Neph1, a Component of the Kidney Slit Diaphragm, Is Tyrosine-phosphorylated by the Src Family Tyrosine Kinase and Modulates Intracellular Signaling by Binding to Grb2*

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Yutaka Harita‡, Hidetake Kurihara†, Hidetaka Kosako†, Tohru Tezuka‡, Takashi Sekine‡, Takashi Igarashi‡, and Seisuke Hattori†‡*

From the †Division of Cellular Proteomics (BML) and the ‡Department of Oncology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, the ††Department of Pediatrics, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, the †‡Department of Anatomy, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, and the †§Department of Biochemistry, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

There are several lines of evidence that the podocyte slit diaphragm (SD), which serves as a structural framework for the filtration barrier in kidney glomerulus, also plays an essential role as a signaling platform. Several SD components including nephrin and TRPC6 are known to be phosphorylated by a Src family tyrosine kinase (SFK), Fyn. Here we have characterized Neph1, another SD component, as a novel substrate of SFK. Fyn interacts with and phosphorylates the cytoplasmic domain of Neph1 in vitro and in intact cells. Peptide mass fingerprinting and site-directed mutagenesis identified several tyrosine phosphorylation sites. In pull-down assays using rat glomerular lysates, Neph1 but not nephrin specifically binds to adaptor protein Grb2 and tyrosine kinase Csk in a phosphorylation-dependent manner. Both tyrosine 637 and 638 of Neph1 are crucial for Neph1-Grb2 binding. Phosphorylation of tyrosine 637 is significantly up-regulated in in vivo models of podocyte injury. Furthermore, Neph1 attenuates ERK activation elicited by Fyn, and this inhibitory effect requires the intact binding motif for the Grb2 SH2 domain. Our results shown here demonstrate that Neph1 is a novel in vivo substrate of SFK and suggest that Neph1 modulates ERK signaling through phosphorylation-dependent interaction with Grb2. Thus, SFK orchestrates a wide spectrum of protein-protein interactions and intracellular signaling networks at SD through tyrosine phosphorylation.

Recent studies have identified several molecular components of SD. The first of these molecules to be identified was nephrin (2). Nephrin is a transmembrane protein encoded by the NPHS1 gene, and is a member of the immunoglobulin superfamily. Mutations in NPHS1 cause heavy proteinuria before birth and result in early death (congenital nephrotic syndrome of the Finnish type) (2, 3). Neph1 is structurally related to nephrin, and has five extracellular immunoglobulin-like motifs. Mice deficient in Neph1 develop proteinuria and die within the 8 weeks after birth (4). Several other molecules, including podocin (5), FAT1 (6), and CD2AP (7) are identified as components of SD, and gene disruption of these molecules in human diseases or in genetically manipulated mice results in similar phenotypic conditions; the flattening (effacement) of foot processes, loss of SD, and proteinuria. Nephrin interacts with Neph1 and podocin, forming a trimeric protein complex (8–11). These transmembrane proteins at SD further interact with the junctional scaffolding proteins, ZO-1 (12), CD2AP (13), calcium calmodulin-dependent serine protein kinase (14), and MAGI-1 (membrane-associated guanylate kinase inverted) (14, 15), which anchor the SD complex to the elaborate actin cytoskeleton network. Most of these proteins are crucial to both the development of the glomerulus and the filter function of SD.

In addition to its role as a structural framework of the filtration barrier, SD has been implicated as the signaling platform (16). Nephrin interacts with IQGAP (17), an effector protein of small GTPases Rac1 and Cdc42. Nephrin and CD2AP also interact with phosphatidylinositol 3-kinase p85, which leads to increased Akt activity and reduction in cell death induced by apoptotic stimuli (18).

There are also several lines of evidence that tyrosine phosphorylation may play a key role in the integrity of SD. The cytoplasmic domain of nephrin is tyrosine-phosphorylated by a Src family tyrosine kinase (SFK), Fyn (19). Tyrosine phosphorylation of nephrin results in the recruitment of the SH2-SH3 domain-containing adapter protein Nck to SD, and this nephroepithelial cell

signal-regulated kinase; MAPK, mitogen-activated protein kinase; ZO-1, zona occludens; CD2AP, CD2-associated protein; SH, Src homology; HEK, human embryonic kidney; GST, glutathione S-transferase; PS, protamine sulfate; PAN, puromycin aminonucleoside; MS, mass spectrometry.
Tyrosine Phosphorylation of Neph1

rin-Nck interaction regulates actin polymerization (20, 21). Recently, the TRPC6 cation channel, whose activity is regulated by tyrosine phosphorylation by Fyn (22), was also identified as an SD-associated protein (23, 24). The crucial role of Fyn-mediated phosphorylation of SD in maintaining the integrity of glomerular permselectivity is also indicated by the effacement of foot processes and proteinuria in fyn-deficient mice (19, 25).

Neph1 interacts with nephrin at SD, and this hetero-oligomeric complex is considered to be an important determinant of glomerular permselectivity (26). Recently Neph1 is also considered as a signaling molecule. Neph1 has a longer cytoplasmic domain that contains a larger number of tyrosine residues than nephrin, and the carboxyl-terminal domain of Neph1 interacts with the PDZ domain of ZO-1, which not only connects Neph1 to actin cytoskeleton but also modulates the Neph1-mediated signal transduction by augmenting tyrosine phosphorylation of Neph1 (12). Neph1 is phosphorylated by Tec family tyrosine kinases in cotransfected cultured cells, and the phosphorylation leads to an increase of Neph1-mediated AP-1 activation (10).

Whereas tyrosine phosphorylation of ZO-1 (27) and nephrin (20) is dramatically increased in the rat protamine sulfate (PS) perfusion model, one of the in vivo models of podocyte injury, tyrosine phosphorylation of Neph1 in vivo, has not been reported. We hypothesize that Neph1 is tyrosine-phosphorylated in injured podocytes, and involved in intracellular signaling events.

In this study, we have demonstrated that Neph1 is tyrosine-phosphorylated in podocyte injury in vivo. Neph1 is phosphorylated by SFK in vitro and in intact cells. Tyrosine phosphorylation of Neph1 regulates its interaction with Grb2 and Csk, which may be involved in negative feedback control of tyrosine kinase signaling.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Mouse monoclonal anti-FLAG antibody (M2; Sigma), rabbit polyclonal anti-Grb2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-Csk antibody (BD Biosciences, San Jose, CA), mouse monoclonal anti-phosphotyrosine antibody (4G10; Upstate, Lake Placid, NY), mouse monoclonal anti-His antibody (Qiagen, Hilden, Germany), and rabbit polyclonal anti-Fyn antibody (Cell Signaling Technology, Danvers, MA) were obtained commercially. A rabbit polyclonal anti-Neph1 antibody was raised against a COOH-terminal peptide of 20 amino acids, CSYGQHSDYQGHRFFQRMQTH (the first cysteine is not part of the Neph1 sequence), coupled to keyhole limpet hemocyanin. The antisera was affinity purified using the immunogen coupled to a SulfoLink column (Pierce). A rabbit polyclonal phosphospecific antibody (anti-pY637) was raised against the high pressure liquid chromatography-purified synthetic oligopeptide CDPTNGpYYNVRHA. The antisera was affinity purified by the immunogen described above and absorbed with non-phosphorylated peptide CDPTGYYNVRHA. Western blotting was carried out with these antibodies diluted at 1/2000. PP2 was obtained from Biomol (Plymouth Meeting, PA). SU6656 was obtained from Calbiochem.

**Cell Culture and Transfection**—HEK293T cells were purchased from the ATCC (Manassas, VA). These cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s instructions. A temperature-sensitive rat podocyte cell line, 2DNAlD7, was described previously (28). The cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 10% fetal calf serum and insulin/transferrin/selenium A (Invitrogen).

**Eukaryotic Expression Constructs**—The following plasmids were prepared. For full-length Neph1-Flag, a cDNA fragment coding for full-length rat Neph1 was amplified by PCR using primers (5'-ccggaattccgcctgagttgtctctgtctgtgctc-3' and 5'-ccgctcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctgtcagctg...
Tyrosine Phosphorylation of Neph1

Animals—All the experiments using animal models were carried out according to the guidelines set by the Animal Center of the Institute of Medical Science, the University of Tokyo. Perfusion of rat kidneys with PS was carried out essentially as previously described (32). Six-week-old male Wistar rats were purchased from Charles River Laboratories Japan, Inc. (Atsugi, Japan). The rats were anesthetized with pentobarbital. Kidneys were perfused through the aorta at 5 ml/min with Hanks’ balanced salt solution for 20 min followed by PS solution (500 μg/ml in Hanks’ balanced salt solution) for 20 min. The cryostat sections for immunohistological study and glomerular lysates were prepared as previously described (32). Induction of puromycin aminonucleoside (PAN) nephrosis was carried out as described previously (32). Young male rats (Wistar) were injected intraperitoneally with 10 mg/100 g body weight of PAN (Sigma). Animals were killed on days 2, 5, 7, and 11 for protein samples from glomeruli.

RESULTS

Characterization of Anti-Neph1C Antibody—We prepared a rabbit polyclonal antibody against rat Neph1 carboxyl-terminal 20 amino acid residues. To assess the specificity of the antibody, the full-length rat Neph1 cDNA was cloned and transiently expressed as Neph1-Flag in HEK (human embryonic kidney) 293T cells. A portion of the cell lysates was subjected to immunoblotting with the antibody. As shown in Fig. 1A, the anti-Neph1C antibody recognized a protein with an apparent molecular mass of ~100 kDa in the Neph1-Flag-transfected cells but not in the empty vector transfectants. Rat glomeruli were isolated using a sieving protocol and their extracts were analyzed similarly. A protein with a slightly slower mobility than overexpressed Neph1-Flag in 293T cells, possibly due to some difference in post-translational modification, was identified.

Because COOH-terminal 20-amino acid sequences of Neph1 and Neph2 show 52.4% identity, we examined whether this antibody cross-reacts with Neph2 or nephrin using lysates from 293T cells expressing FLAG-tagged Neph1, Neph2, or nephrin. As shown in Fig. 1B, the anti-Neph1C antibody specifically reacted with Neph1, but not with Neph2 or nephrin. We immunostained kidney sections isolated from normal rats with this antibody (Fig. 1C). Neph1 was highly expressed in podocytes and also in surrounding cells at a relatively low level.

Neph1 Is Phosphorylated by Src Family Tyrosine Kinases in Cultured Rat Podocytes—As has been demonstrated in other cell-cell junctions, SD components are targets for tyrosine phosphorylation. Therefore, we expected that treatment with a tyrosine phosphatase inhibitor, pervanadate, may induce the phosphorylation of Neph1 in cultured rat podocytes (2DNA1D7). Neph1 was immunoprecipitated with anti-Neph1C from the cell lysates, and the immunoprecipitates were immunoblotted with anti-Neph1C or anti-phosphotyrosine (4G10). As shown in Fig. 2A, tyrosine phosphorylation of Neph1 was markedly increased in vanadate-treated podocytes. Previous studies have demonstrated that the SD protein nephrin is tyrosine-phosphorylated by SFKs (19). TRPC6, mutations of which cause familial focal segmental glomerular sclerosis, is also phosphorylated by Fyn (22). Moreover, gene...
targeting of \textit{fyn} or \textit{yes} results in partial effacement of podocyte foot processes (19, 25). These observations prompted us to further investigate the effect of SFK-specific inhibitors on the tyrosine phosphorylation of endogenous Neph1. As shown in Fig. 2B, PP2 completely abolished pervanadate-stimulated tyrosine phosphorylation of Neph1. SU6656, a more specific SFK inhibitor, also largely inhibits tyrosine phosphorylation. These data suggest that a Src family tyrosine kinase or kinases, at least in part, are responsible for the phosphorylation of Neph1 in podocytes.

\textit{Fyn Binds to and Phosphorylates Neph1—}Among the members of SFK, Fyn has been shown to be essential for SD integrity and podocyte structure (19, 25). Thus, we examined whether Fyn phosphorylates Neph1. We transiently cotransfected 293T cells with full-length Neph1 and either a kinase-active form (YF) or a kinase-dead form (KN) of Fyn. When coexpressed with active Fyn, some portion of Neph1 exhibited an upward mobility shift (Fig. 3A). Anti-phosphotyrosine antibody reacted

\textit{FIGURE 1. Detection of Neph1 with the anti-Neph1C antibody.} A, lysates from isolated rat glomeruli, untransfected 293T cells, 293T cells transiently transfected with plasmid encoding Neph1, or a control vector were separated by SDS-PAGE (10%), transferred to nitrocellulose, and immunoblotted with anti-Neph1C (left panel) or anti-Neph1C preabsorbed with the peptide used for immunization (right panel). B, lysates from 293T cells transiently transfected with plasmid encoding FLAG-tagged Neph1, Neph2, or nephrin were immunoprecipitated by anti-FLAG antibody, and immunoprecipitates were immunoblotted with anti-FLAG or anti-Neph1C antibody. C, indirect immunofluorescence microscopy was performed to detect Neph1 in adult rat kidney cryosections with anti-Neph1C antibody. This antibody specifically labels glomerular podocytes.

\textit{FIGURE 2. Tyrosine phosphorylation of endogenous Neph1 in pervanadate-treated cultured podocytes.} A, lysates from cultured podocytes treated with or without 1 mM pervanadate for 15 min were immunoprecipitated with anti-Neph1C antibody, and the immunoprecipitates were immunoblotted with anti-Neph1C or anti-phosphotyrosine (p-Tyr) antibody. B, where indicated, cells were pretreated with 5 \mu M SU6656 for 60 min or 10 \mu M PP2 for 15 min prior to treatment with pervanadate. Anti-Neph1C immunoprecipitates (IP) were immunoblotted with anti-phosphotyrosine or anti-Neph1C antibody. WB, Western blot.

\textit{FIGURE 3. Neph1 is bound to and phosphorylated by the Src family tyrosine kinase Fyn.} A, 293T cells were transfected with the indicated plasmids encoding an active (YF) or inactive (KN) form of Fyn together with wild-type Neph1. Cell lysates were analyzed by immunoblotting with anti-Neph1C antibody or anti-phosphotyrosine (p-Tyr) antibody. B, 293T cells were transfected with the indicated plasmids, and anti-FLAG immunoprecipitates (IP) or cell lysates were analyzed by immunoblotting with the indicated antibodies. C, 293T cells were transfected with Neph1-Flag together with either the inactive or active form of Fyn. Anti-FLAG immunoprecipitates and cell lysates were analyzed by immunoblotting with antibodies as indicated. D, Fyn binds to Neph1 in vitro. GST or GST-tagged Neph1 cytoplasmic domain (GST-Neph1CD; amino acids 585–755) were immobilized on glutathione beads, and phosphorylated by His-tagged Fyn (active form) in vitro. GST pull-downs were immunoblotted with anti-His antibody. WB, Western blot.
with these upward-shifted bands, suggesting that Fyn phospho-
rylated Neph1 under these conditions. Under the same condi-
tions, Fyn was coimmunoprecipitated with Neph1 (Fig. 3
B).

Kinase-dead Fyn neither induced phosphorylation of Neph1
(Fig. 3A) nor bound to Neph1 at all (Fig. 3C), indicating that the
interaction between Neph1 and Fyn depends on Fyn kinase
activity.

To examine whether the binding of Fyn to Neph1 is direct or
not, we performed pull-down assays using GST-Neph1CD
(cytoplasmic domain, amino acids 585–755) and His-tagged
Fyn. As shown in Fig. 3D, Fyn bound to GST-Neph1CD but not
to GST, indicating the direct interaction between these two
proteins.

Identification of Tyrosine Residues of Neph1 Phosphory-
lated by Fyn—We next determined the tyrosine residues
phosphorylated by Fyn. To identify these sites, we performed
in vitro phosphorylation of Neph1 by recombinant active
Fyn, and confirmed that Neph1CD was tyrosine-phospho-
rlyated by Fyn in vitro (Fig. 4A). These samples (phosphory-
lated and non-phosphorylated Neph1CD) were digested with
trypsin and their peptide mass fingerprints were compared. Fig.
4B shows the peptide mass spectra of non-phosphorylated
(upper panel) and phosphorylated (lower panel) Neph1CD.

When a peptide is phosphorylated, its peptide mass should
increase by 80 Da. Occasionally, phosphorylated peptides can-
not be detected due to its low efficiency of ionization. By this
criterion, we could identify phosphorylated peptides by com-
paring these two spectra. Peptides of 970.5 Da (corresponding
to amino acids 651–658) and 1500.7 Da (602–614) (indicated
by arrows) were not detected in the phosphorylated sample,
and a significant decrease in peak intensity was observed for a
peptide of 1198.5 Da (632–641; an arrowhead). Because two of
these three peptides contain two tyrosine residues (Fig. 4C),
we further performed MS/MS analysis to identify the exact tyro-
sine residues that were phosphorylated by Fyn. Phosphoryla-
tion of Tyr637 and Tyr638 (Fig. 5) as well as Tyr604 and Tyr654
(data not shown) was confirmed unambiguously.

To evaluate the phosphorylation of these tyrosine residues in
intact cells, we introduced a series of single phenylalanine sub-
stitutions for tyrosine, Y604F, Y637F, Y638F, Y654F, and Y657F
into FLAG-tagged Neph1CD (amino acids 585–789), and
expressed these proteins together with Fyn in 293T cells (Fig.
6). Tyrosine phosphorylation of Y604F, Y637F, and Y654F was
clearly reduced compared with wild-type cells, suggesting that
these tyrosine residues are phosphorylated by Fyn in intact
cells.
Identification of an SH2 Domain Containing Proteins That Associate with Phosphorylated Neph1—Having established that Neph1 is tyrosine-phosphorylated by Fyn, we searched for molecules that bind to Neph1 upon tyrosine phosphorylation. Because Fyn plays an essential role in the establishment of SD integrity, the binding partner(s) of tyrosine-phosphorylated Neph1 may also play important roles. To this end, we performed pull-down analysis. GST or GST-Neph1CD were immobilized on glutathione-Sepharose beads, and phosphorylated by Fyn in vitro. These samples were used to pull-down binding proteins from rat glomerular lysates. Proteins trapped on the beads were analyzed by immunoblot using antibodies against several SH2 domain containing proteins. Of these, an adapter protein Grb2 and a tyrosine kinase Csk specifically bound to Neph1 in a phosphorylation-dependent manner (Fig. 7A). Binding of phosphorylated Neph1 with Grb2 or Csk was also observed when 293T cell lysates were used (data not shown).

Tyr637 and Tyr638 Are Critical for Grb2-Neph1 Interaction—Neph1 contains a putative Grb2 SH2 binding motif starting at Tyr637 (YYNV), and previously Sellin et al. (10) demonstrated that Neph1 interacts with Grb2 in transiently transfected 293T cells. We performed coimmunoprecipitation experiments to examine whether the interaction between Neph1 and Grb2 is phosphorylation-dependent. 293T cells were transfected with Neph1-Flag or nephrin-Flag together with or without Fyn (Fig. 7B). Grb2 was coimmunoprecipitated with phosphorylated Neph1, but not with non-phosphorylated Neph1 nor phosphorylated nephrin. Neph1 is phosphorylated by Fyn at Tyr637 and Tyr638 (Figs. 4–6) and these tyrosine residues of Neph1 are in the consensus binding motif for Grb2. Therefore, we examined the effect of phenylalanine substitution for these residues on the Neph1-Grb2 interaction. The substitution of either Tyr637 or Tyr638 with phenylalanine completely abolished the Neph1-Grb2 interaction (Fig. 7C), indicating that these two tyrosine residues are essential for this interaction.

Csk Also Directly Binds to Phosphorylated Neph1—Next, the interaction between Neph1 and Csk was investigated. GST or GST-Neph1CD immobilized on glutathione beads was phos-
phosphorylated by Fyn in vitro and incubated with the His-tagged SH2 domain of Csk. Bound proteins were analyzed by SDS-PAGE and immunoblotted with anti-His antibody. As shown in Fig. 8A, the Csk SH2 domain bound directly to phosphorylated Neph1. We also confirmed this interaction in transiently transfected 293T cells. Csk was specifically coimmunoprecipitated with phosphorylated Neph1, but not with phosphorylated nephrin nor non-phosphorylated Neph1 (Fig. 8B). Csk still bound to Grb2 binding-deficient mutants (Y637F and Y638F) (Fig. 8C) and other phenylalanine substitution mutants (Y604F, Y654F, and Y657F) (data not shown) of Neph1, indicating that Csk binds to other tyrosine residue(s) of Neph1.

Neph1 Negatively Regulates ERK Signaling—Grb2 forms a stable complex with Sos, a guanine nucleotide exchange factor for Ras, and activates Ras upon recruitment to appropriate tyrosine-phosphorylated adaptors or autopshosphorylated receptor tyrosine kinases via the SH2 domain (33, 34). Therefore, we examined whether the phosphorylation of Neph1 and recruitment of Grb2 to Neph1 may have some effects on the Ras-ERK pathway. To elucidate this, we evaluated the activation status of ERK in the presence of Neph1. The expression of active Fyn in 293T cells resulted in the activation of ERK (Fig. 9, third lane). Interestingly, the additional expression of wild-type Neph1 abolished this Fyn-induced ERK activation (lane 4). Furthermore, this suppression of ERK activation was partially prevented by the mutations of tyrosine residues that are critical for Grb2 binding (Y637F and Y638F), but not by other mutants, indicating that binding of Grb2 to phosphorylated Neph1 is required for the attenuation of ERK activation.

Tyr637 Is Phosphorylated in Injured Podocytes in Vivo—Several tyrosine-phosphorylated proteins in the PS-induced podocyte injury model have been described by Kurihara and others (20, 32). Therefore, we expected that treatment with PS may induce the phosphorylation of Neph1 in vivo. Neph1 was immunoprecipitated with anti-Neph1C from glomerular lysates of normal or PS-treated rats, and the immunoprecipitates were immunoblotted with anti-Neph1C or anti-phosphotyrosine
antibody specifically recognized phosphorylation of Tyr637 of Neph1 (Fig. 10). As shown in Fig. 10A, Neph1 was weakly phosphorylated in normal rats, and this tyrosine phosphorylation of Neph1 was significantly increased in PS-treated glomeruli.

To develop a reagent that would be useful to investigate the phosphorylation of the critical residue of Neph1, in vivo, a rabbot polyclonal antibody against a phosphopeptide surrounding Tyr637 was raised as described under “Experimental Procedures.” Anti-Neph1C immunoprecipitates (IP) from control or protamine sulfate-treated glomeruli were subjected to immunoblotting with anti-Neph1C or anti-phosphotyrosine antibody. B, recombinant wild-type, Y637F, or Y657F GST-Neph1CD was incubated in vitro with recombinant Fyn, followed by immunoblotting with affinity purified rabbit polyclonal anti-phospho-Neph1 antibody (anti-pY637). C, rat kidneys were perfused with protamine sulfate as in A. Glomerular lysate was immunoprecipitated with anti-Neph1C antibody and the immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-Neph1C or anti-phospho-Neph1. D, immunoblot analysis of glomerular lysates from normal and PAN-treated rat kidneys probed with anti-phospho-Neph1. WB, Western blot.

FIGURE 10. Tyr637 is phosphorylated in injured podocytes in vivo. A, Neph1 is transiently tyrosine-phosphorylated during foot process effacement in the protamine sulfate-induced podocyte injury model. Rat kidneys were perfused with protamine sulfate for 20 min as described under “Experimental Procedures.” Anti-Neph1C immunoprecipitates (IP) from control or protamine sulfate-treated glomeruli were analyzed by Western blotting (WB) with the indicated antibodies.

FIGURE 9. Suppression of Fyn-induced activation of ERK by Neph1. 293T cells were co-transfected with hemagglutinin (HA)-tagged ERK expression vector and a Fyn expression vector together with an empty expression vector, a wild-type Neph1, or each of mutant Neph1 vectors. Anti-hemagglutinin or anti-FLAG immunoprecipitates (IP) were analyzed by Western blotting (WB) with the indicated antibodies.
the members of SFKs, Fyn plays a major role in the barrier function of podocyte. First, Src, Fyn, Yes, and Lyn are expressed in podocytes, and Fyn, but not Yes is coimmunoprecipitated with nephrin (36). Verma et al. (19) reported that Fyn and Yes, but not Src, fractionate with nephrin and podacin in Triton X-100-insoluble membrane fractions in glomerular lysate. They also found that phosphorylation of nephrin is abrogated in Fyn knock-out mice. Furthermore, fyn−/− mice represent proteinuria characterized by podocyte foot process effacement with B and T lymphocyte-independent mechanisms (19, 25), indicating that intrinsic Fyn in glomeruli, possibly in podocytes, might be crucial in the maintenance of podocyte structure. Therefore, we investigated the phosphorylation of Neph1 by Fyn. We further examined whether small interfering RNA of Fyn attenuated the phosphorylation of Neph1 in vanadate-treated cultured podocytes. However, we could not detect a significant decrease in tyrosine phosphorylation of Neph1 upon small interfering RNA for Fyn (data not shown). This result is probably attributed to phosphorylation by other SFKs because vanadate non-selectively inhibits protein-tyrosine phosphatases. During the revision of this manuscript, Garg et al. (37) reported the tyrosine phosphorylation of Neph1, which is greatly but not completely abolished in fyn-deficient mice. These observations altogether suggest that tyrosine phosphorylation of SD components by Fyn plays a physiologically important role in podocyte function.

We identified Grb2 as a binding partner of phosphorylated Neph1. Grb2 is composed exclusively of Src homology domains, one SH2 domain flanked by two SH3 domains. In fibroblasts, a substantial amount of Grb2 is constitutively associated with its SH3 domain with the COOH-terminal proline-rich region of the Ras guanine nucleotide exchange factor, Sos (33, 34). Many other effector molecules can bind to the SH3 domains of Grb2 as well (38). Because recruitment of the Grb2-Sos complex to the plasma membrane by epidermal growth factor receptor stimulation regulates the Ras-ERK pathway, we examined the effect of Grb2 binding to Neph1 on ERK activation. Unexpectedly, the activation of ERK by Fyn was inhibited by phosphorylation of wild-type Neph1, and this inhibitory effect was dependent on Tyr637 and Tyr638, two residues that are critical for Grb2 binding.

The spatiotemporal regulation of ERK activity is an emerging concept in the field of signal transduction (39, 40). Recently, several negative regulators for Ras/ERK signaling have been identified, and their detailed molecular mechanisms have been analyzed (41). Sprotouty 1/2 (42, 43), Dok-3 (44), and DOC-2/DAB2 (45) inhibit the Ras-ERK pathway by sequestration of Grb2, whereas Dok-1/2 (46, 47) act by binding to Ras-GAP or by unidentified mechanisms. Unlike classical negative feedback factors that are transcriptionally induced by stimuli, these factors attenuate Ras activation in a tyrosine phosphorylation-dependent manner. Neph1 may also belong to this type of negative regulators for Ras/ERK signaling.

As a consequence of the high degree of differentiation of podocytes, it has been postulated that they, in analogy to neurons, are unable to proliferate (1). Recent studies revealed the role of ERK as a key regulator of neuronal apoptosis besides its role as a survival factor, because ERK activation promotes neuronal degeneration by causing plasma membrane damage (48). The roles of the MAP kinase family in injured podocytes are not well elucidated. Recent analysis by Koshikawa et al. (49) demonstrated that p38 MAP kinase and ERK are activated in podocyte injury models and complete inhibition of p38 MAP kinase and attenuation of ERK results in suppression of proteinuria, suggesting their deleterious effect in differentiated podocytes. In this regard, it may be an intriguing possibility that the in vivo phosphorylation of the Grb2 binding site of Neph1 (Tyr637/Tyr638) in podocyte injury protects podocytes from further damage by negatively regulating ERK signaling.

Garg et al. (37) also demonstrated that Fyn-dependent phosphorylation of Neph1, which is increased in the disease model, induces Neph1 binding to Grb2, and this complex reorganizes actin polymerization at the plasma membrane in cultured fibroblast. We have shown here using site-specific anti-phospho-Neph1 antibody that phosphorylation of the critical binding sites for Grb2 greatly increases in the disease models. These observations taken together suggest the essential role of Neph1 phosphorylation at Tyr637–Tyr638 to elicit diverse signaling pathways in living animals.

Neph1 is a member of a group of closely related proteins. Recently, other Neph family members, Neph2 and Neph3, have been reported to be involved in odorant receptor-specific segregation of axon termini, by their homophilic interaction (50). Phosphorylation of Neph2 or Neph3 is not well characterized, but the binding motif for Grb2 is not conserved in these two proteins, suggesting that negative feedback control for ERK by binding to Grb2 may be unique to Neph1.

Csk negatively regulates SFKs by phosphorylating a tyrosine residue in their carboxyl-terminal region (51). In brain and fibroblasts, Csk is translocated to the plasma membrane with the aid of a lipid-raft-associated membrane protein known as Csk-binding protein, and the membrane localization of Csk causes sustained inhibition of the SFK activity (30). Caveolin-1 is another adaptor protein that recruits Csk to the plasma membrane (52). Csk binds to caveolin-1 phosphorylated by SFK via its SH2 domain, and this binding is also known as a negative feedback control on SFK activity. We have shown here that Csk directly binds to phosphorylated Neph1. This may present another method of Csk regulation, because Neph1 is mainly localized in Triton X-100-insoluble lipid microdomain (8), where most SFK also localize. The spatiotemporal regulation of SFKs in SD may be very critical to maintain normal podocyte function in the kidney, integrating activating and inhibitory signals, but the mechanism of how tyrosine phosphorylation of SD proteins is regulated is still largely unknown. It is an intriguing hypothesis that membrane-targeted Csk modifies SFK activity at the slit diaphragm. Further studies are needed to determine the role of Csk in the regulation of tyrosine phosphorylation of SD components.

Neph1 and nephrin are essential for establishing selective glomerular filtration. The results presented here demonstrate that Neph1 as well as nephrin is tyrosine-phosphorylated at SD in vivo, which promotes the dynamic signaling complex formation depending on tyrosine phosphorylation. Interestingly, phosphorylated Neph1 binds to Grb2 and Csk, whereas phosphorylated nephrin binds to Nck. Therefore, tyrosine-phos-
phorylated Neph1 and nephrin at SD might regulate distinct signaling pathways through binding to different SH2 domain proteins. Thus, phosphorylation by SFK may orchestrate a wide spectrum of signaling pathways by various protein-protein interactions. Further studies of phosphorylation of SD will contribute to a better understanding of the molecular pathogenesis of proteinuria.

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