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In vivo expression of podocyte slit diaphragm-associated proteins in nephrotic patients with *NPHS2* mutation

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In vivo expression of podocyte slit diaphragm-associated proteins in nephrotic patients with *NPHS2* mutation.

Background. Mutations in *NPHS2,* encoding podocin, are a prevalent cause of autosomal-recessive steroid-resistant nephrotic syndrome (SRNS). Podocin is a protein associated with the slit diaphragm that interacts with nephrin and CD2-associated protein (CD2AP) within lipid rafts.

Methods. Using renal biopsies of six patients, we analyzed the in vivo consequences of different types of *NPHS2* mutations on (*1*) the podocyte expression and distribution of podocin using in situ hybridization and immunohistology and (*2*) the distribution of related podocyte proteins and glomerular extracellular matrix components.

Results. In two patients with homozygous 855_856delAA or 419delG mutation, absence of podocyte labeling with the antibodies against the C-terminal domain contrasted with the normal expression of the N-terminal domain of the protein along the glomerular basement membrane (GBM). In patients carrying compound heterozygous mutations or variants (R168S/467_468insT, R138Q/V180M, and R291W/R229Q), or single heterozygous 976_977insA, podocin transcription appeared unchanged but the distribution of the protein was modified. Podocin was restricted to the podocyte body in the patient carrying the R168S/467_468insT mutation whereas strong immunolabeling of the podocyte body was associated with discrete labeling along the GBM in the three others. In all cases, podocin defect was associated with changes in the distribution of nephrin, CD2AP, and α-actinin: the proteins were mainly detected in the podocyte body, with mild expression along the GBM. There were no detectable changes in the distribution of other podocyte proteins or glomerular extracellular matrix components.

Conclusion. NPHS2 mutations result in profound alteration of podocin expression and/or distribution. Secondary changes in the distribution of nephrin, CD2AP, and α-actinin are additional evidences for the scaffolding role of podocin in the organization of the slit diaphragm.

Key words: *NPHS2*, podocin, steroid-resistant nephrotic syndrome, nephrin, CD2AP, α-actinin.

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Autosomal-recessive steroid-resistant nephrotic syndrome (SRNS) associated with *NPHS2* mutations, belongs to the heterogeneous group of hereditary nephrotic syndromes [1]. It was initially characterized by childhood onset proteinuria, progression to end-stage renal disease (ESRD), absence of recurrence after renal transplantation, and, at the histologic level, minimal glomerular changes on early biopsy samples and focal segmental glomerulosclerosis (FSGS) at later stages [2]. Subsequently, *NPHS2* mutations were described in sporadic cases of SRNS [3, 4], in late onset FSGS [5], and in congenital forms of the disease, possibly associated with *NPHS1* heterozygous mutation [6, 7]. Moreover, delayed recurrence of mild proteinuria, responsive to therapeutic approach, has been observed, after renal transplantation, in two patients with *NPHS2* mutations [8]. *NPHS2*, mapped to 1q25–31, is a gene consisting of eight exons [2]. The protein product, a podocyte protein named podocin, is an integral membrane protein of 383 amino acids, with a single membrane domain (amino acids 105 to 121) forming a hairpin-like structure placing both N- and C- terminal domains in the cytosol. It belongs to the stomatin family of lipid-raft–associated proteins [2, 9]. Podocin plays an important role in the maintenance of the glomerular filtration barrier as confirmed by the occurrence of massive proteinuria and death in the first days of life in *Nphs2−/−* mice [10].

By immunoelectron microscopy, using antibodies to the C- and N- terminal domains of the protein, we and others previously showed that podocin was located at the cytoplasmic face of the slit diaphragm [9, 11]. Slit diaphragms are thin structures connecting interdigitated podocyte foot processes along the glomerular basement membrane (GBM). Their existence and structure had been described 30 years ago, at the ultrastructural level [12]. But, their importance in the control of glomerular filtration has been recognized only recently. In addition to podocin, several other molecules participating in the formation of slit diaphragms have been identified through the genetic characterization of hereditary nephrotic syndrome, or the production of animal models.

Table 1. Clinical parameters, pathologic, and genetic findings in the six patients with *NPHS2* mutations

Patient Gender	Age at onset NS *years*	Age at ESRD *years*	Renal biopsy I	(Age *years*) II	Mutation			
					Nucleotide change	Effect on coding sequence	Exon	Mutation status
1F	0.5	5.5	MGC (0.6)	FSGS (2)	419delG	Frameshift	3	Hom
2F	0.4	12	MGC(0.6)	FSGS (8)	855_6delAA	Frameshift	7	Hom
3M	0.5	7.9	DMP (1.9)		502C→A	R168S	4	C Het
					467_468insT	Frameshift	4	
4F	7.5	17	MGC (8.6)		413G→A	R138Q	3	C Het
					538G→A	V180M	5	
5F	0.3	9.3	MGC (0.3)		871C →T	R291W	7	C Het
					686G→A	R229Q	5	
6F	9	13.7	FSGS (11.9)		976_977insA	Frameshift	8	S Het

Abbreviations are: NS, nephrotic syndrome; DMP, diffuse mesangial proliferation; MGC, minimal glomerular changes; FSGS, focal segmental glomerularsclerosis; Hom, homozygote; C Het, compound heterozygote; S Het, single heterozygous mutation.

Nephrin, a transmembrane cell adhesion molecule of the immunoglobulin superfamily, is a major component of the slit diaphragm complex [13–16]. Mutations of the nephrin gene *NPHS1* result in congenital nephrotic syndrome of the Finnish type [14] and *Nphs1* knock-out mice have no slit diaphragm and die at birth with massive proteinuria [17, 18]. Nephrin interacts with podocin and CD2-associated protein (CD2AP), an adapter protein that could anchor the nephrin-podocin complex to the submembranous actin cytoskeleton [9]. The role of CD2AP in the maintenance of the glomerular filtration barrier has been demonstrated by the occurrence of congenital nephrotic syndrome in CD2AP knockout mice [19]. In addition, CD2AP haploinsufficiency in humans may be associated with glomerular disease susceptibility [20]. P-cadherin and zona occludens-1 (ZO-1) are other components of the slit diaphragm [21–24]. NEPH1, a podocyte protein related to, and interacting with nephrin in the slit diaphragm [25], is important to podocyte function, since mice lacking NEPH1 develop proteinuria and perinatal lethality [26]. FAT, a protocadherin is also involved in the maintenance of the glomerular filtration barrier as shown by the occurrence of nephrotic syndrome in mFAT1-deficient mice [27, 28]. No mutation of the corresponding genes has been found so far in nephrotic patients. Conversely, mutations in *ACTN4,* the gene encoding α-actinin-4, an actin-binding protein localized in podocyte foot processes, causes an autosomal-dominant form of FSGS in humans [29] and mice [30] whereas α-actinin-4 null mice have severe glomerular disease [31].

In order to determine if and how *NPHS2* mutations affect the podocyte phenotype, we analyzed the podocyte expression of podocin and related proteins in the kidneys of six patients suffering from SRNS and in which *NPHS2* mutations have been characterized. We also examined whether the glomerular distribution of extracellular matrix components was modified in these nephrotic patients.

METHODS

Patients and kidney tissue specimens

Renal tissue from six patients (three biopsies and three nephrectomies performed at the time of transplantation) were available for this study (Table 1). The six patients had early onset SRNS progressing to ESRD between the ages of 5.5 and 17 years. Patients 1 and 2 carried homozygous *NPHS2* deletions resulting in a frameshift. Data on podocin expression in patient 1 have been previously shown [11]. Patient 3 had two different mutations, one missense and one frameshift. Patient 4 had a compound missense mutation, including R138Q, a recurrent mutation. Patient 5 had one missense mutation R291W and the common nonneutral R229Q polymorphism. A single heterozygous frameshift mutation was identified in patient 6. These mutations are described in other papers [2, 7].

Initial renal biopsy showed minimal glomerular changes in four patients (3 months to 8.6 years old), diffuse mesangial proliferation in one (1.9 years old) and FSGS in one (11.9 years old). Repeated renal biopsies performed in patients 1 and 2 showed the development of FSGS. Severe glomerular sclerosis, sparing only a few glomeruli, was observed in all end-stage kidneys. In these end-stage kidneys, sections, including preserved glomeruli, were carefully selected for immunolabeling and in situ hybridization studies. Four normal kidneys (from humans aged 1 to 51 years), unsuitable for transplantation because of vascular problems, were used as controls.

Dubosq-Brazil or formalin–fixed, paraffin-embedded tissues were used for light and immunoperoxidase studies. For immunofluorescence, specimens were immediately snap-frozen in liquid nitrogen using OCT (Tissue-Tek, Nile, Inc., Eckhart, IN, USA) and stored at −80°C until use. Frozen tissue sections were used for the analysis of the podocyte phenotype and the distribution of glomerular extracellular matrix components.

Table 2. Characteristics of antibodies used in the study

Antibodies	Specificity	Dilution	Source
A Anti podocyte proteins			
Rabbit anti-podocin P35	Podocin C-terminal	1:1000	Roselli et al [11]
Rabbit anti-podocin P21	Podocin N-terminal	1:50	Roselli et al [11]
Rabbit anti-nephrin	Nephrin	1:500	Tryggvason et al [32]
Rabbit anti-CD2AP (H-290)	CD2AP	1:50	Santa Cruz Biotechnology, CA
Rabbit anti-ZO-1	ZO-1	1:10	Zymed Laboratories, South San Francisco, CA
Mouse CD49c	α_3 integrin	1:100	Beckman Coulter, Brea, CA
Mouse anti- β-dystroglycan	β-dystroglycan	1:50	Novocastra Laboratories, UK
Mouse mAb 5C11	Glepp1	1:100	Sigma, Saint Louis, MO
Rabbit anti- α-actinin	α-actinin	1:100	Biogenex, San, Ramon, CA
Mouse mAb GID4	Synaptopodin	1:10	Progen Biotechnik GMBH, Heidelberg, Germany
Mouse mAb V9	Vimentin	1:100	Beckman Coulter, Brea, CA
B Anti extracellular matrix			
Rabbit anti-[α1(IV)2 α2(IV)]	Type IV collagen[α1(IV)2 α2(IV)]	1:60	Novotec, Lyon, France
Mouse mAb anti-α1(IV)	α1(IV)NC1	1:2	Wieslab AB, Lund, Sweden
Mouse mAB3	α3(IV)NC1	1:40	Wieslab AB, Lund, Sweden
Rat CFT-45325	α2-α5(IV)NC1	1:1	Shigei Medical Research Institute, Okayama, Japan
Mouse mAb A7	α5(IV)NC1	1:4	Wieslab AB, Lund, Sweden
Mouse mAb 5C6	Type VI collagen	1:1000	Hybridoma Bank, Iowa City, IA
Mouse mAb 1924	Laminin α5	1:100	Chemicon International, Temecula, CA
Mouse mAb 1921	Laminin B1(β1)	1:20	Chemicon International, Temecula, CA
Mouse mAb C4	Laminin β2	1:20	Hybridoma Bank, Iowa City, IA
Mouse mAb 1920	Laminin B2 (γ1)	1:20	Chemicon International, Temecula, CA
Mouse mAb A9 Nidogen	Nidogen/entactin	1:1	Katz et al [33]
Mouse mAb 458	Agrin HSPG	1:100	Chemicon International, Temecula, CA
Mouse mAb 1948	HSPG core protein (Perlecan Domain 4)	1:1000	Chemicon International, Temecula, CA
Rabbit anti-Fibronectin	Fibronectin	1:80	Novotec, Lyon, France

Antibodies

The primary antibodies used in this study, their specificity, dilution, sources, and references are shown in Table 2. We previously characterized rabbit polyclonal P21 and P35 antibodies used for the analysis of podocin expression [11]. P21 was generated against fusion proteins derived from the whole N-terminal region of the human protein, before the membrane-associated domain (amino acids 15 to 89) whereas P35 was made against the C-terminal region downstream of the membrane-associated domain (amino acids 135 to 383). By Western blot analysis of transfected HEK293 cells, both antibodies detected one thick band of 49 kD. They also immunoprecipitated overexpressed podocin in HEK293-transfected cells and specifically labeled normal human and murine podocytes. By immunoelectron microscopy, both antibodies localized to the podocyte slit diaphragm area [11]. The polyclonal antinephrin antibody, a gift from K Tryggvason, was produced against a fusion protein corresponding to immunoglobulin repeats 1 and 2 of the amino terminal extracellular portion of human nephrin (amino acids 22 to 240). By Western blot analysis of extract of isolated normal human glomeruli, it specifically reacted with one 180 kD band and recognized full-length nephrin in enzyme-linked immunosorbent assay (ELISA) as well as in nephrin-transfected COS-7 cells. It specifically labeled the slit diaphagm of glomeruli podocytes [32]. Other studies were performed using commercially available antibodies against other proteins of the slit diaphragm, CD2AP and ZO-1, adhesion molecules α_3-integrin and β-dystroglycan, GLEPP1, a transmembrane protein tyrosine phosphatase specifically expressed in the podocytes [34], α-actinin and synaptopodin [35], two actin-associated proteins, and vimentin, an intermediate filament protein expressed by the podocyte. The specificity of these antibodies has been established by the firms. Briefly, the rabbit anti-CD2AP was prepared against a recombinant protein corresponding to the C-terminus (amino acids 350 to 639) of the human protein. It was shown to give one band in Western blot analysis of CD2AP expression in mouse spleen extract, and to immunoprecipitate the protein. ZO-1 has been prepared against a 69 kD fusion protein corresponding to amino acids 4463 to 1109 of human ZO-1 and reacts, on Western blots, with human, mouse rat, guinea pig, and canine ZO-1. In Western blot analysis of the human colon cancer cell line CACO-2, it specifically reacts with the two ZO-1 α+ and ZO-1 α− isoforms. Its specificity was also confirmed by immunoprecipitation. CD49c, the monoclonal anti-α_3-integrin antibody was raised against human umbilical vein endothelial cells. It recognizes an amino-terminal epitope of the integrin-α_3 chain and immunoprecipitates the 120 to 130 kD fragment under reducing conditions. In addition, it inhibits adhesion of human keratinocytes to laminin-5 in vitro functional assays. The monoclonal anti-β-dystroglycan antibody has been prepared against 15 of the last 16 amino acids of the C-terminus of the human dystroglycan

sequence. By Western blot analysis of human skeletal muscle extract, the antibody detected only one 43 kD band. The monoclonal anti-GLEPP1 antibody was raised against GLEPP1 fusion protein. Its specificity was characterized in immunohistochemistry assays. Only the glomerular epithelium was seen to be positive. No positivity was detected in other kidney cells or in extrarenal epithelial cells. The monoclonal antisynaptopodin antibody was raised against isolated rat kidney glomeruli. In Western blot analysis, it reacts with one 100 kD polypeptide in brain and 110 kD polypeptide in kidney. It also reacts with a 44 kD degradation fragment of synaptopodin. By immunohistochemistry, it recognizes podocytes and a subset of telencephalic synapses. The anti-α-actinin polyclonal antibody was developed in rabbit using purified α-actinin from chicken gizzard as an immunogen. It recognizes the four α-actinin isoforms but it could be used for this study as only α-actinin-4 is significantly expressed in the human kidney [29, 31]. The V9 anti-vimentine antibody is a classical one prepared by Osborn, Bebus, and Weber [36], who established its specificity by Western blot analysis using porcine vimentin as antigen, or total cell extract prepared from a human glioma cell line. Only a single band of molecular weight 57 kD was recognized by the antibody. The antibody was further characterized by immunohistochemical assays [36]. Analysis of the glomerular extracellular matrix was made using antibodies against types IV and VI collagen, the α1, 2, 3, and 5 chains of type IV collagen, fibronectin, laminin α5, β1, β2, and γ1 chains, nidogen, perlecan and agrin cyanine2 or fluorescein isothiocyanate(FITC)-conjugated AffiniPure goat or donkey antirabbit IgG and cyanine3-conjugated AffiniPure donkey antimouse IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The Vectastain Elite ABC Kit, avidin-biotin blocking kit, and diaminobenzidine were from Vector Laboratories (Burlingame, CA, USA).

Immunofluorescence staining

Immunofluorescence labeling was performed on 3 μm thick cryostat sections fixed in acetone for 10 minutes, and incubated for 20 minutes with 10% normal swine serum in phosphate-buffered saline (PBS) containing 0.05% Tween-20 for blocking nonspecific binding. After incubation for 1 hour at room temperature with primary antibodies diluted in the same buffer, the sections were rinsed three times in PBS and incubated for 30 minutes with cyanine2-conjugated antirabbit antibodies diluted 1:100 in PBS, or with cyanine3-conjugated antimouse antibody diluted 1:500 in PBS. A mounting media for immunofluorescence (Fluoprep) (BioMerieux, Lyon, France) was used to delay fluorescence quenching. Labeling was examined with an Orthoplan microscope equipped for light, fluorescence, and phase contrast microscopy (Leica Microscopic Systems, Heezbrugg, Switzerland).

Immunoperoxidase

Immunoperoxidase staining, using the Vectastain Elite ABC Kit (Vector Laboratories), was used for analysis of expression of GLEPP1 and vimentin. Deparaffinized sections (4 μm thick) were pretreated by microwave heating in a urea solution (2 × 5 minutes in 0.8 mol/L urea, pH 6.4). For quenching the endogenous peroxidase, sections were treated with 3% hydrogen peroxide in methanol for 5 minutes and then washed in PBS for 20 minutes. They were then incubated for 1 hour at room temperature in a moist chamber with anti-GLEPP1 or anti-vimentin antibody. After washing in PBS, they were incubated with biotinylated secondary antibodies for 30 minutes. They were then incubated for 30 minutes with Vectastain Elite ABC reagent. Chromogen was diaminobenzidine. No labeling was observed on control sections in which the first antibody was omitted.

Double immunofluorescence labeling and confocal microscopy

For dual fluorochrome labeling, the slides were simultaneously incubated with mouse anti-nidogen antibodies and rabbit anti-podocin, anti-nephrin, anti-CD2AP or anti-α-actinin antibodies. After washing with PBS, the slides were simultaneously incubated with cyanine2-conjugated donkey antirabbit IgG and cyanine3-conjugated donkey antimouse IgG. Sections were examined with an Orthoplan microscope equipped with appropriate filters (Leica Microscopic Systems) and with a Zeiss confocal microscope (Carl Zeiss Microscopy, Jena, Germany).

NPHS2 in situ hybridization

In situ hybridization was carried out on paraffin-embedded sections according to a standard protocol previously reported [2, 11]. Paraffin-embedded 6 μm thick sections were deparaffinized, rehydrated, and treated by microwave heating in sodium citrate buffer (0.01 mol/L, pH 6.0). The *NPHS2* riboprobes were synthesized from a 1065 bp polymerase chain reaction (PCR) product (spaning bases 728 to 1792 in the *NPHS2* cDNA) subcloned into the vector PGEM-Teasy (Promega, Madison, WI, USA). The antisense probe was synthesized after digestion with *Sal*I using T7 RNA polymerase and sense probe after digestion with *Sac*II using *Sp*6 RNA polymerase. The riboprobes were labeled with digoxigenin (DIG)-11-uridine triphosphate (UTP) (Roche Diagnostics, Penzberg, Germany) and subsequently visualized using anti-DIG antibody fragments coupled with alkaline phosphatase. The specificity of this probe has been previously

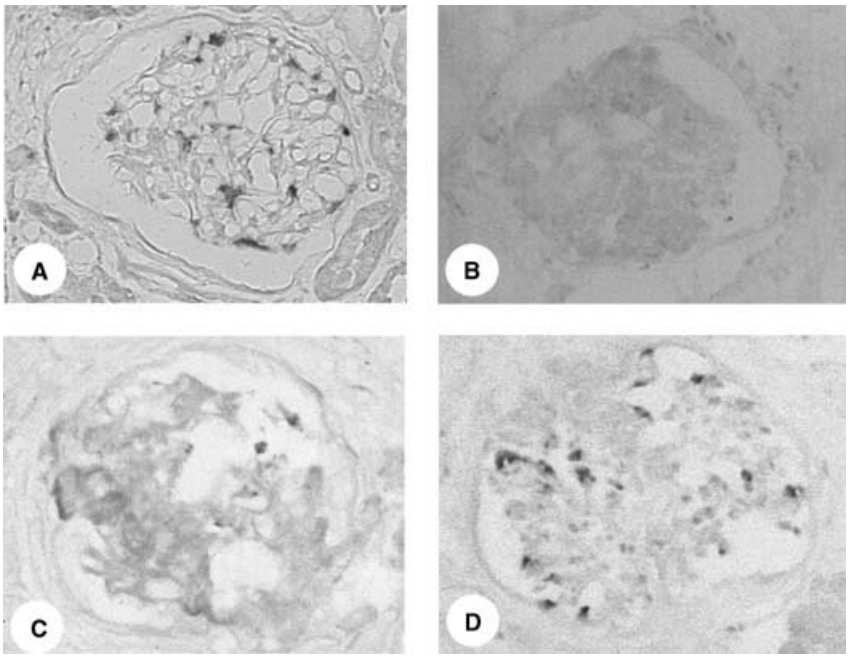

Fig. 1. In situ hybridization of antisense and sense *NPHS2* riboprobe labeled with digoxigenin. In normal kidney, strong signals were observed in podocytes, at the periphery of the glomerular tuft (*A*). No specific signals were observed with the sense probe (*B*). Faint labeling was seen in the patient with homozygous nt 417delG mutation (*C*). In contrast, there were no significant changes in the intensity of podocin mRNA labeling in the other patients (*D*) (magnification × 300).

established by northern blot analysis and in situ hybridization of normal fetal and adult kidneys [2].

RESULTS

Podocyte expression of podocin

In situ hybridization. In normal kidneys, as previously shown [2, 11] strong podocin expression of the transcripts was seen in podocytes, at the periphery of the glomerular tuft (Fig. 1A) and no signal was observed with the sense probe (Fig. 1B). In patient 2, with the homozygous 419delG mutation, faint labeling was seen in the podocytes (Fig. 1C). In contrast, there was no significant changes in the intensity of podocin mRNA labeling in the other patients (Fig. 1D).

Podocin distribution. In normal kidneys, polyclonal antibodies against the C- or the N-terminal regions of podocin gave the same pattern of immunolabeling but the staining was stronger with the anti-C terminal antibodies that could be used at a higher dilution. Both antibodies gave a precise staining of the basal region of the podocyte along the GBM (Fig. 2A and B) [11].

In *NPHS2* patients, the pattern of podocin expression varied according to the type of *NPHS2* mutation. In two patients with homozygous 855_8566delAA or 419delG frameshift mutation leading to the synthesis of a truncated protein, podocyte expression of the N-terminal domain of the podocin contrasted with the absence of labeling with the antibodies against the C-terminal domain (Fig. 2C and D). The expression of the N-terminal domain was strong in the patient with homozygous 855_856delAA, and reduced in the other one. In both of them podocyte labeling was precisely located along the GBM. In patients 3 to 6 carrying different types of point mutations (R168S/467_468insT, R138Q/V180M, R291W/R229Q, and 976_977insA), podocin expression was observed with both antibodies but the distribution of the protein was modified. Podocin was clearly restricted to the podocyte body in the patient carrying the R168S/467_468insT mutation (Fig. 2E) whereas immunolabeling of the podocyte body was associated with labeling along the GBM in the three others (Fig. 2F).

Double immunofluoresence labeling and confocal microscopy. In normal kidneys, double immunofluorescence labeling of podocin (in green) and nidogen, a component of the GBM (in red) showed a nearly complete overlap of the red and green stainings (Fig. 3A). However, high magnification and confocal microscopy examination focally disclosed the red nidogen labeling restricted to the GBM, from the green podocin labeling at the basis of the podocytes. In patients 1 and 2, the pattern of labeling with the anti-N-terminal domain of podocin was similar to the one in controls (Fig. 3B). In patient 3 with the R168S/467_468insT mutation, dual immunolabeling clearly confirmed the selective labeling of the podocyte body with antipodocin antibodies (Fig. 3C), whereas more diffuse distribution, in the podocyte body and along the GBM was observed in patients 4 to 6. The nidogen expression was unchanged.

Expression of other podocyte-specific protein

Immunofluorescence/immunoperoxydase labelings. In normal kidneys, the glomerular distribution of slit diaphragm-associated proteins nephrin, CD2AP and ZO-1, of the adhesion molecules α_3-integrin and

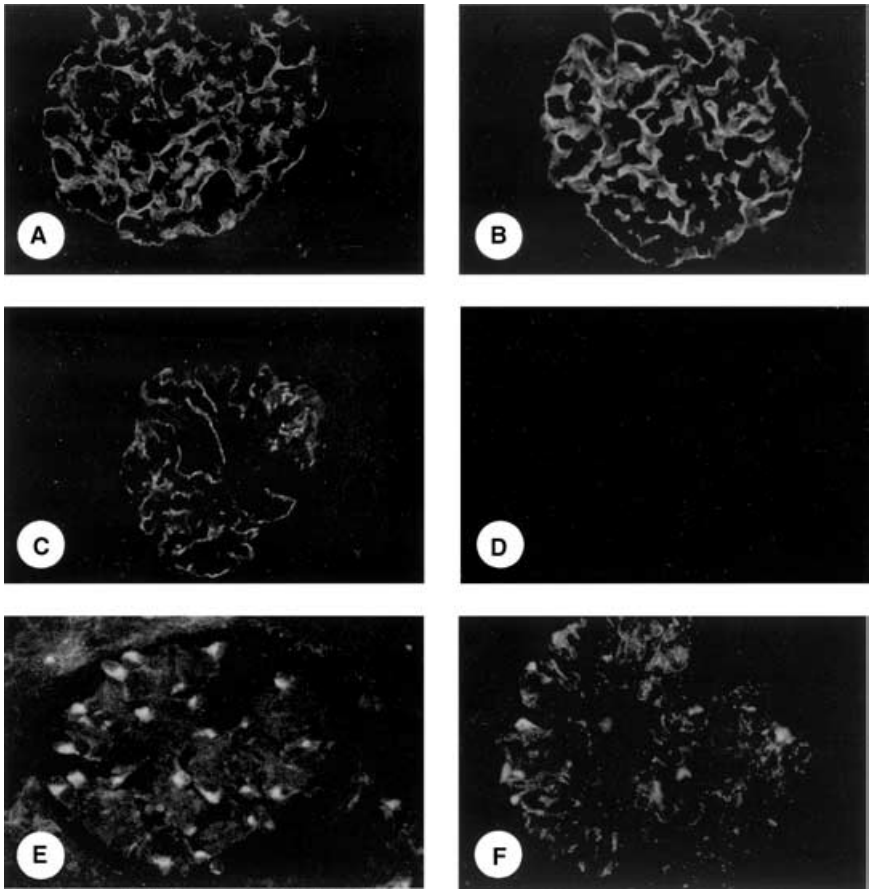

Fig. 2. Immunostaining of podocin in glomeruli from controls and patients carrying *NPHS2* mutations. In normal kidneys, polyclonal antibodies against the N- or the C-terminal regions of podocin gave a precise staining of the basal region of the podocyte along the glomerular basement membrane (GBM) (*A* and *B*). In patients with homozygous 855_856delAA or 417delG frameshift mutation, podocyte expression of the N-terminal domain of the podocin (*C*) was observed whereas no labeling with the antibodies against the C-terminal domain (*D*) could be detected. Podocyte labeling of podocin N-terminal domain was precisely located along the GBM (C). Podocin was clearly restricted to the podocyte body in the patient carrying the R168S/467_468insT mutation (*E*) whereas prominent immunolabeling of the podocyte body was associated with segmental labeling along the GBM in the three other ones carrying mutations of R138Q/V180M, R291W/R229Q, and 976_977insA (*F*) [magnification (A, B, and F) × 300; (C and D) × 250; (E) × 200].

β-dystroglycan, and the other podocyte proteins α-actinin, GLEPP1, and synaptopodin appeared to be similar to that of podocin, by immunofluorescence/immunoperoxidase microscopy. CD2AP and α-actinin were seen in the podocyte along the capillary wall, with focal enhancements toward the podocyte body. ZO-1, α-actinin, and CD2AP were also expressed in tubular cells, and ZO-1 in the glomerular parietal epithelial. Strong labeling of podocyte cytoplasm was observed with anti-vimentin antibodies.

In the six *NPHS2* patients, changes in the distribution of nephrin, CD2AP, and α-actinin were observed. In all of them, nephrin was detected not only along the GBM with a granular and discontinuous distribution, but also in the podocyte body. Labeling of CD2AP and α-actinin was markedly increased and frequently seen in the entire podocyte. No significant changes in the glomerular distribution of other podocyte proteins were detected in the *NPHS2* patients.

Double immunofluoresence labeling and confocal microscopy. To precisely delineate the changes in the distribution of slit diaphragm proteins we have just mentioned, dual labelings were performed with antibodies against nephrin, CD2AP or α-actinin (in green) and nidogen, a GBM marker (in red) and examined by confocal microscopy (Fig. 3). In normal kidneys, partial overlap of dual labeling resulted in a yellow linear staining of the GBM associated with a green labeling following the external aspect of the basement membrane (Fig. 3D, G, and J). In patients with *NPHS2* mutations, the green labeling of the podocyte body was strong with all three antibodies contrasting clearly with the red GBM labeling (Fig. 3E, F, H, I, K, and L). Focal superposition of labelings was revealed by the segmental yellow staining of the GBM.

Glomerular expression of extracellular matrix proteins

Because of the possible consequences of *NPHS2* mutation on the podocyte synthesis of extracellular proteins, we analyzed the distribution of glomerular extracellular matrix components. No difference in their pattern of distribution was detected between normal and NPHS2 patient kidneys. Antibodies to the α1 and α2 chains of type IV collagen or to [α1(IV)2α2(IV)]-labeled the mesangial matrix and gave a thin linear staining of the GBM whereas antibodies to the α3 to α5 (IV) chains strongly stained the GBM.

Antibodies to type VI collagen and fibronectin stained the mesangial matrix and faintly labeled the subendothelial aspect of the GBM. Antibodies against nidogen, and laminin chains α1/α5, β2, and γ1 gave a linear staining

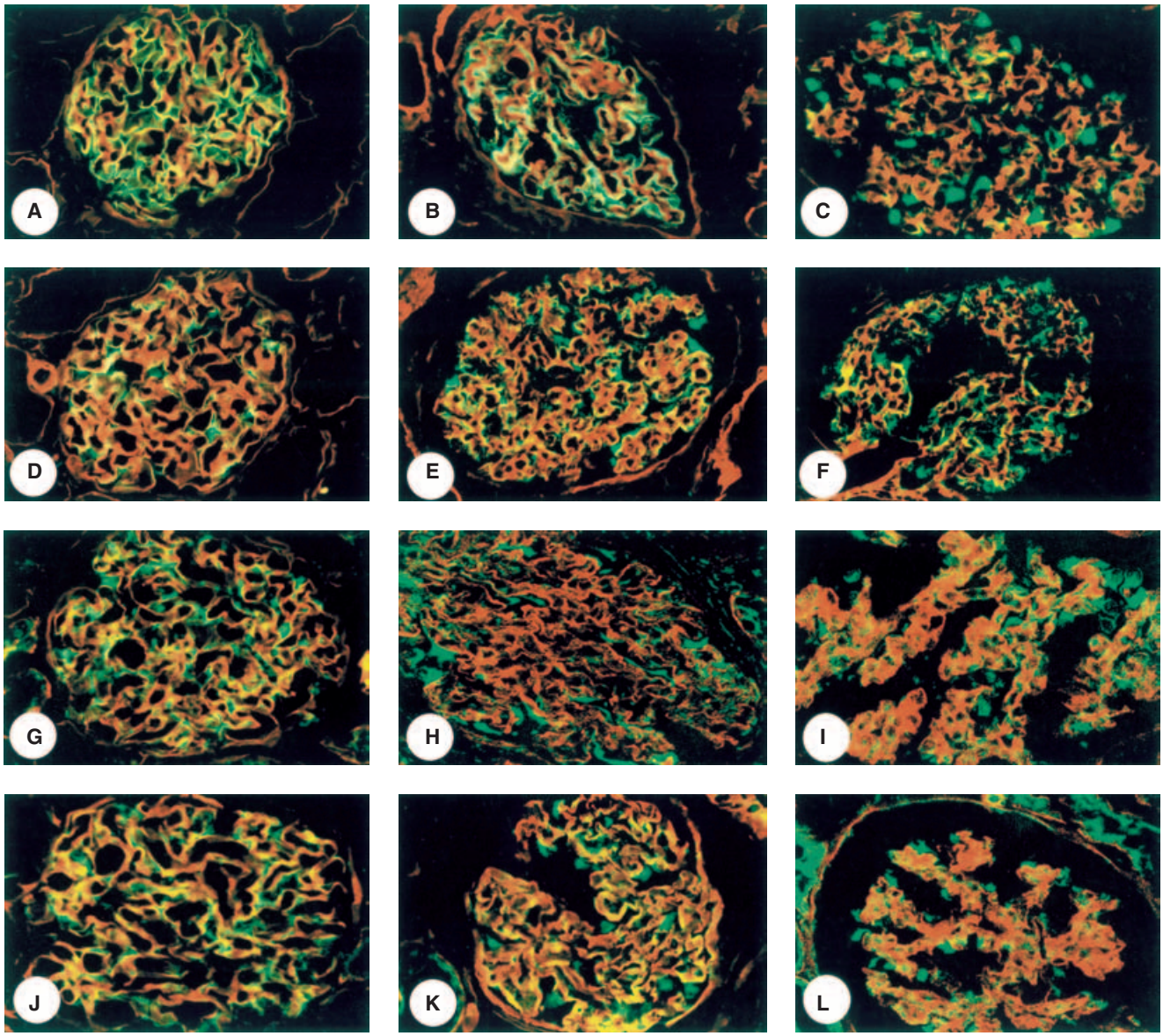

Fig. 3. Double immunofluorescence and confocal microscopy with polyclonal antibodies against podocin (*A* to *C*), nephrin (*D* to *F*), CD2-associated protein (CD2AP) (*G* to *I*), or α-actinin (*J* to *L*) (in green) and monoclonal anti-nidogen (in red). In normal glomeruli, partial overlap of the red and green labelings results in a yellow linear staining of the glomerular basement membrane (GBM) (A, D, G, and I). In patients 1 and 2 with truncating mutation, the pattern of labeling with the anti N-terminal domain of podocin was similar to the one in controls (B). In patients 3 to 6 with other types of mutation, dual immunolabeling clearly confirmed the diffuse cytoplasmic labeling of the podocytes with antipodocin antibodies (C). In all patients, whatever the type of *NPHS2* mutations, green labeling was strong in the podocyte body with antinephrin, CD2AP, and α-actinin antibodies contrasting clearly with the red GBM labeling (E, F, H, I, K, and L). Focal superposition of labelings was revealed by the segmental yellow staining of the GBM (magnification × 200).

of the GBM, whereas β1 chain labeling was faint and restricted to the mesangial matrix. Anti-agrin antibodies gave a strong linear staining of the GBM, whereas antiperlecan antibodies stained the mesangial matrix (data not shown).

DISCUSSION

Podocytes are highly specialized cells located on the external side of the glomerular capillary walls. Their voluminous cell body that protrudes into the urinary space emits thick extensions that divide into pedicels or foot processes and attach to the GBM. Adjacent foot processes, derived from different podocytes, are joined laterally by a thin structure, the slit diaphragm, now regarded the major glomerular size-selective filter [24, 37, 38]. Podocin normally localizes at the insertion site of the slit diaphragm, in lipid rafts, specialized microdomains of the plasma membrane involved in cell signaling processes, and signal transduction pathways [9]. It interacts

with nephrin, CD2AP, and NEPH proteins through its C-terminal cytoplasmic domain and those interactions facilite nephrin signaling [9, 19, 39–41]. Within the foot processes, a contractile cytoskeleton, composed of actin, myosin II, α-actinin-4, talin, and vinculin [42] is linked to the slit diaphragm, and connected to the foot processes' soles and the underlying GBM through the $\alpha_3\beta_1$ integrin and the dystroglycan complexes. Mutations in *NPHS2* encoding podocin, result in severe glomerular disease characterized by early onset proteinuria/nephrotic syndrome with effacement of podocyte foot processes, steroid resistance and progression to ESRD [2–7, 10, 43, 44]. No slit diaphragm was observed in glomeruli of *Nphs2*−/− mice examined from the age of 16.5 embryonic days onward, at a stage when podocytes of mature glomeruli in the deep cortex are fully differentiated in normal control animals [10]. The severe clinical and morphologic consequences of the absence or structural alteration of podocin prompted us to examine the expression pattern of podocin and slit diaphragm-associated proteins in a series of renal biopsies from nephrotic patients with *NPHS2* mutations. We enlarged this study to the analysis of several other podocyte proteins and glomerular extracellular matrix components.

In normal kidneys, by immunofluorescence or immunoperoxidase stainings, podocin, nephrin, CD2AP, and α-actinin-4 appear to be codistributed, along the outer surface of the GBM. This labeling is correlated with the foot process distribution of the proteins. In *NPHS2* patients, the pattern of podocin expression varied according to the type and location of the mutation. Two patients had homozygous deletions of 1 or 2 bp predicted to introduce a frameshift and to lead to the production of a truncated protein. In the first one, with mutation located in exon 7 resulting in the absence of the last 98 amino acids of the protein, no important change in RNA expression was detected by in situ hybridization. As expected, no staining of the C-terminal domain was observed. In contrast, a strong podocyte labeling of the N-terminal domain was seen along the GBM, indicating a normal localization of the truncated protein. Changes were similar in the second patient with frameshift mutation in exon 3 leading to the truncation of 203 amino acids, but the expression of the transcripts and the podocin N-terminal domain was strikingly reduced, a possible consequence of rapid RNA decay and/or degradation of the truncated protein. Along the same lines, as previously reported in a patient with a homozygous nonsense mutation in exon 3, very low RNA expression was detected by reverse transcription (RT)-PCR [2] and no protein was seen by immunofluorescence [11]. In the three patients with compound point mutations, the expression of *NPHS2* transcripts seemed normal but the protein distribution was severely modified. It was restricted to the podocyte body, without any visible labeling of the basis of the cell along the GBM, in the patient with the compound heterozygous mutation R168S/467_468insT. Recently, Roselli et al [45] have shown that the R168S mutant podocin is retained in the endoplasmic reticulum in vitro. Our results strongly suggest that the same defect in intracellular trafficking occurs in vivo in the podocytes of this patient. In patients carrying mutations R138Q/V180M or R291W/R229Q, strong podocin labeling of the podocyte body was associated with moderate labeling along the GBM. Interestingly, in opossum kidney (OK) cells, human podocytes and embryonic kidney cells, the R138Q podocin mutant was shown to be also retained in the endoplasmic reticulum [45, 46]. Moreover, the R291W mutant accumulated in late endosomes, whereas the V180 mutant and the R229Q variant were normally targeted to the plasma membrane [45]. Here, our findings suggest that the same processus occurs in vivo in patients with compound missense mutations, resulting in the presence of podocin in the podocyte body and along the GBM. In the patient in which a single heterozygous frameshift mutation has been identified, the expression of the transcript appeared to be normal but podocin was present in the podocyte body and along the GBM, suggesting a normal expression of the normal allele.

In all *NPHS2* patients, the podocin defect resulted in major changes in the podocyte location of nephrin and CD2AP characterized by switching from a podocyte localization along the GBM in controls to a prominent location in the podocyte body in patients. Podocin, nephrin, and CD2AP have been shown to co-localize and interact within lipid raft domains of the plasma membrane facing the filtration slit [9]. More recently, it was demonstrated, by in vitro experiments, that podocin recruits nephrin in lipid rafts, and that recruitment is abolished in the absence of podocin, or the presence of mutated proteins retained in the endoplasmic reticulum or not targeted to rafts [47]. Our morphologic findings in vivo are consistent with these data: protein interactions demonstrated in vitro likely explain the mislocalization of nephrin and CD2AP observed in patients with mutated podocin. Interestingly, similar changes in nephrin expression, characterized by strong labeling of the podocyte body, associated with increased CD2AP podocyte labeling, were observed in *Nphs2*−/− mice [10]. The distribution of α-actinin-4, a component of the contractile apparatus of the podocyte foot processes, was also modified in the patients. In contrast to the normal distribution along the GBM, α-actinin was also seen in the podocyte body, a possible consequence of the disruption of the slit diaphragm complex that is normally anchored to the actin cytoskeleton, possibly by linkage to CD2AP [48, 49]. Using a human podocyte cell line, Saleem et al [50] provided functional evidence for an intimate relationship between the actin cytoskeleton and the slit diaphram proteins by showing that normal intra cellular co-localization of these proteins

is dependent on intact actin polymers. All of these findings are additional evidences of the tight interdependence of proteins participating in the formation of the foot process cytoskeleton and slit diaphragm structure. ZO-1 is known to be co-localized with nephrin in glomeruli, from the S-shaped body stage, and to concentrate in the slit diaphragm of mature glomeruli [51]. It also interacts with Neph family members and could contribute to the organization of the filtration slit [52]. However, normal expression of ZO-1 along the GBM was observed in the 6 patients with *NPHS2* mutation and disrupted localization of other slit diaphragm-associated proteins. A normal expression of ZO-1 has also been found in the kidneys of nephrin null mice [53] and *NPHS1* fetuses with Finmajor/Finmajor genotype, who completely lack nephrin and slit diaphragm [51]. These findings suggest that ZO-1 does not belong to the same multimolecular complex participating in the formation of slit diaphragm than nephrin/podocin/CD2AP. No changes were detected in the distribution of the adhesion molecules α_3-integrin and β-dystroglycan, implicated in the attachment of the sole of the foot processes to the GBM, the transmembrane protein tyrosine phosphatase GLEPP1, the actin-associated protein synaptopodin, or the intermediary filament vimentin. In the same way, no changes were observed in the composition or the distribution of glomerular extracellular matrix components, a finding consistent with the absence of specific ultrastructural abnormalities of the GBM.

CONCLUSION

Most *NPHS2* mutations result in profound alteration of podocin expression and/or distribution, from normal location of truncated proteins lacking the C-terminal domain, to mislocalization of the mutated protein in the podocyte body. Podocin is normally targeted to the plasma membrane via the classical endoplasic reticulum pathway [45]. It may be suggested that, in vivo as shown in in vitro experiments, some *NPHS2* missense mutations prevent correct folding of the protein which is retained in the endoplasmic reticulum or internalized [45, 46]. Secondary changes in the distribution of nephrin and CD2AP are additional evidences of the scaffolding role of podocin in the organization of the slit diaphragm.

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