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Positionally Cloned Gene for a Novel Glomerular Protein—Nephrin—Is Mutated in Congenital Nephrotic Syndrome

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Summary

Congenital nephrotic syndrome of the Finnish type (NPHS1) is an autosomal-recessive disorder, characterized by massive proteinuria in utero and nephrosis at birth. In this study, the 150 kb critical region of NPHS1 was sequenced, revealing the presence of at least 11 genes, the structures of 5 of which were determined. Four different mutations segregating with the disease were found in one of the genes in NPHS1 patients. The *NPHS1* gene product, termed nephrin, is a 1241-residue putative transmembrane protein of the immunoglobulin family of cell adhesion molecules, which by Northern and in situ hybridization was shown to be specifically expressed in renal glomeruli. The results demonstrate a crucial role for this protein in the development or function of the kidney filtration barrier.

Introduction

The primary barrier for ultrafiltration of plasma in renal glomeruli comprises three layers: a fenestrated endothelium, a 300–350 nm thick glomerular basement membrane (GBM), and slit pores, i.e., diaphragms located between the foot processes of the epithelial podocytes. This barrier is a highly sophisticated size-selective molecular sieve whose molecular mechanisms of function are still largely unclarified. It is anticipated that the GBM, a tightly cross-linked meshwork of type IV collagen, laminin, nidogen, and proteoglycans, contains pores that restrict the penetration of large proteins and cells, and additionally, it has been hypothesized that anionic heparan sulfate proteoglycan components contribute to an electric barrier for macromolecules (Caulfield and Farquhar, 1978; Kanwar and Farquhar, 1979; Kasinath and Kanwar, 1993). The glomerular filter is affected in a large number of acquired and inherited diseases resulting in extensive leakage of plasma albumin and larger proteins leading to nephrotic syndrome and end-stage renal disease. Understanding of the molecular mechanisms of the glomerular filtration process and its pathology is of fundamental importance for clinical medicine, which, in turn, may facilitate novel developments for diagnosis and treatment of complications in primary and secondary diseases of the kidney. Genetic diseases with defects in the filtration barrier as major symptoms can serve as models for providing such knowledge.

Congenital nephrotic syndromes form a heterogeneous group of diseases characterized by massive proteinuria at or shortly after birth (Rapola et al., 1992). Congenital nephrotic syndrome of the Finnish type (CNF, NPHS1, MIM 256300) is a distinct entity among nephrotic syndromes. It is an autosomal-recessive disorder with an incidence of 1:10,000 births in Finland, but considerably less frequent in other countries (Norio, 1966; Huttunen, 1976). The disease manifests itself already at the fetal stage with heavy proteinuria in utero, demonstrating early lesions of the glomerular filtration barrier. The pathogenesis of NPHS1 has remained obscure. There are no pathognomonic pathologic features, the most typical histological finding of NPHS1 kidneys being dilation of the proximal tubuli (Huttunen et al., 1980). The kidneys are also large and have been found to contain a higher amount of nephrons than age-matched controls (Tryggvason and Kouvalainen, 1975). Electron microscopy reveals no abnormal features of the GBM itself, although there is a loss of foot processes of the glomerular epithelial cells, a finding characteristic for nephrotic syndromes of any cause. Analyses of GBM proteins, such as type IV collagen, laminin, and heparan sulfate proteoglycan, have not revealed abnormal findings in NPHS1 (e.g., see Ljungberg et al., 1993; Kestilä et al., 1994a). NPHS1 is a progressive disease, usually leading to death during the first two years of life, the

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only life-saving treatment being kidney transplantation (Holmberg et al., 1995). Importantly, most transplanted patients have, thus far, not developed extrarenal complications, suggesting that the mutated gene product is highly specific for kidney development and/or glomerular filtration function. However, about 20% of the patients have developed posttransplantation nephrosis, the cause of which is unknown (Laine et al., 1993; Holmberg et al., 1995).

Due to its high specificity for the glomerular filtration process, NPHS1 provides a unique model disease for studies on this important kidney function. Since there was no strong candidate gene for the disease, we have used the positional cloning approach in our attempts to identify the *NPHS1* gene, and have localized the gene to a 150 kb region on chromosome 19q13.1 (Kestilä et al., 1994b; Männikkö et al., 1995; this study). In the present study, we have identified a novel gene in the critical region and shown it to be mutated in NPHS1. The gene product is a novel transmembrane member of the immunoglobulin superfamily, which in the human embryo shows a high expression level in renal glomeruli.

Results

Characterization of Genes at the *NPHS1* Locus

Following localization of the *NPHS1* gene to 19q13.1, overlapping cosmid clones from the interval of interest between markers *D19S208* and *D19S224* were isolated (Männikkö et al., 1995; Olsen et al., 1996). Based on the significant linkage disequilibrium observed with *D19S608* and *D19S610*, as well as the new microsatellite markers *D19S1173*, *D19S1175*, and *D19S1176* identified in this study, the *NPHS1* gene was fine-mapped between *D19S1175* and *D19S608*, in close vicinity to *D19S1176* and *D19S610* (Figure 1). Southern hybridization analyses of NPHS1 patients' DNA with genomic clones did not reveal variations, suggesting that the mutations causing NPHS1 do not represent major genomic rearrangements. The 150 kb critical region was sequenced in its entirety, and the sequence was searched for potential candidate genes using exon prediction programs and data-base similarity searches. Based on those analyses, the critical region was estimated to include over 100 potential exons. Similarity searches revealed one previously known gene, i.e., *APLP1* encoding an amyloid precursor-like protein (Lenkkeri et al., 1998), and eight distinct expressed sequence tags (ESTs). Together, the analyses indicated the presence of at least ten novel genes in the critical region.

Using GRAIL and GENSCAN exon prediction programs and sequences from cDNAs, the exon/intron structures of five of the genes, *NPHS1* (Figure 1), *APLP1* (Lenkkeri et al., 1998), *A*, *B*, and *C* (not shown) were determined. Although steady-state transcript levels varied, Northern analyses revealed expression of all of the genes in kidney, and with the exception of *NPHS1*, also in other tissues (not shown). Therefore, none of them could be excluded as the *NPHS1* gene, and all were subjected to mutation analysis.

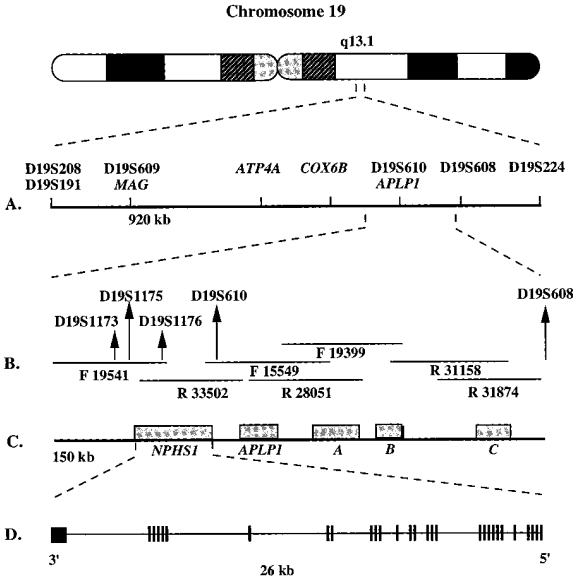


Figure 1. Physical Map of the *NPHS1* Locus at 19q13.1 and Genomic Organization of the *NPHS1* Gene

(A) Physical map of the 920 kb region between *D19S208* and *D19S224*.

(B) Overlapping cosmid clones spanning the 150 kb critical region containing the *NPHS1* gene. Location of polymorphic markers are indicated by arrows.

(C) Location of five genes, *NPHS1*, *APLP1*, *A*, *B*, and *C*, characterized and analyzed for mutations in this study.

(D) Schematic structure of the *NPHS1* gene.

Identification of the *NPHS1* Gene

Haplotype analyses of chromosomes have revealed two major classes in Finnish NPHS1 patients (Männikkö et al., 1995; this study). The first one containing haplotypes 1-1-1-6-G-2-8-9 and 1-1-1-6-G-6-4-2 (markers *D19S1173*, *D19S1175*, *D19S1176*, *D19S610*, RFLP of gene *B*, *D19S608*, *D19S224*, and *D19S220*, respectively) is the most common one found in 78% of Finnish NPHS1 chromosomes. The second haplotype class, 3-5-3-6-A-8-10-x, is found in 14% of cases (Table 1). The remaining 8% of observed haplotypes show totally different allele combinations, and have been thought to represent other mutations. The two major haplotype classes could represent the same mutation, because they both share allele 6 of *D19S610*. However, the present results demonstrated that they represent two different mutations.

Since Southern hybridization analyses did not reveal any major gene rearrangements, we decided to systematically search for the mutations by direct sequencing of PCR-amplified exon regions of, if necessary, all of the genes of this region.

The 17 exon *APLP1* gene located distal to *D19S610* did not show variations between patients and controls, and was excluded as the *NPHS1* gene (Lenkkeri et al., 1998). Also, the novel genes *A*, *B*, and *C* containing 9, 5, and 3 exons, respectively, did not have sequence variants segregating with NPHS1, and could similarly be excluded as genes causing NPHS1 (data not shown). A fourth novel gene *NPHS1* located proximal to *D19S610* encoding a transcript of about 4.3 kb was shown to be strongly expressed in human embryonic and adult

Table 1. Observed Haplotypes and Mutations in *NPHS1* Chromosomes and Effects of Mutations on Nephrin

Haplotype	Number of Chromosomes	Nucleotide Changes in the *NPHS1* cDNA	Effect on Amino Acid Sequence	Mutation Code
1-1-1-6-G-2-8-9	57	Deletion of nucleotides 121-122 (CT) in exon 2	Frameshift→truncated 90-residue protein	nt121(del2), Fin_{major}
x-y-1-6-G-x-y-x	10			
1-1-1-6-G-6-4-2	9			
3-5-3-6-A-8-10-x	14	nt3325 (C→T) substitution in exon 26	Nonsense→truncated 1109-residue protein	R1109X, Fin_{minor}
x-5-2-y-x-y-x-y	2			
ND	2 (NA-1)	AC insertion after nucleotide 1306 in exon 10	Frameshift→truncated 438-residue protein	nt1306(ins2)
ND	1 (NA-2)	G insertion after nucleotide 3250 in exon 24	Frameshift→truncated 1095-residue protein	nt3250(insG)

NPHS1 haplotypes consist of alleles observed with markers *D19S1173-D19S1175-D19S1176-D19S610*-RFLP of gene *B-D19S608-D19S224-D19S220*, respectively. The critical region for the *NPHS1* gene was restricted between *D19S1175* and *S19S608* (150 kb) based on observed recombinations in the founder haplotype 1-1-1-6-G-2-8-9. Out of 49 NPHS1 patients studied, 32 were homozygous for the Fin_{major} mutation, four were homozygous for the Fin_{minor} mutation, and eight were compound heterozygotes. Four patients had the Fin_{major} mutation in one allele, the other potential mutation still being unknown. One patient had neither one of the two mutations. Mutation nt1306(ins2) was found as homozygous in a North American patient (NA-1). NA-2 was compound heterozygote for mutations nt3250(insG) and Fin_{major}.
x and y can represent any allele of observed alleles.
ND, not determined.

kidneys, no clear signals being observed above background in other tissues (Figure 2). Therefore, this gene was a strong candidate for NPHS1. Full-length cDNA for the transcript was constructed using fetal kidney poly(A) mRNA (Clontech), and PCR primers were made based on the predicted exon structure. The gene was found to have a size of 26 kb and to contain 29 exons (Figure 1).

Exon sequencing analyses revealed the presence of two major mutations in over 90% of NPHS1 chromosomes (Table 1, Figure 3). The first mutation, a 2 bp deletion in exon 2, causes a frameshift resulting in the generation of a stop codon within the same exon. This mutation [nt121(del2), Fin_{major}] was found in all NPHS1 chromosomes with the haplotype 1-1-1-6-G-2-8-9 or 1-1-1-6-G-6-4-2 (total of 76 chromosomes). The second sequence variant found in the *NPHS1* gene was a nonsense mutation CGA→TGA in exon 26 (R1109X, Fin_{minor}), present in patients with the haplotype 3-5-3-6-A-8-10-x (14 chromosomes), and two patients with different haplotypes. A total of 108 parents and 54 healthy siblings were analyzed, none of them being homozygous or compound heterozygous for the two mutations identified here. One out of 83 control individuals was heterozygous for the Fin_{major} mutation.

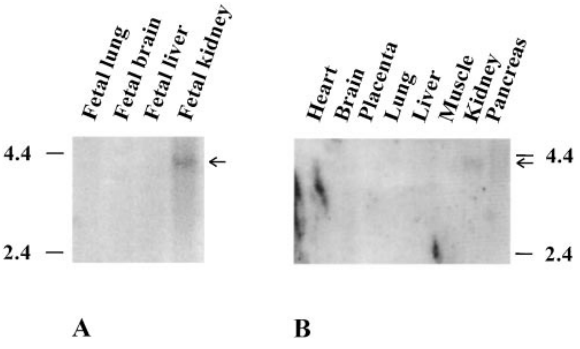


Figure 2. Northern Analysis of Nephrin Expression with mRNA from Human Embryonic and Adult Tissues

The Northern filters containing 2 μg of human poly(A) RNA from four fetal and eight adult tissues (Clontech) were hybridized with a 1371 bp *NPHS1* cDNA probe (exons 1–10) made by RT–PCR from fetal kidney poly(A) RNA.
(A) Distinct expression can be seen only with fetal kidney RNA (arrow).
(B) Using RNA from adult tissues, signal is only observed in a 4.3 kb band with kidney RNA (arrow), the other tissues not exhibiting signals above background.
The tissues studied are marked above the filter, and molecular size markers (kb) are shown to the sides of the filters.

Cases of NPHS1 have also been found outside Finland. In this study, mutation search was carried out in two North American NPHS1 patients. A homozygous two nucleotide insertion was found in exon 10 [nt1306(ins2)] in patient NA-1. Patient NA-2 was heterozygous for one nucleotide insertion in exon 24 [nt3250(insG)], carrying the Fin_{major} mutation in the another allele. Mutations observed in this study and their consequences in the gene product are described in Figure 3 and Table 1.

Characterization of the *NPHS1* Gene Product

The cDNA-predicted amino acid sequence of nephrin is 1,241 residues (Figure 4A), with a calculated molecular mass of 134,742 without posttranslational modifications. Based on several similarity comparison and protein structure prediction programs, it was predicted that the NPHS1 protein would be a transmembrane protein of the immunoglobulin superfamily. There is a tentative 22-residue N-terminal signal peptide, an extracellular domain containing eight immunoglobulin-like modules, and one fibronectin type III–like module, followed by a single putative transmembrane domain–like sequence, and a cytosolic C-terminal end. In spite of the presence of known structural modules (Figure 4B), the sequence identity with corresponding domains of proteins in the data base was relatively low. The tentative extracellular portion of the protein contains ten NXS or NXT consensus triplets for N-glycosylation. Furthermore, there are seven SG doublets that are potential attachment sites for heparan sulfate.

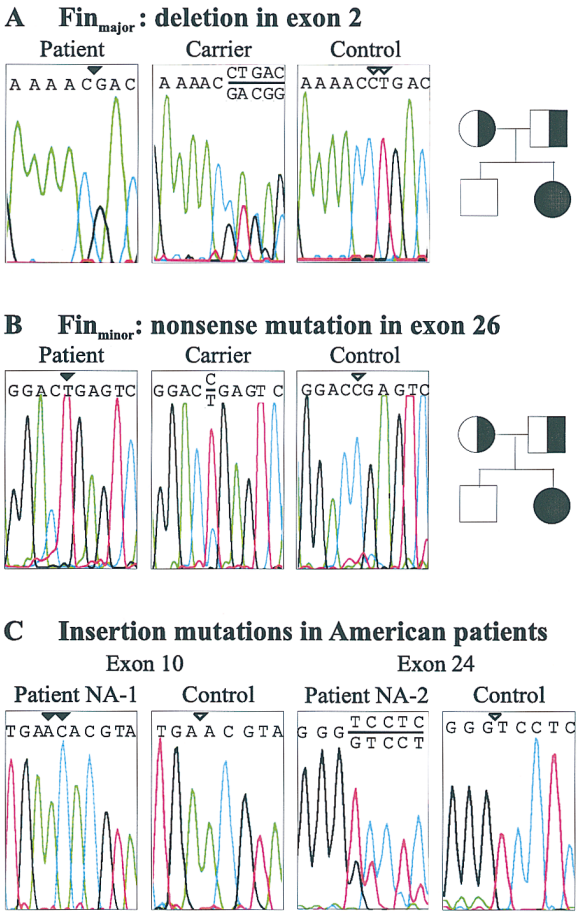


Figure 3. Mutation Analysis of the *NPHS1* Gene

(A) Pedigree of an NPHS1 family with an affected child having the Fin$_{major}$ mutation, a CT deletion in exon 2. Sequences of the deletion region are shown from patient (homozygous), parent (heterozygous carrier), and a healthy sibling. Open arrows indicate bases in normal sequence; the closed arrow indicates site of deletion in the patient DNA.

(B) Pedigree of an NPHS1 family with an affected child having the Fin$_{minor}$, a nonsense mutation (CGA→TGA) creating a premature translation termination codon in exon 26. Sequences of the mutated site are shown from a patient (homozygous), parent (heterozygous carrier), and a healthy sibling. One C nucleotide in normal DNA (open arrow) is replaced by a T in the patient (closed arrow).

(C) Sequences of two North American patients (NA-1 and NA-2) with different insertion mutations in the *NPHS1* gene and the corresponding sequences from controls. Left: Patient NA-1 has an AC insertion in exon 10. Right: Patient NA-2 has a G insertion in exon 24. The sites of insertion into the DNA are depicted in the control sequence by open arrows, and the inserted bases in the patient DNAs are shown by closed arrows.

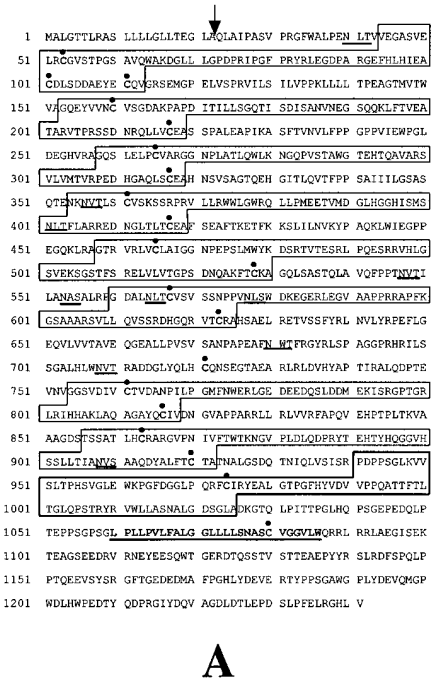
Northern hybridization analysis carried out with poly(A) mRNA from four human embryonic and eight adult tissues revealed a high steady-state level of the *NPHS1* gene transcript in the kidney, but not notably in other tissues (Figure 2). In situ hybridization carried out on a kidney sample from a 23-week-old human embryo revealed intense expression signals in the glomeruli (Figure 5A). At higher magnification (Figure 5B), the signals could be seen in the periphery of mature and developing glomeruli, while the central mesangial regions are negative. It is apparent that the positive cells are epithelial podocytes. No specific signals were obtained with the antisense control probe (data not shown).

Discussion

Several lines of evidence obtained in the present study suggest that the gene affected in congenital nephrotic syndrome of the Finnish type has been positionally cloned. First, the defective gene is located in the critical 150 kb region on chromosome 19q13.1 to which the gene has been localized using linkage disequilibrium analyses (Kestilä et al., 1994b; Männikkö et al., 1995; this study). Second, the two mutations identified in the study were shown to be present, either as homozygous or compound heterozygous mutations, in 90% of Finnish patients studied. Of the remaining patients, 8% had the major mutation in one allele, the mutation in the other allele being, as yet, unidentified. One patient who did not have either of the two mutations, has a unique haplotype and, therefore, probably carries a different mutation. In addition, two different frameshift mutations were found from North American NPHS1 patients. Third, individuals homozygous or compound heterozygous for the mutations were not found in control DNAs. Additional, indirect evidence was the strong and practically renal glomeruli-specific expression of the gene, which implies involvement of the gene product in glomerular development or function.

Identification of the *NPHS1* Gene

The present identification of the gene causing NPHS1 demonstrates the power of linkage disequilibrium analysis and direct DNA sequencing in the positional cloning of disease genes containing small mutations. Here, linkage disequilibrium mapping (Hästbacka et al., 1994) used with DNA from individuals of a homogenous population, such as the isolated Finnish population (de la Chapelle, 1993), was utilized to localize the *NPHS1* gene to a 150 kb genomic segment. To find genes located in this region, the entire segment was first sequenced, and using a combination of exon prediction programs and homology comparison analyses, we could construct remarkably accurate gene structures that were verified from cDNAs. These cDNAs could be isolated either with the use of EST clones or by using the predicted exon sequences to construct cDNAs by PCR from mRNA. In this manner, we could quickly identify 11 genes within the 150 kb *NPHS1*-containing genomic segment. Since none of the genes was an obvious candidate for *NPHS1*, and no major gene rearrangements, such as deletions, insertions, or inversions, were found in patient DNAs, search for small mutations had to be initiated, if necessary, in all of the 11 genes. Having determined the exon and cDNA sequences for the genes, methods such as SSCP and DGGE, which are frequently used for identification of small mutations, were potential alternatives. However, our experience from search for small mutations in Alport syndrome (Barker et al., 1990; Tryggvason, 1996) suggests that these methods can frequently yield false negatives. For example, SSCP analyses in quite large patient populations have revealed only a 35%–50% mutation detection rate (Kawai et al., 1996;



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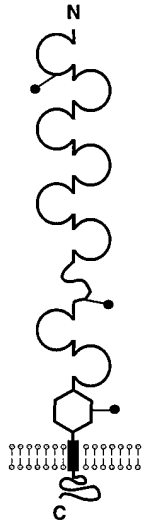

B

Figure 4. Nucleotide-Derived Amino Acid Sequence of Nephrin and Predicted Domain Structure

(A) The predicted N-terminal signal sequence is 22 residues, the cleavage site being marked with an arrow. The putative transmembrane domain (residues 1059–1086) is shown in bold and underlined. The putative extracellular part of the protein contains eight Ig-like modules (boxed), and one fibronectin type III–like module (boxed with a bold line) adjacent to the transmembrane domain. Cysteine residues are indicated by closed dots, and the ten putative N-glycosylation sites in the extracellular part of the protein are underlined.

(B) Predicted domain structure of nephrin. The Ig-like modules are depicted by partial circles, and the fibronectin type III–like motif is represented by a hexagon. The transmembrane domain is shown as a closed rectangle located in a membrane lipid bilayer. The locations of three free cysteine residues are indicated by closed dots.

Knebelmann et al., 1996; Renieri et al., 1996), while direct sequencing of PCR-amplified exon regions has yielded over 80% detection (Martin et al., personal communication). We therefore decided to use direct sequencing of exon regions to find the *NPHS1* mutations. Although we had to sequence numerous exons of several genes, this relatively soon resulted in the identification of two small mutations in one gene.

Genetics of *NPHS1*

Crucial components in the successful positional cloning of the *NPHS1* gene were the small isolated population, good clinical records, and an equal, high-quality health care system that made it possible to reliably collect family samples. A typical situation in population isolates is that close to 100% of cases are caused by the same mutation, and this phenomenon can already be seen in haplotype analysis. Observed changes in the founder haplotype, caused by historical recombinations, can be used to restrict the critical chromosomal region to a short genomic segment. Thus, differences in the major NPHS1 haplotype 1-1-1-6-G-2-8-9 enabled substantial narrowing of the interval, leading to the isolation of the *NPHS1* gene. The Fin_{major} mutation causes only 78% of cases, in contrast to many other "Finnish diseases" with 95%–98% prevalence of major disease alleles (Ikonen et al., 1991; Vesa et al., 1995; Höglund et al., 1996). However, the two main NPHS1-causing mutations characterized in this study together represent 94% of Finnish cases.

NPHS1 is enriched in the Finnish population, but several cases have been found elsewhere. Considerable immigration from Finland to Minnesota has also caused the spread of NPHS1 to the USA (Norio, 1966; Mahan et al., 1984). In addition, several NPHS1 cases have been diagnosed in different European countries, and linkage studies have supported association of analyzed families to the same chromosome 19 locus (Fuchshuber et al., 1996). Two frameshift mutations were identified in this study in North American patients, and a spectrum of different mutations can be expected to be found in other NPHS1 patients of non-Finnish origin.

The identification of the *NPHS1* gene and disease-causing mutations has immediate clinical significance, as they have enabled the development of exact DNA-based diagnosis for NPHS1 and carrier screening. This is particularly important, as we have recently demonstrated that the screening method for NPHS1 based on measurements of alpha-fetoprotein levels in amniotic fluid can lead to false-positive results and subsequent abortions of healthy NPHS1 carriers (Männikkö et al., 1997).

Nephrin—A Putative Glomerulus-Specific Cell Adhesion Receptor

Due to the high association of expression and pathology with glomeruli, the proximal part of the nephron, we have named the *NPHS1* gene product nephrin. The role of nephrin remains unknown, but it is likely to be an adhesion receptor and a signaling protein, as its domain structure resembles that of a large group of cell adhesion receptors belonging to the immunoglobulin superfamily (Brümmendott and Rathjen, 1994).

The Ig-like modules of nephrin are all of type C2, which is particularly found in proteins participating in cell–cell or cell–matrix interactions. The cytosolic domain has no significant homology with other known proteins. However, it contains nine tyrosines, some of which could become phosphorylated during ligand binding of nephrin. The crucial role for the intracellular domain of nephrin is emphasized by the fact that the Fin_{minor} mutation, which results in the loss of 132 out of 155 residues of this domain, results in full-blown NPHS1.

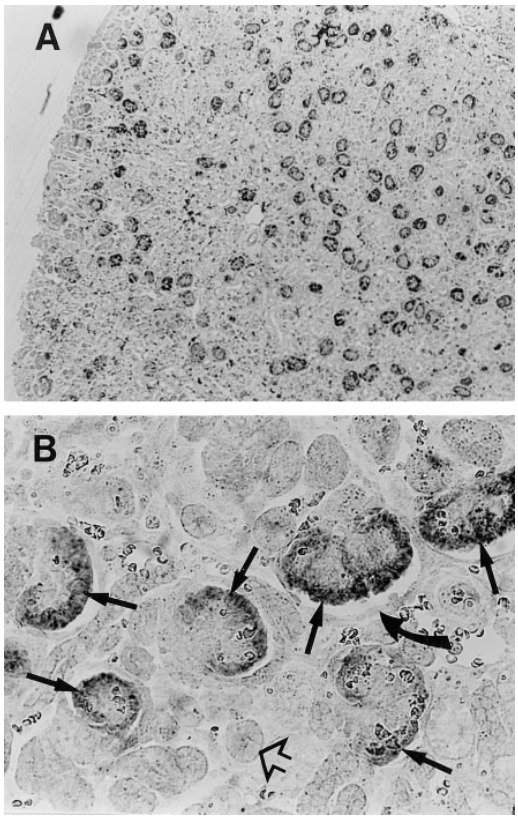

Figure 5. Expression of Nephrin mRNA in Human Embryonic Kidney by In Situ Hybridization

(A) Intense expression is seen in glomeruli throughout the renal cortex, little if any specific expression being observed in other structures (4× objective magnification).

(B) Higher magnification reveals intense expression in the periphery of individual glomeruli (straight arrows), probably mainly in epithelial cells. No expression is observed in the Bowman's capsule (bent arrow), proximal tubuli (open arrows), or endothelial cells of vessel walls (20× objective magnification).

How does nephrin function and what is its role in glomerular function? A vast majority of similar receptors interact with other membrane proteins in a homo- or heterophilic manner. However, some of the receptors have been shown to interact with extracellular matrix (ECM) proteins. For example, the myelin-associated glycoprotein MAG, whose extracellular domain contains five Ig-like modules, interacts with different types of collagens and glycosaminoglycans (Fahrig et al., 1987). Furthermore, the axonal glycoprotein F11 and the deleted in colorectal cancer (DCC) protein have both been shown to bind tenascins and netrins, respectively (Zisch et al., 1992; Pesheva et al., 1993; Keino-Masu et al., 1996). Since it is possible that nephrin either binds another membrane protein or a protein of the ECM, which in this case would be the GBM, it will be important to localize nephrin by immunoelectron microscopy before embarking on the search for a specific ligand.

Whatever its function, the in situ hybridization analyses strongly suggested that nephrin is produced in glomerular epithelial cells that form the foot processes partially covering the outside of the glomerular capillaries. The ultimate filtration barrier for plasma macromolecules is located in the diaphragm covering the slit pores between the foot processes (Kasinath and Kanwar, 1993). In NPHS1 and nephrotic syndromes of other causes, fusion of the foot processes is a general finding, and the structure or function of the slit pores is somehow affected with proteinuria as a result. It is possible that the plasma membrane protein nephrin is important for maintaining the integrity of the foot processes of glomerular epithelial cells, or is crucial for their anchorage to components of the GBM.

Conclusions

The identification of the *NPHS1* gene will immediately find applications for diagnosis of the disease. Studies on the gene product nephrin, a putative cell-adhesion and signaling receptor, may also provide a key to new fundamental knowledge on the molecular mechanisms of glomerular filtration, which despite decades of research are still poorly understood. As abnormal function of the filtration barrier is a major complication in many clinically important kidney diseases, such as diabetic nephropathy, nephrotic syndromes, and glomerulonephritides, the present work is likely to have a significant impact on clinical nephrology. However, more information about nephrin and its function needs to be generated. Immediate questions relate to the developmental expression and location of the protein, which would require the generation of antibodies and nucleotide probes for studies in animal and cell culture systems. Other questions are whether nephrin is important for maintaining the structure of the epithelial foot processes, or is involved in their anchorage to the GBM with some specific role in the actual filtration process. It would also be interesting to find out whether and how nephrin is involved in the development of complications of numerous acquired kidney disorders where glomerular filtration is affected. Generation of a mouse model for NPHS1 by gene targeting in embryonic stem cells would be of great value for addressing these and other questions.

Experimental Procedures

Sequencing of Cosmid Clones

Isolation of cosmid clones spanning the region between *D19S208* and *D19S608* has been reported previously (Olsen et al., 1996). DNA of cosmid clones F19541, R33502, F15549, R28051, F19399, R31158, and R31874 was mechanically sheared by nebulization, and fragments of 1000–2000 bp were isolated and subcloned into M13 phage, prior to random sequencing using ABI377 automated DNA sequencers.

Analysis of Sequence

To develop new microsatellite markers, repeat regions were searched from the sequence, and three of them (*D19S1173*, *D19S1175*, and *D19S1176*) were found to be polymorphic. Detailed information on markers is presented elsewhere (Pekkarinen et al., personal communication). Homology comparisons were performed using BLASTX and BLASTN programs (Altschul et al., 1990). Prior to BLASTN analyses, the nucleotide sequence was filtered using CENSOR (Jurka et al., 1996) to mask out repeat regions like Alu sequences. Exon prediction was made using GRAIL (Uberbacher and Mural, 1991), GENSCAN (Burge and Karlin, 1997), and FGENEH and HEXON (Solovyev et al., 1994) programs, and prediction of the protein structure was made using BLASTP (Altschul et al., 1990) and EXPASY

molecular biology server (Appel et al., 1994). The mutation search was performed by comparing patient sequences to the normal genomic sequence using the FASTA program of the GCG package (Genetics Computer Group, 1996).

Isolation of cDNAs

cDNAs were generated by PCR from poly(A) RNA from different tissues using primers based on the exon sequences. The PCR fragments were sequenced and used for screening of cDNA libraries. Marathon ready cDNA kits (Clontech Laboratories) were also used to characterize the 5′ and 3′ extremities of the cDNAs. Comparisons of the cDNA and genomic sequences were made to establish the sizes of introns, as were intron sequences at acceptor and donor splice sites.

Southern and Northern Blots and In Situ Hybridization Analyses

For Southern analyses, samples containing 10 μg of genomic DNA were digested with different restriction enzymes and electrophoresed on 1% agarose gels, transferred to nylon membranes, and hybridized with the cDNA probe. In multiple-tissue Northern analysis, poly(A) RNAs from 8 adult and 4 fetal tissues were studied (Clontech). Hybridization was done in ExpressHyb buffer at 65°C using a cDNA clone containing exons 1–10.

For in situ hybridization, a fragment from the *NPHS1* cDNA clone (corresponding to exon 10) was labeled with digoxigenin (Boehringer Mannheim), cut to about 150 base-pair fragments by alkaline hydrolysis, and then used as a probe. Tissue sections of 7 μm from a 23-week human embryonic kidney were treated with 0.2 M HCl, 0.1 M triethanolamine buffer (pH 8.0), containing 0.25% (v/v) acetic anhydride and 100 μg/ml proteinase K. The sections were hybridized with the probe at 62°C for 16 hr. After rinsing in 50% formamide and standard sodium citrate, the probe was immunologically detected with an antibody to digoxigenin conjugated to alkaline phosphate enzyme (Boehringer Mannheim). The color was developed with NBT and BCIP.

Mutation Analysis

In this study, we analyzed 49 Finnish NPHS1 patients, their parents, and a total of 54 healthy siblings. The diagnosis of NPHS1 is based on severe proteinuria, a large placenta (>25% of birth weight), nephrotic syndrome during the first weeks of life, and exclusion of other types of congenital nephrotic syndrome (Koskimies, 1990). Additionally, samples from 83 control individuals were analyzed.

The *NPHS1* gene was analyzed by PCR-amplifying and sequencing all exon regions from genomic DNA. The sequences of the primers for exon 2 were 5′-GAGAAAGCCAGACAGACGCAG-3′ (5′ UTR) and 5′-AGCTTCCGCTGGTGGCT-3′ (intron 2), and the sequences of the primers for exon 26 were 5′-CTCGGGGAGACCCACCC-3′ (intron 23) and 5′-CCTGATGCTAACGGCAGGGC-3′ (intron 26). PCR reactions were performed in a total volume of 25 μl, containing 20 ng of template DNA, 1× AmpliTaq buffer (Perkin-Elmer), 0.2 mM of each nucleotide, 50 ng of primers, and 0.5 U of AmpliTaq Gold DNA polymerase. The reactions were carried out for 30 cycles with denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. In the first cycle, denaturation was carried out for 12 min, and extension in the last cycle was for 8 min. PCR products were separated by 1.5% agarose gel, sliced off, and purified by the QiaexII system (Qiagen). The purified PCR product was sequenced using specific primers employing dRhodamine dye-terminator chemistry and an ABI377 automated sequencer (Perkin-Elmer).

When screening for the NPHS1 Fin_{major} mutation from parents, siblings, and controls, a 100 bp PCR product containing the exon 2 deletion site was amplified using a radioactively end-labeled primer and electrophoresed on 6% polyacrylamide gels. The NPHS1 Fin_{minor} mutation could be screened for using a novel restriction site for DdeI. The 170 bp amplified PCR product was digested with DdeI, and the products (140 bp or 90 bp + 50 bp) were separated on an agarose gel (1% SeaKem agarose - 3% NuSieve agarose).

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