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Nephrin mediates actin reorganization via phosphoinositide 3-kinase in podocytes

J Zhu[1], N Sun[1], L Aoudjit[1], H Li[1], H Kawachi[2], S Lemay[1] and T Takano[1]

[1]*Department of Medicine, McGill University, Montreal, Quebec, Canada and* [2]*Department of Cell Biology, Institute of Nephrology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

Nephrin is a slit diaphragm protein critical for structural and functional integrity of visceral glomerular epithelial cells (podocytes) and is known to be tyrosine phosphorylated by Src family kinases. We studied the role of phosphoinositide 3-kinase (PI3K), activated via the phosphorylation of nephrin, in actin cytoskeletal reorganization of cultured rat podocytes. Phosphorylation of rat nephrin by the Fyn kinase markedly increased its interaction with a regulatory subunit of PI3K. Stable transfection of rat nephrin in the podocytes with podocin led to nephrin tyrosine phosphorylation, PI3K-dependent phosphorylation of Akt, increased Rac1 activity, and an altered actin cytoskeleton with decreased stress fibers and increased lamellipodia. These changes were reversed with an inhibitor of PI3K and not seen when the nephrin-mutant Y1152F replaced wild-type nephrin. Rac1 and Akt1 contributed to lamellipodia formation and decreased stress fibers, respectively. Finally, in the rat model of puromycin aminonucleoside nephrosis, nephrin tyrosine phosphorylation, nephrin-PI3K association, and glomerular Akt phosphorylation were all decreased. Our results suggest that PI3K is involved in nephrin-mediated actin reorganization in podocytes. Disturbed nephrin–PI3K interactions may contribute to abnormal podocyte morphology and proteinuria.

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Correspondence: *T Takano, Department of Medicine, McGill University, 3775 University Street, Room 236, Montreal, Quebec, Canada H3A 2B4. E-mail: tomoko.takano@mcgill.ca*

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Visceral glomerular epithelial cells (GEC, also known as podocytes) play a central role in maintaining the selective filtration barrier of the renal glomerulus. Podocytes project numerous actin-rich processes called 'foot process.' Foot processes from adjacent podocytes form tight interdigitation and surround and support glomerular capillaries. Nephrin is a transmembrane protein, which belongs to the Ig superfamily and is localized at the slit diaphragm, which connects foot processes from adjacent podocytes.[1] Mutations of nephrin cause congenital nephrotic syndrome of the Finnish type;[2] thus nephrin has a pivotal role in glomerular permselectivity. Nephrin molecules from adjacent foot processes interact with each other in an antiparallel, homophilic manner, serving as a structural backbone of the slit diaphragm.[3] In addition to its structural role, research efforts in the recent years unraveled the importance of nephrin as a component of the slit diaphragm protein complex, which transmits signals into the cells.[4] The cytoplasmic domain of nephrin consists of approximately 150 amino acids and contains several tyrosine residues, six of which are conserved among human, mouse, and rat.[5] We and others have reported that the cytoplasmic domain of nephrin is tyrosine phosphorylated by the Src family kinase Fyn.[5–7] Tyrosine phosphorylation modulates the interaction of nephrin with other proteins such as another slit diaphragm protein, podocin,[5] adaptor protein, Nck,[8–10] and phosphoinositide 3-kinase (PI3K).[11] It is noteworthy that many of the nephrin-interacting proteins are known for their roles in actin regulation, suggesting the important role of nephrin in regulating the actin cytoskeleton and podocyte morphology.[12]

PI3K phosphorylates phosphatidylinositol lipids at the D-3 position of the inositol ring and converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate $PI(4,5)P_2$ (PIP_2) into phosphatidylinositol-3,4,5-trisphosphate $PI(3,4,5)P_3$ (PIP_3).[13] PI3K consists of the two subunits, that is the regulatory p85 subunit (PI3K-p85) and the catalytic p110 subunit (PI3K-p110). The p85 interacts with phosphotyrosine containing motifs of activated growth factor receptors or adaptor proteins, bringing the p110 to the plasma membrane, the site of its enzymatic action.[13] PIP_3 generated by PI3K recruits pleckstrin homology containing proteins such as Akt and Rac guanine nucleotide exchange

factors (GEF) to the plasma membrane, initiating downstream signaling cascades.[13] Akt, generally considered as one of the main effectors of PI3K, is best known for its antiapoptotic/prosurvival actions.[14] In addition, a growing body of evidence indicates that Akt regulates the actin cytoskeleton and cell motility; Akt promotes the formation of lamellipodia and cell migration via its binding partner, Girdin, in Vero fibroblasts.[15] Similarly, activation of PI3K leads to decreased stress fibers and increased lamellipodia/filopodia via Akt and p70S6K in chicken embryo fibroblasts.[16] Akt also stimulates cell migration via modulating the interaction between Pak1 and Nck in HeLa cells.[17] A recent proteomics approach confirmed the direct interaction of Akt and actin in MCF-7 breast cancer cells.[18] In this study, the authors also demonstrated that the cortical remodeling of actin associated with cell migration was reversed by small interfering RNA directed against Akt. Furthermore, recent studies suggest the isoform-specific actions of Akt; Akt1-deficient cells showed increased stress fibers and decreased cell migration, whereas Akt2-deficient cells showed increased membrane ruffling and migration. Akt2 appeared to contribute to the inhibition of Pak1 and Rac1 as well.[19] Rac1 belongs to the Rho family of small GTPases. As many Rac GEFs (for example, Tiam1) are activated by PIP_2 or PIP_3,[20] Rac1 can also be activated by PI3K. Rac1 is one of the key regulators of the actin cytoskeleton in mammalian cells. In particular, Rac1 has been shown to mediate lamellipodia formation and membrane ruffling in response to growth factor stimulation.[20] In addition, Rac1 activity appears to be essential in regulating cell–extracellular matrix interaction and cell migration.[20]

A previous study by Huber *et al.*[11] showed that nephrin interacts with PI3K in a tyrosine phosphorylation manner, leading to the activation of Akt and increased cell survival. However, in this study, the precise mapping of phosphotyrosine-containing motifs in nephrin responsible for its interaction with PI3K was not reported. Also, potential consequences of PI3K activation other than cell survival were not addressed. In this study, we characterized a tyrosine phosphorylation-dependent interaction between rat nephrin and PI3K-p85 and focused on the impact of PI3K activation on the actin cytoskeleton. We demonstrated that nephrin–PI3K interaction leads to the activation of the Akt and Rac1 pathways, resulting in the remodeling of the actin cytoskeleton in cultured rat GEC.

RESULTS

Tyrosine 1152 is responsible for interaction of rat nephrin and PI3K-p85

We and others have reported that the cytoplasmic domain of nephrin is tyrosine phosphorylated by Src family kinases.[5–7] It was also reported that human nephrin interacts with PI3K in a tyrosine phosphorylation-dependent manner.[11] We first confirmed these results with rat nephrin. Wild-type, full-length rat nephrin and PI3K-p85 were transiently expressed in Cos-1 cells with or without the Src family kinase Fyn. Nephrin was strongly tyrosine phosphorylated only in the presence of Fyn (Figure 1a), consistent with our previous results.[5] Nephrin co-immunoprecipitated with PI3K-p85 in the presence but not in the absence of Fyn (Figure 1a). We also obtained similar results using the chimeric construct, in which the extracellular domain of the human interleukin-2 receptor is connected to the transmembrane/cytoplamic domain of rat nephrin (Tac/nephrin),[5] in the place of wild-type nephrin (data not shown). These results indicate that the cytoplasmic domain, but not the extracellular domain of nephrin, interacts with PI3K-p85 in a tyrosine phosphorylation-dependent manner.

To identify the binding site(s) for PI3K-p85, we analyzed the protein sequence of the cytoplasmic domain of nephrin by Motif scan analysis (http://scansite.mit.edu). This program identifies potential interacting proteins for various protein motifs in a given molecule. The results suggested that a motif containing Y1152 of rat nephrin (corresponding to Y1153 in mouse and Y1139 in human) is a likely binding site for PI3K-p85. Thus, we generated a nephrin Y1152F mutant and expressed it in Cos-1 cells with p85 and Fyn. In contrast to wild-type nephrin, the Y1152F mutant failed to co-immunoprecipitate with PI3K-p85 even in the presence of Fyn (Figure 1b). We showed previously that the Y1204F mutant of nephrin is tyrosine phosphorylated by Fyn significantly less than wild type and that its ability to interact with podocin is also significantly reduced.[5] Thus, for comparison, we studied the Y1204F mutant for its ability to interact with PI3K-p85. The Y1204F mutant co-immunoprecipitated with p85 in a Fyn-dependent manner similar to wild-type nephrin (Figure 1b). We have also tested other nephrin mutants including Y1127F (Figure 1b), Y1171F, and Y1194F (data not shown). All the mutants tested other than Y1152F co-immunoprecipitated with p85 in a Fyn-dependent manner. These results indicate that tyrosine phosphorylation of Y1152, but not the other tyrosine residues, plays a critical role in the nephrin (rat)–p85 interaction.

Nephrin activates Akt via PI3K

Nephrin is a transmembrane protein. Thus, by analogy to growth factor receptors, its interaction with PI3K-p85 is likely to activate the catalytic activity of p110, leading to the increased local concentration of PIP_3 and the activation of downstream signaling cascades such as the Akt pathway. To study whether nephrin activates Akt, we used cultured rat GEC and studied the activity of Akt using the antibody specific to the active form of Akt, which is phosphorylated at Ser473. In a subclone of GEC, which stably overexpress podocin and nephrin (GEC-P/N), nephrin was clearly tyrosine phosphorylated without any stimulation (Figure 2a). In these cells, phosphorylation of Akt was increased, as compared with GEC-neo (vector-transfected control; Figure 2a) or with GEC-P (overexpressing podocin alone; Figure 2b). Akt phosphorylation was not different between GEC-neo and GEC-P, suggesting that overexpression of podocin alone does not have an impact on Akt phosphorylation/