Molecular Structure-Function Relationship in the Slit Diaphragm

By Sumant S. Chugh, Beenu Kaw, and Yashpal S. Kanwar

The past 5 years have witnessed an exponential increase in our understanding of the structure and function of the glomerular slit diaphragm. The identification of nephrin as the first transmembrane slit diaphragm protein was a watershed event in slit diaphragm biology. This article correlates some of the observations of the prenephrin era with more recent studies, and elaborates on the individual characteristics of each slit diaphragm protein. Recent studies on protein-protein interactions related to slit diaphragm permeability and cell signaling are elaborated, along with observations on their expression in human disease and experimental models of proteinuria. Developmental expression of components of the slit diaphragm in normal and knockout mice also is discussed. Finally, some areas of future investigation are proposed.

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The slit diaphragm (SD) is a transmembrane structure spanning a gap of approximately 40 nm between adjacent foot processes of visceral glomerular epithelial cells (or podocytes), and forms the final size-selective permeability barrier of the glomerular capillary loop (Fig 1). Some of the earliest images of the SD go back to the early 1950s. For convenience, the SD can be divided into an extracellular segment (comprising the extracellular segments of transmembrane proteins spanning the SD), an intramembrane segment (comprising portions of transmembrane- and membrane-associated proteins passing through the foot process cell membrane), and an intracellular segment (comprising cytoplasmic tails of transmembrane proteins, and of SD proteins and portions of membrane-associated proteins present in the cytoplasm). Our knowledge of the SD is best divided into 2 eras based on the discovery of nephrin, the first protein known to span the extracellular segment of the SD.

One of the leading observations in SD biology in the prenephrin era was the publication of its ultrastructure by using electron microscopy (EM) on tannic acid and glutaraldehyde-fixed tissue sections by Rodewald and Karnovsky. The so-called zipper structure was suggested to comprise a midline core, with ladder-like processes stretching to adjacent foot processes that enclose the filtration pores. This zipper subsequently was shown to be displaced from its usual location in proteinuric conditions associated with foot process effacement, such as puromycin aminonucleoside nephrosis. The first known marker of the extracellular segment of the SD was monoclonal antibody (Mab) 5-1-6, which when injected intravenously into rats induced massive complement- and leukocyte-independent proteinuria in the absence of significant foot process effacement. Zonula occludens-1 (ZO-1) subsequently was localized to the cytoplasmic segment of the SD. Although the identity of the Mab 5-1-6 antigen p51 remained elusive in the prenephrin era, an interaction, direct or indirect, with ZO-1 was suggested by a reduction of ZO-1 protein in the podocytes of Mab 5-1-6–injected proteinuric rats.

The current era of SD biology starts with the positional cloning of NPHS1, the gene that encodes nephrin, and the demonstration that the majority of children with congenital nephrotic syndrome (CNS) of the Finnish type have mutations in this gene. Based on the size and structure of nephrin, it then was proposed that the SD is formed by the interaction of nephrin molecules from adjacent foot processes. The discovery of NPHS1 was followed by a cascade of novel gene discovery. Shortly thereafter, the monoclonal antibody (Mab) 5-1-6 antigen was identified to be nephrin, and CD2-associated protein (CD2AP) was localized to the cytoplasmic aspect of the SD. Although the identity of the Mab 5-1-6 antigen p51 remained elusive in the prenephrin era, an interaction, direct or indirect, with ZO-1 was suggested by a reduction of ZO-1 protein in the podocytes of Mab 5-1-6–injected proteinuric rats.

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with autosomal-recessive steroid-resistant nephrotic syndrome. Antignac and colleagues discovered mutations in NPHS2, a novel gene that encodes for podocin, a critical component of the cytoplasmic aspect of the SD.\(^{18}\) Inoue et al\(^{19}\) showed the presence of FAT, a cadherin superfamily member, in the SD. By using high-throughput mutagenesis in mouse embryonic stem cells, Donoviel et al\(^{20}\) mutated a novel gene neph1, and showed a nephrotic phenotype in neph1\(^{-/-}\) mice. Nephrin now has been characterized extensively by us\(^{21}\) and others,\(^{22-25}\) and encodes for a transmembrane protein present in the SD.

**INDIVIDUAL PROTEINS IN THE SD**

**Nephrin**

Human nephrin is a 1241 amino acid product of the NPHS1 gene, a member of the immunoglobulin (Ig) superfamily organized into an extracellular segment with 8 Ig-like domains and a fibronectin domain, a transmembrane region, and a cytoplasmic segment with 9 tyrosine residues at potential phosphorylation sites.\(^{26-29}\) It is expressed in the kidney, brain, and pancreas, and expression in the kidney is podocyte specific.\(^{30}\) Humans and rodents also express a splice variant that excludes the transmembrane region.\(^{28}\) The extracellular segments from adjacent foot processes span the SD, interacting with the extracellular segments of neph1 and with other nephrin molecules as well.\(^{21,24,25}\) The cytoplasmic tail interacts with CD2AP,\(^{14,15}\) podocin,\(^{31,32}\) and the actin cytoskeleton via CD2AP.\(^{33}\) A variety of mutations have been noted in patients with CNS of the Finnish type.\(^{11,34-38}\) Some mutations, including the 2 common Fin major (2 bp deletion in exon 2) and Fin minor (nonsense mutation in exon 26), result in truncated proteins, whereas several missense mutations result in misfolding and defective intracellular transport with a consequent lack of the mutant fully formed protein in the podocyte plasma membrane.\(^{39,40}\) Nephrin is glycosylated heavily, and migrates at 185 kd on reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In some species (eg, the rat), a double band of nephrin is seen, of which the upper band likely represents the SD fraction and the lower band likely represents the endoplasmic reticulum fraction.\(^{41}\)

**Podocin**

Human podocin is a 383 amino acid product of the NPHS2 gene, a member of the band-7-stomatin family.\(^{18}\) Podocin has a membrane-associated hairpin-like structure, with a transmembrane region between a short N-terminal domain and a longer C-terminal domain, both of which are cytosolic.\(^{31,32,42}\) Podocin molecules associate with each other to form dimers and homo-oligomers, and its C-terminal domain interacts with nephrin.\(^{31,32}\)
Mutations in the NPHS2 gene have been implicated in childhood-onset autosomal-recessive steroid-resistant focal and segmental glomerulosclerosis (FSGS)\(^{18,38}\) sporadic steroid-resistant FSGS\(^ {43-46}\) and late-onset FSGS.\(^ {47,48}\) At least some podocin mutations result in the formation of an improperly folded protein that stays in the endoplasmic reticulum and does not reach the SD.\(^ {49}\) Because NPHS2 is expressed exclusively in podocytes, its promoter has been used to generate transgenic mice that can be used for podocyte-specific expression of candidate genes in the future.\(^ {50,51}\) Podocin migrates at 42 kd on reducing SDS-PAGE.

**CD2AP**

Human CD2AP is a 639 amino acid adaptor protein with 3 SH3 domains initially discovered in association with CD2 in lymphocytes.\(^ {52}\) In the podocyte, CD2AP binds to the cytoplasmic tail of nephrin\(^ {14,15}\) and also interacts directly with the actin cytoskeleton.\(^ {33}\) CD2AP\(^ {−/−}\) mice develop proteinuria at 2 to 3 weeks of age, along with foot process effacement, followed by extensive mesangial deposits, worsening of nephrotic syndrome, and death from kidney failure at 6 to 7 weeks of age.\(^ {15}\) CD2AP\(^ {+/−}\) mice, on the other hand, develop lesions suggestive of FSGS at 9 months of age.\(^ {53}\) Mutations in CD2AP as a cause of FSGS have been described recently.\(^ {53}\) In the kidney, CD2AP is expressed in podocytes, proximal and distal tubules, and the collecting duct.\(^ {54}\) Murine CD2AP migrates at 80 kd on reducing SDS-PAGE.

**ZO-1**

Human ZO-1 is a 1736 amino acid peripheral membrane protein,\(^ {55,56}\) and is a member of the MAGuK protein family.\(^ {56,57}\) It exists as 2 isoforms, \(α^+\) and \(α^−\), depending on the presence or absence of an 80 amino acid internal domain.\(^ {58}\) Similar to other members of its family, it has 3 PDZ domains, an SH3 domain, and a guanylate kinase domain.\(^ {56,57}\) and interacts with the SD complex via the PDZ motif at the tip of the cytoplasmic tail of nephrin.\(^ {21,23}\) ZO-1 is present in tubular epithelial cells, glomerular epithelial cells, and endothelial cell junctions in the kidneys. The expression of the \(α^−\) variant, however, is restricted to the SD, glomerular endothelial, and peritubular capillary endothelial cell junctions.\(^ {59}\) ZO-1 \(α^−\) migrates at 225 kd on reducing SDS-PAGE.

**FAT1**

The kidney expression of FAT1 (previously known as FAT), a giant protocadherin with a size of approximately 500 kd, was detected initially in glomerular messenger RNA (mRNA) by reverse-transcription polymerase chain reaction (RT-PCR), and subsequently was localized to the SD by immunogold EM.\(^ {19}\) The extracellular segment contains 34 cadherin repeats along with 5 epidermal growth factor (EGF) and 2 laminin–A-G domains, and the cytoplasmic segment contains 2 potential \(β\) catenin binding regions, although these only weakly are conserved with classic cadherins.\(^ {60}\) It has been suggested that FAT1 acts both as an adhesion as well as a spacer molecule in the SD, and helps to maintain an extracellular gap wider than normally found in adhesion junctions.\(^ {61}\) FAT1\(^ {−/−}\) mice are born without SD and with effaced foot processes, and die within 48 hours after birth, suggesting a critical role for FAT1 in normal SD development.\(^ {61}\)

**P Cadherin**

Human P Cadherin is a 829 amino acid transmembrane protein with multiple cadherin domains in the extracellular segment, initially noted to be a component of the developing podocyte\(^ {16}\) and subsequently localized to the SD.\(^ {37}\) It has been proposed that adjacent P cadherin molecules interact
with each other via their cadherin domains. Unlike nephrin, Neph1, CD2AP, and FAT1, P cadherin–deficient mice do not appear to have a significant renal phenotype, suggesting that P cadherin may not be involved critically in the development of a normal glomerular capillary loop. Another recent study was unable to show an in vivo or in vitro association of P cadherin with nephrin or neph1. Members of the catenin family have been shown by immunofluorescence (IF) to be present in the glomerular capillary loop distribution, and are presumed to bind the cytoplasmic segment of P cadherin.

In addition to the physical components of the SD described earlier, at least 2 transcriptional factors significantly influence the expression of some of the SD proteins. Three studies on newborn Lmx1b−/− mice show reduced expression of podocin, CD2AP, and perhaps nephrin as well, along with the presence of Lmx1b-binding sites in these genes in sequences flanking exon 1. This suggests that Lmx1b influences the expression of podocin, CD2AP, and perhaps nephrin in the SD. In another study, partial rescue of WT1 expression in Wt1 null mice using 2 copies of a human transgene in a 430-kb YAC construct revealed significantly reduced expression of nephrin by real-time PCR when compared with wild-type controls. Although this study does not address issues of WT1 binding directly to NPHS1 regulatory elements, it does suggest that NPHS1 expression occurs downstream of WT1.

DEVELOPMENTAL EXPRESSION OF SD PROTEINS

Most of our knowledge of the developmental expression of SD proteins comes from animal studies. In the mouse (gestation period, 22 d.), development of the definitive kidney begins on embryonic day 11 and continues for 2 to 3 weeks after birth. After the formation of the renal vesicle by condensation of the metanephric mesenchyme around the tip of the ureteric bud, the developing glomerulus passes through 4 stages: the comma-shaped body, the S-shaped body, the capillary loop stage, and the mature glomerulus. The expression of nephrin in the developing glomerulus has been looked at in 2 studies by immunogold or immunoperoxidase EM, whereas the expression of neph1, podocin, CD2AP, ZO-1, and P cadherin have been evaluated by conventional IF or immunoperoxidase staining. Although traces of immunostaining are noted in the S-shaped body in future podocytes, nephrin makes its distinctive appearance in the early capillary loop stage on or near intercellular junctions between forming foot processes. No immunogold particles were noted in the lateral or apical surfaces of podocytes above these regions or in the basal surfaces facing the underlying glomerular basement membrane. CD2AP also makes its appearance during the capillary loop stage, and in the podocyte tends to colocalize with nephrin, although some staining was noted elsewhere as well.

The extracellular segments of nephrin, neph1, FAT1, P cadherin, and perhaps several other as yet unidentified proteins form the core of the SD. Recent studies on the interactions among some of these proteins help us understand their molecular organization within the SD. There is now conclu-
sive evidence using both native rodent glomerular nephr1 as well as the recombinant protein to show that nephr1 molecules form dimers and multimers via all of their Ig-like domains and the intracellular segments within the SD. At least 3 studies suggest that nephr1 and nephrin heterodimers exist in the SD and actually may be the dominant protein interaction governing SD permeability characteristics. This heterologous interaction is specific for these 2 proteins and is not shared by either of these proteins with P-cadherin. The interaction of nephr1 with nephrin possibly is influenced by, but is not entirely dependent on, glycosylation because bacterially expressed murine nephr1 extracellular segments incubated overnight with native nephrin extracted from rodent glomeruli appear to associate with each other in co-immunoprecipitation studies.

Two studies looking at recombinant nephrin protein interactions also strongly suggest the existence of nephrin dimers within the SD mediated via the extracellular segments and were preceded by velocity gradient ultracentrifugation studies showing high molecular weight complexes containing nephrin (see later). Whether the homodimeric and heterodimeric interactions of nephrin and nephr1 occur among molecules of the same foot process (ie, cis), or adjacent foot process (ie, trans), or both, cannot be concluded with any certainty at present (Fig 2). Although one study suggested the nephr1-nephrin interaction at the level of the extracellular segments only (thereby favoring a predominantly trans interaction), another study showed an interaction between the intracellular segments of these proteins as well (thereby favoring a predominantly cis interaction). Whether one or both of these arrangements dominates may need to be answered in the future by using advanced imaging techniques currently under development. Protein interactions of FAT1 have not as yet been investigated.

Multiple studies over the past 4 years have helped to clarify the protein interactions at the cytoplasmic aspect of the SD. The cytoplasmic segment of nephrin binds to the C-terminal region of CD2AP, the C-terminal region of podocin, and the SH3 domain of the Src family kinase Fyn. The cytoplasmic segment of nephr1 and not FAT1 binds to the first PDZ domain of ZO-1. Also, the C-terminal region of podocin binds with CD2AP and nephr1. The latter interaction appears to be regulated by an as-yet unidentified protein kinase that recognizes a PxxxY motif in nephr1 common to neph family members and phosphorylates tyrosine 637 (mouse nephr1). In addition, the C-terminal region of CD2AP binds directly with filamentous actin, thereby linking the SD complex with the actin cytoskeleton.

There also is now evidence suggestive of raft formation in the podocyte cell membrane at the site of, or in the vicinity of, the SD. When rodent glomerular lysates are analyzed by sucrose density ultracentrifugation or Optiprep flotation gradients (Nycomed Pharma, Oslo, Norway), a fraction of the nephrin and CD2AP, and most of the podocin appear to be present on detergent-resistant membranes that also contain raft markers. When analyzed by velocity gradient ultracentrifugation, nephrin and podocin, but not CD2AP, individually appear to exist as monomers as well as heavier complexes suggestive of oligomer formation. There are 2 opposing viewpoints at to whether the SD is raft associated. The presence of most of the podocin in the detergent-resistant membranes fraction on sucrose density ultracentrifugation suggests that it clusters other SD proteins on cholesterol-rich lipid rafts. However, previous work by Orci et al using filipin labeling and freeze-fracture EM showed an abrupt decrease in the cholesterol content of the podocyte plasma membrane at the level of the SD, with the apical membrane having a high level and the basal membrane being relatively depleted of cholesterol, suggesting the presence of rafts in the vicinity, but not at the actual site of the SD.

**CELL SIGNALING IN THE SD**

Before discussing the developments in cell signaling in the SD over the past 4 years, it is important to acknowledge the observations of 2 specific studies from the prenephrin era. A study on protamine sulfate-treated rats showed a dramatic increase in phosphotyrosine staining in the podocyte specifically at the cell-cell and cell-matrix interface. Of the 3 tyrosine-phosphorylated glomerular proteins noted exclusively in the protamine sulfate group, the 225-kd band was identified as ZO-1, whereas a 180-kd and a 100-kd band remained unidentified. Based on our current knowledge of SD protein size, interaction, and phosphorylation, it is tempting to speculate that the 180-kd
and 100-kd phosphorylated proteins may have been nephrin and neph1, respectively. A biopsy study using quantitative IF on biopsy specimens of patients with minimal change disease (MCD) and early membranous glomerulonephritis for the first time showed increased glomerular phosphotyrosine in human glomerular disease, but was unable to pinpoint the specific proteins involved.73

The cytoplasmic segments of nephrin and neph1 have been the focus of investigation into cell signaling pathways involved in SD function. Both proteins appear to be signaling molecules that can activate mitogen-activated protein kinase cascades. Tyr 1176, Tyr 1193, and Tyr 1210 in nephrin are predicted to serve as potential docking sites for SH2 domain–containing adaptor and signaling molecules. Assays for the AP-1 transcriptional factor activity during cotransfection studies of the nephrin cytoplasmic segment with dominant-negative mutants of protein kinases and small G-proteins in HEK 293T cells reveal that nephrin activates stress-activated p38 protein kinase as well as c-Jun N-terminal protein kinase JNK.32 In cotransfection studies of C-terminal nephrin with either podocin or CD2AP, nephrin-induced AP-1 activation is enhanced greatly by podocin and not...
CD2AP. The same group also showed the direct interaction of the p85 regulatory subunit of phosphoinositide 3-OH kinase (PI3K) with nephrin and CD2AP. Although podocin does not interact directly with PI3K, both podocin and CD2AP strongly stimulate nephrin-induced PI3K-dependent AKT signaling in podocytes.74 This leads to the phosphorylation of several podocyte proteins, thereby creating a putative 14-3-3 binding site. One of the phosphorylated proteins was Bad, a proapoptotic Bcl-2 family member, that after phosphorylation dissociates from Bcl2 and associates with 14-3-3, thereby promoting cell survival at a postmitochondrial level. This suggests that the nephrin-CD2AP-podocin complex triggers anti-apoptotic signaling in the podocyte.

Another recent study showed that the Src family kinase Fyn binds to the cytoplasmic tail of nephrin via its SH3 domain, and phosphorylates nephrin.69 Fyn−/− mice develop coarsening of foot processes with partial effacement by age 4 weeks and have marked attenuation of nephrin phosphorylation. In contrast, Yes−/− (another Src family kinase) mice have normal foot processes and increased nephrin phosphorylation. Fyn−/− Yes−/− mice have reduced nephrin phosphorylation comparable with Fyn−/− mice, but at 4 weeks of age developed a more severe phenotype than Fyn−/− mice, with extensive foot process effacement, endothelial swelling, expansion of the subendothelial space, and focal accumulation of platelets and fibrin. This indicates that Yes may have a role in the regulation of nephrin phosphorylation, but the mechanisms have yet to be clarified. Antibody-induced clustering of cell surface nephrin in stably transfected HEK293 cells also induces phosphorylation of nephrin and another as yet unidentified protein via Src family kinase members.75

The presence of a Grb2 SH2 binding site in the cytoplasmic tail of neph1 suggests that neph1 can activate signaling cascades. Studies conducted in transiently transfected HEK 293T cells with the cytoplasmic segment of Neph1 suggest that, similar to nephrin, neph1 induces activation of AP-1, although the effect is less dramatic with neph1.22 Itk substantially increased neph1-induced AP-1 activation, suggesting that Itk or other members of the Tec family present in the podocyte are required for efficient downstream signaling of Neph1. Unlike with nephrin, the association of podocin with neph1 causes only a minor increase in AP-1 activation.22 In contrast, the association of ZO-1 with neph1 enhances tyrosine phosphorylation of the cytoplasmic tail of neph1 and augments neph1-induced AP-1 activation.23

FUNCTIONAL STUDIES OF THE SD

Knockout mouse models for SD-related genes help us understand the role of each gene in the development and function of the SD. Several groups have generated nephrin−/− mice either by insertion of a targeting vector into exon 130,66 or insertion of a gene-trap vector in exon 8.76 Nephrin−/− mice have extensive foot process effacement, absent SD, and severe proteinuria at birth, and most die within 24 to 48 hours, indicating that nephrin expression is critical to the development of a normal SD. In contrast, nephrin+/− mice are not proteinuric and are morphologically similar to +/+ mice,30,66 except for focal foot process effacement in trap mice.76 Interestingly, the protein expression of CD2AP, ZO-1, P cadherin, and FAT1 is normal in nephrin−/− glomeruli,66 indicating that the lack of nephrin does not influence the expression of these proteins in the developing SD.

CD2AP−/− mice develop extensive foot process effacement by age 1 week, proteinuria by age 2 weeks, followed by extensive mesangial deposits and sclerosed glomeruli by age 4 weeks, and die by age 6 to 7 weeks.15 In CD2AP−/− mice, nephrin expression is indistinguishable from control +/+ mice until adult life, when reduced nephrin expression develops secondary to extensive renal disease.54 This indicates that CD2AP expression does not influence the expression of nephrin during SD development. Studies on the expression of other SD proteins in neph1, FAT1, and P cadherin−deficient mice are pending.

One recent study specifically addresses functional aspects of protein-protein interactions in SD in adult rodents.21 This study showed as well as disrupted the association between neph1 and nephrin by intravenously injecting combinations of antibodies against the extracellular segments of these proteins in small doses to induce subtle SD dysfunction (ie, proteinuria) in rodents. Increasing doses of these antibodies initially were injected separately into rats until the highest subnephriogenic dose (ie, beyond which rodents developed mild proteinuria) was identified. This was followed by the injection of a combination of 50% of the
highest subnephritogenic dose for anti-neph1 and anti-nephrin, or 100% of the highest subnephritogenic dose of individual antibodies or preimmune serum into different groups of rats. An SD leak in the form of mild proteinuria was noted in the combination group within 24 hours of injection. Proteinuria was complement and leukocyte independent, associated with intact foot processes, and decreased over the next 2 days. Mild reduction in the podocyte nephrin and neph1 protein content, and dramatic reduction in podocyte ZO-1 protein content in the absence of changes in mRNA expression of nephrin, neph1, and ZO-1 at 24 hours also were noted in the proteinuric group. The results of this study suggest that of 3 protein interactions identified in the extracellular segment of the SD (ie, neph1-neph1, nephrin-nephrin, and neph1-nephrin), the most important interaction in the pathogenesis of a SD leak may be the neph1-nephrin interaction. The dramatic reduction in podocyte ZO-1 content in this novel model may be a marker of SD-related proteinuria because it represents disruption of the nephrin-neph1-ZO-1 macromolecular complex, and is reminiscent of a similar phenomenon noted in proteinuric rats injected with Mab 5-1-6 (now redefined as an antinephrin antibody).10,13 Interestingly, the proteinuria in the Mab 5-1-6 model is massive and occurs in the absence of significant foot process effacement, and is associated with a significant reduction in the amount of nephrin in the SD.77 The impact of the disruption of this complex on other SD proteins is pending further investigation.

**SD PROTEINS IN ACQUIRED HUMAN GLOMERULAR DISEASE**

Because nephrin was the first transmembrane protein to be identified on the SD, most studies on human glomerular disease focus on the expression and distribution of this protein and/or its mRNA in primary and secondary glomerular disorders. Three human kidney biopsy studies have assessed nephrin mRNA expression in MCD, FSGS, and membranous nephropathy (MN) either by RT-PCR or by in situ hybridization (ISH). The first study78 found reduced nephrin mRNA expression in single glomeruli plucked from kidney biopsy specimens of patients with MCD. Even though glyceraldehyde-3-phosphate-dehydrogenase mRNA expression was used to normalize mRNA expression in this study, the interpretation of the results of this study is complicated by the fact that RT-PCR is at best semiquantitative, and that only 3 patients were studied. The second study79 was in children, and using ISH found no change in nephrin mRNA expression in MCD, FSGS, and MN, whereas the third study80 used both ISH and RT-PCR, and found no change in MCD but found a significant reduction in FSGS and MN. Five biopsy studies, including the second and third mRNA studies mentioned earlier, have studied nephrin protein expression by immunostaining. Protein expression in the 2 mRNA studies79,80 ran parallel with the results of mRNA expression. A study by Doublier et al81 showed reduced nephrin expression in MCD, MN, FSGS, and documented shedding of nephrin from the surface of cultured human GECs. The study by Wang et al82 showed reduced nephrin expression in MN, membranoproliferative glomerulonephritis, systemic lupus erythematosus, and cryoglobulinemic GN, and another study by Wernerson et al83 showed reduced nephrin expression in MCD with redistribution from the SD to the podocyte cytoplasm. A change from the normal interrupted linear staining pattern of nephrin by IF to a granular pattern has been reported in several studies of patients with MCD. The protein expression of ZO-1 in MCD was studied in the prenephrin era and was found to be unchanged.73 This is complemented by the fact that ZO-1 expression remains unchanged in animal models of human glomerular disease that do not primarily target the SD.10,72,84,85 Overall, the results of these studies in human glomerular disease are quite mixed, and may be related to differences in the patient population and disease duration in various studies. It is more likely that nephrin-protein/mRNA expression is altered as an effect of the disease because no clear-cut pattern pointing toward causality has as yet been noted.

Two studies have looked at the expression of nephrin in kidney biopsy specimens of diabetic patients. One study looked at nephrin mRNA expression by ISH in 14 patients with type 2 diabetes randomized to receive an angiotensin-converting enzyme inhibitor or placebo for 2 years.86 Control human tissue sections were obtained from normal renal cortex after nephrectomy for malignancy. In this study, placebo-treated patients had significantly lower nephrin mRNA expression (P = .0003) compared with normal controls, whereas angiotensin-converting enzyme inhibitor–treated
patients had levels similar to control. Also, an inverse correlation was noted between the magnitude of nephrin gene expression and the degree of proteinuria in both placebo and treated groups. Therefore, the modulation of nephrin expression appeared to correlate with the extent of proteinuria in diabetic nephropathy. In another study on both type 1 and type 2 diabetics with either nephrotic syndrome or microalbuminuria, significant reduction in nephrin protein by IF was noted in both groups. The investigators also studied the effects of glycated albumin and angiotensin II on nephrin expression in cultured human GECs, and noted that the former reduced nephrin expression by engaging the receptor for advanced glycation end products, whereas the latter caused cytoskeletal redistribution and shedding of nephrin from the cell surface. There are also several interesting studies of the effect of diabetes on nephrin expression in diabetic rat models.88,89

Some interesting phenomena have been observed recently in patients with mutations in SD genes who develop recurrent disease after transplantation. A group in Finland studied the genetics, serologic, and kidney biopsy specimen characteristics of 15 episodes of recurrent nephrotic syndrome in 13 of 51 grafts (incidence of 25%) transplanted to 45 children with CNS of the Finnish type. All 9 patients with recurrence had a Fin-major/Fin-major genotype, which lead to the absence of nephrin in the native kidney. Thirteen of these episodes were treated with cyclophosphamide, of which only 7 were successful. Antiglomerular antibodies were noted in 8 patients, and 4 patients had high titer of antinephrin antibodies. Foot processes were effaced on EM in some of these patients, along with reduced nephrin mRNA expression and granular nephrin staining. This study suggested that circulating antinephrin antibodies may be involved in the pathogenesis of recurrent posttransplant nephrotic syndrome in some patients with CNS. Another group from Italy has shown increased glomerular permeability activity (Palb) in 5 patients with mutations in NPHS2.91 Four of these patients received a renal transplant, with recurrence of proteinuria responsive to plasmapheresis and cyclophosphamide in 2 patients. A decrease in Palb with plasmapheresis coincided with reduction in proteinuria in these patients. Also, co-incubation of serum from all 5 patients with homologous urine, but not control urine, abolished Palb, indicating that an unknown inhibitor of permeability in these patients was lost in the urine.

A retrospective analysis of renal pathology and NPHS1 genotype of fetuses from 21 terminated pregnancies shows that increased maternal serum and amniotic fluid levels of α-fetoprotein, used as a screening tool for this disease, did not distinguish between fetuses that were homozygous or heterozygous for NPHS1 mutations.92 Although adults heterozygous for NPHS1 mutations do not have renal problems, the fetuses do have microscopic changes suggestive of congenital nephrosis, and consequently have proteinuria in utero leading to increased levels of α-fetoprotein. This study strongly suggested that mutational analysis of the NPHS1 gene should be used to confirm the α-fetoprotein results in the prenatal diagnosis of CNS of the Finnish type. The study investigators also suggested that shortage of nephrin in heterozygous fetuses caused temporary dysfunction of the developing SD, and once glomerulogenesis was complete, the amount of nephrin synthesis no longer was critical.

SUMMARY AND FUTURE DIRECTIONS

Despite over 5 decades of research, our knowledge of the SD remains in its infancy. We are just starting to understand the structure-function relationship among some SD proteins. In the extracellular segment, the critical contributions of nephrin, neph1, and FAT1 to the development of a normal SD are quite clear. In adult human kidney disease, only nephrin expression has been investigated so far in some detail, and as yet changes in nephrin expression cannot be implicated as a causative factor in any acquired primary glomerular disease, except perhaps selected cases of recurrent nephrotic syndrome in posttransplant CNS patients. This tends to suggest that other proteins or groups of proteins may be more important in the pathogenesis of these disorders. Similar to NPHS1, neph1 and FAT1 genes also are interesting candidates for mutational analysis in families with heritable proteinuric glomerular disorders. Whether any of these proteins has a dominant role in pathogenesis of various forms of adult glomerular disease remains to be seen. Our knowledge of animal models suggests that an important protein interaction in the extracellular segment controlling SD permeability is the nephrin-neph1 interaction.

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However, it is quite likely that nephi-nephi and nephrin-nephrin interactions also play a major role, in addition to putative interactions of FAT1 and P cadherin with other as yet undiscovered SD proteins.

In the intracellular segment of the SD, a cluster of proteins interacting with the intracellular segments of nephrin and nephi forms the critical interface between the SD and the rest of the podocyte. CD2AP binds nephrin and ZO-1 binds nephi, whereas podocin monomers and homo-oligomers link nephrin-CD2AP with nephi. Fyn interacts with this protein complex via nephrin, and the actin cytoskeleton via CD2AP. Interactions among proteins within this complex activate AP-1 transcriptional factor activity and PI3K-dependent AKT signaling. Protein interactions at the intracellular aspects of FAT1 and P cadherin require further studies.

There are now at least 2 animal models of direct SD injury that can induce proteinuria in the absence of foot process effacement, one targeting nephrin (resulting in massive proteinuria) and the other targeting both nephrin and nephi (subtle injury, mild proteinuria). We do not as yet know if more severe injury in the nephrin-nephi model would preserve or efface foot processes. The concept of proteinuria related primarily to a SD leak is likely to develop further over the next few years, since many patients with less than 1.5 to 2.0 g of proteinuria/albuminuria have morphologically preserved foot processes on kidney biopsy examination. Investigation of this phenomenon would have to focus initially on this population, or on animal models, and reduction in podocyte ZO-1 protein expression potentially could be used as a marker of SD injury. This would have to be complemented with the development of high-resolution imaging techniques that allow actual visualization of the SD pores. Finally, we need to combine the experience of the past with innovative technology to develop an in vitro model of the SD, which will help simplify our understanding of this complex structure.

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