Interaction with Podocin Facilitates Nephrin Signaling*

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Mutations of NPHS1 or NPHS2, the genes encoding for the glomerular podocyte proteins nephrin and podocin, cause steroid-resistant proteinuria. In addition, mice lacking CD2-associated protein (CD2AP) develop a nephrotic syndrome that resembles NPHS mutations suggesting that all three proteins are essential for the integrity of glomerular podocytes. Although the precise glomerular function of either protein remains unknown, it has been suggested that nephrin forms zipper-like interactions to maintain the structure of podocyte foot processes. We demonstrate now that nephrin is a signaling molecule, which stimulates mitogen-activated protein kinases. Nephrin-induced signaling is greatly enhanced by podocin, which binds to the cytoplasmic tail of nephrin. Mutational analysis suggests that abnormal or inefficient signaling through the nephrin-podocin complex contributes to the development of podocyte dysfunction and proteinuria.

Hereditary nephrotic syndrome is a heterogeneous disease, characterized by heavy proteinuria and renal failure. The most severe disorder is the congenital nephrotic syndrome of the Finnish type, caused by mutations in NPHS1, the gene encoding for nephrin. The disease manifests itself with massive proteinuria in utero and nephrosis at birth (1). The nephrotic syndrome usually leads to death during the first 2 years of life unless the patient undergoes renal transplantation (2). Nephrin is an integral membrane protein located at opposing sites of the secondary foot processes formed by podocytes, a specialized epithelial cell that ensures size- and charge-selective ultrafiltration (reviewed in Ref. 3). The precise function of nephrin is unknown; however, it appears to form a zipper-like filter structure within the ~40-nm-wide slit between two foot processes (4).

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‡ Cloning of Mouse Podocin—The human podocin cDNA sequence was used to search for mouse EST clones containing podocin nucleotide sequence (standard nucleotide BLAST, www.ncbi.nlm.nih.gov/BLAST/). Clone AW106985 was identified to contain the complete coding region of mouse podocin (GenBank™ accession number AY050309).

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Materials and Methods

Materials and Methods—To generate C-terminally FLAG-tagged full-length human nephrin the 5-prime sequence was isolated by polymerase chain reaction from a human kidney library (CLONTECH, Palo Alto, CA) and inserted into clone NPHS902 kindly provided by Dr. Karl Tzara (Karolinska Institute, Stockholm, Sweden); the FLAG-tag was inserted at the 3’ end of the coding sequence to create a C-terminally FLAG-tagged construct (denoted as Nephrin.F). Membrane-bound fusion proteins of the C-terminal and N-terminal cytoplasmic domains of podocin and the C-terminal cytoplasmic domain of nephrin were generated using a pCDM5 cassette that contained the leader sequence of CD5 fused to the CH2 and CH3 domains of human IgG, followed by the transmembrane region of CD7 (10).

Co-immunoprecipitation—Co-immunoprecipitations were performed as described (11). Briefly, HEK 293T cells were transiently transfected by the calcium phosphate method. After incubation for 24 h, cells were washed twice and lysed in a 1% Triton X-100 lysis buffer. After centrifugation (15,000 × g, 15 min), cell lysates containing equal amounts of total protein were incubated for 1 h at 4 °C with the appropriate antibody, followed by incubation with 40 µl of protein G-Sepharose beads for ~3 h. The beads were washed extensively with lysis buffer, and bound proteins were resolved by 10% SDS-PAGE. Nephrin Binding—HEK 293T cells seeded in 12-well plates were transiently transfected with a luciferase reporter construct, a β-galactosidase expression vector (kindly provided by C. Cepko), and vectors directing the expression of the proteins as indicated. Total DNA amount was 1.5–2.0 µg/well. Cells were serum-starved for 12 h, harvested in cold phosphate-buffered saline, and lysed in 100 µl of reporter lysis buffer (Applied Biosystems, Norwalk, CT) for 10 min at 4 °C. Lysates were incubated with the luciferase substrates and the relative firefly luciferase activity assayed as described (11, 12). The results represent the mean ± S.D. of triplicate samples. The pCDM5 control vector was transfected into HEK 293T cells in parallel as a control. The translational efficiencies of the luciferase reporter constructs were controlled by co-transfection with a β-galactosidase expression vector. Each experiment was repeated at least three times with similar results.
Nephrin Signaling

RESULTS AND DISCUSSION

The domain architecture of the extracellular domain of nephrin, containing eight immunoglobulins, a fibronectin type III-like domain repeat, and a single transmembrane domain, suggests that nephrin is as an adhesion molecule involved in cell-cell or cell-matrix interactions. Based on the structure of the glomerular podocyte slit diaphragm (12) and the electron microscopic localization of nephrin (4), it was suggested that the N-terminal six immunoglobulin repeats of nephrin form interdigitating zipper-like homophilic interactions (4, 13). The C-terminal domain of nephrin is relatively short but contains several potential tyrosine phosphorylation sites. In particular, Tyr1176, Tyr1193, and Tyr1210 are predicted to serve as potential docking sites for SH2 domain-containing adapter and signaling molecules (cansite.bidmc.harvard.edu/, medium stringency).

Because overexpression of surface receptors can initiate signaling in a ligand-independent fashion (14, 15), we assessed the activity of AP-1 transcription factor in HEK 293T cells expressing nephrin. Fig. 1 demonstrates that nephrin caused a substantial increase (~20-fold) in AP-1-dependent luciferase activity. In contrast, serum triggered a 3-fold increase (data not shown), whereas podocin and CD2AP alone had only marginal effects on AP-1 activation (Fig. 1). Interestingly, podocin synergistically augmented nephrin-mediated AP-1 activation, yielding a nearly 40-fold transactivation of the luciferase construct. Western blot analysis revealed that this augmentation was not the result of altered protein levels (Fig. 1C). In contrast, CD2AP had only a marginal effect on nephrin-mediated AP-1 activation (Fig. 1B) and did not further augment the synergism between nephrin and podocin (data not shown).

AP-1 is composed of homodimers and heterodimers of Jun, Fos, or activating transcription factor family members and modulates a variety of cellular programs, including proliferation, differentiation, and apoptosis (reviewed in Ref. 16). Using AP-1 as a downstream target of nephrin signaling in combination with dominant-negative mutations of protein kinases and small G-proteins, we determined that nephrin activates stress-activated p38 protein kinase as well as c-Jun N-terminal protein kinase JNK (17, 18). As demonstrated in Fig. 2A, dominant-negative mutants of small G-proteins (Cdc42, Rac1, and RhoA (SOS DN), as well as MKK4 (MKK4 DN) and a combination of MKK3 and MKK6 (MKK3/6 DN). The dominant-negative mutant of MEK1 (MEK1 DN) did not affect nephrin-mediated AP-1 signaling. These results suggest that the nephrin-mediated AP-1 activation involves the activation of p38 and JNK but not ERK1/ERK2. B, nephrin/podocin-mediated AP-1 activation was inhibited by the same set of dominant-negative protein kinases, indicating that the signaling pathways activated by nephrin and the nephrin/podocin combination are identical. C, monitoring of phosphorylation of p38 and c-Jun confirms that nephrin activates p38 and JNK. HEK 293T cells were transfected with expression plasmids as indicated. Cellular lysates were separated on SDS-PAGE. Western blot analysis was performed, using the FLAG-specific M2 monoclonal antibody.

FIG. 1. Podocin augments the nephrin-mediated AP-1 activation. A, HEK 293T cells were transfected with an AP-1-dependent luciferase construct and expression plasmids as indicated. Podocin triggered a modest (~less than 5-fold) increase in luciferase activity, whereas nephrin stimulated an ~20-fold increase. The combination of nephrin and podocin was synergistic, reaching a nearly 40-fold increase in luciferase activity (***, p < 0.001). B, CD2AP had no significant effect on nephrin-mediated AP-1 activation. HEK293T cells were transfected with an AP-1-dependent luciferase construct and expression plasmids as indicated. CD2AP triggered only a marginal increase in luciferase activity and did not increase the nephrin-mediated AP-1 activation.

C, augmentation of nephrin signaling by podocin was not the result of altered protein expression. HEK 293T cells were transfected with FLAG-tagged proteins as indicated. Cellular lysates were separated on SDS-PAGE. Western blot analysis was performed, using the FLAG-specific M2 monoclonal antibody.

were centrifuged at 14,000 rpm for 5 min to remove insoluble material. Luciferase activity was determined using a commercial assay system (Applied Biosystems, Norwalk, CT) and normalized for -galactosidase activity to correct for transfection efficiency. Equal expression of proteins was ensured by Western blot analysis.

Mitogen-activated Protein Kinase Phosphorylation—To determine the phosphorylation status of p38 and c-Jun, dually phosphorylated p38 and phosphorylated c-Jun were visualized by Western blot analysis, using phosho-specific antiserum (New England Biolabs). Equal loading was confirmed by reprobing the membrane with the non-phospho-antibodies and by Amido Black staining. The degree of phosphorylation of p38 and c-Jun was quantified by densitometry of non-saturated radiographs with the NIH Image software.

The nephrin-mediated AP-1 activation involves activation of the stress-activated protein kinases p38 and JNK. A, the nephrin-mediated AP-1 activation was significantly inhibited by dominantly-negative (DN) mutants of Cdc42, Rac1, and RhoA (SOS DN), as well as MKK4 (MKK4 DN) and a combination of MKK3 and MKK6 (MKK3/6 DN). The dominant-negative mutant of MEK1 (MEK1 DN) did not affect nephrin-mediated AP-1 signaling. These results suggest that the nephrin-mediated AP-1 activation involves the activation of p38 and JNK but not ERK1/ERK2. B, nephrin/podocin-mediated AP-1 activation was inhibited by the same set of dominant-negative protein kinases, indicating that the signaling pathways activated by nephrin and the nephrin/podocin combination are identical. C, monitoring of phosphorylation of p38 and c-Jun confirms that nephrin activates p38 and JNK. HEK 293T cells were transfected with expression plasmids as indicated. Cellular lysates were separated on SDS-PAGE. Western blot analysis was performed, using the FLAG-specific M2 monoclonal antibody.
combination with podocin (Fig. 2B), suggesting that podocin increases the efficiency of nephrin signaling without recruiting additional signaling molecules. To confirm these results, we monitored the phosphorylation status of p38 and c-Jun, using phosphospecific antisera. As demonstrated in Fig. 2C, nephrin triggers phosphorylation of p38 and c-Jun that is significantly augmented by podocin. Taken together, these results strongly suggest that nephrin is a signaling molecule that can activate canonical mitogen-activated protein kinase cascades.

The structural similarities of podocin to other stomatin-like proteins suggest that podocin forms homo-oligomers to facilitate recruitment of nephrin to specialized membrane domains. We speculated therefore that the synergistic effect of podocin on nephrin signaling results from a direct interaction between podocin and nephrin in vitro. To determine the significance of AP-1 signaling in congenital nephrotic syndrome caused by nephrin mutations, we truncated nephrin at amino acid 1160, corresponding to the most C-terminal nephrin truncation reported in patients to date (9, 19). This truncation deletes the three potential SH2-binding sites but retains several other potential tyrosine and threonine phosphorylation sites. As demonstrated in Fig. 3C, the truncated nephrin bound only marginal amounts of podocin, indicating that a sufficient interaction between podocin and nephrin requires the C-terminal 81 amino acids of nephrin. In contrast, neither nephrin nor podocin interacted with CD2AP (Fig. 3D), and CD2AP did not affect the binding between nephrin and podocin (data not shown). Surprisingly, the nephrin R1160X truncation promotes a high level of AP-1 activation that was not significantly different from wild-type nephrin (Fig. 3E). Because this nephrin mutation failed to bind podocin, we speculated that one important function of podocin is the augmentation of nephrin signaling. To test this hypothesis, the nephrin R1160X truncation was co-expressed with podocin. As demonstrated in Fig. 3E, podocin failed to significantly augment the AP-1 activation mediated by the nephrin R1160X mutant, whereas both podocin and the nephrin mutation were well expressed (Fig. 3F).

Our results suggest that the function of nephrin is contingent on three requirements: expression of functionally intact protein, targeting of nephrin to the secondary foot processes, and efficient signaling of nephrin originating at the slit diaphragms. Most nephrin mutations, including the Fin-major and Fin-minor mutations, result in the absence of protein (20), thereby completely abrogating nephrin function. In contrast, the nephrin R1160X mutation appears to be well expressed, at least in a heterologous expression system, and retains the ability to activate AP-1. However, it fails to interact with podocin. Based on the predicted structure of podocin and its potential to form membrane-associated homo-oligomers, podocin may serve two closely related functions: the recruitment and/or stabilization of nephrin at the podocyte foot process, and the augmentation of nephrin signaling, perhaps by organizing specialized nephrin-containing microdomains. It is therefore conceivable that nephrin signaling is preserved in patients with NPHS2 mutations but greatly reduced. We assayed the forma-
tion of AP-1 transcriptional activity as a marker for cellular activation; however, it is likely that signaling by the nephrin-podocin complex triggers additional cellular signaling cascades and programs aimed to preserve the structural integrity of the podocyte slit diaphragm. CD2AP did not contribute to the AP-1 activation mediated by the nephrin-podocin complex. However, it is possible that CD2AP is ultimately required to activate the specific downstream target of nephrin-podocin signaling, essential for normal podocyte function. Overexpression of nephrin±podocin in a heterologous cell system may prove to be an efficient way to identify crucial signaling cascades and target genes that maintain podocyte function and integrity.

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