Fyn Binds to and Phosphorylates the Kidney Slit Diaphragm Component Nephrin*

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Recent investigations have focused on characterizing the molecular components of the podocyte intercellular junction, because several of these components, including Nephrin, are functionally necessary for development of normal podocyte structure and filter integrity. Accumulating evidence suggests that the Nephrin-associated protein complex is a signaling nexus. As such, Nephrin-dependent signaling might be mediated in part through Nephrin phosphorylation. Described are biochemical and mouse genetics experiments demonstrating that membrane-associated Nephrin is tyrosine-phosphorylated by the Src family kinase Fyn. Nephrin fractionated in detergent-resistant glomerular membrane fractions with Fyn and Yes. Fyn directly bound Nephrin via its SH3 domain, and Fyn directly phosphorylated Nephrin. Glomeruli in which Fyn, Yes, or Fyn and Yes were genetically deleted in mice were characterized to explore the relationship between these kinases and Nephrin. Fyn depletion resulted in coarsening of podocyte foot processes and marked attenuation of Nephrin phosphorylation in isolated glomerular detergent-resistant membrane fractions. Yes deletion had no identifiable effect on podocyte morphology but dramatically increased Nephrin phosphorylating activity. Similar to Fyn deletion, simultaneous deletion of Fyn and Yes reduced Nephrin phosphorylating activity. These results demonstrate that endogenous Fyn catalyzes Nephrin phosphorylation in podocyte detergent-resistant membrane fractions. Although Yes appears to effect the regulation of Nephrin phosphorylation, the mechanism by which this occurs requires investigation.

Diseases of the renal glomerulus that result in the nephrotic syndrome are important causes of morbidity and mortality affecting both adults and children. Unfortunately, the molecular mechanisms governing development of the nephrotic syndrome remain poorly understood (1, 2).

Glomerular visceral epithelial cells appear to play a central role in maintaining the selective filtration barrier of the renal glomerulus. These cells are also termed podocytes to describe the foot-like appearance of numerous interdigitating processes that arise from their cell bodies and cover glomerular capillary walls. Glomerular filtrate passes across the specialized intercellular junction—also termed the “slit diaphragm” formed at the interface of these interdigitated foot processes (3).

In response to glomerular injury, podocytes undergo a dramatic change in morphology termed “foot process effacement” resulting in retraction and spreading of foot processes and alteration in their intercellular junctions (4). Foot process effacement is a fluid and reversible process that correlates closely with the development of proteinuria both in human disease and in experimental models (2, 5). Clearly, foot process effacement requires precise interplay of multiple cellular processes, including alterations in the structure of the cytoskeleton, movement of foot process over the basement membrane, and disassembly or re-assembly of the intercellular junction that comprises the slit diaphragm. Each of these unique but interdependent cellular processes are in and of themselves complex.

The cellular and molecular mechanisms governing podocyte morphology and filter integrity are incompletely defined. Recent investigations have focused on identifying and characterizing the interrelationships and functions of the molecular components of the foot process intercellular junction, because several of these components, including Nephrin (6), Podocin (7), and CD2ap (8), have been demonstrated to be necessary for development of normal podocyte structure and filter integrity.

Nephrin is encoded by NPHS1, the gene mutated in congenital nephrotic syndrome of the Finnish type, a rare autosomal recessive developmental disorder manifest at birth by heavy proteinuria and diffuse podocyte foot process effacement (9, 10). Deletion of Nephrin by gene targeting in mice results in a similar phenotype (11). In the kidney, Nephrin expression is podocyte-specific, and the protein is targeted to the lateral aspect of foot process cell membranes in the region of the slit diaphragm (12, 13). Nephrin is a member of the immunoglobulin superfamily. For this reason, it has been proposed that Nephrin is a cell adhesion molecule (CAM)3 that participates in forming the glomerular filter via interactions involving its extracellular domain (12).

More than simply mediating cellular interactions, various Ig superfamily CAMs behave as signal transducing molecules. Depending on the particular CAM involved, Ig superfamily

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3 The abbreviations used are: CAM, cell adhesion molecule; GST, glutathione S-transferase; RIPA, radioimmune precipitation assay; PBS, phosphate-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxyethyl)methyl]glycine; DRM, detergent-resistant membrane fraction; CD, cytoplasmic domain; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine; SFK, Src family kinase.
CMs can play a role in either “outside-in” signaling in which a signal is transduced into the cell following ligand binding or can participate in “inside-out” signaling in which signals originating within the cell result in changes in binding characteristics of the CAM. Like other transmembrane proteins, Nephrin participates in a protein complex at the cytoplasmic face of the plasma membrane. To date, Nephrin has been shown to associate with Podocin, the protein product of NPHS2 that is mutated in an inherited form of steroid-resistant focal and segmental glomerulosclerosis (7). Nephrin also binds CD2ap, and pedalpge and control mouse and rabbit immunoglobin (Sigma Chemical Co.) were obtained commercially. Plasmid vectors encoding GST-FynSH3SH2, GST-FynSH3, and GST-FynSH2 were previously described (16). Recombinant GST-Fyn fusion proteins and hexahistidine-tagged Nephrin cytoplasmic domain (13) were prepared and purified from bacterial lysates as described previously (17, 18). Mammalian expression plasmids encoding wild type Fyn, Yes, and FynSH3 were previously described (16). Mammalian expression plasmid encoding mouse FLAG-Nephrin was prepared in a manner similar to that previously described (19). The mammalian expression construct encoding a FLAG-tagged form of the entire cytoplasmic domain of mouse Nephrin (F-NephrinCD) was prepared by a standard PCR cloning technique using pDNA3.1 (Invitrogen). PPP2, a specific inhibitor of Src kinases (F-NephrinCD) was prepared by a standard PCR cloning technique

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EXPERIMENTAL PROCEDURES

Reagents—Rabbit polyclonal antibodies to the cytoplasmic domain of mouse Nephrin (13) and to the COOH-terminal 17 amino acid residues of human or mouse Podocin (15) were described previously. Rabbit antibody to a GST fusion protein containing the first and second SH3 domains of mouse CD2ap was raised by immunizing rabbits (performed at Lampire Biological Laboratories Inc., Pipersville, PA). Anti-FLAG epitope monoclonal antibody (M2, Sigma) and antibodies to phosphotyrosine (PY66, Sigma Chemical Co.), pan-Src (Santa Cruz Biotechnology), Fyn (Transduction Laboratory or Santa Cruz Biotechnology), Yes (Santa Cruz Biotechnology), and Fyn (Becton Dickinson) were obtained commercially. Recombinant GST-Fyn, GST-FynSH3SH2, GST-FynSH3, and GST-FynSH2 were previously described (16). Recombinant GST-Fyn fusion proteins and hexahistidine-tagged Nephrin cytoplasmic domain (13) were prepared and purified from bacterial lysates as described previously (17, 18). Mammalian expression plasmids encoding wild type Fyn, Yes, and FynSH3 were previously described (16). Mammalian expression plasmid encoding mouse FLAG-Nephrin was prepared in a manner similar to that previously described (19). The mammalian expression construct encoding a FLAG-tagged form of the entire cytoplasmic domain of mouse Nephrin (F-NephrinCD) was prepared by a standard PCR cloning technique using pDNA3.1 (Invitrogen). PPP2, a specific inhibitor of Src kinases (F-NephrinCD) was prepared by a standard PCR cloning technique using pDNA3.1 (Invitrogen). PPP2, a specific inhibitor of Src kinases

Cell Culture—Transient transfections were carried out in COS7 cells. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Invitrogen) and 200 units/ml penicillin and streptomycin (Roche Applied Science). Transfections were performed using FuGene-6 (Roche Applied Science). Where indicated, cells were cultured in the presence of 50 μM pervanadate for 40 min prior to harvesting cells. For orthophosphate metabolic labeling of cells, 24 h after transfection, cells were washed and incubated with phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 10% diazylated fetal bovine serum containing 1 mM ω-3ω-3ATP orthophosphate (Amersham Biosciences) for 5 h at 37 °C.

Immunoprecipitation and Immunoblotting—Illumonoprecipitation and immunoblotting experiments were performed using the indicated antibodies as described elsewhere (19). Where indicated Nephrin and indicated Src family protein kinases were extracted from plasma membranes in RIPA buffer (phosphate-buffered saline (PBS) containing 0.1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate). Phosphoamino Acid Analysis—Phosphoamino acid analysis was performed essentially as described elsewhere (17). Following metabolic labeling, Nephrin was immunoprecipitated, separated by SDS-PAGE, and stained with polyvinylidene difluoride membrane and then exposed to diodoacetate of the appropriate molecular weight was excised. Phosphoamino acids were separated by one-dimensional electrophoresis. Phosphoamino acid standards were visualized by ninhydrin staining, and radioactivity was detected by using a PhosphorImager (Storm, Amersham Biosciences).

Mouse and Rat Husbandry—Male and female homozygous null breeding pairs for Yesc−/− mice (129-Yesc−/−; stock number 002380) and Fyn−/− mice (B6;129-Fyn−/−; stock number 002385) were obtained from Jackson Laboratory (Bar Harbor, ME). Yes−/− mouse and Fyn−/− mouse colonies were obtained by inbreeding, and Fyn−/− mouse born from mixing Yesc−/− and Fyn−/− mice. Genomic DNA was made from tail tips clipped at weaning using a QIAamp tissue kit (Qiagen, Valencia, CA). A PCR assay was established to genotype mice as described on the Jackson Laboratories website. For Yesc−/− mice, the following primers were employed: 5′-CTT TGG TGA AGC AGG TAC TC-3′ (Neol generic neo forward primer), 5′-AGG TGA GAT GAC AGG AGA AGC TC-3′ (Neol generic neo reverse primer), 5′-TTA CCC TCT GAG CAT AC-3′ (Fyn intron forward primer from Paul Stein, Wistar Institute), 5′-GCA AAA CAA CCC ACA CAT G-3′ (Fyn reverse primer). The size of the expected Yesc-specific amplification product is 600 bp from the wild type allele. For Yesc−/− mice, the following primers were employed: 5′-TGT GTG TCT TAC TGT GAA ACC C-3′ (Yes KO forward primer), 5′-GCA TCC TTG ACC TAG TTT CAC-3′ (Yes KO reverse primer), and 5′-GAT CTC GTC GAA ACC CAT G-3′ (Yes KO forward primer). 2). A 103-bp product was expected from the wild type Yes allele. A 600-bp and a 385-bp product were expected from the targeted allele. Sprague-Dawley mice (100–120 g) were obtained from Harlan, Inc. The University of Michigan Committee on Use and Care of Animals approved all procedures using rats and mice. All work was conducted in accordance with the principles outlined in the National Institutes of Health Guidelines for the Care and Use of Experimental Animals.

Glomerular Isolation—Adult kidneys were obtained where indicated from rats or mice. Glomeruli were isolated by sieving as described elsewhere (13). The average purity of the glomerular preparations was ~60% for mice and 90% for rat preparations. Glomeruli were lysed in the indicated buffer containing protease inhibitors at a concentration of 10,000 glomeruli per milliliter of extraction buffer.

Flotation Gradient Preparations—Isolated glomeruli from rat or mouse kidneys were homogenized at 4 °C with 14 strokes of a Dounce homogenizer in a buffer containing 250 mM sodium chloride, 5 mM sodium phosphate, 1 mM sodium orthovanadate, and 0.1 mM sodium pyrophosphate, and protease inhibitor mixture tablet (In vitrogen). To the homogenate, Triton X-100 was added to a final concentration of 1% and mixed well. The mixture was maintained on ice for 15 min before being mixed with 80% Optiprep (Nycomed Pharma AS) to obtain a final density of 40%. Five milliliters of this mixture was added to pre-cooled centrifuge tubes then layered with 3 ml of 30% Optiprep and 4 ml of 5% Optiprep. Optiprep dilutions were obtained by diluting 60% stock Optiprep with Tricine buffer containing 20 mM Tricine, 0.25 mM sucrose, and 1 mM EDTA. The gradient was centrifuged at 169,044 × g in a Beckman SW41 Ti rotor for 3 h at 4 °C. The Triton X-100 immunosorbable fraction (detergent-resistant membrane fraction, DRM) was observed at the interface between 5 and 30% Optiprep densities. Top to bottom 1-ml fractions were collected and analyzed as indicated.

Spot Urine Albumin/Creatinine Ratios—Spot urine samples collected from mice after physical stimulation were analyzed for albumin concentration using a commercial murine microalbuminuria enzyme-linked immunosorbent assay kit (Albuwell, Exocell Inc., Philadelphia, PA) and for creatinine concentration by the alkaline picrate method (Sigma, catalog no. 555, St. Louis, MO).

In Vitro Kinase Assay on Glomerular DRM—Glomerular DRM fractions were isolated, resuspended in kinase buffer (25 mM Hepes, pH 7.2, 10% glycerol, 100 mM NaCl, 5 mM MgCl2, 5 mM MnCl2, 0.1 mM sodium orthovanadate, and a mixture of protease inhibitors) and centrifuged at 16,000 × g at 4 °C. These pellets were resuspended in kinase buffer containing 25 μM ATP, 5 μCi of [γ-32P]ATP (3000 Ci/ mmol), and incubated at 25 °C for 15 min. Reactions were carried out in the presence of 10 μM PP2 or PP3 where indicated. Pellets were pelleted and washed once with kinase buffer lacking ATP MgCl2 and MnCl2. Protein was extracted with 0.1% Triton X-100, 50 μg/ml proteinase K, and the obtained supernatants were immunoprecipitated or further analyzed as indicated in the text.

Pull-down Assay—Indicated GST fusion proteins were incubated either with isolated glomerular extract (RIPA buffer extraction) or with lysate obtained from COS7 cells transfected with plasmid encoding Myc-tagged Nephrin. The mixtures were incubated with 500 μl 50 μg/ml glutathione agarose added to each tube. The tubes were further incubated at 4 °C for 1 h. Following incubation beads in the tubes were washed five times using PBS (phosphate-buffered saline, pH 7.4, and 1% Triton X-100) to remove the unbound protein. The bound protein

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was then eluted using 5 mM reduced glutathione. The eluates were separated on SDS-PAGE and analyzed by immunoblotting as indicated.

**In Vitro Kinase Assay**—One microgram of hexahistidine-tagged Nephrin cytoplasmic domain was incubated with 1 μg of partially purified Fyn or enolase in kinase buffer containing 25 μM ATP, 5 μCi of [γ-32P]ATP (3000 Ci/mmol) and incubated at 25 °C for 15 min. Reactions were carried out in the presence of 10 μM PP2 or PP3 where indicated. Reactions were terminated with SDS loading buffer and boiled for 5 min. Samples were separated by reducing SDS-PAGE, transferred to nitrocellulose, and detected by autoradiography. 

Electron Microscopy—Preparation of samples for transmission electron microscopy was performed by standard methods using pieces of boiled for 5 min. Samples were separated by reducing SDS-PAGE, fractionated in the same DRM fraction.

**Statistics**—Statistical significance for urine albumin/creatinine ratios was assessed using one-way analysis of variance, and Bonferroni’s multiple comparison test was used for post-hoc analysis.

**RESULTS**

The Nephrin Cytoplasmic Domain Is Tyrosine-phosphorylated—Sequence analysis of the cytoplasmic domain of Nephrin suggested that Nephrin might be tyrosine-phosphorylated. To initially examine this possibility, plasmid encoding FLAG-epitope-tagged Nephrin cytoplasmic domain (Neph-C) was expressed by transient transfection in COS7 cells. Neph-C was examined as described in Fig. 1 by immunoprecipitation and immunoblotting. Tyrosine phosphorylation of the Nephrin-C was detected following treatment with the tyrosine phosphatase inhibitor pervanadate (Fig. 1A). Tyrosine phosphorylation of Nephrin-C was not observed in the absence of pervanadate pretreatment (not shown). Phosphoamino acid analysis of Nephrin-C expressed in COS7 cells that were metabolically labeled with [32P]orthophosphate confirmed that Nephrin phosphorylation occurred on tyrosine and not on serine and threonine residues (Fig. 1B). Given these preliminary observations, we examined the phosphorylation state of endogenous Nephrin expressed in adult rat glomeruli. Prior to removal of kidneys, rat renal arteries were perfused with physiologic buffer containing or not containing pervanadate. Endogenous Nephrin obtained by immunoprecipitation from the lysates of isolated glomeruli pre-treated with pervanadate was tyrosine-phosphorylated (Fig. 1C). In most experiments, tyrosine phosphorylation was not detected on Nephrin obtained from kidney perfused with buffer lacking pervanadate. However, in some experiments, prolonged exposure of immunoblots to film or the use of a more sensitive enzymatic substrate (Femto, Pierce) to develop immunoblots allowed the detection of Nephrin tyrosine phosphorylation (data not shown).

**Nephrin Fractionates with Fyn and Yes Protein Kinases in Triton X-100-insoluble Glomerular Membrane Fractions**—In part, Nephrin associates with a Triton X-100-insoluble membrane fraction obtained from isolated glomeruli (21). In a similar fashion, tyrosine-specific Src family protein kinases associate with detergent-resistant membrane (DRM) fractions in a number of cell types (22, 23). Given this association, we investigated the hypothesis that an Src-family kinase or kinases are responsible, at least in part, for Nephrin tyrosine phosphorylation. Initially, the co-fractionation of Nephrin and several Src family kinases in detergent-resistant glomerular lipid membrane fractions was examined (Fig. 2). Isolated rat glomeruli were lysed at 4 °C in buffer containing 1% Triton X-100, and the lysate was fractionated by flotation gradient centrifugation. Like Nephrin, Src family kinases Fyn and Yes but not c-Src were detected in the detergent-resistant membrane fraction.
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To strengthen the hypothesis that Nephrin serves as a substrate for Src family protein kinase, the capacity of endogenous Nephrin to interact with endogenous Fyn or Yes was investigated (Fig. 4). Lysate obtained from isolated rat glomeruli was immunoprecipitated with anti-Nephrin antibody. These immune complexes were resolved by SDS-PAGE and examined by immunoblotting for the presence of Fyn or Yes. Fyn and Yes both co-immunoprecipitated with Nephrin. In reciprocal experiments, Nephrin co-immunoprecipitated with Fyn. However, attempts to co-immunoprecipitate Nephrin with anti-Yes antibody did not succeed despite trials with two distinct anti-Yes antibodies.

The Fyn domain sufficient for interaction with Nephrin was mapped in pull-down experiments (Fig. 5). Isolated recombinant GST-Fyn-SH2 or -SH3 or composite -SH2SH3 domains were purified from bacterial lysates. Prior to lysis, COS7 cells transfected with plasmid encoding wild type Nephrin were treated without or with pervanadate to increase Nephrin phosphorylation. In these experiments, Fyn SH3 domain but not SH2 domain interacted with Nephrin obtained from COS7 cells pretreated with pervanadate (Fig. 5A). In contrast, Nephrin obtained from COS7 cells that were not pretreated with pervanadate did not interact with either the Fyn SH3 or its SH2 domain. In similar pull-down experiments, GST-Fyn SH3 but not Fyn SH2 interacted with Nephrin obtained from lysates of isolated glomeruli (Fig. 5B). The binding affinity of Nephrin for Fyn or Yes was also examined in co-transfection experiments (Fig. 6). As anticipated, wild type Nephrin co-immunoprecipitated with wild type Fyn and Yes. Consistent with results of the pull-down experiments described above, Fyn or Yes deleted of their SH3 domains had significantly attenuated affinity for Nephrin in these co-immunoprecipitation experiments. Of note, pervanadate treatment of transfected cells prior to lysis resulted in attenuation of the affinity of Fyn and Yes for Nephrin. Taken together, these results demonstrate that Fyn and/or Yes can interact with Nephrin via their SH3 domains. As discussed below, regulation of this interaction is complex but appears to be phosphorylation-dependent.

That Nephrin can serve as a direct substrate of Fyn was confirmed in an in vitro protein kinase assay (Fig. 7). Recombinant hexahistidine-tagged Nephrin cytoplasmic domain expressed in bacteria was purified, combined with purified Fyn, and incubated at 30 °C for 20 min in a buffer containing Mg2+ identified when incubation of DRM was carried out in the presence of PP2. It was concluded that DRM-associated Nephrin might be phosphorylated by a Src family protein kinase located in a podocyte detergent-resistant membrane fraction.

Fig. 2. Nephrin fractionates in part with Src kinases in Triton X-100 insoluble membrane. Rat glomeruli isolated by sieving were extracted in buffer containing 1% Triton X-100 at 4 °C. The lysate was subjected to flotation gradient centrifugation. Lipid fraction was identified in fraction #5 at the interface between 5 and 30% OptiPrep densities. Indicated 1-ml fractions were collected from top to bottom and were analyzed by immunoblotting with the indicated antibodies. This representative experiment was repeated five times with similar results.

Fig. 3. Nephrin obtained from DRM fraction of isolated glomeruli is associated with kinase catalytic activity. Lanes 1–4, the DRM fraction was pelleted, resuspended, and incubated in kinase buffer containing radiolabeled ATP at 30 °C for 20 min. PP2 or PP3 was added prior to kinase reactions as indicated. Samples were re-extracted in RIPA buffer and immunoprecipitated with anti-Nephrin antibody. Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose, and autoradiographed. Lanes 5 and 6, DRM fractions incubated in kinase buffer as above were separated by SDS-PAGE without immunoprecipitation. This representative experiment was repeated five times. Another example is shown as a control in Fig. 10.

remained phosphorylated. Following incubation in kinase buffer, unextracted DRM fractions were also examined by SDS-PAGE and autoradiography. A prominent set of proteins with a mobility consistent with that of Src family kinases were identified at ~58 kDa; phosphorylation of these proteins was not
and Mn\(^{2+}\) and [\(^{32}\)P]ATP. Under these conditions, Nephrin was readily phosphorylated. When this reaction was carried out in the presence of the Src-inhibitor PP2, Nephrin was not phosphorylated. In similar experiments, recombinant c-Src was substituted for Fyn; here, c-Src-phosphorylated enolase used as a control substrate did not phosphorylate Nephrin (data not shown). Therefore, Fyn can directly phosphorylate Nephrin in a specific fashion.

**Genetic Evidence That Fyn and Yes Participate in Determining the Phosphorylation State of Nephrin**—The glomerular phenotypes of mice deleted of Fyn (Fyn\(^{-/-}\)) or of Fyn and Yes (Fyn\(^{-/-}\)/Yes\(^{-/-}\)) by homologous recombination have been described previously. Fyn\(^{-/-}\) and Fyn\(^{-/-}\)/Rag1\(^{-/-}\) mice developed proteinuria and foot process effacement (24). Fyn\(^{-/-}\)/Yes\(^{-/-}\) mice were reported to develop proteinuria, foot process effacement, and marked mesangial matrix expansion with mesangial hypercellularity and mesangial deposition of electron dense deposits (25). An abnormal glomerular phenotype was not previously described for Yes\(^{-/-}\) mice. In preparation for examining Nephrin phosphorylation in these mouse models, Fyn\(^{-/-}\) and Yes\(^{-/-}\) mouse colonies were raised and examined by light microscopy (data not shown). Kidneys obtained from 4- and 7-week post-gestation mice were also examined using a

and Mn\(^{2+}\) and [\(^{32}\)P]ATP. Under these conditions, Nephrin was readily phosphorylated. When this reaction was carried out in the presence of the Src-inhibitor PP2, Nephrin was not phosphorylated. In similar experiments, recombinant c-Src was substituted for Fyn; here, c-Src-phosphorylated enolase used as a control substrate did not phosphorylate Nephrin (data not shown). Therefore, Fyn can directly phosphorylate Nephrin in a specific fashion.

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endothelial swelling and disruption were evident in many arrested epithelial mass with absence of structurally typical slit.

Characterization of Fyn, Yes, and Fyn/Yes null podocyte capillary loop segments. Shown are higher magnification transmission electron photomicrographs of representative morphologies observed in foot process segments in mouse models at 4-week post-gestation. (A, wild type; B, Yes−/−; C, Fyn−/−; D, Fyn−/−Yes−/−). Note particularly that several types of morphologic changes are observed in Fyn−/− mice foot processes. In Fyn−/−Yes−/− mice, endothelial swelling and disruption are noted in addition to foot process effacement.

FIG. 8. Characterization of Fyn, Yes, and Fyn/Yes null glomeruli. A, spot urine samples from indicated mice were analyzed to determine albumin/creatinine ratios. Shown are pooled data from wild type mice of genetic backgrounds matching the three experimental groups, including B6129SF2/J, 129/SvImJ, and wild type littermates derived from mice of genetic backgrounds matching the three experimental groups, mice foot processes. In Fyn−/−Yes−/− mice, endothelial swelling and disruption are noted in addition to foot process effacement.

FIG. 9. Characterization of Fyn, Yes, and Fyn/Yes null podocyte capillary loop segments. Shown are higher magnification transmission electron photomicrographs of representative morphologies observed in foot process segments in mouse models at 4-week post-gestation. (A, wild type; B, Yes−/−; C, Fyn−/−; D, Fyn−/−Yes−/−). Note particularly that several types of morphologic changes are observed in Fyn−/− mice foot processes. In Fyn−/−Yes−/− mice, endothelial swelling and disruption are noted in addition to foot process effacement.

A kinase activity assay similar to that described above was used to examine the role of Fyn or Yes in catalyzing Nephrin phosphorylation in isolated glomerular detergent-resistant membrane fractions of Fyn, Yes, and Fyn/Yes null mouse mutants (Fig. 10A and pooled data obtained from multiple independent experiments summarized in Fig. 10D). In these experiments, Triton X-100-resistant plasma membrane fractions from isolated glomeruli obtained from kidneys of eight mice were pooled prior to assay. Deletion of Fyn was reproducibly associated with significant attenuation of Nephrin phosphorylation in glomerular DRM fractions. In contrast, deletion of Yes did not result in attenuation of Nephrin phosphorylation under the conditions of this assay. Rather, deletion of Yes reproducibly resulted in increased Nephrin phosphorylation in four independent experiments. When this assay was carried out using DRM isolated from Fyn−/−Yes−/− mice, no significant additional attenuation in Nephrin phosphorylation was detected beyond that observed in glomerular DRM isolated from Fyn−/− mice (Fig. 10, B and E). Together with the observations reported above, these results provide genetic evidence that endogenous Fyn catalyzes Nephrin phosphorylation in podocyte detergent-resistant membrane fractions. Although Yes appears to effect the regulation of Nephrin phosphorylation, the mechanism by which this occurs requires further investigation.

Emerging evidence suggests that the molecular composition of the intercellular junction between podocyte foot processes is complex. This complexity is not unexpected, because, like better-studied typical epithelial cell intercellular junctions, the podocyte intercellular junction is necessarily a dynamic structure. Macroscopically, this dynamic nature is obvious during podocyte differentiation or during the process of foot process effacement that occurs in response to podocyte injury. At the molecular level, these dynamic properties must require integration of multiple cellular processes via intracellular signaling.

Several observations suggest that Nephrin, or more likely, the complex of proteins with which it is physically associated at the podocyte intercellular junction, serve as a signaling nexus. Deletion of Nephrin either in human disease (6) or in genetically manipulated mice (9) results in profound phenotypic effects on multiple cellular processes that are linked via undefined signal transduction pathways. Although it is unlikely...
that all of the components of the Nephrin-associated protein complex have been defined, it is remarkable that deletion of Podocin and CD2ap, the two confirmed Nephrin-interacting proteins identified to date, result in a phenotype similar to that of Nephrin deletion (7, 8). That Nephrin and its associated protein complex are involved in signaling has also been suggested by Huber et al. (26) who observed that overexpression of Podocin with Nephrin in HEK 293 cells augmented the tendency of overexpressed Nephrin alone to increase AP-1 transcriptional activity possibly via activation of a MAPK module. Despite these observations, our understanding of the composition, assembly, and signaling function of the Nephrin complex remains primitive.

The work presented herein suggests that phosphorylation-mediated signaling mechanisms participate in Nephrin complex-mediated signal transduction. Employing both biochemical and genetic approaches, this work shows that the Src family kinase Fyn is a component of the Nephrin-associated protein complex. Fyn can bind directly to the Nephrin cytoplasmic domain via its SH3 domain and can directly phosphorylate Nephrin both in vitro and in vivo.

Our work corroborates the work of others that the expression of Fyn is necessary for the development or maintenance of normal foot process morphology (24). In contrast to the findings of Yu et al. (24), abnormalities in Fyn null mouse foot process morphology did not concomitantly result in increased albuminuria; the etiology of these differing observations is unknown but may be related to differences in genetic background. It is tempting to speculate that deletion of Fyn results in the observed podocyte morphologic alterations due to resultant abnormalities in Nephrin phosphorylation. However, this remains to be proved because Fyn deletion could effect podocyte structure at multiple additional sites within the cell.

The functions of Fyn and Yes in the glomerulus are clearly distinct. Despite the fact that Yes can be shown to physically associate with Nephrin in biochemical experiments, the results of genetic experiments with Yes do not allow one to conclude that Yes lies immediately proximal to Nephrin in a signaling pathway. Rather, deletion of Yes reproducibly results in increased phosphorylation of Nephrin in isolated Triton X-100 insoluble lipid membrane fractions. The assays used here to evaluate the role of Yes in Nephrin phosphorylation were carried out in isolated membrane fractions containing Nephrin. Therefore, it is reasonable to surmise, but not yet possible to conclude, that Yes is indeed present in the Nephrin-associated protein complex. That deletion of both Fyn and Yes also results in attenuated Nephrin phosphorylation in these DRM fractions suggests that Fyn lies in an epistatic relationship between Yes and Nephrin. In this model, Yes activity would be predicted to inhibit Fyn activity either directly or indirectly. The mechanism by which this occurs requires further investigation.

The mechanism by which Src family kinases (SFKs) are regulated has been intensively studied (reviewed in Ref. 27). SFKs are inactive when they are phosphorylated on a defined COOH-terminal tyrosine. This results in a conformational change in which the intrinsic SH2 domain of the SFK binds to the phosphorylated COOH-terminal domain of the same molecule preventing activation of SFK catalytic activity and pre-

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**Fig. 10.** Genetic deletion of Fyn or Yes from mice effect Nephrin phosphorylation in detergent-resistant membrane fractions isolated from glomeruli. A and B, glomeruli obtained from the kidneys of eight mice of the indicated genotype were pooled, and DRM were isolated by flotation gradient centrifugation. DRM were subjected to kinase assay described in text. Where indicated, assays were carried out in the presence of PP2 or PP3. DRM were re-extracted with RIPA buffer, and Nephrin was immunoprecipitated. Immune complexes were separated by SDS-PAGE and transferred to nitrocellulose. The same blots were then immunoblotted with anti-Nephrin antibody. Experiments are representative of several experiments. C, to confirm genotyping, lysates from isolated glomeruli of indicated animals were separated on SDS-PAGE and immunoblotted with either anti-Fyn or anti-Yes antibody as indicated. D and E, representation of pooled data from several experiments. Graphs indicate mean relative Nephrin-specific activity normalized for Nephrin protein and compared with wild type control. Emitted counts were quantified using a phosphorimaging. The relative amount of Nephrin protein present in each sample was evaluated by densitometry using Image (National Institutes of Health). Error bars indicate standard deviation. The number of independent experiments is indicated below each column.
venting association of the SFK with its substrate. The SFK is activated when a protein phosphatase dephosphorylates the COOH-terminal tyrosine. This mechanism might explain the observation that treatment of transfected COS7 cells with the nonspecific protein phosphatase inhibitor pervanadate results in decreased affinity of wild type Nephrin for wild type Fyn. By similar reasoning, the isolated recombinant Fyn SH3 domain may have higher affinity for Nephrin obtained after treatment with pervanadate, because endogenous Fyn, which might compete for binding of recombinant Fyn SH3 domain, should not be associated with immunoprecipitated Nephrin under these conditions.

The work presented herein raises many new questions. For example, the functional significance of Nephrin phosphorylation remains undefined. Kurihara et al. (28) observed a correlation between increased tyrosine phosphorylation of proteins at the podocyte intercellular junction and the induction of podocyte effacement and loss of glomerular filter integrity in a rat model. Therefore, Fyn activation and Nephrin phosphorylation might occur as a result of a podocyte stimulus that results in foot process effacement. The mechanism by which Fyn activation is induced in this setting is also undefined: activation could occur via an outside-in stimulus via the Nephrin “receptor” complex or via an inside-out signal initiated outside of the junctional complex. The mechanistic significance of Nephrin complex phosphorylation similarly requires investigation. Nephrin phosphorylation might result in protein complex assembly or dissociation, the recruitment of specific signaling proteins that initiate signaling along specific pathways, or alterations in the affinity of Nephrin for its extracellular ligand, or it might regulate Nephrin complex-mediated endocytosis and recycling (29). Examples of these types of mechanisms are abundant in nature but are best described for the immune-cell receptor complex (30, 31) or at the neuronal synapse (32). Each of these possibilities requires further investigation.

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Fyn Binds to and Phosphorylates the Kidney Slit Diaphragm Component Nephrin
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Fyn binds to and phosphorylates the kidney slit diaphragm component Nephrin.

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Pages 20719 and 20720 (Fig. 5): Fig. 5 in this paper was inadvertently mislabeled. A revision of this figure is shown below. In Fig. 5, A, and B, the labels GST-FynSH2 and GST-FynSH3 were reversed. As a result, the conclusion reached in the text on page 20719 (second full paragraph) is incorrect. It should properly read: “In these experiments, Fyn SH2 domain but not its SH3 domain interacted with Nephrin obtained from COS7 cells pretreated with pervanadate (Fig. 5A) …. In similar pull-down experiments, GST-Fyn SH2 but not Fyn SH3 interacted with Nephrin obtained from lysates of isolated glomeruli (Fig. 5B).”

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