two missense mutations, P20L and G92C, located at the N-terminus part of the molecule, were also present at the plasma membrane, indicating that these mutations did not affect the subcellular localization of the mutated podocin molecules. In contrast, subcellular localization of three other missense mutants located in the proximal C-terminus part of the protein was drastically altered, in which R138Q was retained in the endoplasmic reticulum (ER), V180M formed inclusion bodies in the cytoplasm, and the R291W mutant was trapped both in the ER and in small intracellular vesicles. Interestingly, this abnormal subcellular localization of podocin missense mutants also resulted in alteration in protein trafficking of wild-type nephrin in cotransfected cells through the strong protein binding between both molecules.

Conclusion. In patients with SRN, some missense mutations in the NPHS2 gene not only lead to misfolding and mislocalization of the mutated podocin, but they can also interfere with slit diaphragm structure and function by altering the proper trafficking of nephrin to the plasma membrane.

Pathomechanism of the proteinuria in congenital and acquired forms of glomerular diseases are diverse and dependent on different underlying factors. Characterization of the recently identified gene NPHS1 [1], mutated in the congenital nephrotic syndrome of the Finnish type (CNF), has shed much light onto the pathomechanism of nephrotic syndromes. The discovery of the gene and localization of its product has dramatically increased our understanding of the nature of the glomerular ultrafiltration barrier. The gene product, termed nephrin, is a transmembrane glycoprotein belonging to the immunoglobulin superfamily molecules [2]. Nephrin is currently thought to be a major component of the slit diaphragm (SD) structure, which connects the interdigitating foot processes of the glomerular podocytes [3–5]. In the past few years, intensive research focused on the molecular basis of the SD structure and function has resulted in identification of several novel SD-associated proteins such as NEPH1, CD2-associated protein (CD2AP), and podocin. NEPH1, similar to nephrin,
is also a member of the immunoglobulin superfamily with five extracellular Ig-like domains and a cytoplasmic domain [6]. It has recently been shown that NEPH1 can form heterodimer with nephrin at its extracellular domain [7]. CD2AP is an 80-kD protein that was originally found to be associated with the cytoplasmic domain of CD2, a transmembrane protein also belonging to the immunoglobulin superfamily, expressed on T cells and natural killer cells [8]. Subsequent work by Shaw et al clearly demonstrated that this protein was also expressed by podocytes and localized at the glomerular SD area connecting the cytoplasmic domain of nephrin with the actin cytoskeleton [9, 10]; however, nephrotic syndrome with a mutated CD2AP gene has not yet been identified. On the other hand, NPHS2, mutated in patients with autosomal-recessive steroid-resistant nephrotic syndrome (SRN), has been recently identified [11]. The product of this gene, termed podocin, is a membrane-integral protein belonging to the stomatin family. In the kidney, it is exclusively localized at the cytoplasmic face of the SD [12]. Pull-down studies have shown that podocin interacts with the cytoplasmic part of nephrin, thus being an important component of the glomerular filtration barrier [13].

SRN is a specific type of familial steroid-resistant idiopathic nephrotic syndrome characterized by an autosomal-recessive inheritance, with onset between three months to five years. The massive proteinuria in these patients does not respond to glucocorticoid treatment, resulting in rapid progression to end-stage renal disease. The histologic findings are quite interesting in that minimal glomerular lesions are seen at early stages, followed by focal segmental glomerulosclerosis (FSGS) at later stage. It was originally reported that many nonsense, insertion, deletion, and missense mutations are responsible for SRN [11]. However, rapidly accumulated reports have clarified broad implication of these podocin mutations, not only in the pathophysiology of children with SRN, but also in the cases with nonfamilial FSGS mutations, not only in the pathophysiology of children with SRN, but also in the cases with nonfamilial FSGS [14, 15], glucocorticoid-sensitive frequent relapses [16], and with sporadic FSGS in adulthood [17]. These results point to the central role of podocin together with nephrin for a functional perme selectivity of the SD.

Recently, we have reported that the majority of missense mutated nephrin are retained in the endoplasmic reticulum (ER), and do not appear on the plasma membrane [18]. In the present study, we have explored a potential pathomechanism in development of steroid-resistant nephrotic syndrome in patients with missense podocin mutations. We have generated cDNA constructs with five different disease-causing missense mutations commonly found in SRN patients and analyzed the subcellular localization of these mutants in human embryonic kidney cells (HEK293). Three out of these five mutations, located at the proximal C-terminal part of podocin molecule, failed to localize to the plasma membrane. Interestingly, we also found that the normal localization of wild-type nephrin at the plasma membrane was also altered in cells coexpressing these three podocin mutants. Our findings point to a potential pathomechanism in massive proteinuria in some patients with SRN, in which the proper slit diaphragm localization of both podocin and nephrin molecules are disrupted.

METHODS

Antibodies

Antihuman podocin polyclonal antibody (KYJ2) was generated against a synthetic peptide corresponding to KRAKAERSGGRGRQE (amino acids 25–40) of the human podocin amino acid sequence. A rabbit was immunized with 0.5 mg of this peptide conjugated to the carrier protein keyhole hemocyanin, and boosted three times with 0.5 mg of the immunogen each time. The rabbit was sacrificed and bled 10 days after the last immunization. The antisemur was affinity-purified with the corresponding peptide linked to CNBr-activated Sepharose 4B column (Pharmacia, Piscataway, NJ, USA). Horseradish peroxidase (HRP)–labeled anti-podocin antibody was generated according to the procedure of Wilson and Nakane [19], with minor modifications. Mouse monoclonal antibody raised against the eighth Ig domain (mAb2) and rabbit polyclonal antibody against the whole intracellular domain (pAb2) of nephrin, as described before [20], and mouse monoclonal antibody against human podocalyxin (3D3) [21] were used for immunofluorescence study or immunoblotting. The following antibodies were purchased from the suppliers as indicated: monoclonal anti-GM130 antibody (Becton Dickinson Transduction Laboratories, Franklin Lakes, NJ, USA); monoclonal anti-KDEL (GRP78/94) antibody (Stressgen, Victoria, British Columbia, Canada), HRP-labeled goat antirabbit immunoglobulins (Dako; Carpinteria, CA, USA); Alexa Fluor 488-conjugated goat antimouse IgG and antirabbit IgG, and Texas Red-X goat antimouse IgG (Molecular Probes, Eugene, OR, USA), colloidal gold (12 and 18 nm)-labeled donkey antirabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA).

Normal kidney samples and isolation of glomeruli

Four human kidney cortex samples were obtained from histologically normal regions of fresh kidneys from patients undergoing nephrectomy with renal cancer or ureter cancer at the Department of Urology, Kyorin University Hospital. Informed consent was obtained from all patients. The isolation of glomeruli was performed by a differential sieving technique as described previously [22,
The purity of the glomerular preparations, as assessed by phase-contrast microscopy, was above 95%.

Immunoperoxidase staining

Immunoperoxidase staining was performed using frozen kidney cortex samples [22, 23]. Briefly, the slides were washed with phosphate-buffered saline (PBS) and incubated with 1% \( \text{H}_2\text{O}_2 \)/PBS for 30 minutes to quench endogenous peroxidase activity. After washing with PBS, the slides were incubated with blocking buffer (2% bovine serum albumin, 5% goat serum, and 0.05% Tween-20 in PBS), and then reacted for 60 minutes at room temperature with KYJ2 antiserum or preimmune rabbit serum (1:500). After washing with PBS, the slides were incubated at room temperature with HRP-labeled goat antirabbit antibody, and then the slides were developed by immersion in 1.4 mmol/L 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, USA) in PBS.

Immunoelectron microscopy

Two percent formaldehyde PBS-fixed specimens of normal kidney cortex were dehydrated through a series of graded ethanol, and embedded in London Resin (LR) white resin (Polysciences, Warrington, PA, USA), which was polymerized by ultraviolet irradiation at 4°C with benzoin methylether as initiator according to the manufacturer’s instructions. Ultrathin sections were cut and mounted on nickel grids. The grids were first incubated in 5% donkey serum/PBS to prevent non-specific binding. They were then immersed in drops of either KYJ2 antiserum (1:250) or preimmune rabbit serum (1:20) diluted in 5% donkey serum for 20 minutes, followed by incubation with KYJ2 antiserum (1:500), and developed with HRP-labeled goat antirabbit antibody. After treatment with 1.4 mmol/L 3,3′-diaminobenzidine tetrahydrochloride \( \text{H}_2\text{O}_2 \), the glass cover slips were treated with 1% osmium tetroxide in 0.1 mol/L phosphate buffer for 10 minutes, dehydrated by passage through a series of graded ethanol, and embedded in Epon on glass slides. Ultrathin sections were made, stained with 0.1% lead citrate for 7 minutes, and examined with a transmission electron microscope.

<table>
<thead>
<tr>
<th>Nucleotide exchange</th>
<th>Exon #</th>
<th>Effect on coding sequence</th>
</tr>
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<tbody>
<tr>
<td>59C→T</td>
<td>1</td>
<td>P20L</td>
</tr>
<tr>
<td>274G→T</td>
<td>1</td>
<td>G92C</td>
</tr>
<tr>
<td>413G→A</td>
<td>3</td>
<td>R138Q</td>
</tr>
<tr>
<td>538G→A</td>
<td>5</td>
<td>V180M</td>
</tr>
<tr>
<td>871C→T</td>
<td>7</td>
<td>R291W</td>
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Table 1. List of podocin missense mutations

Construction of the full-length and missense mutated podocin cDNA

A partial clone of human podocin cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from human kidney poly (A)⁺ RNA (Clontech, Palo Alto, CA, USA) using oligonucleotide primers (5′-GTG GTG GAC GTG GAT GAG GT-3′ and 5′-CGT CTC CTA GCA CAT CGA TCC-3′) based on the human podocin sequence (Genbank accession no. AJ279254), and was subcloned with a TA cloning kit (Invitrogen, Carlsbad, CA, USA). The digoxigenin (DIG)-labeled probe was made using a PCR DIG probe synthesis kit (Roche Diagnostics, Basel, Switzerland). Human kidney cDNA library constructed from human kidney poly (A)⁺ RNA (Clontech) was screened with DIG-labeled probe from the partial clone of human podocin cDNA. The full-length clone of podocin cDNA was excised using Not I, and was subcloned into the Not I site of pcDNA3.1 Zeo (+) (Invitrogen). The sequencing of the human podocin cDNA clone was done by use of a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with synthetic oligonucleotide primers using ABI 310 DNA sequencer.

Podocin missense mutations (Table 1) were created using the human podocin full-length cDNA clone as template and a Chameleon® Double-Stranded Site-Directed Mutagenesis Kit according to the manufacturer’s protocol (Stratagene, La Jolla, CA, USA). After mutagenesis, the entire mutated podocin cDNA inserts were recloned back into the original expression vector pcDNA3.1 Zeo (+). Finally, the entire mutated podocin cDNA inserts were resequenced to ensure that no undesired mutations had occurred during the mutagenesis.

Construction of the human podocalyxin cDNA

A full-length cDNA encoding the human podocalyxin was cloned into an Xbal/BamHI-cleaved mammalian
expression vector pcDNA3.1/Hygro (−) (Invitrogen). Using PCR and a human podocalyxin cDNA clone [21] as template, a PCR fragment of approximately 1600 base pairs was amplified using a 5'-upstream primer (5'-GCTCAGAGCCACCATGCCTGCGGCGGTGC-3') with a XbaI restriction site (underlined), followed by a Kozak sequence (double underlined) overhang tail, and a 3'-downstream primer (5'-CGGGATCCCTAGAGGTGTTGTC) with a BamHI site (underlined). The PCR amplified cDNA fragment was gel-purified and cleaved with the restriction enzymes (XbaI and BamHI), and was purified again. The cleaved PCR fragment was cloned into the gel-purified XbaI/BamHI-cleaved pcDNA3.1 vector. The final construct, pcDNA3.1-HPC1, having the insert (1587 base pair long, including the ATG start- and TAG stop codon), encoded a full-length cDNA clone for the human podocalyxin.

Construction of the missense mutated nephrin

Two nephrin missense mutations described previously [18], one in the fourth Ig domain (S366R), and another one in the intracellular region part (R1140C), were used for the cotransfection with wild-type podocin construct as below.

Cell lines and DNA transfection

The human embryonic kidney cell line QBI293A (Qbiogene) (HEK293 cell) was grown in Dulbecco's modified Eagle's medium (D-MEM) (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin, in 37°C cultured in the medium containing 200 μg/mL geneticin (Sigma) for one hour at 4°C to avoid nonspecific binding of the lysates to protein A-Sepharose beads. After washing and centrifugation, the supernatants were incubated with peptide-purified KY2 antibody overnight at 4°C, and then again incubated with protein A-Sepharose beads for one hour at 4°C. The pellets of Sepharose beads were washed four times with lysis buffer, and then applied for immunoblotting of nephrin and podocin.

Immunoprecipitation

To determine the protein binding of the missense podocin mutants to wild-type nephrin, NPH15 cell line was transiently transfected with the cDNAs of wild-type or each missense mutation of podocin, and then lysed on ice with a Dounce homogenizer in lysis buffer (10 mmol/L Tris-HCl pH 7.4, 1% Triton-X 100, 0.2% saponin, 0.5% NP40, 150 mmol/L NaCl, 1 mmol/L sodium vanadate, 20 mmol/L sodium fluoride, 1 mmol/L EDTA, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride). The lysates were first incubated with protein A-Sepharose CL-4B (Amersham Biosciences Co., Piscataway, NJ, USA) for one hour at room temperature with KYJ2 antiserum (1:5000) or pAb2 nephrin (0.2 μg/mL). The filters were then incubated for 60 minutes at room temperature with or without a 1:2000 diluted HRP-labeled goat antirabbit antibody and developed by using a Chemiluminescence kit (Life Science Products, Boston, MA, USA) according to the manufacturer’s instructions.

Indirect immunofluorescence and confocal microscopy

To determine the intracellular localization of wild-type and all missense podocin mutants described above, HEK293 cells were cultured on glass cover slips and transiently transfected with the plasmid cDNAs of wild-type or missense mutants, and further cultured for 48 hours. The cells were washed and fixed in fixing solution (3% formaldehyde in PBS) for 15 minutes at room temperature and permeabilized with 0.3% Triton X-100 in PBS for 5 minutes. After incubation with blocking buffer (3% bovine serum albumin and 5% goat serum in PBS) for 60 minutes, the cells were incubated with KY2 antibodies for 60 minutes at room temperature. After

Immunoblotting

Samples of freshly isolated glomeruli, HEK293 cells transiently transfected with wild-type or missense mutated podocin constructs, and the immunoprecipitated samples mentioned above were separated by 5% to 15% sodium dodecyl sulphate (SDS) gradient gel electrophoresis under nonreducing or reducing conditions, respectively. They were then transferred to polyvinilidene difluoride membranes and incubated for one hour at room temperature with KY2 antiserum (1:5000) or HRP-labeled anti-podocin antibody (0.1 μg/mL) and pAb2 nephrin (0.2 μg/mL). The filters were then incubated for 60 minutes at room temperature with or without a 1:2000 diluted HRP-labeled goat antirabbit antibody and developed by using a Chemiluminescence kit (Life Science Products, Boston, MA, USA) according to the manufacturer’s instructions.
washing with PBS, the slides were incubated with Alexa Fluor 488-conjugated goat antirabbit IgG (10 μg/mL) for 40 minutes at room temperature. For double staining of wild-type or mutated-podocin at the proximal C-terminus and the ER or the cis-Golgi, HEK293 cells were transiently transfected with each plasmid and cultured for 48 hours with or without treatment with monensin (500 nmol/L) for the last 20 hours. Cells were fixed and permeabilized as above, and reacted with the KYJ2 antiserum and mouse monoclonal antibody against KDEL (GRP78/94) (5 μg/mL) or GM130 (5 μg/mL), respectively.

For double staining of wild-type nephrin and wild-type or mutated podocin, NPH15 cells were transiently transfected with each podocin plasmid cDNAs. Cells were cultured for 48 hours and incubated with KYJ2 antiserum and mAb2 nephrin (1 μg/mL), followed by the incubation with Alexa Fluor 488-conjugated goat antirabbit IgG and Texas Red-X goat antimouse IgG (5 μg/mL).

For double labeling of podocin mutants and wild-type podocalyxin, HEK-HPC1 cells were transiently transfected with the plasmids of podocin mutants (R138Q, R291W) and cultured for 48 hours, and prepared for immunofluorescence using KYJ2 antiserum and 3D3 antibody (1:100). The positive signals of the podocin mutants and wild-type podocalyxin were visualized with Alexa Fluor 488-conjugated goat antirabbit IgG and Texas Red-X goat antimouse IgG, respectively.

For double labeling of wild-type podocin and missense nephrin mutants, PDN8 cells were transiently transfected with the plasmid DNAs of missense mutated nephrin (S366R, R1140C) and cultured for 48 hours, reacted with KYJ2 antiserum and mAb2 nephrin, and then the positive signals of wild-type podocin and nephrin mutants were visualized with Alexa Fluor 488-conjugated goat antirabbit IgG and Texas Red-X goat antimouse IgG, respectively. All samples were mounted in 1 mg/mL p-phenylenediamine in PBS/glycerol (1:1) and examined under a confocal laser scanning microscope equipped with a Krypton/Argon laser (MRC1024, Bio-Rad; Hercules, CA, USA). Serial optical sections were obtained at 0.5 μm increments under the same image-capturing conditions.

RESULTS
Generation and characterization of polyclonal antibody against human podocin and colocalization of podocin with nephrin at the slit diaphragm

To study subcellular localization of podocin in cultured cells and human kidney sections, a rabbit polyclonal antiserum was generated against a synthetic peptide corresponding to amino acid residues 25 to 40 of human podocin (see Methods). A full-length cDNA clone for human podocin was obtained by screening a human kidney cDNA library (see Methods). A mammalian expression vector containing the full-length cDNA clone was used to generate stable HEK293 cell line expressing the human podocin.

The specificity of polyclonal antibody (KYJ2) was first tested by immunoblotting using samples of isolated human glomeruli and PDN8 cell line. As shown in Figure 1A, this antibody detected a specific protein band of molecular mass of human podocin was detected (Fig. 1A). Under reducing conditions, in addition to the 49 kD band, multiple protein forms of 98 and 147 kD corresponding to dimer and trimer were also recognized by the antiserum (B). Preimmune rabbit serum as negative control did not show any reactivity with protein samples from isolated glomeruli or PDN8 cells (C).

![Fig. 1. Characterization of the polyclonal antibody KYJ2 against human podocin. Immunoblot showing the specificity of KYJ2 antibody to human podocin with glomerular extract and cellular lysate from a human embryonic kidney (HEK)293 cell line transfected with full-length human podocin cDNA construct (PDN8). Fifty μg protein of lysates from isolated human glomeruli and PDN8 cells were separated on 5% to 15% gradient gels under reducing or nonreducing condition, and immunoblotted with KYJ2 antiserum (1:5000). Under reducing condition, a specific immunoband of 49 kD corresponding to the calculated molecular mass of human podocin was detected (A). Under nonreducing conditions, in addition to the 49 kD band, multiple protein forms of 98 and 147 kD corresponding to dimer and trimer were also recognized by the antiserum (B). Preimmune rabbit serum as negative control did not show any reactivity with protein samples from isolated glomeruli or PDN8 cells (C).](image-url)
glomerular-specific localization of podocin. On the other hand, preimmune serum showed no reactivity (Fig. 2C and D). Specific localization of podocin at the SD of podocyte foot processes was also shown by post-embedding immunoelectron microscopy (Fig. 2E and F). Furthermore, double/labeled immunoelectron microscopy using KYJ2 and antibody against the intracellular part of nephrin clearly showed colocalization of podocin and nephrin at the SD (Fig. 2H).

Subcellular localization of wild-type and missense mutated podocin in transfected cells

To study the effect of missense mutations in podocin, five different disease-causing missense mutations found in SRN patients (Table 1) with severe proteinuria were introduced into the full-length construct for human podocin. Two of these missense mutations (P20L and G92C) were located at the N-terminus part of the molecule, while the other three (R138Q, V180M, and R291W) were located at the C-terminus. These cDNA constructs were used to transfect wild-type HEK293 cells for transient expression. To compare the molecular mass of podocin missense mutants expressed in HEK293 cells with that of wild-type podocin, cellular lysates transiently transfected with each podocin constructs were examined by immunoblotting. The molecular mass of all podocin mutants was detected to be 49 kD, similar to that of cells expressing the wild-type construct and glomerular extract (Fig. 3A). To further determine the localization of wild-type and mutated podocin in HEK293 cells, we performed immunofluorescence staining followed by confocal microscopy with transiently transfected cells. As shown in Figure 3B, wild-type podocin was clearly observed at the plasma membrane, including the tip of cellular processes, together with small dotted pattern throughout the cytoplasm. In contrast to this, there were clear differences of subcellular localization among podocin missense mutants. The localization of missense mutants at the proximal N-terminus (P20L, G92C) were shown to be basically the same as that of wild-type, although its plasma membrane expression tended to decrease compared to wild-type. On the other hand, subcellular localization of the mutants at the proximal C-terminus part (R138Q, V180M, R291W) was found to be considerably different from that of wild-type. While R138Q and R291W mutants showed a more similar diffuse-reticular staining pattern in the cytoplasm, the
V180M podocin mutant showed vesicular pattern staining with irregular shape and sizes. What was even more noticeable for V180M mutant was that the staining pattern for this mutant was time-dependent, changing from smaller and dispersed vesicular staining at 24 hours post-transfection (not shown) to larger irregular vesicular staining with aggregated inclusion bodies in the cytoplasm and some very weak staining at the plasma membrane when stained 48 hours post-transfection (Fig. 3B).

To further study the subcellular localization of these mutants, we performed double-immunostaining using an ER-specific monoclonal antibody against the peptide sequence KDEL, which is present at the C-terminus of all ER-resident proteins, and a Golgi-specific antibody against the Golgi-resident protein GM130. As shown in Figure 4A, and also reported by others [25–27], the R138Q mutant was clearly localized to the ER compartment. Colocalization with the Golgi marker, GM130, in cells transiently transfected with each plasmid without monensin treatment. Prominent Golgi localization was seen only with wild-type podocin (B). Colocalization with the Golgi marker, GM130, 20 hours post monensin-treatment (C). V180M and R291W podocin mutants and the wild-type podocin were clearly retained in the Golgi, while subcellular localization of R138Q was not changed by monensin treatment.
ABCD

Fig. 5. Pre-embedding immunoelectron microscopy of podocin mutants. Human embryonic kidney (HEK)293 cells expressing plasmid constructs encoding the wild-type and C-terminus podocin mutants were fixed and processed for immunoelectron microscopy by using antihuman podocin–specific antibody as described in the text. Wild-type podocin is predominantly accumulated in the plasma membrane (arrows) (A). R138Q mutant shows perinuclear endoplasmic reticulum (ER) localization in addition to specific localization on the ER-associated tubular structures (arrows) (B). Plasma membrane localization was not observed (arrowheads). V180M mutant shows inclusion body staining within vesicular structures (arrows) (C). Staining for R291W mutant shows both vesicular localization (arrows) (D), and ER-associated tubular structures (arrowheads). Bar indicates 1 μm.

We next performed pre-embedding immunoelectron microscopy of the cells to identify the precise subcellular localization of each podocin mutants. As it was expected, wild-type podocin was mostly localized at the plasma membrane, but it also showed colocalization with the ER marker and a distinct localization in the Golgi, indicating its normal traffic through ER and Golgi to the plasma membrane (Fig. 4A and B). In contrast to the wild-type, this clear localization in the Golgi was not observed for the C-terminus podocin mutants (Fig. 4B). When cells were further treated with the Golgi-perturbing agent monensin [28], both V180M and R291W mutants, as well as the wild-type podocin were clearly colocalized with the Golgi marker, suggesting that their traffic was perturbed in the Golgi (Fig. 4C). In contrast, the localization of R138Q was not altered by monensin treatment (Fig. 4C), indicating that this mutant was already retained in the ER, as shown in Figure 4A.

We next performed pre-embedding immunoelectron microscopy of the cells to identify the precise subcellular localization of each podocin mutants. As it was expected, the wild-type podocin was predominantly localized at the plasma membrane (Fig. 5A), whereas the R138Q mutant clearly showed a strong perinuclear localization (Fig. 5B), consistent with its retention in the ER as shown in Figure 4A. V180M mutant showed an inclusion body staining pattern within irregular vesicular structures outside of ER and Golgi compartments (Fig. 5C). And finally, cells expressing the R291W mutant appeared to accumulate the mutant protein both in small vesicular structures outside the ER and in the tubular-like structure within the ER.

Podocin missense mutants interfere with the proper traffic of wild-type nephrin

It has been reported that podocin interacts with intracellular nephrin, and both molecules can be isolated from specialized lipid rafts at the SD structure [13]. Therefore, we next studied whether these podocin missense mutants may affect the proper protein trafficking of wild-type nephrin. To this end, a stable transfected cell line expressing wild-type nephrin, NPH15, was transiently transfected with wild-type or each missense mutated podocin cDNAs, followed by immunostaining with KYJ2 antibody and antinephrin monoclonal antibody. Wild-type nephrin colocalizes with C-terminal mutated podocin mutants R138Q, V180M, and R291W. Double-labeling immunofluorescence of podocin mutants, R138Q and V180M, and podocalyxin (B). Human podocalyxin-expressing cell line (HEK-HPC1) was transiently transfected with R138Q and V180M cDNAs, followed by immunostained with KYJ2 antibody and 3D3 monoclonal antibody. The proper localization of podocalyxin on the plasma membrane was not affected by coexpression of the podocin mutants. Double-labeling immunofluorescence of nephrin missense mutants and wild-type podocin (C). PDNS8 cell line was transiently transfected with nephrin mutants, S366R and R1140C, followed by immunostaining with KYJ2 antibody and antinephrin monoclonal antibody. The subcellular localization of wild-type podocin was not altered by nephrin mutants, S366R and R1140C. Bar length is 50 μm.
the plasma membrane; however, as indicated before, intracellular staining of both proteins were also seen in the ER and Golgi-like pattern. Expression of the N-terminal mutants, P20L and G92C, in the NPH15 cells did not alter the plasma membrane localization of wild-type nephrin, indicating that these missense mutations had no effect on nephrin localization (Fig. 6A). In contrast, and interestingly, when C-terminus podocin mutants were expressed in NPH15 cells, the normal membrane localization of wild-type nephrin was dramatically altered. In cells expressing the R138Q mutant, previously shown to be retained in the ER, wild-type nephrin was also found abundantly in the ER, and was completely absent from the plasma membrane (Fig. 6A). Normal localization of wild-type nephrin was also altered in cells expressing V180M and R291W mutants, in which nephrin was absent at the plasma membrane, but instead colocalized with the mutant proteins in the same intracellular compartments.

To find out whether this colocalization of wild-type nephrin and podocin mutants was not due to ER overloading in transient expression system, we chose to test the localization of yet another podocyte cell membrane protein, podocalyxin. Podocalyxin is a transmembrane sialoglycoprotein known for its localization on the podocyte plasma membrane, but not at the SD region. Stable HEK293 cells were established to express a full-length cDNA clone encoding human podocalyxin [21]. Double-staining of such cells showed that the plasma membrane localization of podocalyxin was altered neither by expression of R138Q nor V180M podocin mutants (Fig. 6B). It was concluded that the interruption of wild-type nephrin trafficking was due to the interaction of nephrin and mislocalized podocin mutants.

We finally extended this finding to ask whether missense mutated nephrin molecules may affect the protein trafficking of wild-type podocin. We have previously shown that a number of missense mutations in the nephrin molecule are retained in the ER and do not appear on the plasma membrane [18]. Two such nephrin missense mutants were selected; one for its ER localization (S366R, located in the fourth extracellular Ig-like domain of nephrin), and the other one with no alteration in the trafficking (R1140C, located in the intracellular C-terminus). The PDN8 cells, expressing the wild-type podocin, were cotransfected with plasmid constructs encoding the nephrin mutations. The transport and localization of wild-type podocin and mutated nephrin molecules were followed by double immunostaining. As shown in Figure 6C, coexpression of nephrin mutants did not affect the localization of wild-type podocin.

Finally, we performed a communoprecipitation study to determine whether the direct protein interaction between mutated podocin and wild-type nephrin underlies this defect of nephrin trafficking. The result demonstrated that nephrin was apparently pulled down by anti-podocin antibody in the samples from nephrin-expressing cells transfected with all the proximal C-terminal mutants of podocin, as well as wild-type and N-terminal mutant P20L, indicating a tight association between nephrin and podocin molecules (Fig. 7).

**DISCUSSION**

Defects in intracellular protein trafficking, resulting in the accumulation and aggregation of misfolded proteins in the cells, are a common causative factor in development of many human diseases [29, 30]. In most cases, this alteration in protein trafficking occurs due to missense mutations, leading to secondary protein structures and misfolding of the protein as the consequence.

The ER is a very specialized subcellular compartment, providing the cell a selective environment for complex protein folding and assembly [31]. Protein complex formation and assembly of the newly formed polypeptides are stringently orchestrated by the process of conformation-dependent molecular chaperones, also called quality control system [32]. In the ER, several cotranslational (such as signal-peptide cleavage) and post-translational modifications (such as glycosylation and disulphide bond formation) take place to ensure that newly synthesized secreted or membrane-integrated proteins are correctly transported to their final destinations. Along with this the presence of molecular chaperones, such as the glucose-regulated protein 78 (GRP78) [33], the glucose-regulated protein 94 (GRP94) [34], lectin-like chaperones (calnexin, calreticulin) [35, 36], and disulphide-isomerase [37] are critical for proper protein folding and assembly.

The aim of the present study was to investigate whether and how the disease-causing missense mutations in the NPHS2 gene are involved in the pathomechanism of the proteinuria in patients carrying such mutations. In order to address this question, we introduced five missense mutations into a full-length cDNA construct encoding the wild-type human podocin, and studied the localization of the mutant proteins in transfected cells.
As a tool, we further generated a polyclonal antibody against a synthetic peptide corresponding to amino acid residues 25 to 40 in the human podocin. The specificity of this polyclonal antibody was demonstrated by Western blot analysis on protein extracts from human kidney and transfected cells expressing the wild-type podocin construct (Fig. 1). As expected, immunohistochemistry and electron microscopy studies on human kidney sections demonstrated that polyclonal KYJ2 antibody localized its antigen podocin specifically to the podocyte SD region in the vicinity of nephrin (Fig. 2). Transfection studies using the HEK293 cells revealed that in contrast to the wild-type podocin, several podocin mutants were not targeted to the plasma membrane. Similar results have also been recently reported by others [25–27]; however, some discrepancies remain to be clarified. In their report, Roselli et al investigated subcellular localization of a number of disease-causing podocin mutants using HEK293 cells, in which V180M mutant was observed in the plasma membrane. Our extensive studies on this mutant showed that only a minor fraction of the mutant appeared on the plasma membrane, and the majority of the mutant remains as intracellular inclusion bodies in a time-dependent manner. Since with monensin treatment the inclusion body staining pattern of this mutant was abolished and the mutant was retained in the Golgi, it is most likely that the traffic of V180M podocin mutant is altered in the endosomes or lysosomes after exiting from the Golgi, and not degraded by the cytoplasmic-ubiquitin-proteasome pathway such as aggresomes [38]. Perhaps higher expression levels in our transiently transfected studies compared to cell lines with stable expression used by Roselli et al may underlie the discrepancy in localization of this particular mutant. In fact, the difference in expression levels may explain why the other C-terminus mutant (R238S) also reached to the plasma membrane [27], while in our studies the C-terminus mutants did not reach to the plasma membrane. For the fate of R291W mutant, our data are in good agreement with that reported by Roselli et al, suggesting that this mutant was retained in the late endosomes [27]. However, as shown by our immunoelectron microscopy studies, we also concluded that this mutant, at least partially, retains the ER localization (Fig. 5). As the protein quality control by the chaperones occurs at different stages through ER and Golgi, and artificial overexpression of mutant proteins in cells may cause overloading of this system, it is difficult to unequivocally determine the fate of some missense mutations that may not cause an immediate aggregation or misfolding of the mutated polypeptide.

Although many missense mutations are known to interfere with trafficking and proper subcellular destinations [29, 30], it is little known what amino acid substitutions are generally responsible for this defect of intracellular trafficking. It has been shown that several ER resident motifs, such as KDEL [39] and dilsyne [40], confer ER localization of membrane proteins; however, the amino acid substitutions of R138Q and R291W do not contain these motifs. Many misfolded integral proteins inappropriately expose hydrophobic surfaces that are normally buried inside the protein or at the interface with other subunits [41]. Perhaps the substitution of the arginine residue in both R138Q and R291W mutants, in which the positively charged arginine residue is replaced with an uncharged glutamine residue or a bulky aromatic side chain such as tryptophan, leads to interruption of an important intra- or intermolecular interaction within or between the mutant podocin molecules. In fact, as shown by Schwarz et al [13], oligomerization of podocin molecules and its association with other SD components such as nephrin and CD2AP in the lipid rafts isolate from glomerulus and transfected cells, suggesting that intermolecular interaction between podocin molecules may be essential for its localization and function. In this regard, we found that normal trafficking of wild-type nephrin was dramatically affected in cells coexpressing nephrin together with podocin mutants carrying mutations in the proximal C-terminal part. Our pull-down study clearly demonstrated that such mutations did not affect the intermolecular interaction between nephrin and the podocin mutants, as nephrin could be pulled down from the cellular lysates (Fig. 7). Colocalization of nephrin in cells expressing these podocin mutants, rather, points to the possibility that after binding to podocin mutants, nephrin molecules are also recruited to other cellular compartments than the plasma membrane.

CONCLUSION

An important unresolved issue, however, is to find out whether there is a correlation between NPHS1 and NPHS2 mutations (genotype) and the severity or the onset of proteinuria (phenotype) in patients with hereditary nephrotic syndrome. Koziell et al have recently reported on such potential genotype/phenotype correlations between these two genes in patients diagnosed as CNF, SRN, or congenital FSGS [42]. For example, coexistence of both NPHS1 and NPHS2 mutations was found in all tested patients with congenital FSGS, suggesting a potential functional and corroborative effect between these two genes. Two out of five typically severe CNF patients were also found to lack mutations in NPHS1 gene; however, the patients carry homozygous mutations in the NPHS2 gene [42]. Taken together with our present data, one may speculate that some mutations in NPHS2 gene may not necessarily lead to nephrotic syndrome characterized by SRN phenotype, but instead, the disease may be manifested as severe CNF because the fate of wild-type nephrin may also be affected in such patients.
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REFERENCES