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Unraveling the Mechanisms of Glomerular Ultrafiltration: Nephrin, a Key Component of the Slit Diaphragm

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Glomerular Filtration Barrier

Ultrafiltration of plasma during formation of the primary urine in the glomeruli is a major function of the kidney. The glomerulus is a tuft of anastomosing capillaries located within Bowman's capsule. The glomerular capillary wall has a complex morphology that facilitates a unique filtration function. This filtration barrier consists of three layers: a fenestrated endothelium, the glomerular basement membrane (GBM), and the outermost epithelial podocyte foot processes with their interconnecting slit diaphragms. Despite rapidly accumulating knowledge on the molecular nature of the GBM, there has been little information on the nature of the slit diaphragm, the last barrier to glomerular permeability.

It is well established that the glomerular filtration barrier behaves as a size-selective filter that restricts the passage of plasma macromolecules based on their size, shape, and charge (1–3). Negatively charged molecules are filtered in smaller amounts than neutral molecules of comparable size, and the traversal of positively charged molecules is actually facilitated. The exact locations of the filtration functions within the barrier have been a matter of debate. Farquhar and coworkers demonstrated the presence of anionic sites in the GBM (4,5) and proposed that they contain heparan sulfate (6). The GBM is a molecular scaffold composed of tightly cross-linked type IV collagen, laminin, nidogen, and proteoglycans (7,8). Type IV collagen and laminin form two apparently independent structural networks that are linked through nidogen. In addition to providing strength to the GBM, type IV collagen and laminin probably also have adhesion roles for the endothelial cells and podocytes. The basement membrane-specific heparan sulfate proteoglycans perlecan (9) and agrin (10) are components of the GBM. After perfusion of kidneys with heparinase, which removes all glycosaminoglycans except keratan sulfate, Kanwar *et al.* (11) showed increased permeability of the GBM to ferritin (molecular weight, 470 kD), and Caulfield and Farquhar (12) have also shown loss of anionic sites from the GBM in aminonucleoside nephrosis. These and similar data from other laboratories support the role of negative glycosaminoglycans as an anionic filtration barrier.

Although it is generally acknowledged that the GBM can restrict the traversal of large plasma proteins, there is also evidence that the ultimate barrier for proteins of the size of albumin resides in the slit diaphragm (13,14). However, there is very limited knowledge about the structure and molecular properties of this membrane, which has a remarkably constant width of about 40 nm. Several investigators have attempted to gain insight into the ultrastructure of the slit diaphragm by electron microscopy (15). By analyses of sections obtained in the plane of the diaphragm, Rodewald and Karnovsky in 1974 suggested that it has an isoporous, zipper-like structure (Figure 1) (16). According to their hypothesis, the pores (4×14 nm) had a size slightly smaller than that of albumin. Because these studies used thin-sectioning requiring harsh chemical treatments for sample preparation, the zipper structure has been questioned (15). Although most investigators seem to agree that the slit diaphragm is a rigid structure with a fairly constant width, a few electron microscopic studies have indicated that the width may vary between 20 and 50 nm (17,18). It has recently also been suggested that the slit area might increase with increasing intraglomerular pressure (19,20), indicating that the slit diaphragm is partially elastic.

The molecular nature of the slit diaphragm has hitherto been a mystery. Characterization of glomerular proteins conducted for more than two decades has not yielded any slit membrane-specific components. Monoclonal antibodies are often a useful tool for identifying novel proteins, and this approach has also been used to identify proteins in the slit diaphragm. Orikasa *et al.* (21) generated monoclonal antibodies against glomeruli, one of which (5-1-6) recognized a 51-kD protein based on Western immunoblotting analysis. By using immunoelectron microscopy, this protein was localized exclusively to the slit diaphragm (22). However, attempts to identify this slit diaphragm-specific protein have been unsuccessful. Farquhar and coworkers have localized the α-isoform of the intracellular tight junction protein ZO-1 in the glomerulus primarily to sites where the slit diaphragm is inserted into the plasma membrane of the foot process (23,24). They have proposed that the slit diaphragm is a modified tight junction, where ZO-1 connects the components of the diaphragm to the cytoskeleton.

Our research group has been interested in the molecular properties of the GBM and in the nature of the ultimate

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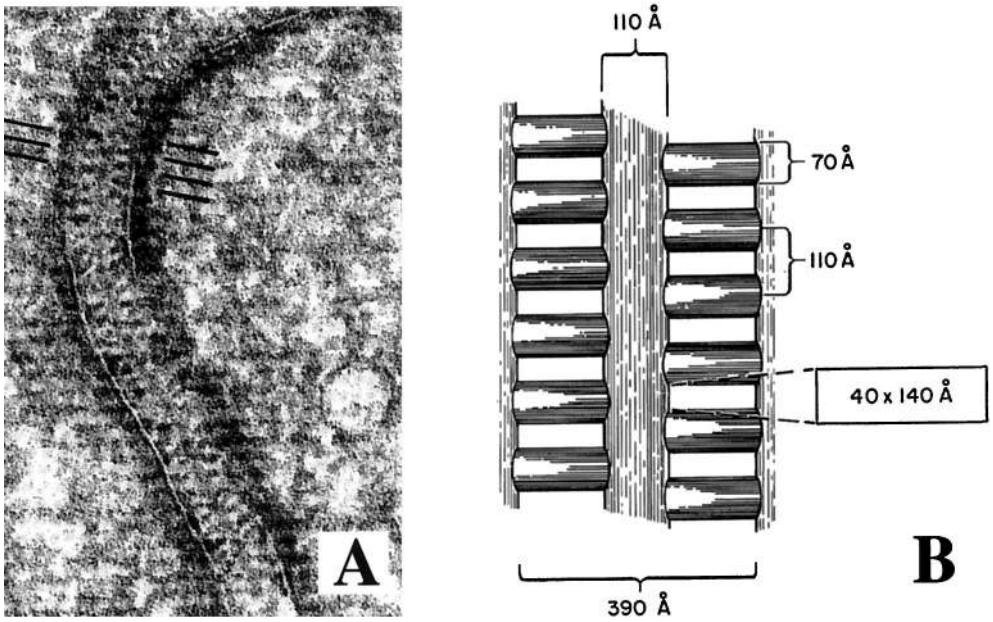

Figure 1. Zipper-like morphologic structure of the glomerular podocyte slit diaphragm as revealed by transmission electron microscopic studies of Rodewald and Karnovsky in 1974 (16). (A) Tannic acid-stained and glutaraldehyde-fixed sections obtained in the plane of the slit diaphragm of a rat glomerulus exhibit a central filament with cross bridges connecting it to the plasma membrane of the foot process. Density of the cytoplasm opposite the points of attachment of the slit diaphragm can be observed. (B) Schematic model of the slit diaphragm predicted based on the results in A (16). The average cross-section dimensions of the pores between the cross bridges are indicated within the rectangle. Reproduced from reference (16).

filtration barrier, the slit diaphragm, over an extended period of time. Being fascinated by the power of molecular genetics, we wished to examine whether identification of gene(s) mutated in diseases of the filtration barrier could provide a new understanding of this important physiologic function. The congenital nephrotic syndrome (NPHS1) is such a disease, and we decided to determine its underlying gene defect.

The Congenital Nephrotic Syndrome Gene

In numerous primary and secondary diseases of the kidney, the filtration barrier is affected resulting in proteinuria, with edema and the nephrotic syndrome as a consequence. Congenital nephrotic syndrome (NPHS1) is one such disorder that is inherited as an autosomal trait. NPHS1 is particularly frequent in Finland, where the so-called Finnish type has an incidence of 1:10,000 births (25–27). The disease manifests itself 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, and the most typical histologic finding of NPHS1 kidneys is dilation of the proximal tubules (28). The kidneys are also large and have been found to contain a greater number of nephrons than age-matched controls (29). 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 of the nephrotic syndrome of any cause (25–28). Chemical analyses carried out on the composition of the GBM of NHPS1 patients in the 1970s did not reveal any typical changes (30), and later studies on GBM proteins, such as type IV collagen, laminin, and heparan sulfate proteoglycan, or their genes, did not reveal any association with NPHS1 (31,32). NPHS1 is a progressive disease, usually leading to death during the first 2 years of life, with kidney transplantation the only life-saving treatment (33). Because of the apparent kidney specificity of NPHS1 and because the disease appears to affect the filtration barrier only, we considered NPHS1 a model for elucidating the nature of the actual kidney glomerular filter.

For the isolation of the NPHS1 gene, samples from all known 29 Finnish families were collected. Of those families, 17 were large enough to be suitable for linkage analyses. We first examined the association of known basement membrane genes with the disease, but they were quickly excluded (32). Subsequently, we applied the genome-wide screening approach using markers for all autosomal chromosomes, and the gene was localized to the 13.1 region on the long arm of chromosome 19 (34). The critical region was narrowed down to a 1 Mb segment (35), which was subsequently sequenced in its entirety, after which the disease gene region could be narrowed further to only a 150-kb segment (36). With the use of exon prediction programs and database similarity searches, and through characterization of transcripts such as ESTs and cDNAs, 10 potential novel genes and one previously known gene *APLP1* encoding an amyloid precursor-like protein were identified (37). The *APLP1* gene was immediately excluded as the disease gene. However, one of the novel genes turned out to be specifically expressed in the kidney as determined by Northern hybridization. This gene, *NPHS1*, was subsequently shown to contain two types of mutations segregating with the disease in the Finnish patients, establishing that it was the

congenital nephrotic syndrome gene. We have now identified more than 40 mutations in *NPHS1* in congenital nephrotic syndrome patients from numerous countries (38). Interestingly, some of the mutations led to a slower progression to end-stage renal disease than is characteristic for patients with the more severe Finnish type of NPHS1.

Nephrin—A Specific Component of the Slit Diaphragm

By using *in situ* hybridization, the NPHS1 gene product was shown in the kidney to be specifically expressed in podocytes of developing human glomeruli (36). Due to this nephron-specific expression, the protein has been termed nephrin (36). The cDNA-deduced amino acid sequence predicted that nephrin is a transmembrane protein of the Ig superfamily (36). As shown in Figure 2, nephrin has eight extracellular Ig-like modules and one fibronectin type III-like module. The Ig-like modules are of the so-called type C2 that is found predominantly in proteins participating in cell–cell interactions (39,40). The intracellular domain has no significant homology with other known proteins, but it has nine tyrosine residues, some of which might become phosphorylated during ligand binding of nephrin.

Polyclonal antibodies were generated against the two N-terminal Ig-like modules produced in *Escherichia coli* (41). By using these antibodies, strong staining was shown in the GBM region (Figure 3A), while the cell bodies of podocytes appear to be negative (41). Immunoelectron microscopic analysis of human glomeruli with this antibody (Figure 3B) demonstrated that the epitope is located exclusively in the slit diaphragm region (41). This is an important observation, because it was the first demonstration of a known protein to be located in the slit. The fact that nephrin appears to be specific for the slit also indicates a crucial role of nephrin for the filter structure.

Is the Slit Diaphragm an Isoporous Zipper-Like Filter Structure?

The results of the immunolocalization studies raise the fundamental question of how a protein such as nephrin, either alone or together with other slit membrane protein(s), can contribute to the molecular structure of a porous filter. Several lines of evidence indicate that nephrin may assemble into a zipper-like isoporous filter structure similar to that presented by Rodewald and Karnovsky (Figure 1) (16). First, our studies have demonstrated that nephrin is specifically located at the slit diaphragm (41). Second, nephrin must be crucial for the structural integrity of the slit diaphragm, because the absence of the protein or different amino acid substitutions cause nephrosis and lack of the slit diaphragm with massive proteinuria as a result (36,38). Third, nephrin molecules extending toward each other from two adjacent foot processes are likely to interact in the slit through homophilic interactions, as has been shown for other Ig cell adhesion molecules, such as N-CAM (42), C-CAM (43), and L1 (44). Fourth, such homophilic assembly of nephrin molecules in the slit could have a zipper-like arrangement, essentially like that proposed based on electron microscopic studies (Figure 1). Our hypothesis on the head-to-head assembly of nephrin in the slit diaphragm through homophilic interactions and covalent cross-linking as depicted in Figure 4 has been reported recently (41).

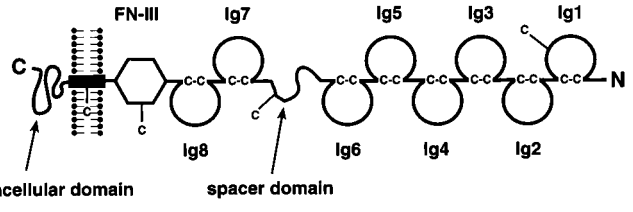

Figure 2. Schematic domain structure of human nephrin. The amino terminus (N) is located in the extracellular space, and the carboxy terminus (C) is intracellular. The Ig repeats are depicted as incomplete circles connected by disulfide bridges (C-C), and numbered from the N terminus. The locations of free cysteine residues are indicated by a -C.

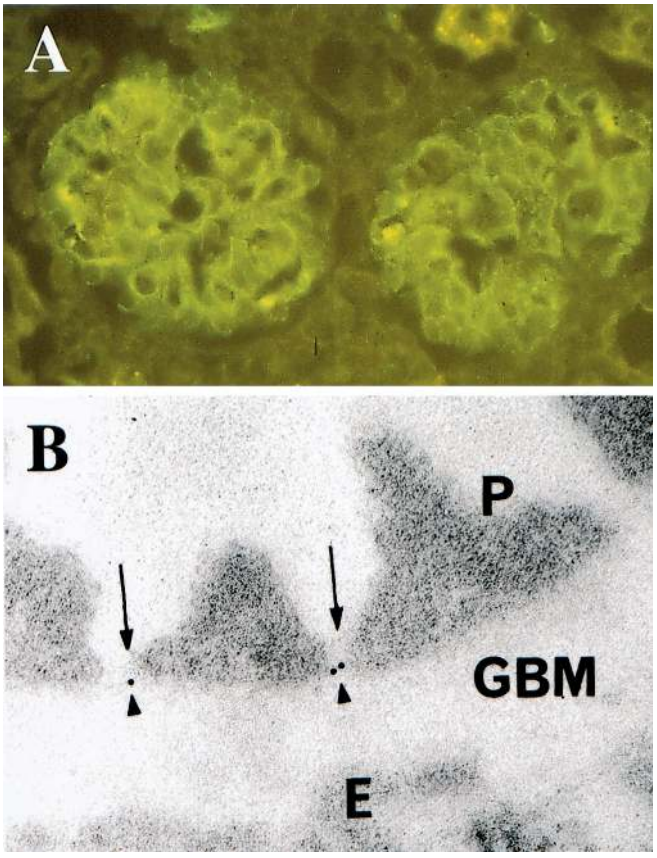

Figure 3. Immunolocalization of nephrin in human kidney. (A) Indirect immunofluorescence staining of a 2-mo-old human kidney with antibodies against the extracellular part of recombinant human nephrin shows glomerular basement membrane (GBM)-like immunoreactivity in the glomerulus. (B) Immunoelectron microscopy using affinity-purified IgG against the extracellular region of recombinant human nephrin and a 10-nm gold-coupled secondary antibody. The label is located in the central area of the slit (arrowheads) between the podocyte foot processes (P), close to the faintly visible slit diaphragm (arrows) above the glomerular basement membrane (GBM). The endothelium (E) is unlabeled. Reproduced from reference 41.

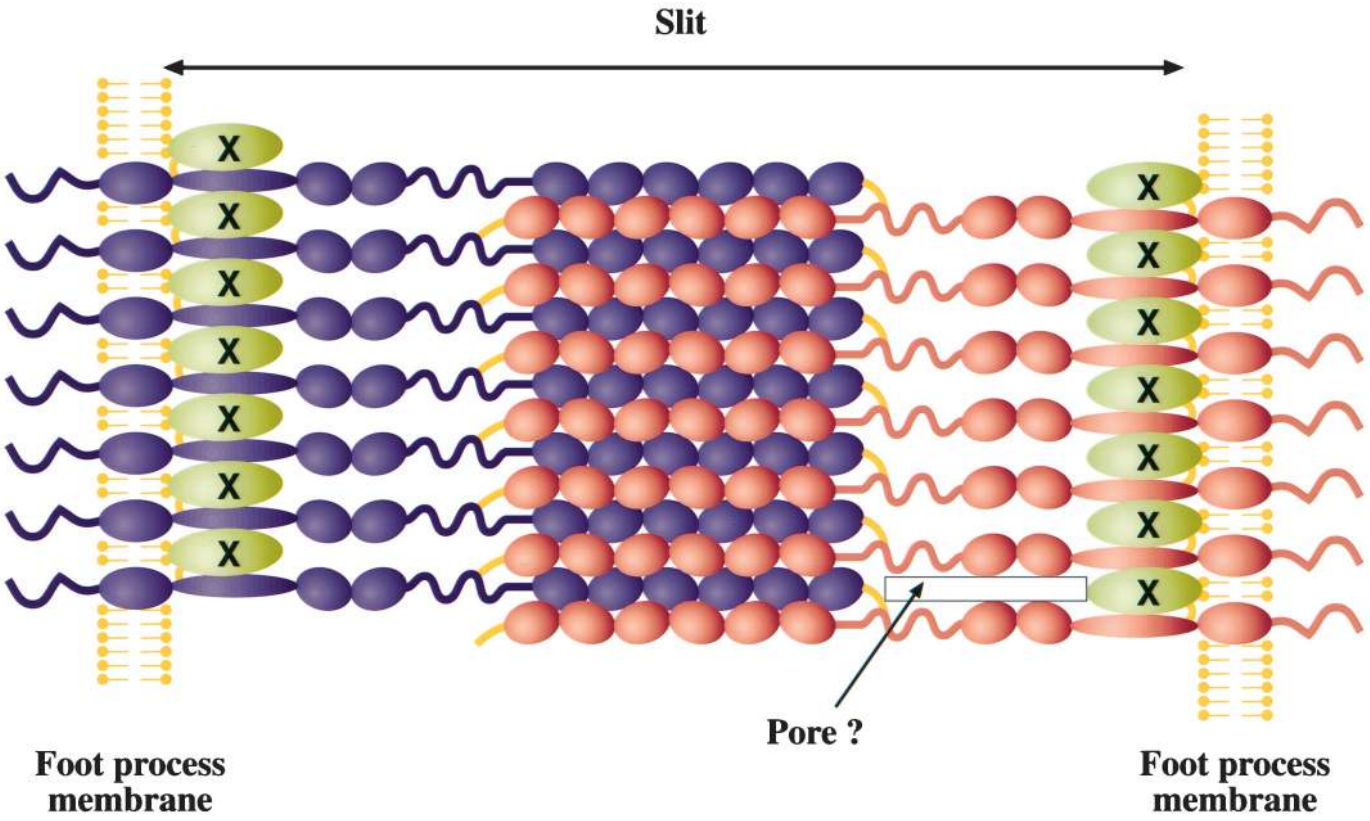

Figure 4. Hypothetical assembly of nephrin assembly into an isoporous filter of the podocyte slit diaphragm where molecules from opposite foot processes are predicted to interact with each other in the center of the slit. For clarity, nephrin molecules from opposite foot processes are shown in different colors. In this model, it is assumed that Ig repeats 1 through 6 of a nephrin molecule from one foot process associate in an interdigitating manner with Ig repeats 1 through 6 in neighboring molecules reaching out from the opposite foot process. Disulfide bonds predicted to be formed between cysteine residues in Ig repeat 1 and in the region between Ig repeats 6 and 7 are depicted by yellow lines. The free cysteine present in the fibronectin domain may interact with a neighboring nephrin molecule, or as depicted with another, as yet unknown protein (X), that may connect with the plasma membrane or cytoskeleton.

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 (Figure 2). Each Ig motif contains two cysteine residues that—similar to corresponding motifs in other proteins (40)—can be assumed to form a disulfide bridge within the repeat structure (Figure 2). Ig motifs have been shown to adopt a globular or ellipsoid structure with an average axis length between 24 and 47 Å, averaging 35 Å. 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 molecule could extend through most of the width of the 35- to 45-nm-wide slit diaphragm.

In addition to the two cysteine residues in each Ig motif, nephrin contains four free cysteines: one in Ig motif 1, one in the spacer region between Ig motifs 6 and 7, one in the fibronectin domain, and one in the transmembrane domain. The three free cysteines are likely to play a role in forming intermolecular disulfide bridges that provide strength to the slit diaphragm. These cysteines are important because their absence results in proteinuria and congenital nephrotic syndrome (38). According to our hypothesis, the free cysteine of Ig motif 1 in one molecule interacts with a cysteine residue of the spacer in another nephrin molecule (Figure 4). 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 (Figure 1). Depending on the actual size and orientation of the Ig motifs, the width of the central aggregate could be between 14.5 and 28 nm by assuming a linear chain arrangement of six Ig repeats, 24 to 47 Å each. The free cysteine in the fibronectin-like domain could form a disulfide bond with a neighboring nephrin, or with another as yet unknown protein (protein X, Figure 4) that might connect the slit diaphragm to the cytoskeleton. According to measurements made by Rodewald and Karnovsky (16), the central filament is 11 nm wide. The model presented in Figure 4 can even account for the 4×14 nm slit membrane pores proposed by Rodewald and Karnovsky. Such pores could be located between neigh-

boring nephrin molecules extending from the same foot process. The Ig motifs are compact, cross-linked motifs that probably are not stretchable. However, the spacer domain and possibly also the fibronectin domain might allow for the stretching that has been attributed to the slit membrane (17–20).

The slit membrane model presented in Figure 4 needs to be tested. It will be particularly important to search for other potential components that together with nephrin build up the slit diaphragm filter.

Future Research Questions

The identification of nephrin, both as a first and as an apparent key component of the kidney ultrafiltration barrier, opens a wide array of research opportunities that should lead to a better understanding of glomerular filtration and the pathomechanisms of proteinuria, and provide novel targets for its treatment. The list of questions to explore could be quite extensive, but some of the most apparent ones are pointed out below.

First, it will be important to have tools such as a collection of monoclonal antibodies directed against different regions of nephrin and nucleotide probes for nephrin from animal species. The monoclonal antibodies would be important for analyzing the properties of the nephrin protein itself, its functional domains, and its involvement in human diseases. Animal DNA and RNA probes would facilitate analysis of nephrin in experimental animal models, particularly for studying models of proteinuria and kidney complications in diabetic nephropathy. The availability of nephrin knockout mice could shed light on the development of the glomerular podocytes and on the pathogenesis of congenital nephrosis. Such mice would also be particularly interesting to use for so-called knock-in experiments, in which one can replace the abnormal nephrin gene with nephrin cDNA containing different mutations, to study what is needed and when for normal differentiation of the epithelial cells to develop into normal podocytes with their secondary foot processes and slit membrane. Such constructs might also be used to generate animals with a tendency to develop proteinuria, *e.g.*, in a hypertensive state.

One of the most important questions is to determine whether the slit membrane contains proteins other than nephrin. This requires a search for an extracellular ligand(s) that can participate in building up the filter, and/or proteins that connect nephrin to the plasma membrane and the cytoskeleton. Intriguing questions also relate to the potential for intracellular signaling of nephrin. This work could shed light on how the podocyte reacts to slit membrane injury, *i.e.*, proteinuria, and might provide leads toward novel targets for treatment of proteinuria. The nephrin gene is also extremely interesting by itself, because it is so restricted with regard to expression. It would be interesting to know what kind of gene regulatory elements (enhancers) drive expression so specifically to the podocyte. Enhancer(s) of the nephrin gene might be used in the future to express specific proteins in podocytes, possibly a part of glomerular disease treatment by gene therapy.

Conclusion

The cloning of the nephrin gene is an example of how studies on defects in rare genetic diseases can generate new knowledge of general importance and interest. The 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. Our model for nephrin assembly into a slit diaphragm supports the model for slit diaphragm ultrastructure presented more than two decades ago based on transmission electron microscopy. However, additional studies are needed to validate this model and examine whether other proteins contribute 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 congenital nephrotic syndrome, but also may help to clarify the pathogenic mechanisms of proteinuria in several other genetic and acquired kidney diseases that lead to proteinuria and renal failure. Considering the previously limited knowledge on the molecular structure of the slit diaphragm, the discovery of nephrin represents a significant advance that may help to completely unravel the mystery of this important extracellular structure.

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