Nephrin Dissociates from Actin, and Its Expression Is Reduced in Early Experimental Membranous Nephropathy

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Abstract. These studies examined the expression of the podocyte slit diaphragm protein nephrin and its association with actin at the onset of proteinuria in passive Heymann nephritis (PHN), a rat model of human membranous nephropathy. Four days after immunization, 58% of PHN rats had mild proteinuria. At that time, most slit diaphragms were still visible on electron microscopy; however, in those locations where the deposits encroached on the filtration slits, the slit diaphragms were either displaced or absent. On day 7, the PHN rats were severely proteinuric, and most slit diaphragms were either absent, displaced, or replaced by occluding-type junctions. Immunofluorescence microscopy with antibodies to the extracellular and cytoplasmic domains of nephrin showed a progressive loss of staining and a change in the distribution of nephrin from an interrupted linear pattern in normal controls to a more dispersed and clustered pattern in PHN. In contrast, the intensity of staining for ZO-1 and CD2-associated protein (CD2AP), two other proteins that are located on the cytoplasmic face of the slit diaphragm, was undiminished. Immunogold electron microscopy confirmed the progressive disappearance of nephrin from podocyte foot processes and retention of CD2AP. Glomeruli and glomerular cell membranes were extracted sequentially with Triton X-100, followed by DNase I or potassium iodide to depolymerize actin. Western blot analysis of the extracts showed a progressive decline of total nephrin on days 4 and 7 of PHN as well as a reduction in the actin-associated fraction. These findings show that nephrin partly dissociates from actin at the onset of podocyte injury in PHN. This is accompanied by a progressive loss of nephrin from the podocyte foot processes and prominent changes in the morphology of the slit diaphragms. These events may underlie the loss of podocyte barrier function in membranous nephropathy.

Membranous nephropathy (MN) is an autoimmune disease characterized by subepithelial immune deposits, effacement of podocyte foot processes, and expansion of the glomerular basement membrane. The primary clinical manifestation is proteinuria, which is often associated with nephrotic syndrome. Although the target antigen/s responsible for human MN are unknown, it is likely that they reside on the basal plasma membrane of the podocyte foot processes, as in Heymann nephritis, a rat model of MN (1,2). Podocyte injury and proteinuria in the rat model are mediated by complement C5b-9 (3,4), and it is likely that a similar process is at play in human MN (5).

During the development of proteinuria in passive Heymann nephritis (PHN), as well as in many cases of active MN, the actin cytoskeleton is condensed at the base of the effaced podocyte foot processes (6). In vitro studies have suggested that this is due to C5b-9–mediated disruption of actin microfilaments and dissolution of integrin-associated focal adhesion complexes (7).

Given that the podocyte foot processes and the slit diaphragms that bridge the intervening filtration slits form the final barrier to macromolecular permeability, it is not surprising that severe podocyte injury in PHN and MN would give rise to proteinuria. Yet the actual mechanisms responsible for proteinuria in these conditions remain uncertain. Possibilities include focal areas of detachment of foot processes from the glomerular basement membrane or disruption of the slit diaphragms. Although podocyte detachment during the acute phase of injury has not been entirely excluded as a cause of proteinuria, there is good reason to examine a role for the slit diaphragm more closely. Morphological alterations in the filtration slits have been well described in human and experimental MN (8–10). In addition, several investigators have examined the integrity of the slit diaphragm in acquired proteinuric diseases, using the slit diaphragm protein nephrin as a marker. Thus, it has been reported that nephrin mRNA levels are reduced in the glomeruli of patients with MN (11), and it has been recently reported that nephrin is reduced in amount and altered in distribution in patients with MN (12). In addition, Benigni et al. (13) reported that the glomeruli of rats with PHN show a progressive decline in nephrin mRNA and protein content from 1 wk to 8 mo after disease induction, which is preventable by angiotensin II blockade. At the stage at which
all these observations were made, however, the disease was well established. Therefore, it is impossible to determine whether the observed changes in nephrin were the primary cause of proteinuria or the result of advanced podocyte injury. This question is examined best at the onset of podocyte injury and proteinuria, which can be predicted precisely in the PHN model.

Nephrin is a member of the Ig superfamily of transmembrane cell adhesion molecules (14). It is the protein that is mutated in certain forms of congenital nephrotic syndrome and is known to reside in the slit diaphragm (14,15). CD2-associated protein (CD2AP) is a ubiquitous adapter that appears to link Ig superfamily membrane proteins to the actin cytoskeleton (16). It is essential for normal glomerular permeability and has been found to bind the cytoplasmic tail of nephrin when it is expressed in a heterologous cell system (17). We have also shown that nephrin is associated with the podocyte actin cytoskeleton (18). Therefore, considering that the actin cytoskeleton is disrupted by complement-mediated podocyte injury (7), we examined the glomeruli of rats with PHN to determine whether the expression of nephrin and its association with actin are altered at the onset of proteinuria.

Materials and Methods
Experimental Protocol
Sheep anti-Fx1A was prepared as described elsewhere (19). PHN was induced in 180 to 200 g adult male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) by two intravenous injections of anti-Fx1A, 0.25 and 0.5 ml, on successive days. Controls were injected with an equal amount of normal sheep globulin. Urine was collected from all rats overnight in individual metabolism cages on days 3 to 4 and 6 to 7 after the first injection and analyzed for protein by a sulfosalicylic acid method, as described elsewhere (19). On days 4 and 7, the kidneys were removed under anesthesia. A 3- to 4-mm coronal slice of cortex was embedded in Tissue-Tek O.C.T. Compound (Sakura, Torrance, CA) and snap-frozen at −80°C without prior fixation for immunofluorescence. The rest was frozen immediately in liquid nitrogen and stored at −80°C until used for glomerular isolation (vide infra). The kidneys of three additional control and PHN rats were perfusion fixed in situ with periodate-lysine-paraformaldehyde (20) on days 4 and 7 for ultrastructural studies.

Antibodies and Reagents
Rabbit antibody to the complete cytoplasmic domain of mouse nephrin (21) was a gift from Dr. Larry Holzman (University of Michigan, Ann Arbor, MI) and was used for Western blots. A rabbit anti-nephrin antibody was produced by immunization with a 21 amino acid peptide (DRD TRS STV STA EVD PNY YSC) from the extracellular domain of rat nephrin, mAb 5–1–6 (23), was kindly supplied by Drs. Hiroshi Kawachi and Fujio Shimizu (Niigata University, Niigata, Japan). Rabbit anti-CD2AP provided by Dr. Andrey Shaw (Washington University School of Medicine, St. Louis, MO) was used for Western blots, and rabbit anti-CD2AP from Santa Cruz Biotechnology, Inc. (SC9137; Santa Cruz, CA) was used for immunofluorescence. Rabbit anti–ZO-1 was from Zymed Laboratories, Inc. (South San Francisco, CA). Rabbit anti-rat actin (A2066) and secondary antibodies—goat anti-rabbit IgG—horseradish peroxidase (A8275) and fluorescein-conjugated goat anti-rabbit IgG (F0382)—were purchased from Sigma-Aldrich (St. Louis, MO). CY3-conjugated goat anti-rabbit IgG (AP132C), donkey anti-mouse IgG (AP129C), and rabbit anti-sheep IgG (AP147C) were from Chemicon (Temecula, CA). Chemicals and reagents, including DNase I (EC 3.1.21.1, from bovine pancreas, D4263) were from Sigma-Aldrich unless stated otherwise.

Isolation of Glomeruli
Glomeruli were isolated from individual rat kidneys by differential sieving (19) that used phosphate-buffered saline (PBS, 10 mM phosphate buffer [pH 7.4] and 100 mM NaCl) with a cocktail of protein inhibitors (PI) (1 mM phenylmethylsulfonyl fluoride, 5 µg/ml soybean trypsin inhibitor, 4 mM N-ethylmaleimide, and 5 mM benzamidine hydrochloride).

Detergent Extraction of Glomeruli and Depolymerization of Actin with DNase I
The isolated glomeruli from each animal were extracted on ice for 30 min with extraction buffer composed of 0.5% Triton (Tx-100) in 20 mM phosphate buffer (pH 6.2), 10 mM NaCl, and 1.5 mM MgCl2. The Tx-insoluble material was removed by centrifugation at 15,000 × g at 4°C for 10 min and then incubated with 100 µg/ml DNase I in PBS at 37°C for 30 min to depolymerize F-actin (24). The soluble proteins released by DNase I were separated from the DNase I-insoluble fraction by centrifugation at 15,000 × g at 4°C for 10 min. The protein concentration of the Tx-100 extracts was measured with the BCA protein assay (Pierce Inc., Rockford, IL). The volume of each sample was adjusted so that 30 µg of the Tx-100 extracts and equivalent volumes of the DNase I-soluble fractions were analyzed by Western blotting.

Preparation of Cell Membranes
Cell membranes were prepared by use of a modification of the method of Lockwich et al. (25). Glomeruli were pooled from three rats, and 1-ml aliquots of each pool were frozen overnight in lysis buffer (100 mM Tris-HCl [pH 8.0], 1 mM MgCl2, and PI), thawed, and homogenized on ice with a Sonifier Cell Disrupter (S250A, Branson Ultrasonics Corp., Danbury, CT) at output 6 and duty cycle 50% for 3 × 10 bursts with 10-s intervals. A 0.4-ml aliquot of each homogenate was diluted in an equal volume of 2× homogenizing buffer (0.5 M sucrose, 20 mM Tris-Hepes [pH 7.4], 2 mM dithiothreitol, and 2× PI) and centrifuged at 3000 × g for 15 min at 4°C to remove cellular debris. The supernatants were ultracentrifuged at 50,000 × g for 30 min at 4°C to separate glomerular cell membranes (pellet) from cytosolic proteins (supernatant). The cell membranes were resuspended in the same buffer, and both preparations were stored at −80°C.

Extraction of Glomerular Cell Membranes
Glomerular membranes were extracted sequentially as follows. Aliquots of 60 µl from each pool were thawed on ice, sonicated, and incubated for 30 min at 4°C with 1% Tx-100 in 88 µl of 20 mM phosphate (pH 6.2), 10 mM NaCl, 1.5 mM MgCl2, and PI. The
Transmission Electron Microscopy

Immunoelectron Microscopy

The cortex of perfusion-fixed kidneys was cut into 1-mm³ blocks, washed with PBS, dehydrated in graded ethanol (30%, 50%, and 70%), and embedded in LR White resin (catalog number 14381, Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin sections were transferred to Formvar-coated nickel grids, blocked with 1% bovine serum albumin and 1% normal goat serum in PBS, incubated with anti-nephrin (1:100) or anti-CD2AP (1:50) in DAKO antibody diluent (catalog number S3022, DAKO Corporation, Carpenteria, CA) followed by goat anti-rabbit IgG coupled to 10-nm colloidal gold (catalog number 15731-1, Ted Pella Inc., Redding, CA). The sections were postfixed with 1% glutaraldehyde, contrasted with uranyl acetate, and examined with a Philip CM10 electron microscope.

Statistical Analyses

Analysis of variance and paired t test were performed with Microsoft Excel 2001.

Results

Proteinuria, Electron Microscopy, and Immunofluorescence

Preliminary immunofluorescence studies of day 7 PHN kidneys with mAb 5–1–6 showed a remarkable redistribution of the staining pattern (Figure 1). This antibody identifies an extracellular epitope of nephrin (23) and normally decorates the slit diaphragm in an interrupted linear pattern (Figure 1). In contrast, the staining of day 7 PHN glomeruli was more dispersed and granular (Figure 1). The evolution of these changes was examined in detail in the experiments described below.

Urine protein excretion was measured on overnight collections from day 3 to 4 and from day 6 to 7 after the first dose of normal sheep globulin or anti-Fx1A in control and PHN rats, respectively. Day 4 is the earliest time point at which proteinuria is detectable and represents the onset of complement-mediated heterologous phase injury (27,28). Urine protein excretion was elevated above control values in 58% of PHN rats on day 4, and 90% had severe proteinuria on day 7 (Figure 2). Transmission electron microscopy showed many subepithelial electron-dense deposits on day 4 with effacement of podocyte foot processes. In several locations, the immune deposits encroached on the filtration slits, and in these locations the slit diaphragms were displaced or not visible (Figure 3). Immunofluorescence at this time with an antibody to the cytoplasmic domain of nephrin showed a change in the distribution of nephrin in PHN kidneys from an interrupted linear pattern, as seen in controls injected with normal sheep globulin, to a more granular appearance (Figure 4A). On day 7, electron microscopy of PHN kidneys showed extensive and severe foot process effacement overlying large subepithelial deposits, accompanied by more pronounced displacement and disruption of slit diaphragms (Figure 3). In some locations, the filtration slits were replaced by occluding-type junctions and, in others, “ladder-like” structures were present (Figure 3). Early basement membrane “spike” formation was present in some locations on day 7 (Figure 3). Immunostaining for nephrin on day 7 was even more attenuated and clustered, and the overall intensity of staining was reduced (Figure 4A). Quanti-
tative analysis revealed a significant reduction in the intensity
of nephrin fluorescence in PHN glomeruli on days 4 and 7
(Figure 4B). In contrast to nephrin, immunostaining for ZO-1
was unchanged in PHN in comparison to controls on day 4 or
7 (Figure 4A). The immunofluorescence for CD2AP was also
undiminished in PHN, but the staining appeared to be more
condensed on both days 4 and 7, whereas it was more diffusely
distributed in controls (Figure 4A). Cross-reactivity of the
CY3-conjugated anti-rabbit IgG secondary antibody with
sheep and rat IgG in the glomerular immune deposits was
excluded by the absence of staining when the primary rabbit
anti-nephrin, anti–ZO-1, or anti-CD2AP were excluded (not
shown).

Immunogold electron microscopy with an antibody to the
cytoplasmic domain of nephrin revealed gold particles on the
cytoplasmic face of podocyte foot processes in many filtration
slits of control glomeruli (Figure 5). In contrast, day 4 PHN
glomeruli contained fewer gold particles in their normal loca-
tion (Figure 5). Where present, they were associated with
preserved, albeit displaced, slit diaphragms. On day 7, how-
ever, there was an almost complete absence of gold particles
from filtration slits, including those that were devoid of visible
slit diaphragms and those that appeared occluded (Figure 5).
Gold particles were rarely, but not consistently, seen on the
apical plasma membrane and in subepithelial immune deposits
but not in lysosomes (Figure 5). In keeping with the findings
from immunofluorescence, there did not appear to be a differ-
ence between control and PHN glomeruli in the number of
CD2AP-bound gold particles in the foot processes, although in
some places they appeared to be less intimately associated
with the plasma membrane bordering filtration slits (Figure 6).

Figure 1. Representative immunofluorescent micrographs of glomeruli from control rats and those with passive Heymann nephritis (PHN) on
day 7. Cryosections were stained with mAb 5–1-6, which detects an epitope on the external domain of nephrin. Control glomeruli illustrate the
normal interrupted linear staining pattern of the slit diaphragms with this antibody. The staining of PHN glomeruli is more dispersed and
granular. Original magnification, ×400.

Figure 2. Urine protein excretion in control and PHN rats on days 4
and 7. Mild proteinuria was present in 7 of 12 PHN rats on day 4. The
differences in the mean values between the controls and PHN rats was
significant at both times (analysis of variance [ANOVA]). Values are
mean ± SEM. n = 12/group on day 4 and 8/group on day 7.
Increased Nephrin Solubility in PHN

After extraction with Tx-100 at 4°C, a substantial fraction of nephrin remains insoluble and can be released by further extraction with agents that depolymerize F-actin. This suggests that nephrin is associated with the actin cytoskeleton. In addition, CD2AP, a known adapter protein with actin-binding properties, associates with nephrin (17). To determine whether the association of nephrin with the actin cytoskeleton is altered during the development of PHN, glomeruli were isolated from the kidneys of six PHN and control rats on days 4 and 7 and extracted sequentially with 1% Tx-100 and DNase I, as described above. The Tx-100 and DNase I extracts from day 4 PHN and control and day 7 PHN and control rats were selected randomly and run on six separate gels, transferred to nitrocellulose membranes, and immunoblotted for nephrin, CD2AP, and actin. Densitometric readings of the Western blots from each membrane were compared by pairwise analysis to control for completeness of transfer and film exposure time. As shown in Figure 8, the ratio of actin-associated (DNase I–soluble) to Tx-100–soluble nephrin was 0.48 on day 4 and 0.61 on day 7 in controls (the difference between day 4 and day 7 controls was NS by paired t test) and was significantly reduced to 0.28 on day 4 and 0.30 on day 7 in PHN glomeruli. There was no significant difference in CD2AP and actin levels in control and PHN glomeruli at either time point (not shown).

In the second set of experiments, we further fractionated the glomeruli to obtain membrane-associated nephrin and cortical content of glomeruli and its association with the actin cytoskeleton. In the first, glomeruli were isolated from the kidneys of six PHN and control rats on days 4 and 7 and extracted sequentially with 1% Tx-100 and DNase I, as described above. The Tx-100 and DNase I extracts from day 4 PHN and control and day 7 PHN and control rats were selected randomly and run on six separate gels, transferred to nitrocellulose membranes, and immunoblotted for nephrin, CD2AP, and actin. Densitometric readings of the Western blots from each membrane were compared by pairwise analysis to control for completeness of transfer and film exposure time. As shown in Figure 8, the ratio of actin-associated (DNase I–soluble) to Tx-100–soluble nephrin was 0.48 on day 4 and 0.61 on day 7 in controls (the difference between day 4 and day 7 controls was NS by paired t test) and was significantly reduced to 0.28 on day 4 and 0.30 on day 7 in PHN glomeruli. There was no significant difference in CD2AP and actin levels in control and PHN glomeruli at either time point (not shown).

In the second set of experiments, we further fractionated the glomeruli to obtain membrane-associated nephrin and cortical

Figure 3. Representative transmission electron micrographs of glomeruli from control and PHN rats selected randomly on days 4 or 7. (A) Control rat glomerulus on day 4 shows normal podocyte foot process (Ep) morphology with slit diaphragms visible in most filtration slits. An arrow indicates one filtration slit in which the slit diaphragm is not visible. (B) PHN rat glomerulus on day 4 demonstrates subepithelial electron dense deposits (*) and focally effaced foot processes. In several locations, the deposits encroach on the filtration slits, and in these locations the slit diaphragms are displaced (fine arrows) or absent (bold arrows). (C) PHN glomerulus on day 7 shows growth of the immune deposits. The foot processes are distorted and more extensively effaced. Normal slit diaphragms are rarely visible, and those that are present are displaced (fine arrows). In some places, the filtration slits are narrowed and replaced by occluding-type junctions or “ladder-like” structures (bold arrows). In others, the slit diaphragms are not visible (▼). Similar observations were made in the glomeruli of two other rats from each group. Magnification, ×27,600.
Figure 4. (A) Representative immunofluorescent micrographs of glomeruli from control and PHN rats on days 4 and 7 stained for nephrin, ZO-1, and CD2-associated protein (CD2AP). Staining with an antibody to the cytoplasmic domain of nephrin shows a progressive change in the distribution of nephrin from an interrupted linear pattern in controls, to a less abundant and more dispersed granular appearance on days 4 and 7 of PHN. In contrast, the intensity of staining for ZO-1 and CD2AP is undiminished in PHN, although CD2AP appears to be more condensed on both days 4 and 7, whereas it is more diffusely distributed in controls. (B) Quantification of the intensity of fluorescent staining for nephrin shows a progressive and significant decline in PHN glomeruli on days 4 and 7 (ANOVA). Mean ± SEM. n = 5 to 7/group. Original magnification, ×400.
Figure 5. Representative immunogold electron micrographs of glomeruli from (A) control and (B) PHN day 4 and (C) PHN day 7 rats stained for the cytoplasmic domain of nephrin. Gold particles are seen labeling nephrin on the cytoplasmic face of podocyte foot processes (Ep) bordering several filtration slits (↑) in the control glomerulus. Progressively fewer gold particles are visible in PHN days 4 and 7 glomeruli, especially in those filtration slits that contain deposits (*). Magnification, ×39,700.

Figure 6. Representative immunogold electron micrographs of glomeruli from (A) control and (B) PHN day 4 and (C) PHN day 7 rats stained for CD2AP. Gold particles labeling CD2AP are seen in the foot processes of control and PHN day 4 and 7 glomeruli, despite the presence of immune deposits (*). Magnification, ×39,700.
actin. In these studies, F-actin was depolymerized with KI so that all extractions could be done at 4°C. Because the amounts of cell membrane that can be isolated from the glomeruli of individual rats is too small for analysis, we pooled the glomeruli from three rats to give three different sets of glomeruli from nine controls and nine day 4 and nine day 7 PHN rats. Four samples were analyzed from each pool as shown in Figure 9A. These included (1) the supernatant remaining after ultracentrifugation to prepare the membrane fraction, (2) the Tx-100–soluble extract of the membrane fraction, (3) the KI-soluble fraction of the Tx-insoluble membrane fraction, and (4) the KI-insoluble pellet. Because KI solubilization is carried out in the presence of 1% Tx-100, we established that Tx-100 alone did not extract any additional nephrin from the Tx-insoluble fraction. The major findings from this analysis were that total nephrin was significantly reduced in PHN glomeruli (Figure 9B) and that the amount of actin-associated (Tx + KI–soluble) nephrin in the membrane fraction was relatively decreased in PHN (Figure 9C). As expected, the amount of Tx-soluble nephrin in the membrane fraction was reciprocally increased (densitometry not shown). In addition, the residual nephrin in the Tx + KI–insoluble pellet was reduced in PHN membranes (densitometry not shown). These changes in PHN were present at 4 d and were more severe at 7 d, in keeping with the immunofluorescent findings. Immunoblots for actin on the same membranes showed that KI was effective in solubilizing actin and that the changes in nephrin could not be explained by differences in sample loading.

Discussion

These studies in PHN show that the glomerular distribution of nephrin is altered and that there is a reduction in total glomerular content of nephrin coincident with or before the onset of proteinuria on day 4. In addition, the fraction of membrane-associated nephrin that is bound to actin is diminished. These changes correspond to an alteration in podocyte morphology, including displacement and loss of slit diaphragms. The loss of slit diaphragms is accompanied in some locations by a narrowing of filtration slits and replacement with occluding-type junctions and ladder-like structures. The changes in the amount and distribution of nephrin appear to be specific and are not simply a consequence of podocyte damage.

Figure 7. Representative Western blot demonstrates the association of nephrin and CD2AP with actin in control glomeruli and reduction in the amount of actin-associated nephrin in days 4 and 7 PHN glomeruli. Glomeruli were isolated from the kidneys of days 4 and 7 control and PHN rats and extracted sequentially with 1% Tx-100 (Tx) at 4°C, followed by extraction of the Tx-insoluble fraction with DNase I, which is known to depolymerize F-actin. The Tx- and DNase I–soluble fractions were resolved by 4% to 20% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) under reducing conditions, transferred to nitrocellulose membranes, and immunoblotted sequentially with rabbit antibodies to nephrin, CD2AP, and actin.

Figure 8. Quantitative Western blot analysis of control and PHN glomeruli reveals a reduction in actin-associated nephrin in PHN. (A) Glomeruli were isolated from the kidneys of individual days 4 and 7 control and PHN rats and extracted and analyzed as described in the legend to Figure 6. (B) Densitometry of the individual bands indicates that the ratio of actin-associated (DNase I–soluble) to detergent-extractable (Tx-100–soluble) nephrin is significantly reduced in whole glomeruli from days 4 and 7 PHN rats (paired t test). Mean ± SEM. n = 6/group.
Thus, the amount of CD2AP and ZO-1, two proteins located on the cytoplasmic face of the slit diaphragm, was not reduced. Furthermore, the association of CD2AP with actin was unaffected. If, indeed, CD2AP is an adapter that binds nephrin to actin in vivo, this latter finding suggests that the link between nephrin and CD2AP is disrupted in PHN and that CD2AP may condense with actin at the base of the effaced podocytes. Although this could explain the reduced association of nephrin with actin, it remains possible that other as-yet-unidentified adapter proteins, or even a direct actin-nephrin link, might be perturbed in PHN.

In our analysis of whole rat glomeruli, we found that the actin-associated fraction of nephrin was substantially (and similarly) reduced on days 4 and 7 of PHN as determined by DNase I sensitivity. As seen in Figures 7 and 8, the Tx-soluble fraction of nephrin is detectable as two closely spaced bands, whereas the DNase I–soluble band in the control lanes appears to correspond to the lower band. This raises the possibility that only the smaller protein, said to be a splice variant that lacks the transmembrane-spanning domain (29), is the component that is bound to actin. To examine this further, we conducted an additional set of experiments with glomerular cell membranes and using KI to depolymerize actin (Figure 9). The use of KI instead of DNase I allowed us to do the extractions at 4°C. These studies revealed a substantial amount of nephrin in the KI-soluble (actin-associated) cell membrane fraction in control glomeruli and a progressive decline in this component on days 4 and 7 of PHN. We can only speculate that the marked reduction in actin-associated nephrin on day 7 in the cell membrane fraction (Figure 9) and the lesser reduction seen on day 7 in whole glomeruli (Figure 8) might be due to retention of an intracellular pool. It is also noteworthy that there was residual nephrin present in the KI-insoluble cell membrane pellet (Figure 9). This may be the result of incomplete depolymerization of actin by KI or represent nephrin in a detergent resistant membrane fraction—so-called lipid rafts (18,30). This fraction was also relatively depleted in the PHN glomerular cell membranes (quantification not shown).

It is noteworthy that filtration slits are often narrowed, and occluding-type junctions appear when nephrin and slit diaphragms are lost (this study and reference [31]). This loss of filtration surface area accounts for the reduction in GFR in MN (9), but it is possible that these abnormal slits are narrowed but not completely occluded. If so, bulk flow would be reduced, but substantial protein permeation could still occur. In this regard, ultrastructural tracer studies showed that the greatest permeation of macromolecules occurred in the vicinity of immune deposits in Heymann nephritis (32), and the most prominent changes in the slit diaphragms in this study were evident in filtration slits that contained electron-dense immune deposits. It is also interesting to speculate on the explanation for the narrowed filtration slits seen in this study and in nephrin...
knockout mice (31). It has been proposed that patency of the filtration slits is maintained by the repulsive negative charge generated on the surface of opposing foot processes by podocalyxin (33). It is possible that the slit diaphragm may also hold adjacent foot processes apart while forming a barrier to plasma protein leakage.

The immunohistological and immunochemical studies reported here clearly establish that the amount of podocyte nephrin is substantially diminished in PHN, but it remains unknown whether this is due to reduced synthesis, increased degradation, shedding from the cell surface, or a combination of these factors. Studies elsewhere have offered support for each of these possibilities. Thus, reduced nephrin mRNA expression has been documented in the glomeruli of patients with MN (11) and in rats with nephropathies induced by purine aminonucleoside, mAb 5–1-6, and mercuric chloride, as well as in advanced PHN (13,22,34,35). We also found that nephrin and mAb 5–1-6 undergo coordinate redistribution and that the antibody can be identified in podocyte lysosomes at the onset of proteinuria induced by mAb 5–1-6 (36), which suggests that the protein is endocytosed and degraded. Finally, it has been reported that the truncated alpha splice form of nephrin can be detected in the urine of rats with purine aminonucleoside nephrosis (35). Although we cannot exclude the possibility that suppressed nephrin synthesis may account for the reduced abundance of the protein on day 4 of PHN, the substantial decline in the fraction bound to actin suggests other possibilities. Thus, it may be that loss of anchorage to the cytoskeleton allows membrane-associated nephrin to redistribute and to be shed from the cell surface into the urinary space or to be endocytosed and targeted for degradation.

The mechanism(s) by which antibody and complement alter the distribution of nephrin and its association with actin are still unknown. Several cellular processes are activated in glomerular epithelial cells by sublethal C5b–9–mediated injury, including influx and release of calcium from intracellular stores, phospholipase activation, adenosine triphosphate depletion, and generation of free radicals, as well as disruption of actin microfilaments (7,37–40). In addition, it has recently been reported that insertion of C5b-9 into the plasma membrane of cultured human podocytes induces the shedding of nephrin with a loss of surface expression (12). These findings need to be considered in the light of recent observations that have shown that at least a fraction of cell membrane nephrin resides in lipid rafts, where it can be tyrosine phosphorylated in response to a glycolipid-specific antibody (30), and that nephrin forms a signaling complex with podocin when the two proteins are coexpressed in cultured cells (41). Although it is premature to attempt to construct an all-embracing scheme to explain our findings and all these important observations, we propose the following possible model. Rather than signaling the podocyte via nephrin from the outside, we suggest that the insertion of C5b-9 into the podocyte plasma membrane triggers a series of signaling events that disrupt the association of nephrin with the cytoskeleton and its stability within the cell membrane. Whether this involves alterations in the phosphorylation status of nephrin, its association with podocin and/or CD2AP, or changes in the lipid composition of the cell membrane has yet to be established. In any event, the outcome appears to be the dislocation or dissolution of slit diaphragms and the loss of barrier function.

In summary, these studies in experimental MN document the redistribution and loss of nephrin from glomerular podocytes and show that this is an early event that coincides with or even precedes the onset of proteinuria. These changes are most likely due to reduced anchorage of nephrin to the actin cytoskeleton, which either allows it to be shed from the plasma membrane or targets it for endocytosis and degradation. The result is that the slit diaphragms become displaced or disrupted, thereby removing the final barrier to plasma protein permeation. It is important to emphasize that these changes need not involve every filtration slit to result in massive proteinuria. In fact, complete loss of barrier function in a small fraction of filtration slits could account for the levels of proteinuria seen in adult humans with nephrotic syndrome from MN.

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