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Decreased tyrosine phosphorylation of nephrin in rat and human nephrosis

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Phosphorylation of tyrosine residue (Y1204) of rat nephrin by Fyn kinase allows Nck adaptor protein binding to nephrin motifs, which include the phosphorylated tyrosine. This phosphorylation-dependent switch induces actin polymerization in a cell culture system. Here, we generated an antibody recognizing phosphorylated nephrin at the Nck binding sites pY1204 and pY1228 to determine the phosphorylation status of nephrin using a rat model of puromycin aminonucleoside-induced nephrosis. Changes in globular actin (G-actin) and filamentous actin (F-actin) contents in isolated glomeruli were measured by western blot. Before experimental nephrosis, both Y1204 and Y1228 were phosphorylated, and most of the actin was filamentous. Before the onset of overt proteinuria, however, phosphorylation of both Y1204 and Y1228 rapidly decreased and became almost undetectable. During this period, the amount of F-actin in glomeruli began to decrease, whereas G-actin increased. Phosphorylation of nephrin at Y1228 in glomeruli of patients with minimal change nephrosis was significantly decreased compared with that in normal glomeruli. Our study suggests that tyrosine phosphorylation of nephrin by regulating F-actin formation may be important for the maintenance of normal podocyte morphology and function.

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Nephrin is a gene product of *NPHS1*,[1] the gene mutated in Finnish-type nephrosis. It is a transmembrane protein belonging to the immunoglobulin superfamily, and it is a major component of the slit diaphragm in glomeruli. Several proteins, such as podocin,[2] CD2AP,[3] and CASK,[4,5] bind to the cytoplasmic domain of nephrin. This binding suggests that nephrin is not only a structural protein in the slit diaphragm but also a protein that transmits signals from the slit diaphragm into the interior of podocytes. Recently, nephrin was found to be phosphorylated by an Src family kinase, Fyn, at Y1204 in rats and Y1208 in mice.[6,7] Nck, an adaptor protein with three SH3 and one SH2 domains, was then found to bind to the motif containing tyrosine phosphorylated by Fyn through its SH2 domain. As the SH3 domains of Nck bind various molecules, including p21-activated kinase[8] and Wiskott–Aldrich syndrome protein,[9] nephrin phosphorylation was speculated to initiate an important signal cascade in podocyte biology. In fact, Fyn knockout and podocyte-specific gene knockout of Nck1 and Nck2 in mice caused proteinuria and abnormalities in the foot process formation.[6,10] However, the downstream events in this signal-transduction pathway are largely unknown. Cell culture studies using an overexpression system suggested that the nephrin–Nck interaction led to localized actin polymerization.[6,7] However, the role of nephrin phosphorylation in glomeruli *in vivo* remains to be determined. Verma *et al.*[7] used an antibody to detect the phosphorylation of mouse nephrin at Y1208 and found that nephrin phosphorylation in the normal glomeruli of adult mice was minimal, but transiently increased during foot process effacement in protamine sulfate-induced podocyte injury. Li *et al.*[11] reported that tyrosine phosphorylation in nephrin was increased in rats with passive Heymann nephritis. These data suggest that nephrin phosphorylation is involved in the pathogenesis of glomerular diseases. However, Li *et al.*[12] reported in a different study that tyrosine phosphorylation of nephrin was detected in normal rat glomeruli and was decreased 7 days after puromycin injection. This finding suggests that nephrin phosphorylation is physiologically important for maintaining the morphology and function of podocytes. However, Li *et al.*[12] used anti-phosphorylated tyrosine antibody to detect nephrin phosphorylation. There are about 10 possible tyrosine phosphorylation sites in the

cytosolic region of nephrin. Accordingly, it is not known whether the changes in nephrin phosphorylation observed by Li *et al*. occurred at Y1204.

To clarify this issue, we generated antibodies to detect nephrin phosphorylation at Y1228 and at Y1204 (corresponding to mouse Y1208). Although the reports by Li *et al.*[11] and Verma *et al.*[7] showed that involvement of Y1228 in the phosphorylation of nephrin by Fyn was inconclusive, Jones *et al.*[6] reported that pY1228 was the only Nck binding site other than pY1204 among the conserved tyrosine residues of rat, mouse, and human nephrin. Accordingly, Y1228 could be another important phosphorylation site *in vivo* in the Nck-mediated signal cascade, which was missed by phosphorylation assays *in vitro* and in cultured cells.

In this study, we found that Y1228 and Y1204 were phosphorylated *in vivo* in normal rat glomeruli. These phosphorylations rapidly decreased after puromycin aminonucleoside (PAN) injection, which was followed by a transient decrease in filamentous actin (F-actin) and an increase in globular actin (G-actin). These data indicate that the nephrin–Nck signal cascade is physiologically important in the actin cytoskeletal dynamics of podocytes.

RESULTS

Generation of anti-phosphorylated nephrin antibodies

Rabbit anti-pY1204 and anti-pY1228 rat nephrin antibodies were generated. After affinity purification with the phosphopeptide and immunoabsorption against non-phosphopeptide, no reactivity to non-phosphopeptide was detected by enzyme-linked immunosorbent assay (data not shown). To check whether these phosphospecific antibodies recognized phosphorylated nephrin, we performed an immunoprecipitation and immunoblot of 3xFLAG-tagged nephrin transiently expressed in COS cells. As shown in Figure 1a, a 180 kDa protein was immunoprecipitated and detected by the anti-FLAG M2 antibody in the presence or absence of pervanadate, a stimulator for tyrosine phosphorylation. Antibodies to pY1208 and pY1228 detected the 180 kDa band immunoprecipitated by M2 antibody only in the presence of pervanadate treatment, indicating that these antibodies recognized tyrosine-phosphorylated nephrin. Then, we checked whether these antibodies detected the native phosphorylated nephrin in glomeruli. We chose protein samples from glomeruli before and after injection of PAN, because at this point we did not know the phosphorylation status of nephrin in normal and disease states. As shown in Figure 1b, a 180 kDa band was detected with both antibodies. This band was more abundant in a sample from normal glomeruli than in glomeruli 4 days after PAN injection, which was confirmed in Figure 3. The band disappeared when the antibodies were applied to the membranes with the phosphorylated antigen peptide, but did not disappear with the corresponding non-phosphorylated peptide, indicating that the band detected with these antibodies was phosphorylated nephrin. An antibody to podocin was also successfully generated as shown in Figure 1c.

Nephrin phosphorylation in PAN nephrosis

We prepared protein samples from the glomeruli of rats with PAN nephrosis at multiple time points. We confirmed the successful generation of the rat nephrosis model by measuring urine protein excretion and performing electron microscopy. Figure 2a shows the development of proteinuria after the injection of puromycin. Proteinuria began to develop 4 days after injection, peaked at day 7, and recovered to almost a normal level at day 28. Electron microscopy (Figure 2b) showed the appearance of diffuse foot process effacement 4 days after injection, which is before the development of overt proteinuria.

Phosphorylation of nephrin at Y1208 and Y1228 showed similar patterns during the course of PAN nephrosis; both phosphorylations were observed in normal glomeruli on day 0, began to decrease rapidly after PAN injection on day 1, became almost undetectable on days 7 and 14, and finally recovered on day 28 (Figure 3a and b). This result clearly indicates that Y1228 is also a phosphorylation site in nephrin, in addition to Y1208. In contrast to the dynamic changes in phosphorylated nephrin, the magnitude of decrease in total nephrin content was at most 40% on day 14, and podocin content was not significantly changed during PAN nephrosis. Data from the immunoblot analysis were confirmed by immunofluorescence microscopy (Figure 4). Strong pY1208 (Figure 4a) and pY1228 (Figure 4b) nephrin immunofluorescence was detected on day 0, significantly decreased on days 4 and 7, and recovered on day 28. Immunofluorescence of total nephrin (Figure 4c) changed from linear pattern (day 0) to cytoplasmic pattern (days 4 and 7), and the fluorescent signal appeared to be decreased significantly on day 7. However, the magnitude of decrease in total nephrin appeared to be less than that of phosphorylated nephrin on days 4 and 7, indicating that the decrease of phosphorylated nephrin was mostly due to the decrease of phosphorylation itself, not to the decrease of total nephrin. Podocin staining was relatively constant during PAN nephrosis (Figure 4d).

G-actin/F-actin contents in PAN nephrosis

As phosphorylation of nephrin at Y1208 was previously linked to the regulation of actin dynamics, we investigated the changes in the G-actin and F-actin contents during PAN nephrosis. As shown in Figure 3c and d, shortly after PAN injection, G-actin increased and F-actin decreased along with the decrease in nephrin phosphorylation. Phalloidin staining of glomeruli (Figure 5) showed that the decrease in F-actin mostly occurred not in the mesangial area but in the peripheral capillary loop. These results suggest that phosphorylation of nephrin may be involved in the maintenance of F-actin in podocytes.

Nephrin phosphorylation in human minimal change nephrosis

Finally, we investigated the status of nephrin phosphorylation in human minimal change nephrosis ($n = 7$). We used only the antibody to pY1228, as its sensitivity was better than the antibody to pY1208. In contrast to the intense