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Discovery of the congenital nephrotic syndrome gene discloses the structure of the mysterious molecular sieve of the kidney

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ABSTRACT The molecular nature of the glomerular slit diaphragm, the site of renal ultrafiltration, has until recently remained a mystery. However, the identification of the gene affected in congenital nephrotic syndrome has revealed the presence of a novel protein, possibly specific for the slit diaphragm. This protein, which has been termed nephrin, is a transmembrane protein that probably forms the main building block of an isoporous zipper-like slit diaphragm filter structure. Defects in nephrin lead to abnormal or absent slit diaphragm leading to massive proteinuria and renal failure. The discovery of nephrin sheds new light on the glomerular filtration barrier, provides new insight into the pathomechanisms of proteinuria, and even opens up possibilities for the development of novel therapies for this common and severe kidney complication.

KEY WORDS: *congenital nephrosis, nephrin, glomerular basement membrane, slit membrane*

Glomerular filtration barrier

Ultrafiltration of blood during formation of the primary urine in the glomerulus is one of the central functions of the human kidney (Tisher and Madsen, 1996). Structurally, the glomerulus is a tuft of anastomosing capillary loops surrounded by the Bowman´s capsule leading the primary urine to the tubular system. The glomerular filtration barrier is formed by three layers: the innermost fenestrated vascular endothelium, the glomerular basement membrane (GBM), and the podocyte layer. The podocytes form a tight web on top of the GBM with their interdigitating foot processes joined by a slit diaphragm.

It is generally acknowledged that the molecules passing through the glomerular filtration barrier are selected according to their size, charge and shape (Bohrer *et al.*, 1978; Brenner *et al.*, 1978; Batsford *et al.*, 1987; Kanwar *et al.*, 1991; Ghitescu *et al.*, 1992; Fujigaki *et al.*, 1993; Remuzzi and Remuzzi, 1994). The exact locations of the various selective functions in the barrier are, however, more controversial. The charge-selective filter has been thought to be located in the GBM, a cross-linked meshwork of type IV collagen, laminin, nidogen and proteoglycans (Hudson *et al.*, 1993; Yurchenco and O´Rear, 1993). The anionic charge of heparan sulfate side chains of proteoglycans is believed to hinder the traversal of anionic plasma proteins (Caulfield and Farquhar, 1978; Kanwar and Farquhar, 1979; Kanwar *et al.*, 1991). The location of the size-selective property of the filtration barrier has been attributed to the GBM alone or, alternatively to the slit diaphragm (Latta, 1970; Karnovsky and Ainsworth, 1972; Kanwar *et al.*, 1991).

Concerning the molecular composition of the slit diaphragm, monoclonal antibody 5-1-6 (Orikasa *et al.*, 1988) that recognizes a 51 kDa protein has been shown in immunoelectron microscopy to react exclusively with the slit diaphragm (Kawachi *et al.*, 1995). However, the nature of this protein is still unknown. The α-isoform of the tight junction protein ZO-1 (Schnabel *et al.*, 1990) has been localized in the glomerulus, predominantly to points where the slit diaphragm is inserted into the lateral cell membrane of the foot process (Kurihara *et al.*, 1992). The ZO-1 protein possibly connects the slit diaphragm, directly or indirectly, to the cytoskeleton.

In numerous primary and secondary diseases of the kidney, the filtration barrier is affected resulting in proteinuria, i.e. leakage of albumin and larger plasma proteins into the urine, with edema and nephrotic syndrome as a consequence. Many cases also include an immunological component in their etiology which, however, is not the case in the congenital nephrotic syndrome of the Finnish type (NPHS1) (Rapola, 1987). We have recently identified the gene mutated in NPHS1 (Kestilä *et al.*, 1998; Lenkkeri *et al.*, 1999). The disease specifically affects the kidney and is characterized by

Abbreviations used in this paper: GBM, glomerular basement membrane; NPHS1, nephrotic syndrome 1; CNF, congenital nephrotic syndrome of the Finnish type (=NPHS1); APLP1, amyloid precursor-like protein 1; CAM, cell adhesion molecule.

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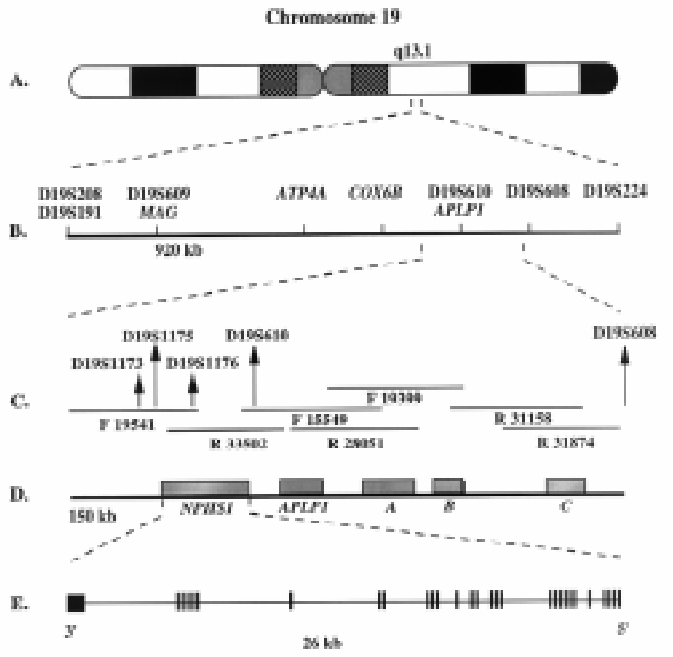

Fig. 1. Positional cloning of the congenital nephrotic syndrome gene. **(A)** *The gene was first localized to the long arm of chromosome 19 using microsatellite markers.* **(B)** *The gene was shown to reside between markers D19S208 and D19S224, in a 920 kb region containing a few known genes MAG, ATP4, COX6B and APLP1.* **(C)** *Using new markers from the region, the NPHS1 gene could be further mapped to a 150 Kb region for which overlapping cosmid clones were isolated. Location of the polymorphic markers within the clones are indicated by arrows.* **(D)** *Four novel genes and APLP1 located in the 150 kb region were analyzed for mutations in patients with the congenital nephrotic syndrome and mutations were found in the NPHS1.* **(E)** *The NPHS1 gene that codes for a novel protein called nephrin is 26 kb and contains 29 exons (Kestilä et al., 1998).*

massive proteinuria already *in utero*. Electron microscopic examination of NPHS1 patient kidneys reveals thinner lamina densa of the GBM than in controls, but no structural abnormality of the GBM has been detected (Autio-Harmainen, 1981; Autio-Harmainen and Rapola, 1983). In kidneys of patients with NPHS1, the podocyte foot processes are absent, and no slit diaphragms have been described in the podocyte cell-cell adhesions. This finding is typical for nephroses of any cause.

The gene mutated in NPHS1 codes for a putative transmembrane protein termed nephrin that belongs to the immunoglobulin (Ig) superfamily. It has an extracellular portion containing eight Ig-motifs and one type III fibronectin domain (Kestilä *et al.*, 1998). This, together with the sequence of the intracellular domain which contains eight tyrosines, suggested that nephrin is a signaling adhesion molecule. Using *in situ* hybridization, nephrin was shown to be exclusively expressed in glomerular podocytes (Kestilä *et al.*, 1998).

Recently, we localized nephrin in immunofluorescence microscopy to the GBM region of newborn human glomeruli using antibodies generated against recombinant antigen (Ruotsalainen *et al.,* 1999). Moreover, we demonstrated by immunoelectron microscopy that nephrin is located in the podocyte slit region. We propose that nephrin is most likely a component of the slit diaphragm. The fact that the protein is mutated in NPHS1, further indicates an essential role for the slit diaphragm in the maintenance and size selectivity of the glomerular filtration barrier.

Congenital nephrotic syndrome of the Finnish type

Congenital nephrotic syndrome of the Finnish type, is a distinct entity among nephrotic syndromes. It is the first disease to be described to belong to the so-called Finnish disease heritage (Hallman *et al.,* 1956). This Finnish variant of congenital nephrotic syndrome has traditionally been referred to in the literature as CNF, but has now been designated as NPHS1. 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. Chemical analyses carried out on the composition of the GBM of NHPS1 patients in the 1970s did not reveal any typical changes and later studies on GBM proteins, such as type IV collagen, laminin, and heparan sulfate proteoglycan, have not revealed abnormal findings in NPHS1 (Tryggvason, 1977, Ljungberg *et al.*, 1993, Kestilä *et al.*, 1994b). NPHS1 is a progressive disease, usually leading to death during the first two years of life, the only life-saving treatment being kidney transplantation (Holmberg *et al.*, 1995).

An important feature with the disease is that most transplanted patients have not developed extrarenal complications. This suggested that the mutated gene product is highly specific for kidney development and/or glomerular filtration function. However, about 20% of the patients have developed post-transplantation nephrosis, the cause of which is unknown (Laine *et al.*, 1993; Holmberg *et al.*, 1995). Because of the apparent kidney specificity of NPHS1 and, because the disease practically only seems to affect the filtration barrier, we considered it as a model for solving the nature of the actual kidney glomerular filter.

The hunt for the *CNF* gene

Since numerous studies on CNF had given no clues concerning the molecular defects in the diseases, we decided to use a molecular genetic approach. In 1989 we initiated the actual gene hunt. Due to the relatively high frequency of the disease in Finland and due to the homogeneity of the Finnish population, we decided to only use Finnish families for the gene search. For that purpose, we collected samples from all CNF families we could identify in Finland. DNA was isolated from peripheral blood, but we also established cell lines from all CNF patients to ensure that we had sufficient amounts of DNA at any time. Samples from a total of 29 CNF families were collected. Of those, only 17 were large enough to be suitable for genetic linkage analyses. This work was initially

in the hands of a graduate student, Marjo Kestilä, who was soon joined by another graduated student, Minna Männikkö. Having collected the patient material they could now start the gene hunt.

Basically two possibilities were ahead of us for identifying the unknown CNF gene. The candidate gene approach or positional cloning. In the candidate gene approach one tries to make educational guesses about possible genes, and the obvious genes to examine in this regard, were genes coding for basement membrane proteins, as the disease seemed to primarily affect the filtration barrier. For this purpose, we first used genetic markers for chromosomal regions where all known BM genes were known to be located. However, we could soon demonstrate absence of linkage to all then known BM genes, such as those for the collagen IV alpha 1-4 chains, laminin alpha 1, 3, etc.

Since the candidate gene approach did not give us a lead, we initiated positional cloning, which basically means to carry out a genome wide screening with markers for all autosomal chromosomes and examine if linkage could be found between any chromosomal region and CNF. At that time, a panel of about 400 markers had been established and after using about 60 of them, Kestilä and Männikkö could demonstrate significant lod scores with markers in the long arm of chromosome 19 (19q13.1) (Kestilä *et al*., 1994a; Männikkö *et al*., 1995).

Having localized the gene to 19q13.1 in 1994 (Fig. 1), we searched data bases to find out if any groups were particularly involved in characterization of chromosome 19, and found out that a genomics group at the Lawrence Livermore Laboratory in California was mapping the entire chromosome for eventual sequencing. Surprisingly, a senior investigator in the group, Anne Olsen, had worked with Karl Tryggvason ten years earlier in the same laboratory at Rutgers Medical School, New Jersey, USA. It was therefore not difficult to establish collaboration with the Livermore group which provided us with overlapping cosmid clones from the critical region between markers *D19S208* and *D19S224* (Olsen *et al*., 1996). This region turned out to be about 1000 kilo-bases which was sequenced in its entirety by the Livermore group. With this information the work started to become real hot, and we got two new graduate students, Ulla Lenkkeri and Heli Putaala, to help us fine comb the CNF gene region and isolate the gene. Based on the significant linkage disequilibrium observed with *D19S608* and *D19S610*, as well as the new microsatellite markers, *D19S1173, D19S1175*, and *D19S1176*, that we identified in the cosmid clones, the CNF gene was fine-mapped to only a 150 kilo base region between markers *D19S1175* and *D19S608*, in close vicinity of *D19S1176* and *D19S610* (Fig. 1). The work now became very exciting and Southern hybridization analyses of CNF patient DNA with genomic clones did not reveal variations, suggesting that the mutations causing NPHS1 do not represent major genomic rearrangements. Having the sequence of the 150 kilo base critical region, we 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 pro-

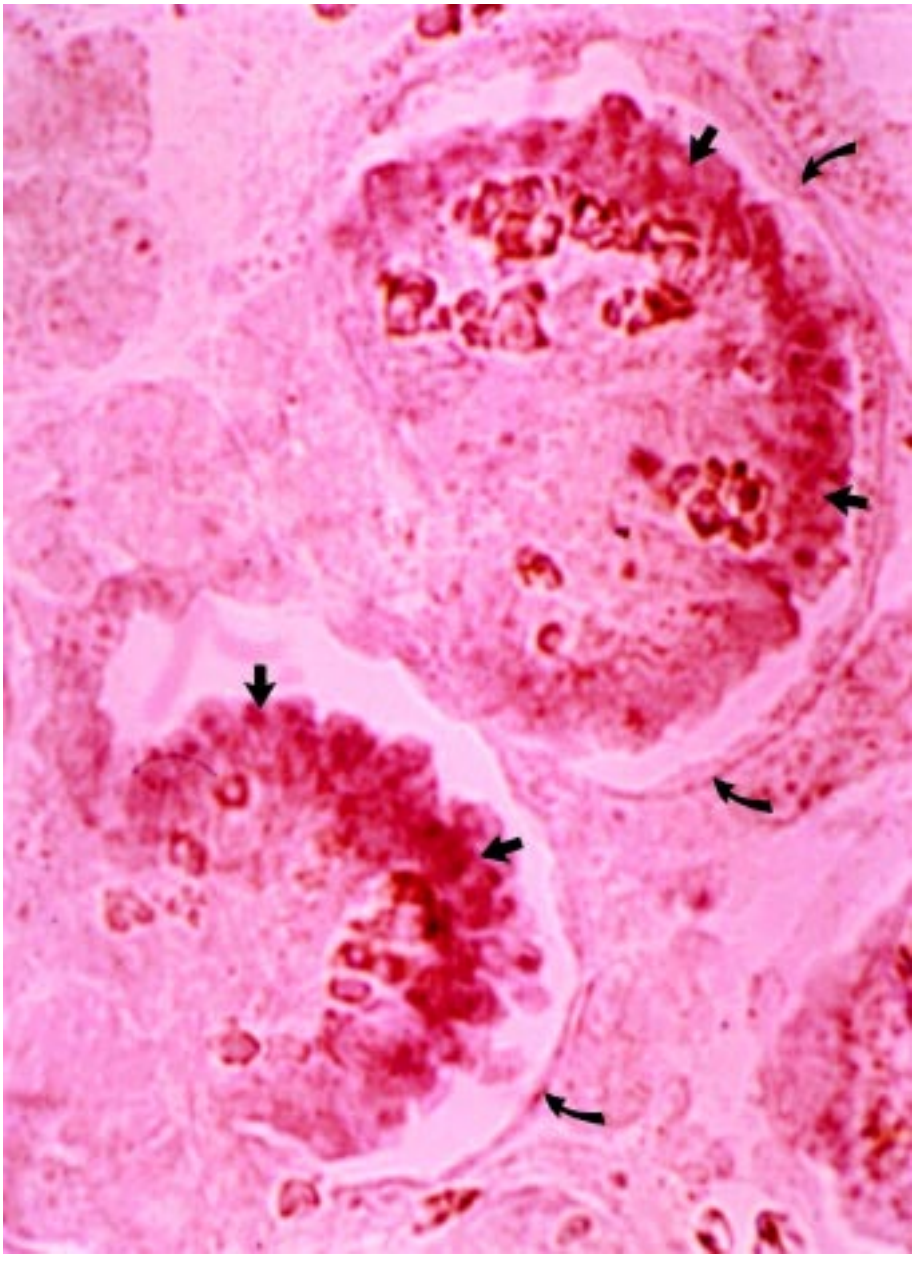

Fig. 2. Expression of nephrin mRNA in human embryonic kidney by *in situ* hybridization. *Expression was only observed in the glomerular regions. A high magnification strong expression was observed in the periphery of individual glomeruli (straight arrows), probably mainly in epithelial cells. No expression is observed in the Bowman's capsule (bent arrow), proximal tubuli or endothelial cells of vessel walls.*

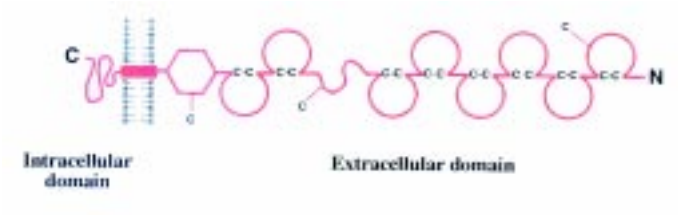

Fig. 3. Schematic domain structure of nephrin. *The red box represents the transmembrane domain. The Ig repeats are shown by incomplete circles connected by disulfide bridges (C-C). The locations of free cysteine residues are indicated by a -C. N= extracellular amino terminal, C= intracellular carboxyl terminal.*

tein (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* (Fig. 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 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.

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 located proximal to *D19S610* encoding a transcript of about 4.3 kb was shown to be strongly expressed in human embryonic and adult kidneys, while no clear signals were observed above background in other tissues (Fig. 2). Therefore, this gene was a strong candidate for CNF. Full-length cDNA for the transcript was constructed using fetal kidney poly(A) mRNA and PCR primers made based on the predicted exon structure. The gene was found to have a size of 26 kb and to contain 29 exons (Fig. 1).

Exon sequencing analyses revealed the presence of two major mutations in over 90% of NPHS1 chromosomes. The first mutation, a 2bp deletion in exon 2 causes a frame shift resulting in the generation of a stop codon within the same exon. The second sequence variant found in the *NPHS1* gene was a nonsense mutation CGA→TGA in exon 26 (R1109X, Fin_{minor}). 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.

The nine-year hunt for the CNF gene was now completed, as we had now definitely demonstrated that the *NPHS1* gene was the disease gene. This gene has now been confirmed to be mutated in a large number of other patients worldwide with congenital nephrotic syndrome (Lenkkeri *et al.,* 1999), as we have now found a total of over 40 mutations in the gene.

Nephrin

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 (Fig. 3). The role of nephrin is still hypothetical, but it is a transmembrane protein with a domain structure resembling that of a large group of cell adhesion receptors belonging to the immunoglobulin superfamily (Brümmendorf and Rathjen, 1994). It has eight extracellular Ig-like modules and one fibronectin type III-like module.

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.

Nephrin is a component of the slit membrane

The remarkable interdigitating pattern of adjacent podocyte foot processes in the kidney glomerulus has implied an essential role of the podocytes for the integrity of the glomerular filtration barrier (Daniels, 1993; Kriz *et al.*, 1994; Mundel and Kriz, 1995). Much interest in the study of this barrier has been focused on the cell junction between the foot processes, the so called slit diaphragm. However, the actual molecular structure of the diaphragm has remained unraveled. Vesa Ruotsalainen generated antibodies against nephrin using remobinant extracellular domain of the protein as antigen. By using these antibodies we could show strong immunostaining of the GBM region in light microscopy (Fig. 4). More importantly, Päivi Ljungberg and Jorma Wartiovaara could localize nephrin to the slit diaphragm region by immunoelectron microscopy (Fig. 5), providing the first evidence for a specific protein in this size-selective molecular filter of the kidney (Ruotsalainen *et al.*, 1999). The fact that mutations in the nephrin

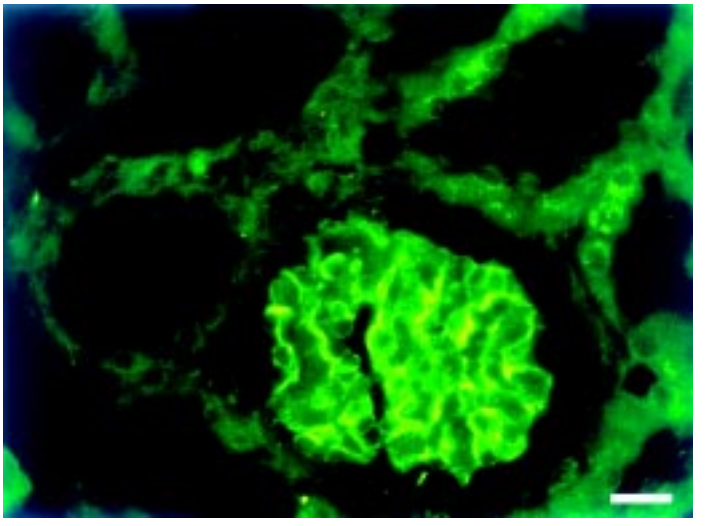

Fig. 4. Immunohistochemical localization of nephrin in human kidney. *Immunofluorescence staining was carried out on a 2-month-old human kidney with antibodies against recombinant human nephrin. Immunoreactivity is seen in the glomerulus, presumably at the podocyte-GBM junction. No staining is present in mesangial or endothelial cells. Bar, 20 μm.*

gene lead to massive proteinuria (Kestilä *et al.*, 1998; Lenkkeri *et al.*, 1999), furthermore indicates that nephrin has a direct role or actual filtration function in this size-selective barrier.

Concerning nephrin localization, it should be stressed that, in its extracellular labeling, nephrin was found only between the foot processes. It was completely absent from the interspaces between the foot processes and the GBM, a site with very much larger potential labeling surface than present in the narrow slit. The occasional cases of gold label observed in the cytoplasm of foot processes may represent newly synthesized nephrin molecules.

The results of the immunolocalization studies raise the fundamental question as to how a protein like nephrin, either alone or together with other slit membrane protein(s), can contribute to the molecular structure of a porous filter. The ultrastructure of the podocyte slit diaphragm has been studied extensively by electron microscopy (Mundel and Kriz, 1995). Rodewald and Karnovsky (1974) were the first to suggest a zipper-like organization of this structure. As these studies used the conventional thin-sectioning method requiring harsh chemical treatments in sample preparation, it has later been suggested that the zipper-looking structure might be due to an artifact. Since then, the podocyte-podocyte junction has also been studied by several other electron microscopic methods (Furukawa *et al.*, 1991; Ohno *et al.*, 1992). These studies have indicated that the width of this junction varies between 20 and 50 nm. The actual slit diaphragm is considered to be a rather rigid structure. However, it has recently been suggested that at least the slit area might increase with increasing perfusion pressures of the glomerulus (Kriz *et al.*, 1996; Yu *et al.*, 1997). Since the actual molecular structure of the slit diaphragm is, as yet, unresolved, it is precocious to speculate on a molecular assembly allowing change in the width of the slit. Our results, however, provide evidence that nephrin is a component of such an assembly. This calls for further studies on the molecular structure of the slit diaphragm.

Several lines of evidence indicate that nephrin may assemble into a zipper-like isoporous filter structure similar to that presented by Rodewald and Karnovsky (1974). First, the present study demonstrated that nephrin is specifically located at the slit diaphragm. Second, nephrin must be crucial for the structural integrity of the slit diaphragm, as absence of the protein or different amino acid substitutions cause congenital nephrosis and lack of the slit diaphragm with massive proteinuria as a result (Lenkkeri *et al.*, 1999). Third, nephrin molecules extending towards each other from two adjacent foot processes are likely to interact with each other in the slit through homophilic interactions, as has been shown for other Ig cell adhesion molecules, such as N-CAM (Kiselyov *et al.*, 1997), C-CAM (Öbrink, 1997) and L1 (Sonderegger and Rathjen, 1992). Fourth, such homophilic assembly of nephrin molecules in the slit could have a zipper-like arrangement, essentially as that proposed based on electron microscopic studies.

A hypothetical head-to-head assembly of nephrin through homophilic interactions is illustrated in Figure 6. The amino terminal extracellular domain of nephrin contains six consecutive Ig repeats, followed by a spacer domain, two additional Ig repeats, and one fibronectin type III -like domain (Fig. 3). Each Ig motif contains two cysteine residues that, similarly to corresponding motifs in other proteins (Chothia and Jones, 1997), can be assumed to form a disulfide bridge within the repeat structure (Fig. 3). Ig motifs have been shown to adopt a globular or ellipsoid structure

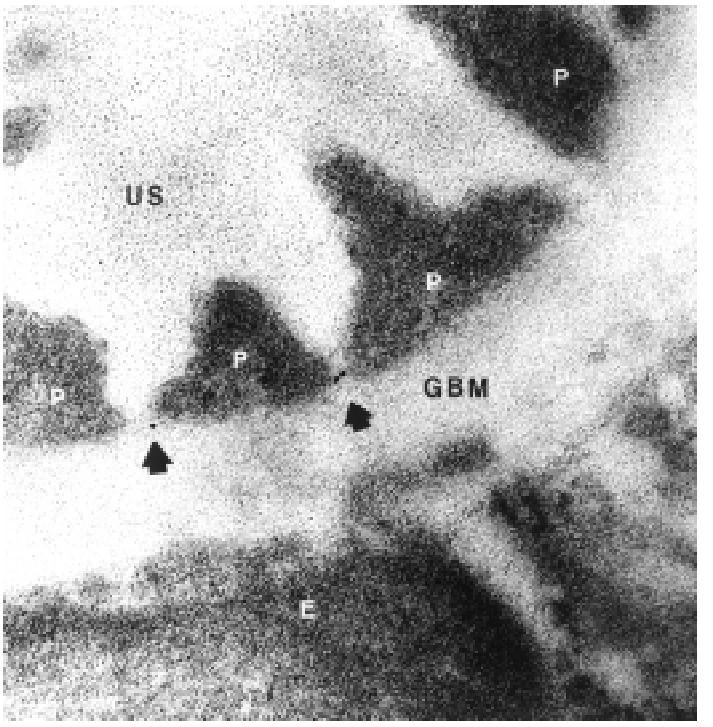

Fig. 5. Immunoelectron microscopic localization of nephrin in human renal glomeruli. *Indirect post-embedding staining for nephrin using affinity purified IgG against the extracellular region of recombinant human nephrin and 10 nm gold -coupled secondary antibody. Gold label (arrowheads) was observed between foot processes of podocytes (P). The label is located in the central area of the slit, between the glomerular basement membrane (GBM) and the slit diaphragm. Endothelium (E) is unlabeled. US, urinary space (Ruotsalainen et al., 1999).*

with an average axis length between 24 and 47 Å, averaging 35 Å (Holden *et al.*, 1992). If the Ig repeats were to form a chain-like structure, as has been proposed for Ig cell adhesion molecules, all eight motifs would contribute to a length of about 28 nm. The region between Ig repeats 6 and 7 and fibronectin type III -like domain would add more length to the protein. Consequently, a single nephrin molecule can extend through most of the width of the 35-45 nm wide slit diaphragm.

In addition to the two cysteine residues in each Ig motif, nephrin contains three free cysteines, one in Ig motif 1, one in the spacer region between Ig motifs 6 and 7, and one in the fibronectin domain close to the plasma membrane. The three free cysteines are likely to have a function in forming intermolecular disulfide bridges that provide strength to the slit diaphragm. These cysteines are important as their absence results in proteinuria and congenital nephrotic syndrome (Lenkkeri *et al.*, 1999). In the hypothetical model presented here, the free cysteine of Ig motif 1 in one molecule interacts with the cysteine residue of the spacer in another nephrin molecule. Such disulfide bonds could “lock” the homophilic unit of six Ig repeats of one nephrin molecule to similar units of two adjacent nephrin molecules. A centrally located aggregate of numerous nephrin molecules along the slit between two foot processes could constitute the central filament visualized by Rodewald and Karnovsky (1974). The width of the central aggregate would be 21 nm (6x35 Å) by simply assuming a linear chain arrangement of six

Fig. 6. Hypothetical model showing how nephrin may assemble in the slit to form the isoporous filter of the podocyte slit diaphragm. *Possible mode of interdigitating association of four nephrin molecules in the slit. For the sake of clarity, nephrin molecules from opposite foot processes are illustrated in different colors. In this model it is assumed that Ig repeats 1-6 of a nephrin molecule of one foot process associate in an interdigitating fashion with Ig repeats 1-6 in a neighboring molecule from the opposite foot process. Cysteine residues are depicted by black lines and two potential disulfide bridges crosslinking four nephrin molecules in the center of the slit are illustrated. The remaining single free cysteine present in the fibronectin domain may react with another nephrin molecule, or some other, as yet unknown molecule, that may connect with the plasma membrane or cytoskeleton (Ruotsalainen et al., 1999).*

Ig repeats, 35 Å each, and this would not agree with the 11 nm width of the central filament reported by Rodewald and Karnovsky (1974). However, this difference could be attributed to the shrinkage of samples, extraction of components, or failure to stain. Also, the mode of packing of Ig modules may well be different from that presented in the model, so that it is still possible that nephrin forms the basis of the slit diaphragm through homophilic interactions.

In conclusion, the recent identification of nephrin and its present specific localization to the podocyte slit diaphragm may accelerate the elucidation of the molecular structure of the size-selective glomerular filtration barrier. The model for nephrin assembly into a slit diaphragm proposed in this study supports the model for slit diaphragm ultrastructure presented over two decades ago based on transmission electron microscopy. However, further studies are needed to validate this model and examine other proteins contributing to the slit diaphragm structure. Also, other functions of nephrin, such as its potential signaling role, need to be investigated. The elucidation of the molecular structure of the filtration barrier can have significant clinical value. It not only explains the absence of slit diaphragms in NPHS1, but may also help to understand the pathogenic mechanisms of proteinuria in several other genetic and acquired kidney diseases that lead to proteinuria and renal failure. Considering the limited knowledge on the structure of the slit diaphragm, our recent results represent a noteworthy advance that may help to unravel the nature of this important extracellular structure.

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