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Molecular basis of the functional podocin–nephrin complex: mutations in the *NPHS2* gene disrupt nephrin targeting to lipid raft microdomains

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Hereditary nephrotic syndrome is a heterogeneous disease, characterized by heavy proteinuria and renal failure. Mutations of *NPHS1* or *NPHS2*, the genes encoding for nephrin and podocin, lead to early onset of heavy proteinuria, and rapid progression to end-stage renal disease, suggesting that both proteins are essential for the integrity of the glomerular filter. Podocin is a stomatin protein family member with a predicted hairpin-like structure localizing to the insertion site of the slit diaphragm of podocytes, the visceral glomerular epithelial cells of the kidney. Here we investigate the pathomechanisms of different disease-causing podocin mutations. We show that wild-type podocin is targeted to the plasma membrane, and forms homo-oligomers involving the carboxy and amino terminal cytoplasmic domains. The association of podocin with specialized lipid raft microdomains of the plasma membrane was a prerequisite for recruitment of nephrin into rafts. In contrast, disease-causing mutations of podocin (R138Q and R138X) failed to recruit nephrin into rafts either because these mutants were retained in the endoplasmic reticulum (R138Q), or because they failed to associate with rafts (R138X) despite their presence in the plasma membrane. None of the mutants did augment nephrin signaling, suggesting that lipid raft targeting facilitates nephrin signaling. Our findings demonstrate that the failure of mutant podocin to recruit nephrin into lipid rafts may be essential for the pathogenesis of NPHS2.

INTRODUCTION

The recent description of gene defects of the podocyte resulting in hereditary nephrotic syndrome has provided a completely new understanding of the glomerular filter and unraveled important aspects of the pathogenesis of proteinuric kidney diseases (1,2). The most severe hereditary disorder is the congenital nephrotic syndrome of the Finnish type, caused by mutations in *NPHS1*, the gene encoding for nephrin. Nephrin is an integral membrane protein of the immunoglobulin super-family located at opposing sites of the foot processes formed by podocytes, specialized epithelial cells that ensure size and charge selective ultrafiltration (reviewed in 3–5). The precise function of nephrin is unknown; however, nephrin is a critical structural component of the slit diaphragm, an ultra-thin zipper-like structure that bridges the ~40 nm wide slit between interdigitating podocyte foot processes. Recent studies have emphasized the central role of the slit diaphragm in the function of the size-selective filtration barrier of the kidney (3,4). In addition to the function as adhesion protein at the slit diaphragm nephrin triggers signal transduction in podocytes. We have recently shown that the cytoplasmic tail of nephrin is tyrosine phosphorylated and recruits signaling intermediates that bind to the phosphorylated tyrosine residues (6,7). We could also show that nephrin is a signaling protein and that signal transduction can be augmented by another podocyte protein called podocin (6). Podocin is a hairpin-like protein at the slit diaphragm encoded by *NPHS2*, the gene disrupted in a steroid-resistant hereditary nephrotic syndrome (SRN) (8–10). Genetic evidence has suggested a functional inter-relationship

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between *NPHS1* and *NPHS2* (11). In addition to nephrin and podocin, other podocyte proteins including CD2AP (12,13), α-actinin 4 (14), Neph1 (15–18) and FAT (19) have recently been associated with the development of proteinuria. Although many of the key components of the slit diaphragm have now been identified, the fundamental question remains of how the different components are organized at this specialized cell–cell junction.

Multiple evidence suggests that the plasma membrane of the filtration slit contains lipid rafts (20–22). Lipid rafts are specialized microdomains of the plasma membrane with a unique lipid composition that are highly concentrated in signal transduction molecules. Rafts have been proposed to form platforms for many important cellular processes, such as polarized sorting of membrane proteins and signal transduction (23,24). It has been shown that podocin is a lipid raft-associated protein at the filtration slit (25). These data suggested that podocin may be required for the structural organization of the slit diaphragm and the regulation of its filtration function (25). However, the exact function of podocin, as well as the structural determinants for lipid raft association, has not been clear so far. Here we show that podocin serves to recruit nephrin into lipid raft microdomains. Lipid raft targeting of nephrin is required for the proper initiation of nephrin signaling. Disease-causing podocin mutations may still interact with nephrin but fail to recruit nephrin into rafts either because they either do not enter the transport pathway to the plasma membrane or they get to the plasma membrane but are not targeted to rafts. Therefore these mutants cannot augment nephrin signal transduction. These data underscore the critical role of podocin-mediated nephrin targeting into rafts in the regulation of glomerular permselectivity.

RESULTS

Homo-oligomerization of podocin involves the amino and carboxy terminal domains

It has been suggested that podocin may form homo-oligomers at the glomerular slit diaphragm (6,25). However, structural requirements and molecular mechanisms involved in podocin oligomerization have not been clear so far. We therefore tested whether podocin may interact with itself to form homo-oligomeric complexes in a well-defined model system. As depicted in Figure 1A, podocin is a hairpin-like protein with a short amino terminal cytoplasmic tail (amino acids 1–106 in mouse podocin), a transmembrane region (amino acids 107–127), and a longer carboxy terminal cytoplasmic domain (amino acids 125–385) containing a PHB domain (amino acids 125–284). PHB domains are protein interaction modules of unknown function. Various parts of podocin were fused to the CH2 and CH3 domains of human IgG, followed by the transmembrane region of CD7 as described previously (6) and tested for their interaction with FLAG-tagged podocin in transiently transfected HEK 293T cells (Fig. 1B–G). Figure 1B demonstrates that both the amino terminus and the carboxy terminus are involved in homophilic interactions. The carboxy terminus interacted with carboxy terminus (Fig. 1C), whereas the amino terminus of podocin specifically bound to the amino terminus (Fig. 1C and D). Although the PHB domain of podocin showed strong homophilic interactions (Fig. 1E and F), C-terminal interactions could not be solely attributed to this domain but did also involve a region distal to

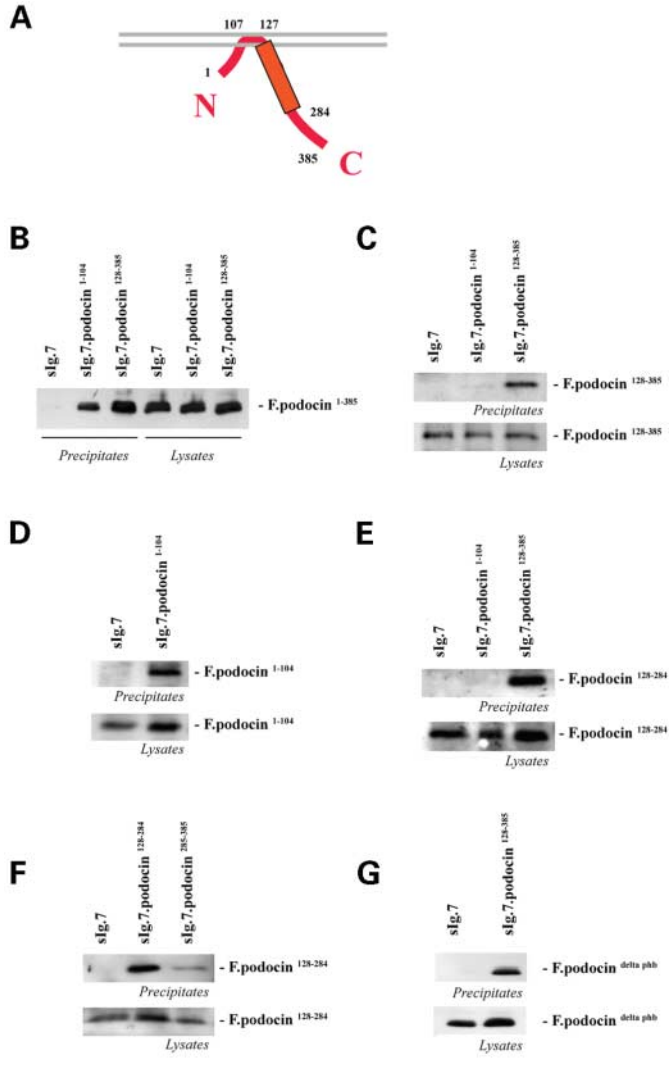

Figure 1. Homo-oligomerization of podocin involves the amino and carboxy terminal domains. (**A**) Podocin is a hairpin-like protein with N-terminal and C-terminal domains facing the cytoplasm. The C-terminal cytoplasmic tail of podocin contains a PHB (prohibitin homology) domain (orange), a protein interaction module of unknown function. Of note, the mouse podocin amino acid sequence is highly homologous to its human counterpart but contains a two-amino acid insertion in the amino terminal tail. Mouse podocin was used for all the studies in the paper. (**B–G**) Lysates were prepared from HEK 293T cells, cotransfected with FLAG-tagged podocin constructs (F.podocin) and truncations of podocin fused to human immunoglobulin and the transmembrane domain of CD7 (sIg.7.podocin), as indicated, precipitated with protein G, and resolved by SDS–PAGE. (B) F.podocin coprecipitates with sIg.7.podocin but not with the control protein (sIg.7). (C) F.podocin$^{128–385}$ coprecipitates with the carboxy terminus of podocin but not with the amino terminus or a control construct (upper panel). Expression levels in cell lysates are shown in the lower panel. (D) F.podocin$^{1–104}$ coprecipitates with the amino terminus of podocin but not with a control construct. (E) F.podocin$^{128–284}$ corresponding to the PHB domain of podocin coprecipitates with the carboxy terminus of podocin but not with the amino terminus or a control construct. (F, G) Carboxy terminal interactions of podocin are mainly but not exclusively mediated by the PHB domain.

the PHB domain (Fig. 1G), suggestive of the fact that high-affinity binding requires larger parts of the C-terminal cytoplasmic tail of podocin. In contrast, binding of podocin to nephrin (and neph proteins, data not shown) almost exclusively required the PHB domain of podocin (Fig. 2A and B). Deletion of the PHB domain as well as truncation of nephrin at amino acid 1109 (protein corresponding to the disease-causing *Fin minor* mutant of nephrin) abrogated binding of nephrin to podocin (Fig. 2B and C), demonstrating that the PHB domain of podocin and the most carboxy terminus of nephrin are required for the interaction. A missense mutation resulting in the substitution of arginine at position 138 for the uncharged amino acid glutamine (R138Q) is one of the most common disease-causing mutations in SRN (9). Interestingly, R138Q, corresponding to R140Q in the mouse protein used in this study, did not influence the interaction with nephrin or the ability to engage in homophilic interactions (Fig. 3A and B). In contrast, a premature stop with an almost complete loss of the PHB domain in the disease-causing mutant R138X (R140X in the mouse protein) resulted in the loss of both homo-oligomerization and interaction with nephrin (Fig. 3C and D).

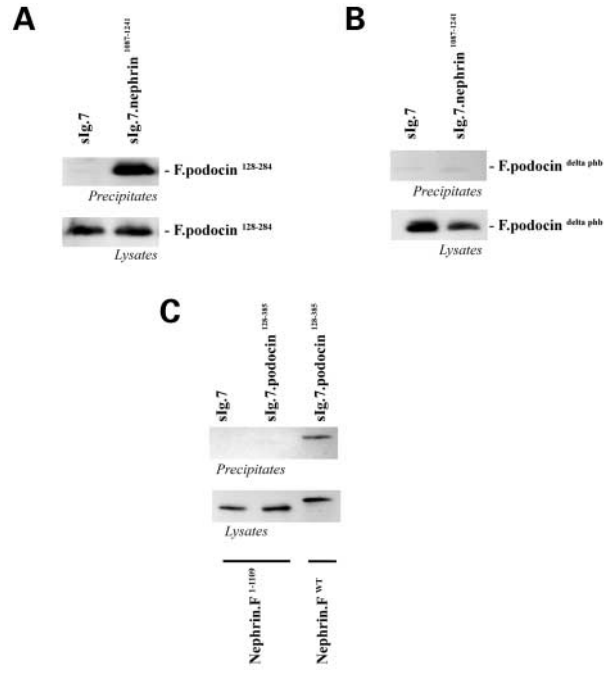

Figure 2. The PHB domain of podocin is required for nephrin interaction. (**A**, **B**) Lysates of HEK 293T cells cotransfected with FLAG-tagged podocin (F.podocin) and fusion constructs of the cytoplasmic tail of nephrin fused to the CH2 and CH3 domains of human immunoglobulin and the transmembrane domain of CD7 (sIg.7.nephrin) were precipitated with protein G, and resolved by SDS–PAGE. Human immunoglobulin fused to the transmembrane domain of CD7 without a cytoplasmic tail served as control (sIg.7). F.podocin$^{128–284}$ corresponding to the PHB domain of podocin coprecipitates with sIg.7.nephrin but not with the control construct sIg.7 (A). Deletion of the PHB domain of podocin (F.podocin$^{delta\ phb}$) results in the loss of interaction (B). (**C**) Lysates of HEK 293T cells cotransfected with C-terminally FLAG-tagged nephrin (nephrin.F) and with fusion constructs of the C-terminal tail of podocin fused to the CH2 and CH3 domains of human immunoglobulin and the transmembrane domain of CD7 (sIg.7.podocin$^{128–385}$) were precipitated with protein G and resolved by SDS–PAGE. The C-terminal tail of podocin interacts with wild-type nephrin but not with a truncation lacking the last 132 amino acids of the carboxy terminus of nephrin. This mutant (R1109X) corresponds to the disease-causing *Fin minor* mutant.

Podocin is a lipid raft protein and recruits nephrin in these specialized microdomains of the plasma membrane

Recently, it has been shown that the plasma membrane of the glomerular slit region contains specialized lipid raft microdomains and that podocin is a raft-associated component of the slit diaphragm colocalizing with raft-resident tyrosine kinases (22,25,26). To address the structural and functional requirements for raft association of slit diaphragm proteins and to study pathomechanisms of podocin mutations HEK 293T cells were transiently transfected with various podocin constructs and cellular lysates were subjected to floatation gradient centrifugation as described (27–29). It has been shown that lipid rafts of the plasma membrane are highly enriched in detergent-resistant membranes (DRMs) prepared by this technique (27,28,30). As demonstrated in Figure 4, preparations of DRM completely excluded the non-raft plasma membrane protein transferrin receptor (TfR) and were highly enriched in the raft-associated protein podocin and caveolin-1 (Fig. 4A). In the absence of podocin, nephrin was excluded from the DRM fraction (Fig. 4A, upper panel). Interestingly, nephrin could be detected in the DRM fraction in the presence of podocin, suggestive of a role of podocin in recruiting nephrin in lipid rafts (Fig. 4A, lower panel). In contrast, disease-causing mutations of nephrin (R1160X and R1109X) that do not interact with podocin (Fig. 2C) (6) were excluded from detergent-resistant light fractions and failed to be recruited by podocin (Fig. 4A). These data clearly suggested that podocin may recruit nephrin into lipid rafts.

Since preparation of DRM relies purely on the physicochemical properties of membrane fractions but does not exclude these membrane domains originating from different places in the cell, it would be extremely helpful to confirm the data with appropriate imaging techniques. Until recently, this has not been possible given the fact that rafts are almost evenly distributed over the plasma membrane and cannot be resolved by conventional microscopical techniques (31). However, Harder and colleagues recently developed a method to visualize clustered lipid rafts (32). Resident raft proteins (i.e. glycosylphosphatidylinositol-linked placental alkaline phosphatase, GPI-PLAP) were cross-linked with antibodies to induce clustering of small rafts to larger patches. This patching allowed the visualization of lipid rafts in living cells and was now used for our study. Since GPI-PLAP is a proven raft resident, protein colocalization with PLAP-positive patches (Fig. 4B, red) can prove raft localization, whereas absence of colocalization excludes the presence in lipid rafts. As demonstrated in Figure 4B, upper panel, in the absence of podocin clustered rat nephrin did not colocalize with PLAP patches. In contrast, coexpression of podocin resulted in very strong recruitment of nephrin to the PLAP-positive raft patches as shown by colocalization of nephrin with patched GPI-PLAP (Fig. 4B, lower panel). A blinded quantitative analysis confirmed the dramatic increase of nephrin in rafts from about 6% in the absence of podocin to about 70% in the presence of podocin (Fig. 4C). Importantly, patched raft proteins and

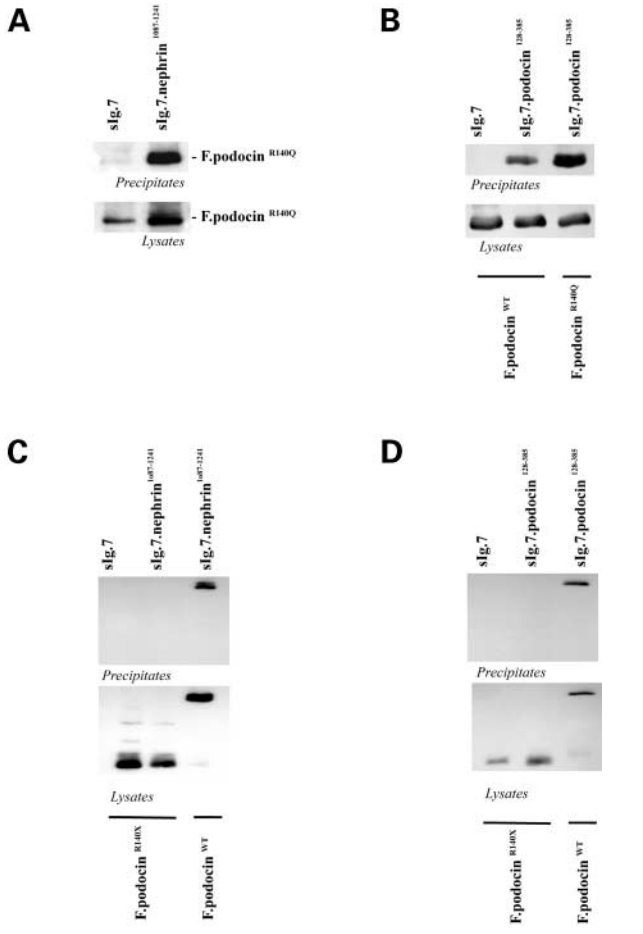

Figure 3. Effect of disease-causing mutations on the ability of podocin to homodimerize and interact with nephrin. (**A**, **B**) The R138Q missense mutation of podocin (corresponding to R140Q in the mouse protein used in this study) still homodimerizes and interacts with nephrin. HEK 293T cells were transfected with the indicated constructs and subjected to immunoprecipitation and immunoblotting as described above. Coprecipitating FLAG-tagged podocin (F.podocin^{R140Q} and F.podocin^{WT}) was detected with anti-FLAG antisera (upper panels). Equal expression of podocin in the lysates is depicted in the lower panels. (**C**, **D**) Mutation of podocin to R138X (corresponding to R140X in the mouse protein used in this study) deletes most of the PHB domain and abrogates carboxy terminal homodimerization (C) and interaction with nephrin (D).

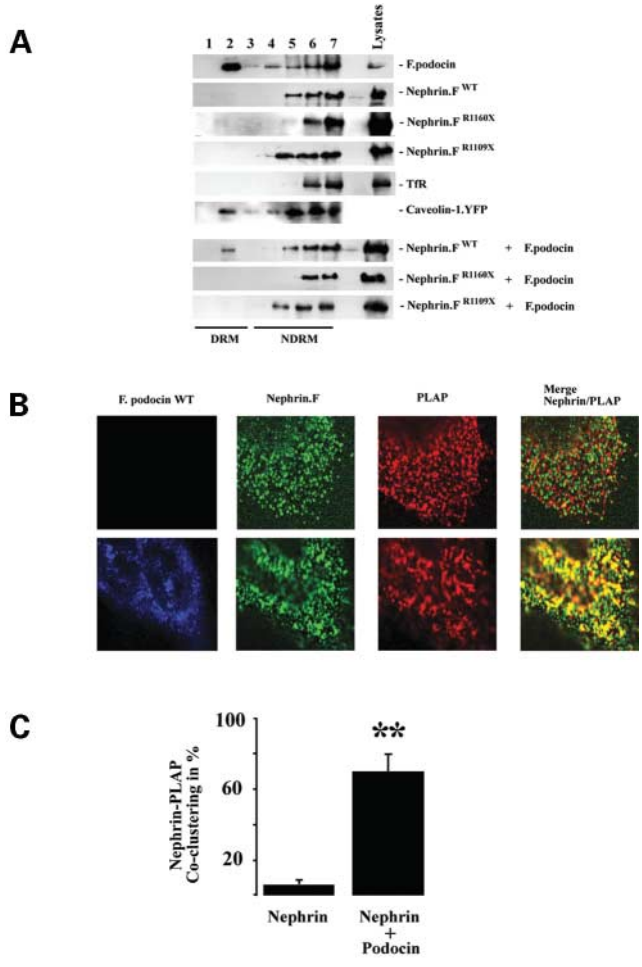

Figure 4. Podocin associates with detergent-resistant membrane domains and recruits nephrin into rafts. (**A**) HEK 293T cells were transfected with the plasmids as indicated, lysed in 1% TX-100 on ice and subjected to floatation gradient centrifugation to prepare DRM. DRMs are known to be highly enriched in lipid rafts (and lipid raft resident proteins such as caveolin-1) and should not contain non-raft proteins. NDRM denotes non detergent-resistant membrane domains. Fractions of the lysates after floatation gradient ultracentrifugation obtained from the top (fraction 1) to the bottom (fraction 7) of the tube were analyzed by western blot with the respective antibodies. Podocin is a lipid raft protein that can be found in DRM (upper panel). In the absence of podocin neither wild-type nephrin (Nephrin.F^{WT}) nor disease-causing mutants of nephrin lacking parts of the cytoplasmic tail (Nephrin.F^{R1160X} or Nephrin.F^{R1109X}) are associated with DRMs. In contrast, coexpression of podocin results in the translocation of nephrin to DRM without altering nephrin expression levels (Nephrin.F^{WT} + F.podocin) but does not influence distribution of nephrin mutants that do not interact with podocin (lower panels, Nephrin.F^{R1160X} + F.podocin or Nephrin.F^{R1109X} + F.podocin). (**B**) To confirm the biochemical data with appropriate imaging techniques the resident raft proteins glycosyl–phosphatidylinositol-linked placental alkaline phosphatase (GPI-PLAP) was cross-linked with antibodies to induce clustering of small rafts to larger patches. This patching allows for the visualization of lipid rafts in living cells. Since GPI-PLAP is a proven raft resident protein colocalization with PLAP-positive patches (red) can prove raft localization, whereas absence of colocalization excludes the presence in lipid rafts. In the absence of podocin clustered rat nephrin did not colocalize with PLAP patches (upper panel). Coexpression of podocin resulted in almost complete recruitment of nephrin to the PLAP-positive raft patches (lower panel). Patched raft proteins and patches of TfR as a non-raft marker protein were completely separated even in the presence of podocin (not shown). (**C**) Quantitative analysis of the raft recruitment of nephrin. Blinded quantitation was performed in 12 independent experiments by counting the nephrin spots that colocalized with PLAP-positive patches (100 patches counted per cell, 12 independent experiments). Podocin expression results in a highly significant recruitment of nephrin into lipid rafts ($^{**}P < 0.01$, $n = 12$) without altering nephrin expression levels (not shown).

patches of TfR as a non-raft marker protein were completely separated even in the presence of podocin (data not shown). These results clearly confirmed our biochemical data and further prove that podocin is involved in the recruitment of nephrin to lipid rafts.

Disease-causing mutants of podocin (R138Q and R138X) fail to recruit nephrin into lipid rafts and lose their ability to augment nephrin signaling

Since these data suggested that podocin may serve to recruit nephrin into lipid rafts, we wondered whether disease-causing mutations of podocin may interfere with the ability of podocin to serve as a lipid raft anchoring protein for nephrin. HEK 293T cells were transfected with podocin mutants and cellular

lysates were subjected to floatation gradient density centrifugation as described above. As shown in Figure 5, both mutants, mouse podocin R140Q and R140X (corresponding to R138Q and R138X in humans), could not be detected in the lipid raft-containing DRM fraction. Consequently, both mutants lost their ability to recruit nephrin into lipid rafts (Fig. 5A and B). The fact that mutant podocin did not reside in rafts could have two explanations. First, it is conceivable that mutant podocin does not enter the pathway involved in the transport of the membrane protein to the plasma membrane. Second, it could be that mutant podocin does reach the plasma membrane but is localized to non-raft parts of the plasma membrane. To solve this issue we expressed and stained wild-type and mutant podocin and tested for the subcellular localization by confocal microscopy. Figure 6 shows that wild-type podocin nicely stained the plasma membrane and did only partially colocalize with the endoplasmic reticulum (ER) marker protein calnexin (Fig. 6, left panel). However, the R140Q mutant of mouse podocin did not stain at the plasma membrane and perfectly colocalized with calnexin, indicative of the fact that this mutant did not leave the ER (Fig. 6, middle panel). In contrast, R140X reached the plasma membrane (Fig. 6, right panel) but was not targeted to rafts (Fig. 5B). These data suggested that defective targeting of podocin molecules can occur at both levels, the level of the transport from intracellular organelles to the plasma membrane or at the level of targeting lipid rafts from non-raft plasma membrane fractions.

We have recently shown that podocin directly interacts with nephrin and that this interaction is required for the podocin-mediated augmentation of nephrin-induced activation of the transcription factor AP-1 (6). We therefore assayed the activity of AP-1 transcription factor in HEK 293T cells expressing nephrin in the presence and absence of wild-type and mutant podocin. Figure 7A demonstrates that nephrin caused a substantial increase (~10-fold) in AP-1-dependent luciferase activity. In contrast, serum triggered a 2- to 3-fold increase (data not shown), while podocin was without effect on AP-1 activation (data not shown). Although wild-type podocin synergistically augmented nephrin-mediated AP-1 activation, yielding a nearly 20-fold transactivation of the luciferase construct, the mutant podocin proteins lost their ability to augment nephrin signaling. Western blot analysis revealed that this was not the result of altered protein levels (Fig. 7A, lower panel), but has to be due to a direct effect of podocin on nephrin signaling. Both biochemical analyses/plasma membrane preparations and imaging studies did not show any difference in nephrin levels on the cell surface in the presence of podocin (Fig. 7B and data not shown). In addition, podocin expression was without effect on the EndoH-resistant or EndoH-sensitive pools of nephrin, indicative of the fact that podocin expression does not influence forward transport of nephrin to the plasma membrane (data not shown). However, podocin significantly influences the recruitment to rafts (see above). Thus, lack of functional podocin does not influence transport of nephrin to the plasma membrane but interferes with the correct targeting to lipid raft microdomains. These data suggested that podocin-dependent recruitment of nephrin to lipid rafts may be required for proper nephrin signaling and that loss of nephrin recruitment in patients with *NPHS2* mutations may contribute to the functional defects of the slit diaphragm protein complex, leading to the development of nephrotic syndrome.

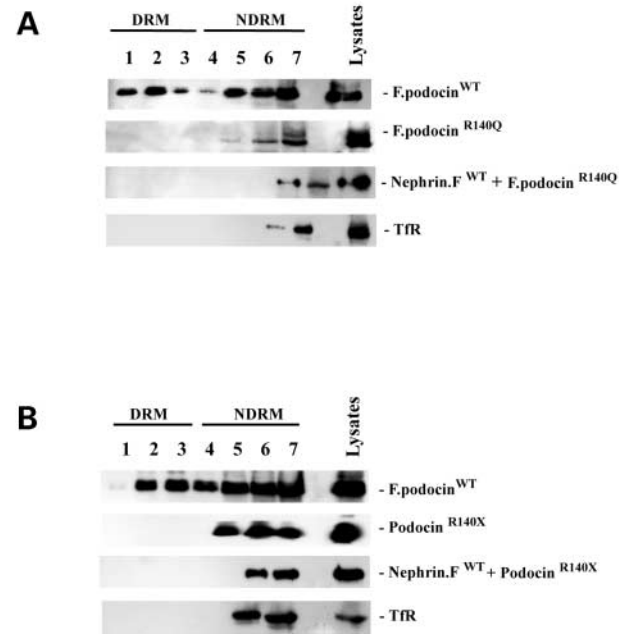

Figure 5. Effect of disease-causing podocin mutations on raft association. (**A**, **B**) HEK 293T cells were transfected with the plasmids as indicated, lysed in 1% TX-100 on ice and subjected to floatation gradient centrifugation to prepare DRM as described. Although expressed at high levels in the lysates both mutants lost their ability to associate with DRM. Consequently, neither mutant was able to recruit nephrin into the DRM fractions. The non-raft plasma membrane protein TfR served as a control to show the cleanliness of the preparation.

DISCUSSION

Diseases of the renal glomerulus, the site of plasma ultrafiltration, make up the majority of kidney diseases leading to end-stage renal failure, a condition that requires the initiation of renal replacement therapies such as dialysis or kidney transplantation. Disappointingly however, the molecular mechanisms involved in the development of proteinuria and glomerular diseases are poorly understood (1). Therefore, the study of the molecular pathogenesis of hereditary nephrotic syndrome has become of greatest interest. Hereditary nephrotic syndrome is a heterogeneous disease, characterized by heavy proteinuria and renal failure. The recent description of gene defects of the podocyte resulting in hereditary nephrotic syndrome has provided a completely new understanding of the glomerular filter and unraveled important aspects of the pathogenesis of proteinuric kidney diseases (1,2). Cloning of the *NPHS2* gene by Antignac's group (9), which followed cloning of the *NPHS1* gene by the Tryggvason group (33) together with the finding that both proteins localize to the glomerular slit diaphragm of podocytes (34–36), suggested that this structure is of critical importance for the function of the glomerular filter. We have previously shown that nephrin, the gene product of *NPHS1*, interacts with the *NPHS2* gene product podocin and that this interaction is required for the augmentation of nephrin signal transduction (6). Here we show

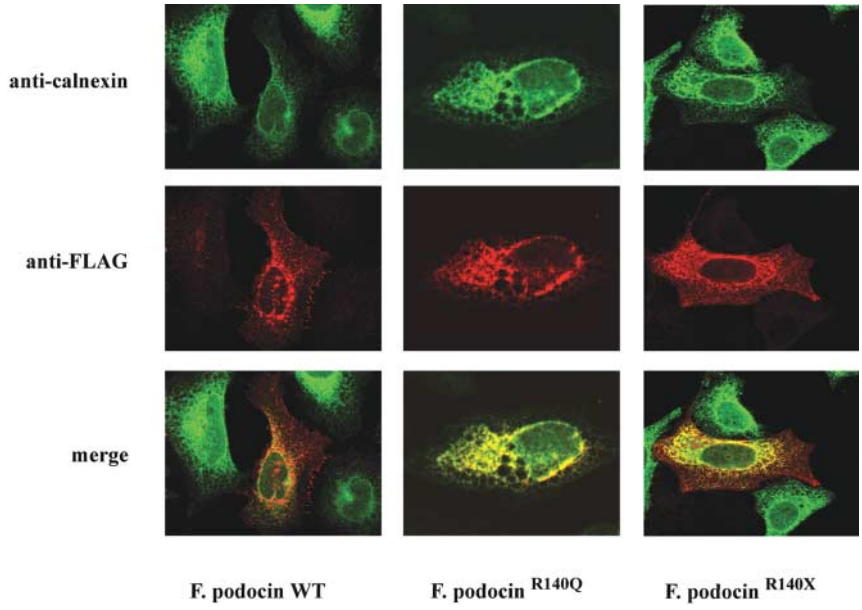

Figure 6. Immunofluorescence localization of podocin mutants. Confocal images showing indirect immunofluorescence staining of FLAG-tagged podocin constructs (red) and the ER resident protein calnexin (green) in HeLa cells. The R138Q missense mutation of podocin (center panels, corresponding to R140Q in the mouse protein used in this study) does not leave the ER and does not reach the plasma membrane in contrast to wild-type protein (left panels) and the R138X mutation (right panels, corresponding to R140X in the mouse protein used in this study).

that podocin mediates the recruitment of nephrin into specialized microdomains of the plasma membrane of the cell. Lipid rafts have been suggested to be important for the function of the glomerular filter (22,37,38). Several groups have shown that nephrin and podocin are associated with detergent-resistant membranes at the filtration slit that may represent special types of lipid rafts (22,25,39,40). Furthermore, resident raft proteins have been shown to localize to the slit diaphragm area in podocytes (20–22,25). Deletion of the raft-associated protein tyrosine kinase fyn results in a severe proteinuria in mice (41), further supporting the importance of raft-associated components for the function of the podocyte. Lipid rafts consist of dynamic assemblies of cholesterol and sphingolipids in the exoplasmic face of the lipid bilayer of the plasma membrane (38). Here we show that podocin provides nephrin with a specialized lipid environment which is required for nephrin signal transduction. Thus, in addition to the protein composition, a special lipid composition of the plasma membrane at the filtration slit may be required for the function, signaling and stability of the glomerular filter. The ability of podocin to augment nephrin signaling was critically dependent on podocin-mediated raft recruitment of nephrin. Although the maximal stimulatory effect of podocin co-expression varied and appeared to reach a plateau of maximal transcriptional activation, this plateau was not due to a limited ability of podocin to facilitate nephrin signal transduction, but rather a result of the design of the AP-1 experiments.

Recent genetic studies suggested a functional inter-relationship of nephrin and podocin *in vivo* (11). This functional inter-relationship may now be explained by our study. To prove our hypothesis that podocin is required for the proper recruitment of nephrin in lipid rafts, we examined two major disease-causing podocin mutations on their ability to recruit nephrin and facilitate nephrin signaling. Podocin R138Q and R138X are located in a mutation hot spot for SRN (9,11) and represent two of the major disease-causing podocin mutations (9,42). Interestingly, both podocin mutants failed to recruit nephrin in lipid rafts, underlining the functional significance of our findings. Although the molecular end-point of disease-causing podocin mutations, namely lack of nephrin recruitment in rafts, was identical, the underlying pathogenic mechanism differed in both mutants. R138Q resulted in a protein that was retained in the ER and did not target the plasma membrane. Thus, this mutant did not reach lipid rafts of the plasma membrane and therefore failed to recruit nephrin and augment nephrin signaling. In contrast, R138X left the ER, did reach the plasma membrane and did not show an obvious trafficking block. However, this mutant was not targeted to lipid rafts and therefore again failed to recruit nephrin and augment nephrin signaling. The finding that R138Q was retained in the ER is in agreement with a recent study that showed ER localization for podocin R138Q in OK cells (43). Defective trafficking caused by missense mutations has also been implicated in CNS due to nephrin mutations (44). Liu *et al.* convincingly showed an altered subcellular distribution in various disease-causing nephrin mutations. Although additional missense mutations of podocin have to be studied, our data clearly indicates that podocin may serve to recruit nephrin in rafts or conversely recruit rafts into close proximity of nephrin complexes.

Since mutations in the *NPHS2* gene do not only account for childhood onset of SRN, but have recently been involved in adult disease and may be among those factors that alter the susceptibility to various forms of secondary renal injury, our data is of particular clinical importance (45–47). While the disease mechanisms of the podocin R138Q and R138X mutations may not be the only way in which disruption of

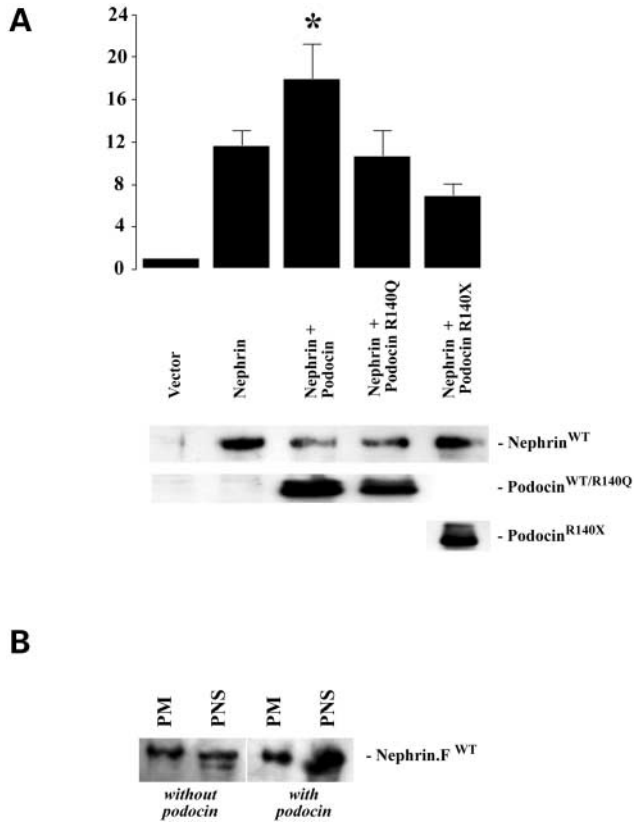

Figure 7. Mutant podocin does not augment nephrin signal transduction. (**A**) HEK 293T cells were transfected with an AP-1 dependent luciferase construct, and expression plasmids as indicated. As described previously, nephrin stimulated an ~12-fold increase in AP-1 activity that was strongly augmented by podocin coexpression (6). Mutation of podocin (R140Q and R140X in the mouse protein corresponding to the disease-causing mutations R138Q and R138X in humans) abrogated the ability of podocin to augment nephrin-mediated AP-1 activity ($*P < 0.05$, $n = 3$), but was without effect on nephrin expression levels (lower panels). (**B**) Podocin expression does not influence nephrin levels on the plasma membrane. HEK 293T cells were transfected with FLAG-tagged nephrin in the absence or presence of podocin. Subcellular fractions were prepared as described and equal amounts of protein from these fractions were separated by SDS–PAGE. Depicted is a representative experiment showing no influence of podocin expression on nephrin levels in the plasma membrane. Purity of the plasma membrane preparation was confirmed by reprobing the blots with antibodies reactive with marker proteins (not shown).

the *NPHS2* gene can cause SRN, our data adds sufficient evidence to support a critical functional inter-relationship between *NPHS1* and *NPHS2* and may guide a strategy to develop new treatments in patients with *NPHS2* mutations.

MATERIALS AND METHODS

Reagents and plasmids

Nephrin and podocin cDNA constructs have been described previously (6). Membrane-bound fusion proteins of the C-terminal cytoplasmic domain of nephrin were generated using a pCDM8 cassette that contained the leader sequence of CD5 fused to the CH2 and CH3 domain of human IgG1 followed by the transmembrane region of CD7 (48). Truncations and mutations of podocin and nephrin were generated by standard cloning procedures. A human nephrin full-length cDNA clone, a rat full-length cDNA clone (kindly provided by Dr H. Holthofer, Helsinki, Finland) and mouse podocin cDNA served as template for all cloning steps. Of note, mouse podocin is highly homologous to the human protein but contains a two-amino-acid insertion in the amino terminal cytoplasmic tail. Therefore, disease-causing mutants R138Q and R138X correspond to R140Q and R140X in mouse podocin. All constructs were verified by automated sequencing. Transferrin receptor and GPI-PLAP cDNA constructs and antibodies were kindly provided by Dr K. Simons (Dresden, Germany). Antibodies were obtained from Sigma (anti-FLAG M2), Santa Cruz (anti-calnexin) and Upstate Biotechnology (anti-PY 4G10).

Coimmunoprecipitation

Immunoprecipitations were performed as described (49). Briefly, HEK 293T cells were transiently transfected by the calcium phosphate method. After incubation for 24 h, cells were washed twice and lysed in a 1% Triton X-100 lysis buffer. After centrifugation (15 000*g*, 15 min, 4°C) and ultracentrifugation (100 000*g*, 30 min, 4°C), cell lysates containing equal amounts of total protein were incubated for 1 h at 4°C with the appropriate antibody, followed by incubation with 40 µl of protein G-sepharose beads for ~3 h. The beads were washed extensively with lysis buffer, and bound proteins were resolved by 10% SDS–PAGE.

Preparation of lipid raft membrane domains and subcellular fractionation

For preparation of low-density Triton X-100-insoluble membrane domains HEK 293T cells were homogenized by 20 strokes in a Dounce homogenizer in 1 ml of MBS buffer (250 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4, proteinase inhibitors) in the presence of 1% Triton X-100 and centrifuged for 10 min at 3000*g* at 4°C. The lysates were incubated for 45 min on ice in the presence of 1% Triton X-100, adjusted to 45% sucrose and pipetted at the bottom of an ultracentrifuge tube. Samples were then overlaid with a sucrose step gradient (2 ml of 30% sucrose and 1 ml of 5% sucrose in MBS) as described previously (50). Gradients were centrifuged for 20 h at 200 000*g* at 4°C in a swing-out rotor, and seven fractions (700 µl each) were collected starting from the top and analyzed by SDS–PAGE.

For subcellular fractionation, HEK 293T cells transfected with the plasmids as indicated were harvested, resuspended in 1 ml of homogenisation buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris, pH 7.4, proteinase inhibitors) and lysed in a glass/glass homogenizer at 4°C. From this lysate, a crude supernatant fraction was prepared by centrifugation at 1000*g* for 10 min. The resulting pellet was resuspended in homogenization buffer, and the homogenization and centrifugation steps were repeated. Both supernatants were combined (PNS, postnuclear supernatant), layered on top of 30% Percoll, and centrifuged at 84 000*g* for 18 min. The purified plasma membrane fraction was collected (PM, plasma membranes), and protein concentration of the different subcellular fractions was determined.

Immunofluorescence and antibody-induced coclustering

Hela cells were transfected using the Fugene6 transfection method (Roche, Mannheim). For immunofluorescence cells were fixed in 80% methanol/20% acetone at −20°C for 5 min. For coclustering experiments cells were additionally prefixed for 10 min in 4% paraformaldehyde/PBS at 4°C (32). Fixed cells were incubated for 30 min in blocking solution (5% BSA/2% FBS) and subsequently incubated at room temperature with primary and secondary antibodies as described (32). After washing in PBS, the cells were mounted in Prolong antifade solution (Molecular Probes) and subjected to immunofluorescence microscopy with an Axiophot 2 microscope (Zeiss, Jena) equipped with a CCD camera or confocal microscopy with a Zeiss laser scan microscope equipped with a 100× oil immersion objective. For the coclustering experiments, antibodies were applied to the cells before fixation as described (32). Briefly, cells were washed in ice-cold PBS and incubated with primary antibodies for 30 min at 12°C, washed with ice-cold PBS and incubated with the respective secondary antibodies for 30 min at 12°C. Primary antibodies included anti-calnexin (Transduction Laboratories), anti-Flag (Sigma, München) as well as Alexa 488-coupled anti-Flag (according to protocol by Molecular Probes), polyclonal anti-PLAP (kindly provided by Dr K. Simons, Dresden, Germany), monoclonal 5-1-6 anti-nephrin (kindly provided by Drs Kawachi and Shimizu, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan) and monoclonal anti-Transferrin Receptor (Zymed Laboratories). Secondary antibodies were Alexa 488-conjugated anti-rabbit (Molecular Probes), Cy3-conjugated anti-mouse and Cy5-conjugated anti-rabbit (Jackson Laboratories). Confocal images were taken with a Zeiss laser scan microscope equipped with a 63× and 100× oil immersion objective. Blinded quantitative analysis of the raft recruitment of nephrin was done in 12 independent experiments by counting the nephrin spots that colocalized with PLAP-positive patches (100 patches counted per cell, 12 independent experiments).

Luciferase assay

As described previously, HEK 293T cells seeded in 12-well plates were transiently transfected with a luciferase reporter construct, a β-galactosidase expression vector (kindly provided by C. Cepko), and vectors directing the expression of the proteins as indicated (6). Total DNA amount was 1.5–2.0 μg/well. Cells were serum starved for 12 h, harvested in cold phosphate-buffered saline, and lysed in 100 μl of reporter lysis buffer (Applied Biosystems, Norwalk, CT, USA) for 10 min at 4°C. Lysates were centrifuged at 14 000 rpm for 5 min to remove insoluble material. Luciferase activity was determined using a commercial assay system (Applied Biosystems, Norwalk, CT, USA), and normalized for β-galactosidase activity to correct for transfection efficiency. Equal expression of proteins was ensured by western blot analysis.

Statistical analysis

Data are expressed as mean ± SEM of n experiments. Statistical evaluation was performed using Student's *t*-test or ANOVA for repeated measures, followed by a Bonferroni test (SigmaPlot, Jandel Scientific and Instat2, GraphPad). Values of $P < 0.05$ were considered to be statistically significant.

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