Predisposition to relapsing nephrotic syndrome by a nephrin mutation that interferes with assembly of functioning microdomains

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Minimal-change disease (MCD) is the most common cause of nephrotic syndrome (NS) and is characterized only by minor morphological alterations in podocytes. A subtype of MCD arises from mutations in nephrin, a major component of the slit diaphragm (SD). Idiopathic MCD is a complex trait where interactions of genetic and immunological factors are implicated. However, the pathogenic mechanisms remain unclear. Here we studied the molecular basis for familial NS characterized by frequent relapses and minimal-change histology. Our previous mutational analysis revealed that the two affected children were compound heterozygotes for nephrin variants C265R and V822M (Kidney Int., 2008). When heterologously expressed, these variants exhibited normal metabolic half-life and raft binding. C265R exhibited substantial ER retention, reflecting an intracellular trafficking defect. In contrast, V822M was able to reach the plasma membrane, but was restricted in lateral diffusion as well as trafficking at the cell surface. Clustering of V822M failed to evoke a maximum tyrosine-phosphorylation and actin reorganization, suggesting the inability to assemble into functioning membrane microdomains. Our results suggest that C265R and V822M compose a dysfunctional SD complex due to their mixed defects comprising reduced cell surface targeting and ineffective assembly of signaling microdomains. The defective SD likely confers a susceptibility to immunogenic stimuli and predisposes to a relapsing phenotype.

INTRODUCTION

Minimal-change disease (MCD) is the leading cause of nephrotic syndrome (NS) among children as well as adults. The typical renal histological appearance of MCD comprises only ultrastructural alterations of podocyte foot processes without any immunological deposits or cellular infiltrates (1–3). MCD occurs as primary (idiopathic), secondary and familial forms. Most patients with the idiopathic form of MCD respond to steroids and have a favorable long-term prognosis without progression to renal failure, thereby comprising a distinct clinical subset of steroid-sensitive NS. Despite such excellent steroid responsiveness, patients with MCD exhibit a considerably high incidence (70–80%) of recurrent NS and thereby risk potential cumulative steroid toxicity. However, the etiology of MCD and its characteristic feature of frequent relapses are not well understood. Patients with MCD generally exhibit a robust response to immunosuppression and often relapse following immunogenic events such as an infection or allergic reaction. Immunological disorders such as atopy, autoimmune disease and lymphoproliferative diseases are also associated with MCD (2–4) and these observations implicate dysfunction of T cell-mediated immunity in the pathogenesis of MCD (2–4). A predominantly type 2 helper T cell (Th2) immunological

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imbalance in MCD patients is thought to increase secretion of cytokines that serve as a circulating glomerular permeability factor. However, the molecular identities of these humoral factors and the mechanisms by which they compromise the filtration barriers are still unknown.

Most cases of MCD occur sporadically and are considered to be multifactorial in origin. A role for genetic factors has been supported by various familial and twin studies (2–5). Recent studies of human familial NS and murine models of NS have highlighted the importance of specialized cells of the glomerular filter, the podocyte, in maintaining the integrity of the glomerular filtration barrier. Podocytes exhibit elaborate long, regularly spaced, interdigitating foot processes that cover the glomerular capillaries and form the slit diaphragm (SD), a highly specialized cell junction (5). The SD bridges over a 20–50 nm wide slit between the neighboring foot processes and serves as the ultimate size-selective barrier for proteins (5–7). The discovery of nephrin, a disease gene product for congenital NS of the Finnish type (CNF) (5–7), resulted in great advances in understanding the molecular composition of the SD. Nephrin, a 180 kDa type I transmembrane protein of the immunoglobulin (Ig) superfamily, is a core structural component of the SD. Nephrin consists of eight extracellular Ig-like domains, followed by a transmembrane domain, and a 154 amino acid cytoplasmic C-terminal tail (5–7). The nephrin ectodomains interdigitate in a homo- and/or heterophilic fashion and bridge over the slits to form a zipper-like intercellular junction (5–7). In addition to its key structural relevance, nephrin transduces a phosphotyrosine signal through its cytoplasmic domain, which contains several putative tyrosine phosphorylation sites for Src-family kinases such as Fyn (8,9). Clustering of nephrin facilitates the recruitment of Fyn and its SH2–SH3 domain-containing adaptor Nck, thereby ensuring a more stable anchorage of the nephrin complex to actin cytoskeleton (10).

Genetic studies have revealed that additional molecules involved in familial NS such as podocin, CD2AP, Nep1 and TRPC6 also converge upon the podocyte (5,11–13). These membrane proteins are primarily expressed at the SD and are spatially organized within the so-called lipid rafts; liquid-ordered structures enriched in cholesterol and sphingolipids that form specialized membrane microdomains. Clustering of nephrin ectodomains leads to the sequential coalescence of pre-existing small subdomains containing various adaptors and scaffolding proteins, thereby forming a large, stable signal platform (14). These observations support a new concept that the SD is a lipid-protein supercomplex (15–18).

More than 70 mutations have been reported in CNF patients worldwide and include various types of mutations such as nonsense and nonsense mutations that may occur at a splicing site or promoter as well as small deletions and insertions (19,20). The two mutations of Fin-major (nt121delCT) and Fin-minor (R1109X) are highly prevalent (>90%) in the Finnish CNF patients and generally cause a severe and early-onset phenotype (6,19,20). In contrast, nephrin missense mutations have been implicated not only in severe Finnish CNF but also in milder NS with a later onset and minimal-change histology, thereby broadening the phenotypic spectrum of nephrin-associated disease (21,22). However, the basis for the variability in disease onset and severity caused by different mutations has not yet been fully elucidated.

We found an unusual occurrence of early-onset familial MCD in which the two affected children manifested NS in the first year of life and subsequently had frequent relapses of NS (23). The relapses were always followed by spontaneous remission in which nephrotic proteinuria reduced to a trace or 1+ within a few weeks as the infection resolved. This relapsing nature closely resembled that of a common sporadic MCD. Genetic analysis revealed that the patients are compound heterozygotes for the nephrin variants of C265R and V822M (23). These variants are rare, non-conservative amino acid substitutions thereby supporting the notion that these mutations are indeed causative and not mere polymorphisms.

We are interested in how these nephrin variants lead to a clinical phenotype that resembles the common frequently relapsing MCD. The aim of this study is to define the biological properties of C265R and V822M following heterologous expression in cultured cells. By using a combination of live-cell imaging and biochemical approaches, we have elucidated the previously uncharacterized phenotype of V822M that is unable to effectively assemble into functioning microdomains on the plasma membrane due to its aberrant dynamic behavior. The data suggest that these nephrin variants constitute a defective SD that permits minimal protein leakage under the steady-state conditions. The labile SD does, however, become vulnerable during infection-induced immunological disturbances resulting in nephrotic range proteinuria and thereby conferring a predisposition to relapsing NS in affected patients. Our observations raise the possibility that mild genetic defects in the SD complex may play a wider role in increasing the risk of disease in the more common, non-familial form of NS that is traditionally considered idiopathic.

RESULTS

Familial relapsing NS are caused by nephrin mutations

We studied familial NS, in which two affected children exhibited a similar clinical picture of early-onset, frequently relapsing NS as reported elsewhere (23) (Fig. 1A). The patients first manifested NS early in life at ages from birth (II-2) to 10 months (II-3), but the initial nephrotic proteinuria spontaneously fell to modest levels. Of note, the patients relapsed frequently two to three times per year with relapses triggered by an upper respiratory infection. The proteinuria, however, spontaneously returned to a trace to 1+ on dipstick within 1 or 2 weeks with this spontaneous improvement coinciding with the resolution of the infection. They had persistent mild proteinuria during the remission periods between nephrotic episodes. The incidence of relapses gradually declined toward adolescence. The affected older brother (II-2) underwent two renal biopsies, both of which revealed the histologic appearance of MCD. The clinical features of early-onset relapsing NS are remarkably concordant between the affected siblings, whereas the parents (I-1, I-2) and eldest sibling (II-1) did not exhibit proteinuria. In light of these observations, we first considered that an inherent recessive defect in the SD complex could be the molecular basis for this familial NS. This hypothesis led us to analyze the NPHS1 gene encoding nephrin as a good candidate and mutation analysis revealed that the patients were compound heterozygotes for C265R.
and V822M (Fig. 1A) (23). Since the Cys 265 residue forms a disulfide-bond within the globular portion of third Ig-like domain, C265R is likely to alter the protein folding of the Ig-like domain (Fig. 1B). On the other hand, the Val 822 residue is located in the spacer region between the seventh and eighth Ig-like domains. To predict the structural consequence of the V822M mutation, we performed homology modeling of seventh Ig-like domain with the corresponding homologous portion of the crystal structure of Hemolin, an insect Ig-like molecule (24). The Val822 residue is predicted to lie within the loop connecting the neighboring β-strands.

Figure 1. Missense nephrin variants cause frequently relapsing NS. (A) Pedigree of the family with nephrin mutations. Affected children with recurrent NS (filled symbols, II-2 and II-3) are compound heterozygotes for the nephrin variants of C265R and V822M. The heterozygous obligate carrier patients (I-1, I-2) and mutation negative brother (II-1) have no proteinuria (open symbols), indicating a recessive co-segregation. (B) Domain structure of nephrin and locations of nephrin mutations. NPHS1 gene is 26 kb in size and encodes a 1241 amino acid protein nephrin. Cysteine at amino acid position 265 forms a disulfide bridge within the third immunoglobulin (Ig)-like domain. In contrast, valine at position 822 lies in the spacer region between Ig-7 and Ig-8 domains. (C) Mapping of V822M with homology-based modeling. The structure of nephrin is modeled from the reported crystal structure of insect H. cecropia Ig molecule Hemolin (PDB 1BIH). The residues at position 822 are highlighted by spheres (yellow) and their solvent accessible surface is shown by blue wire. Val 822 residue is predicted to lie on the loop connecting the neighboring β-strands.

Metabolic stability and surface expression of nephrin variants
We employed a metabolic labeling assay with 35S-methionine in COS-7 cells to determine whether these mutations affected the stability of the nephrin protein. By chasing the radio-labeled nephrin for 24 h following the pulse labeling, we determined that the protein half-lives of C265R and V822M were similar to those of the wild-type protein (4.5–5.5 h, n = 6) (Fig. 2A and B). The unaltered protein half-lives of C265R and V822M variants implied that these missense substitutions do not significantly alter their metabolic stability.

We then performed a surface biotinylation assay in COS-7 cells to seek any abnormalities in intracellular protein processing steps and targeting to the cell surface (Fig. 2C). Wild-type nephrin migrated as a doublet of ~180 kDa. The upper band likely represents a mature glycosylated or phosphorylated form, whereas the lower band is immature core-glycosylated proteins located within the ER (8,9,25,26). Cells expressing C265R displayed only the immature band with little or no detectable mature protein band, presumably reflecting aberrant intracellular trafficking and post-translational processing. The abundance of surface-labeled C265R was significantly decreased compared with wild-type nephrin (31.4 ± 8.9%, n = 3, P < 0.01) (Fig. 2D). In contrast, cells expressing V822M exhibited a doublet pattern and produced the mature band at a slightly reduced level relative to wild-type nephrin.
with a concomitant increase in abundance of the immature band. The overall abundance of surface-labeled V822M was slightly diminished compared with wild-type nephrin (77.2 ± 14.5%, n = 3) (Fig. 2D). We observed similar surface expression profiles in human embryonic kidney 293 (HEK293) cells (data not shown) (8,9). These observations indicate that C265R is largely retained within the cytoplasm due to deficient intracellular transport, while most V822M does traffic to the cell surface though at a slightly reduced level compared with that of wild-type nephrin.

### Intracellular localization and trafficking of nephrin variants

We used transfected COS-7 cells for heterologous expression in order to examine immunocytochemical localization of nephrin variants as this would allow clear interpretation of mutant phenotypes. Wild-type nephrin expressing cells typically exhibited a diffuse reticular staining (reticular pattern, 66.7 ± 5.8%), whereas some cells had discrete punctate staining distributed over the cytoplasm (punctate pattern, 31.5 ± 5.1%) (Fig. 3A and B). Expression of nephrin in other cell lines including cultured podocytes exhibited a similar distribution (data not shown). In contrast, C265R expressing cells showed a striking reticular pattern (91.2 ± 2.6%) with minimal production of the discrete punctate pattern (2.8 ± 1.7%), suggesting substantial retention of misfolded C265R variants in the ER. In contrast, V822M expressing cells exhibited a reticular pattern with a similar extent to cells expressing wild-type nephrin (68.1 ± 9.3%). However, V822M expressing cells tended to produce a vesicular structure with a concomitant increase in the reticular staining (referred as a ‘mixed pattern’, 12.3 ± 4.3 versus 2.5 ± 1.1% in cells expressing wild-type nephrin), rather than the simple generation of cytoplasmic vesicles (‘pure’ punctate pattern, 19.7 ± 4.3%). Notably, V822M vesicles were more variable in size and morphology than wild-type nephrin vesicles and some puncta were larger and brighter. These observations suggest that most V822M exit the ER and traffic to the cell surface, while some V822M are trapped within the ER by the ‘quality control’ surveillance and/or en route during the aggregation process. V822M was then co-expressed with C265R to mimic the compound heterozygous state evident in the patients. The presence of C265R did not affect the overall cellular distribution or the intracellular trafficking of V822M (Fig. 2B). The absence of any interference of one mutant allele with the other was in good agreement with the recessive inheritance pattern of the disease in this family (Fig. 1). These data suggest that, while C265R represents a trafficking-deficient variant, V822M retains the ability to traffic
Figure 3. Immunocytochemical localization of nephrin variants. (A) Cellular localization of mutants in COS-7 cells. COS-7 cells transfected either singly (wild-type nephrin, C265R, V822M) or doubly (C265R and V822M) were fixed, permeabilized and stained with anti-nephrin antibody. Bars; 10 μm. (B) Quantification of mutant phenotypes. Cellular localization is binned into three patterns: a punctate, reticular and mixed pattern. The punctate structures represent a fraction of 50–100 nm of the cell surface, thereby resolving not only lateral diffusion but also endocytic trafficking properties at a single molecular scale (28). Living COS-7 cells transfected with GFP-wild-type or GFP-V822M nephrin were investigated under TIRF illumination (depth of field approximately 100 nm). Most GFP-wild-type nephrin molecules appeared as dynamic and discrete punctate fluorescent spots on the cell membrane (Fig. 5A). A majority of GFP-wild-type nephrin remained within the TIRF field for an extended period of time, typically more than 10 sec, while some appeared only transiently in a few sequential frames (<2 sec). Quantitative analysis indicated that the average residence time was 27.3 ± 4.6 sec (Fig. 5B). The TIRF signal of GFP-wild-type nephrin displayed large diffusive trajectories, moving laterally within an area with a diameter of 1.69 ± 0.1 μm during the 3-min tracking time (n = 200) (Fig. 5C), which is in the range reported for single molecule kinetics of several cell receptors (1.0 μm in diameter) (29). Compared with GFP-wild-type nephrin, GFP-V822M puncta entering the TIRF field were reduced in size and surface brightness (Fig. 5A). Analysis of sequential TIRF time series revealed that most GFP-V822M punctate structures were fast moving and short-lived spots that resided for 0.5 to 10 s (mean resident time 4.5 ± 1.9 s). The average cell-surface lifetime was much shorter than wild-type nephrin (Fig. 5B). The TIRF signals of GFP-V822M displayed a restricted lateral motion, as they were only diffusible within the confined area of 0.78 ± 0.05 μm in diameter (n = 200) (Fig. 5C). These data suggest that V822M forms a less stable structure at the living cell membrane as it exhibits a shorter resident time and more confined lateral motion than wild-type nephrin.

Membrane dynamics of GFP-V822M after photobleaching

We next analyzed the diffusional mobility of V822M on the plasma membrane of live cells by the fluorescent recovery after photobleaching (FRAP) analysis in order to dissect the dynamic behavior of V822M at the cell surface (27). We therefore generated mouse L cells expressing green fluorescent protein (GFP)-tagged wild-type nephrin or the V822M variant (Fig. 4A). After photobleaching in GFP-wild-type nephrin expressing cells, fluorescence signals in the bleached area readily recovered as a result of rapid replenishment via lateral diffusion from the outer margins of the bleached area and reached a steady plateau at 75.6 ± 2.5% of the initial level (n = 6, Fig. 4B and C). The rapid kinetics and homogenous distribution of the recovered fluorescence suggested that nephrin was mobile as single particles or in very small groups. In contrast, fluorescence of the GFP-V822M variant recovered at a slower rate and only reached up to 60.7 ± 4.6% of the initial pre-bleach level (n = 6). Quantification analysis revealed that an immobile fraction of V822M (39.3 ± 4.6%) was significantly increased compared with wild-type nephrin (24.4 ± 2.5%, n = 6, P < 0.05) (Fig. 4D). These data indicate that V822M has a restricted diffusional mobility at the plasma membrane.

Surface diffusive motion and trafficking of single V822M molecules

In an attempt to explore the functional defects in more detail, we next examined the dynamics of nephrin at the cell surface by using total internal reflection fluorescence (TIRF) microscopy. Real-time TIRF microscopy visualizes molecular events that selectively occur in the optical plane within cellular localization is binned into three patterns: a punctate, reticular and mixed pattern. The punctate structures represent a fraction of nephrin being along a vesicular trafficking pathway, while the reticular staining indicates a portion of nephrin retained within the ER. Histograms denote a percentage of each category in the total (n = 100, mean ± SE from three independent experiments). C265R exhibits a more pronounced reticular pattern than wild-type nephrin. V822M vesicles accompany a concomitant increase in the reticular staining ('mixed' pattern) rather than simply presenting a 'pure' punctuate pattern. A proportion of the reticular, ER-retention pattern of V822M is unaffected by co-expression of C265R. Bars; 10 μm.

Aberrant assembly of membrane rafts upon clustering of V822M

Lipid rafts are example of membrane microdomains that function as signaling platforms (14,15). To evaluate the ability of
nephrin variants to efficiently organize within the microenvironment of the plasma membrane, we isolated membrane lipid microdomains of transfected COS-7 cells on the basis of their relative insolubility in non-ionic detergent Triton X-100 and their ability to float in density gradient (Fig. 6A and Supplementary Material, Fig. S1) (30). Both C265R and V822M variants accumulated in the low buoyant, detergent-resistant membrane fractions (DRMs) containing the raft marker caveolin, to a similar level as wild-type nephrin. Co-immunoprecipitation experiments indicated that C265R and V822M physically interact with podocin; a property similar to wild-type nephrin (Supplementary Material, Fig. S2). These data suggest that both variants retain normal binding affinity to lipid rafts as well as resident raft proteins in the non-clustered state. Recent studies have suggested that clustering of microdomains is a key process and mediates their various physiological functions (21,31). In this context, we examined the effects of antibody cross-linking on the raft partitioning of nephrin to examine whether the variants could effectively form a larger raft platform. Under non-clustered conditions, V822M segregated into raft microdomains (17.5±2.8%, n=3) to a similar extent as wild-type nephrin (14.9±3.9%, n=3) (Fig. 6A and B). Ectodomain cross-linking of wild-type nephrin further increased its raft-association by ~1.5-fold relative to un-cross-linking control. In contrast, cross-linking of V822M failed to increase its raft-partitioning (Fig. 6B). The results indicate that V822M fails to coalesce effectively into larger raft clusters, despite its normal partitioning into small separate microdomains in the non-clustered state.

Clustering of V822M fails to activate phosphorylation signaling

Membrane microdomains function as a signaling platform for nephrin and the signaling plays a vital role in its stabilization and remodeling of the SD complex. To assess the competence of V822M to assemble the signaling microdomains, we performed the phosphorylation assay in HEK293 cells as these cells endogenously express Nck, which is an adaptor protein for nephrin (8,9). In HEK293 cells, co-expressing nephrin and Src family kinase Fyn (active form), ectodomain engagement with anti-nephrin antibody rapidly induced its own robust tyrosine phosphorylation. The peak value was reached at 2 min (125.6±0.6%, P<0.001 relative to control n=3) and gradually attenuated over the next 5 to 15 min (73.5±5.4% and 15.4±9.6%, respectively, n=3). The nephrin phosphorylation was mediated mainly via specific interaction with Fyn, since it was inhibited by co-expression of the kinase-dead Fyn and treatment with the Src kinase-specific inhibitor PP2 (data not shown). Irrelevant immunoglobulin did not stimulate nephrin phosphorylation, suggesting the requirement of nephrin clustering in the activation of the signaling process. In contrast, we barely found the fast activation of phosphorylation for V822M as evident by the weak initial peak signal at 2 min compared with wild-type nephrin (104.5±6.6%, n=3, Fig. 7A and B). Instead, despite the minimal fast activation, V822M sustained the initial phosphorylation level for a longer period, thereby giving a greater signal at 5 min in comparison with wild-type nephrin (99.3±4.1%, P<0.05 versus wild-type 73.5±5.4%). These data indicate that V822M invokes a diminished and delayed phosphorytrosine-base signaling response relative to wild-type nephrin, presumably due to impaired molecular interaction through the proper lateral assembly of microdomains.

Clustering of V822M is unable to reorganize actin filaments

Actin cytoskeletal rearrangement is another example of an essential microdomain-mediated biological process that occurs following nephrin clustering. The ectodomain engagement of nephrin alters actin dynamics through lateral segregation of discrete submicrodomains containing the signaling molecules, i.e. Fyn (8,9). The raft-associated scaffolding
protein podocin coordinates the assembly of the signaling complex on the plasma membrane (15,16). In order to determine how the actin cytoskeleton is reorganized following clustering of V822M, we performed a patching assay in COS-7 cells co-expressing nephrin (wild-type or V822M) with Fyn and podocin. Antibody against nephrin ectodomain was used to patch the raft components on the surface of living cells. In control COS-7 cells, nephrin distributed uniformly over the cell surface (Fig. 8A). Upon ectodomain cross-linking by anti-nephrin antibody, wild-type nephrin formed large discrete and bright spots (3.03 ± 0.39 x 10⁻³ μm², n > 10,000) (Fig. 8B). The wild-type nephrin patch clearly exhibited co-accumulation with podocin and F-actin and induced concomitant actin redistribution, altering it from the filamentous...
pattern (stress fiber) to a more condensed comet-like configuration (Fig. 8A) (8,9,27). On the other hand, V822M displayed smaller patches (1.74 ± 0.11 μm², n = 10,000), which were accompanied with less co-accumulation with podocin and F-actin. Since V822M reaches the cell surface at the level of ~70% relative to wild-type (Fig. 2C and D), the diminished size of patched foci is likely due to the impaired lateral segregation of V822M rather than to its reduced surface availability. These data indicate that V822M fails to efficiently induce an interaction network that regulates actin dynamics though lateral segregation of membrane microdomains (Fig. 9).

**DISCUSSION**

The main findings of this study are that an unusual familial case of minimal-change NS with frequent relapses arises from the combined effects of two partially deficient nephrin variants C265R and V822M that result in (i) reduced but not abolished surface availability, (ii) restricted motion and trafficking on the cell surface and (iii) improper dynamic lateral assemblies of microdomains. Some examples of nephrin mutations that could traffic to the cell surface have been reported in patients with NS but the critical question of how these mutations mechanistically cause the disease is still open (22,25). The present study is the first to provide experimental data supporting a plausible mechanism by which nephrin variants lead to a partially defective and labile SD. Accordingly, V822M is unable to effectively cluster, transduce the signaling and organize actin filaments (Figs 6–8). These observations highlight a novel phenotype of a nephrin variant that is unable to efficiently incorporate into the functioning lipid-protein supercomplex at the plasma membrane. The altered microdomain assembly provides a feasible explanation for the subtle instability in the filtration barrier of the patients in whom it maintains almost normal function between nephrotic episodes but transiently becomes much leakier during immunological disturbances (23).

We consider that our patients may be added to the growing list of lipid microdomain-related diseases, in which the membrane microdomain is the primary site for the pathogenesis with defective spatio-temporal regulation of microdomains resulting in the clinical phenotype (31). This view is in accordance with the emerging paradigm of podocyte biology that membrane microdomains play a central role in orchestrating diverse cellular function such as signaling, polarity and cell shape (14,15).

**Clinical features of relapsing NS caused by mild NPHS1 variants**

The present case is a rare familial NS with minimal-change histology. Both patients exhibited a similar mild and early onset NS with the following characteristic features: (i) NS manifested in the first year of life, (ii) spontaneous though partial remissions with no requirement for steroid therapy,
abnormal SD integrity. Our genetic analysis provides clues that increased cytokine levels that exacerbate the already existing extra-renal stresses to induce overt NS such as proteinuria. It is conceivable that the onset of genetically based NS requires the presence of persistent proteinuria from early life. It is consistent with the diagnosis of MCD (23). Moreover, the co-expression study (Fig. 2) suggests that these variants are functionally recessive.

A new phenotype of V822M is defective microdomain assembly

Our results shed light on a novel biological feature of V822M, which interferes with the dynamic assembly of functioning microdomains at the cell surface. In contrast to C265R that exhibits minimal surface expression, V822M is correctly targeted to the cell surface where it has an expression level of ~70% that of wild-type nephrin (Fig. 2), indicating that there must be an appreciable number of V822M that contribute to the formation of the functional complex on the cell membrane. We therefore suspected that V822M is primarily dysfunctional at the cell membrane with a possible defect in the process of assembling dynamic nephrin complexes. The plasma membrane is a lipid bi-layer organized into discrete compartments or microdomains through lipid–lipid, lipid–protein and membrane–cytoskeleton interactions (14,15). These microdomains act as a platform for sequestration of interacting proteins and lipids, selectively involved in vesicular transport, signaling and cell–cell adhesion (14,15,31). The raft microdomains have been the focus of much recent research and are key players in myriad disease processes including vascular, metabolic, neurodegenerative, immunological and infectious diseases (31).

By visualizing and purifying the membrane microdomains, we found that V822M is less able to create the microenvironment required for proper assembly of the signaling complex (Fig. 9). Clustering of membrane microdomains has been implicated as a key process for stable anchorage of the SD complex to the cytoskeleton and the maintenance of podocyte architecture. In this context, the confined diffusive motion and shorter surface lifetime of V822M (Figs 4 and 5) may reflect its inability to properly incorporate into a lipid compartment or to link itself to the cytoskeleton. Clustering of V822M via cross-linking did not rapidly guide additional small rafts into the expanding, larger lipid-protein platforms, thereby making it difficult to create a microenvironment suitable for nephrin signaling and actin reorganization (Figs 6–8). We consider that the aberrant microdomain assembly delays or impairs stable anchorage of the SD complex to the cytoskeleton as well as the remodeling that follows cytokine-mediated insults. Our observations together with others indicate that the dynamic organization of membrane microdomains regulates its own competency of trafficking and signaling and represents a key process to ensure the maintenance of glomerular filtration barrier function.

Phenotypic classes of nephrin variants

The majority of nephrin mutations reported so far typically lead to a severe clinical phenotype of CNF or early-onset
However, our results together with others (21,22) have revealed that a range of allelic differences of nephrin mutations could account for a varying severity of NS. Biological phenotypes of defective plasma membrane proteins have been extensively studied such as the transmembrane regulator (CFTR) in cystic fibrosis (32) and low-density lipoprotein receptors (33). These studies have shown that mutations are categorized into several classes based on their protein stability, cellular localization and function on the plasma membrane. In this context, we propose that nephrin mutations may lead to dysfunction of the SD via at least three distinct mechanisms (Supplementary Material, Fig. S3). Class I mutations, exemplified by a frame-shift mutation Fin-major, disrupt a transcription or translation process. Total absence of functioning nephrin leads to a severe, congenital phenotype (6,19,20). Class II mutations, as observed in most early-onset steroid resistant NS (25), or C265R in this case, result in the miss-trafficking of proteins that are trapped in the ER (34). Previous studies have demonstrated that the majority of nephrin missense mutations in patients with congenital NS (25,26) exhibit a class II phenotype. The observations suggest that ER retention via the quality-control surveillance is a general mechanism underlying severe SD dysfunction. We found that C265R is mostly retained in the ER, despite the ample delivery of V822M to the cell surface. C265R is likely to cause more deleterious conformational changes by altering an intra-molecular, disulfide bridge structure within the Ig-like module than V822M that affects the spacer region between Ig-like domains.

Class III mutations yield mutant proteins that traffic to the cell membrane but are dysfunctional at the plasma membrane. V822M, for example, does not properly assemble into functional SD complexes. In agreement with the previous studies that some mutations may have characteristics of more than one class (32), V822M shows a combined phenotype that mainly corresponds to class III but also has some class II characteristics. Based on these observations, we postulate that a class II trafficking-deficient variant C265R renders the nephrin complex unstable in combination with the class III V822M variant, which is unable to efficiently assemble into functional complexes at the plasma membrane.

Our proposed classification of nephrin mutations provides a framework for understanding the molecular basis of the defects in glomerular filtration barrier and a start point for comprehensive genotype–phenotype correlations. Generally, class III mutations that successfully target to the plasma membrane lead to a milder phenotype than class II mutations with ‘null surface expression’. Other researchers have reported that several children with compound heterozygosity for at least one class III-type nephrin mutation exhibit a later onset and milder course of NS than those who exhibit homo- or compound heterozygosity for only class I and/or class II-type mutations (22).
Notably, the Finnish patients with compound heterozygosity for a Fin-major and missense nephrin mutation show a varying severity of diseases ranging from CNF to MCD (35). The combination of these observations with ours suggests that the presence of at least one ‘mild’ nephrin allele may lead to a milder disease and that such mild recessive alleles may be more widely implicated as a genetic predisposing factor to proteinuria in many kidney diseases.

Relevance of nephrin variants in the etiology of common NS
The question of what environmental factors trigger the relapses in the present case continues to elude us. Spontaneous remission of congenital NS has been described only in rare occasions, but is generally considered to be exceptional. The relapse-remission phenotype of our patients may be ascribed to the complex disease traits involving epistatic interactions of multiple genes and environmental factors. We have discussed various causes for recurrent proteinuria, especially in the context of the T cell hypothesis (4). Numerous studies have suggested that aberrantly regulated T cells release an as-yet-undefined proteinuric circulating factor, thereby leading to the breakage of the ‘charge-barrier’. In our patients, we did not find any immunological abnormalities (23). The present data support the notion that nephrin variants confer a structural weakness to the SD due to the ineffective assembly of the lipid–protein complexes. The defective SD is likely to

Figure 9. Pathogenic models for mild recurrent NS caused by nephrin variants. (A) Normal SD (wild-type nephrin) and regulation of SD integrity. Nephrin is a principal component of the porous filter structure, i.e. SD, between adjacent foot processes. Intracellular nephrin associates with adapter and signaling molecules (Fyn, Nck etc.), and scaffolding proteins (podocin). These SD constituents, under non-clustered conditions, reside in separate small subdomains and float freely within the membrane. Upon clustering of nephrin, they are dynamic and coalesce into a larger and more stable signal platform. Such lateral assembly facilitates nephrin tyrosine phosphorylation and actin reorganization, which ensures stable tethering of the SD complex into the cytoskeleton. At steady-state, most wild-type nephrin is integrated into the SD complex as a dephosphorylated form with no or little lateral dispersion or endocytic removal. Cytokine activation (e.g. infection) triggers podocyte injury that disrupts the SD complex leading to foot process effacement with proteinuria. This promotes nephrin phosphorylation and the subsequent actin polymerization, thereby facilitating the SD reconstitution and cytoskeletal remodeling. As podocytes recover from the injury, nephrin becomes dephosphorylated and the SD complex is reconstituted as normal. (B) Defective SD composed of C265R and V822M leading to mild, relapsing NS. The unstable SD complex comprising C265R and V822M is the molecular basis for mild and recurrent NS with minimal-change histology. C265R is a class II mutation that reduces the surface availability at the SD. V822M is mainly class III, which fails to efficiently assemble into functional nephrin complexes at the plasma membrane. C265R and V822M build up the partially defective, labile SD that permits transient massive protein leakage upon cytokine stimulation (i.e. infection). The dysfunctional SD also appears to be leaky to some extent even during the remission period between nephrotic episodes, as evident by the sustained mild proteinuria. Nephrotic episodes presumably arise from the inability of nephrin variants to fully reassemble into the complete SD complex after cytokine injury.
be more vulnerable to immunological stimuli such as T cell activation and cytokine release. A heterozygous missense nephrin variant was found in 5 of 25 Finnish patients with childhood onset MCD (21), suggesting that even the carrier state of nephrin variants may have an increased risk of NS. Further studies are needed to clarify the potential interactions between the immunologic defects and altered structural property of the SD.

Recent studies have shown that not only common but also rare genetic variants likely contribute to the susceptibility of multifactorial inherited disorders (36). The present case is a good example of a human genetic model by which rare genetic variants contribute to the pathogenesis of complex, multifactorial disorders. The elucidation of the genetic basis for a rare familial NS facilitates understanding the etiology of the more common, non-familial NS. For example, podocin mutations have been found in sporadic patients with NS, which are clinically indistinguishable from common non-familial cases (37,38). Some of the podocin mutations, e.g., R229Q, present with a relatively higher allele frequency (~3%), suggesting that such genetic variants could behave as a common susceptibility factor to NS (38). In light of the fact that nephrin acts as a protein-lipid complex and interacts with other indispensable SD components such as podocin and CD2AP (5,15–18), genetic variants in these proteins may also affect SD integrity to a similar extent as the nephrin mutations. It is therefore plausible that mutations in two or more genes eventually give rise to a common clinical picture of NS and their interactions result in the varying severity of the histological pattern and clinical phenotype. In line with this hypothesis, there have been reported cases of NS with an oligogenic model of inheritance, whereby epistatic interactions among the genes encoding the SD complexes can modify the phenotype (39,40). Taken together, further studies will be required to explore how potential epistatic interactions between genes underlying familial NS as well as effects of gender, ethnicity or other environmental factors influence the clinical phenotype.

Our observations indicate that allelic heterogeneity of nephrin affects the SD integrity to a varying extent and leads to a spectrum of disease severity of NS. Similarly, genetic variants in the SD complex other than nephrin may confer an increased risk to NS. Before our findings are generalized, there is an obvious need for the careful evaluation of similar cases to define the relative importance of intrinsic genetic susceptibilities in podocytes versus external podocyte stress such as immunogenic cytokine stimuli in the pathogenesis of NS. Elucidation of the molecular basis will increase our mechanistic understanding of the pathogenesis of multifactorial, common proteinuric disorders and will guide specific therapeutic approaches.

**MATERIAL AND METHODS**

**Patients**

Families were recruited, with informed, written consent obtained from all participating members. Detailed clinical picture of the patients was reported elsewhere (23). The research ethics committees at our institutions approved all protocols used in this study.

**Constructs and antibodies**

A human nephrin cDNA (Gene Bank; NM_004646) was subcloned into pcDNA3.1 V5-His expression vector (Invitrogen). The mutant constructs for C265R and V822M were generated using QuickChange Site-Directed Mutagenesis Kit (Stratagene). Nephrin-GFP pcDNA3.1 was constructed by inserting an EGFP fragment following the amino position 1093 of nephrin coding sequence. The rabbit anti-nephrin polyclonal antibodies were raised against a synthetic peptide comprising the 17 carboxy-terminal (1104-1125) or the 17 amino-terminal amino acids (199-216) of human nephrin and were affinity-purified by the peptide column. These antibodies were used at 1:80 dilution for immunoblot and 1:200 for immunofluorescent study. pME-B-FynY531F or pME-B-FynK299M were generous gifts from T. Yamamoto (Tokyo University).

**Protein structural modeling**

A molecular model for nephrin was generated by a homology-based method. A portion of nephrin seventh Ig domain is predicted based on the known crystal three-dimensional structure of D3 domain in Hemolin (Protein Data Bank code 1BIH) (24) by the use of Discovery Studio (Accelrys).

**Cell culture and transfection**

COS-7 and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Cansera International Inc.), penicillin and streptomycin (Sigma-Aldrich). Transfections were performed using TransFast (Promega).

**Metabolic labeling**

Transiently transfected COS-7 cells were metabolically labeled by incubating cells in methionine-free medium containing 75 μCi/ml [35S]-methionine for 1 h (pulse). After replacement with the complete medium containing unlabeled methionine, cells were then chased for 0, 6, 12 and 24 h. The cellular lysates were immunoprecipitated with monoclonal anti-V5 antibody. Immunoprecipitates were resolved by an 7.5% SDS–polyacrylamide gel and visualized by fluorography.

**Immunofluorescent staining**

Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min and blocked with 1% BSA in PBS. Primary antibodies were obtained commercially and used at indicated dilutions: anti-podocin [IF: 1/800; western-blot (WB): 1/10 000], anti-V5 (Invitrogen, IF1/100, WB:1/200), anti-caveolin-1 (BD Transduction Laboratories, 1/5000) (27). Secondary antibodies were obtained from Molecular Probes and Jackson ImmunoResearch Laboratories. Slides were mounted on glass slides with Prolong Anti-fade® mounting medium (Molecular Probes). Images were...
obtained with a Zeiss LSM510 and a PASCAL microscope (Carle Zeiss).

Cell surface biotinylation
Surface biotinylation was performed following the manufacturer’s protocol (Pierce). Briefly, transfected cells grown on 60 mm dish were washed with ice-cold PBS (pH8.0) to remove amine-containing culture media and proteins from cells. Surface proteins were labeled by Sulfo-NHS-Biotin reagent (final concentration; 2 mM) for 2 h on ice, and then cells were washed with 100 mM glycine PBS to quench and remove excess biotin reagent and byproducts. Biotin-labeled cells were collected by streptavidin beads (Pierce) and were analyzed by the immuno-blotting.

Immunoprecipitation and immunoblot analysis
Transfected COS-7 cells were lysed in RIPA buffer (50 mM Tris–HCl, pH8.0; 150 mM NaCl; 0.5% sodium deoxycholate; 1% NP-40; protease inhibitor cocktail, Roche Diagnostics Corp.). Lysates (500 μg) were rocked at 4°C for 2 h with the appropriate primary antibody. Then 40 μl of protein G-Sepharose beads were added and rocked at 4°C for 1 h. The bound proteins were resolved by a 7.5% SDS–PAGE, and analyzed by immunoblotting with specific antibodies and ECL-plus detection kit (GE Healthcare Bio-Sciences Corp.).

Fluorescence recovery in photobleaching (FRAP) analysis
Time lapse live-cell images were collected on mouse L cells expressing GFP-nephrin using a Zeiss Pascal confocal microscope (27). Fluorescence of selected 5.0 × 5.0 μm square regions of interest (ROIs) was bleached at 37°C with the 488 nm laser line at full power and full transmission for 2 s. The fluorescence recovery was acquired every 1 ms interval with 1% of laser power, minimizing the photo-toxicity during the scanning period. The recovery values were expressed as pixel intensity and were normalized by the average pre-bleach values of each ROI. The half-time required for the bleached fluorescence to rise to 50% of the full recovery value (t1/2) and mobile fractions (Mf) were estimated by non-linear regression to a function for lateral diffusion.

Phosphorylation assay
HEK293 cells were transfected with nephrin and FynY531F (an active form) or FynK299M (an inactive form). After labeling nephrin on the cell surface with the N-terminus antibody at an active form or FynK299M (an inactive form). After labeling nephrin on the cell surface with the N-terminus antibody at 4°C for 30 min, cells were incubated at 37°C for the indicated periods of time. The cells were lysed in NP40 buffer (150 mM NaCl, 1%NP-40, 50 mM Tris; pH8.0 with protease inhibitor (Roche Molecular Biochemicals) plus 50 mM NaF and 1 mM Na3VO4). Lysates were incubated with 1 μg of phosphotyrosine-RC20: Biotin at 4°C for 2 h and biotinylated protein was isolated with UltraLink Immobilized streptavidin beads (Pierce). Bound proteins were analyzed by immunoblotting with anti-nephrin-Ab.

Total internal reflection fluorescence (TIRF) microscopy
Imaging was carried out using a Nikon ECLIPSE Ti-E microscope with a CFI Apo TIRF 100 × H (NA 1.49) objective, equipped for through-the-objective TIRF illumination (28,29). A 488 nm argon laser was used as the light source. Cells were imaged in DMEM without phenol red containing 10% FCS and 10 mM HEPES (pH 7.4), maintained at 37°C using a temperature-controlled stage. Time-lapse sequences were acquired at a continuous rate of two frames per second using a CASCADE II 512 CCD camera (Nippon Roper). Acquired image sequences were saved as stacks of 16 bit TIFF files and were analyzed by ImagePro software.

Statistical analysis
Data are expressed as mean ± SE of n experiments. Statistical evaluation was performed using Student’s t-test for repeated measures. Values of P < 0.05 were considered to be statistically significant.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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