Nek8 Regulates the Expression and Localization of Polycystin-1 and Polycystin-2

Eisei Sohara,* Ying Luo,* Jingjing Zhang,* Danielle K. Manning,† David R. Beier,† and Jing Zhou*

*Renal Division and †Division of Genetics, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Nek8 is a serine/threonine kinase that is mutated in the jck (juvenile cystic kidneys) mouse, a model of autosomal recessive juvenile polycystic kidney disease, but its function is poorly understood. We used the jck mouse to study the functional relationship between Nek8 and other proteins that have been implicated in polycystic kidney diseases. In the collecting tubules and collecting ducts of wild-type mice, we found that Nek8 was localized to the proximal portion of primary cilia and was weakly detected in the cytosol. In the jck mutant, however, Nek8 was found along the entire length of cilia. Coimmunoprecipitation experiments demonstrated that Nek8 interacted with polycystin-2, but not with polycystin-1, and that the jck mutation did not affect this interaction. Western blot analysis and real-time reverse transcriptase PCR revealed that the protein and mRNA expression of polycystin-1 (PC1) and polycystin-2 (PC2) were increased in jck mouse kidneys. The jck mutation also led to abnormal phosphorylation of PC2, and this was associated with longer cilia and ciliary accumulation of PC1 and PC2. Our data suggests that Nek8 interacts with the signal transduction pathways of the polycystins and may control the targeting of these ciliary proteins. Dysfunction Nek8 may lead to cystogenesis by altering the structure and function of cilia in the distal nephron.

Received September 10, 2006. Accepted August 29, 2007. Published online ahead of print. Publication date available at www.jasn.org.

E.S. and Y.L. contributed equally to this work.

Correspondence: Dr. Jing Zhou, Harvard Institutes of Medicine, Room 522, Brigham and Women’s Hospital and Harvard Medical School, 4 Blackfan Circle, Boston, MA 02115. Phone: 617-525-5860; Fax: 617-525-5861; E-mail: zhou@rics.bwh.harvard.edu

Copyright © 2008 by the American Society of Nephrology.

NIMA (never in mitosis, gene A) is a serine/threonine kinase in Aspergillus nidulans that has been shown to have a role in the progression of mitosis. Defects of NIMA cause cells to arrest in G2, whereas overexpression of NIMA results in the premature onset of mitotic events.1,2 NIMA-related kinase (Nek or Nrk) of Tetrahymena thermophila, Nrk-1 and Nrk-2, localize to the basal bodies and cilia and regulate ciliary length.3 Fa2p is a NIMA homolog from Chlamydomonas reinhardtii and has been shown to have roles in cell cycle progression and microtubule severing during deflagellation.4 Recently, Fa2p was found localized to the proximal end of cilia, in both Chlamydomonas and cultured kidney epithelial cells, and its kinase activity is required for deflagellation.5

To date, 11 Nek family members have been cloned from mouse or human cells, of which Nek2 is considered to be the closest NIMA homolog and therefore has been the most intensively studied. Nek2 is an important player in the coordination of centrosome structure and function with mitotic progression. It is also required for centrosome separation at the G2-M cell-cycle transition.6,7 Nek1, the first family member identified, was found to be mutated in the kat mouse, which develops pleiotropic effects including facial dysmorphism, dwarfin, male sterility, anemia, cystic choroid plexus, and progressive polycystic kidney disease (PKD).8,9 Another Nek family member, Nek8, was found to be...
mutated in the jck (juvenile cystic kidneys) mouse, which develops autosomal recessive juvenile PKD (ARJPKD). Human Nek8 was found overexpressed in primary breast tumors, suggesting that Nek8 is involved in cell proliferation, as are other NIMA family members. Nevertheless, the function of Nek1 and Nek8 and their role in cyst formation in PKD remain unclear.

Autosomal dominant PKD (ADPKD) is the major form of PKD, with a frequency of 1 in 400 to 1 in 1000. Polycystin-1 (PC1) and polycystin-2 (PC2) are the two molecules mutated in almost all ADPKD patients. PC1 is an integral membrane glycoprotein of approximately 460 kD that is located in the plasma membrane and cilia of renal epithelia. Proteins responsible for autosomal recessive PKD (ARPKD) are also found in cilia of renal epithelia, such as cystin for cpk (congenital polycystic kidney) mouse model, polaris for orpk (Oak Ridge polycystic kidney) mouse model, and fibrocystin in human ARPKD.

In this study, we report that the Nek8 protein is located in the proximal region of the primary cilia in mouse kidney tubules and in the same protein complex with PC2. The ciliary localization of Nek8 is observed only in the collecting tubules and collecting ducts where the cysts develop in the jck mice. The transcription for both Pkd1 and Pkd2 genes are upregulated. An increase in the expression of PC1 and PC2 in the primary cilia and abnormal phosphorylation for PC2 was seen in the jck mouse kidney. These data suggest that Nek8 modulates the normal expression of PC1 and PC2 and the phosphorylation of PC2. In addition, Nek8 controls or modulates the ciliary localization of PC1 and PC2. Mutations in Nek8 cause abnormal transcription and localization of polycystins, ultimately resulting in cystogenesis in the jck mouse kidney.

RESULTS

Nek8 Is Located in the Proximal Segment of the Primary Cilia of Renal Tubules

The affinity-purified antibody against Nek8 was used for immunohistochemistry of kidney from 3-mo-old wild-type mice. In kidney tissue, Nek8 colocalized with the primary cilia (Figure 1A). A weak intracellular signal of Nek8 was observed as well. Both cilia and intracellular signals of Nek8 could be blocked by preincubation of Nek8 antibody with its antigen peptide, whereas the signal of cilium marker remained (Figure 1B). Immunohistochemical image at high magnification revealed that Nek8 localization on the primary cilia is limited to the proximal segment (Figure 1, C to E).

Nek8 Is Absent from Primary Cilia of Proximal Tubules

To investigate the characteristic of the ciliary localization of Nek8, we double-stained kidney tissue with Nek8 and Lotus teragonolobus lectin (LTL), a proximal tubule marker or Dolichos biflorus agglutinin (DBA), a collecting tubule and collecting duct marker. Cyttoplasmic signals of Nek8 were most obvious in the LTL-positive tubules that represent proximal tubules (Figure 2, A to C). However, ciliary Nek8 was not detected in those tubules. On the contrary, clear ciliary localization of Nek8 is seen in the DBA-positive tubules that represent collecting tubules and collecting ducts, where the cytosolic signal is weak (Figure 2, D and G). Ciliary localizations of Nek8 were also observed in the collecting ducts of medulla (Figure 2H). To investigate whether there is any correlation between ciliary Nek8 expression and cyst formation in the jck mouse, we stained kidney tissue from jck mice with DBA and LTL (Figure 2, I and J). Most cysts in jck mice were positive for DBA staining and none of the cysts was stained with LTL, suggesting that ciliary Nek8 in collecting tubules and collecting ducts are responsible for cyst formation in jck mice.

Nek8 Protein Complex Contains PC2

To determine whether polycystins play a role in cyst formation in jck mice, we tested the interaction of Nek8 protein with PC1 or PC2 in vivo. Co-immunoprecipitation experiments of Nek8 and PC1 or PC2 were performed in tissue extracts from kidneys of 3-mo-old wild-type and jck mice. PC2 co-immunoprecipitated Nek8 in the lysates of the wild-type mouse kidneys (Figure 3A),
demonstrating that Nek8 was in the same protein complex with PC2 in vivo. A similar amount of Nek8 protein co-immunoprecipitated by PC2 in tissue lysates from the jck and the wild-type mice (Figure 3A) suggested that Nek8 interaction was preserved in the jck mouse. The interaction between PC1 and PC2 was present in tissue lysates of both jck and wild-type mice (data not shown).

Increased Phosphorylation of PC2 in jck Mice

Immunoprecipitation with a PC2 antibody detected an additional band just above the normal PC2 band in the jck mouse kidney, but not in wild-type mouse kidney (Figure 3H). Western analyses confirmed the presence of an additional form of PC2 in jck mice but not in the wild-type mice (Figure 4A) and an increase in PC1 expression levels (Figure 4, B and C). To determine which type of protein modification is responsible for this additional band of PC2, we performed dephosphorylation assay (Figure 4D). Treatment with protein phosphatase 1 resulted in the loss of the additional PC2 in the jck samples, indicating that PC2 is abnormally phosphorylated in jck mice. The increase of PC1 and PC2 expression was also seen in kidney samples from 15 d old mice (Supplemental Figure I). To determine whether the increased expression of PC1 and PC2 protein was caused by an increase in gene transcription or a decrease in protein degradation, we performed real-time RT-PCR of Pkd1 and Pkd2 genes. Pkd1 mRNA levels in jck mice is 9.8 ± 0.15-fold greater than in wild-type mice (P < 0.001, n = 4) (Figure 4E), indicating that increased PC1 expression is caused by increased transcription of Pkd1. This increased transcriptions of Pkd1 and Pkd2 is consistent with previous data.27 On the other hand, mRNA levels of Pkd2 in jck mice were only 1.9 ± 0.5-fold increased compared with wild-type mice (P < 0.03, n = 4) (Figure 4F). These results were consistent with Western blotting results of PC1 and PC2 expression in jck mouse kidneys.
Aberrant Ciliary Localization of Nek8, PC1, and PC2 in jck Mouse Kidney

Having observed an increased level of PC1 protein expression and PC2 phosphorylation in the jck mouse kidney, we went on to investigate their expression and subcellular localization in the kidneys by immunohistochemistry. In contrast to its restricted expression in the proximal segment of primary cilia in wild-type kidneys (Figure 5, A to C), Nek8 expression was seen throughout the length of primary cilia in jck mice (Figure 5, D to F).

Although unable to detect PC1 in primary cilia of kidney from 3-mo-old wild-type mouse kidney (Figure 5, G to I), we observed striking ciliary expression in 3-mo-old jck mice (Figure 5, J to L). PC1 localization in plasma membrane was also detected in the kidneys of jck mice (Figure 5W). We and others have previously shown that PC2 is localized to primary cilia in a variety of cultured cell lines21–24 and in the kidney.21 To our surprise, we did not detect PC2 in the cilia of tubular epithelia from wild-type mouse kidney, using either snap-frozen or perfusion-fixed kidney sections with nicely dilated lumens under our experimental conditions (Figure 5, M to O), although Nek8 was easily detectable in cilia of kidney tubules (Figure 5A). In jck mice, however, PC2 became easily detectable in the cilia (Figure 5, P to R). Consistent with a recent report,27 the cilia in jck kidney also appear to be longer than those in wild-type kidneys (Figure 5). The increased ciliary localizations of polycystins in jck mice were also observed in 15-d-old mice (Supplemental Figure I-D). These data suggest that abnormality in Nek8 results in increased PC1 and PC2 ciliary expression.

DISCUSSION

Although several proteins of the Nek family have been shown to play a role in regulating G2/M cell-cycle progression, the function of Nek8 is still not known. The finding that Nek8 is the protein mutated in the jck mouse linked Nek8 with PKD,10 which is believed to involve abnormal cell proliferation and...
differentiation. A recent study found that Nek8 as measured by RT-PCR is overexpressed in primary human breast tumors, suggesting that Nek8 is important in cell growth control. In this study, we provide the first evidence of Nek8 localization to primary cilia in vivo. Although the ciliary expression of Nek8 was recently reported in a cultured cell line and primary culture of kidney cells, a previous study of Nek8 in kidney reported cytosolic but not ciliary Nek8 expression in collecting ducts. Therefore, it was unknown whether ciliary or cytosolic Nek8 correlates with cyst formation in jck mice. We found the ciliary localization of Nek8 only in the collecting tubules and collecting ducts, which suggests that Nek8 has a function in those locations. Furthermore, DBA and LTL staining revealed that cysts in jck mice are derived from collecting tubules and collecting ducts, correlating ciliary Nek8 expression with cyst formation in these segments in jck mice. This observation is highly significant, given the evidence that a number of genes shown to be mutated in PKD are similarly localized to cilia and that defects in ciliary proteins can cause PKD. We could detect the Nek8 signal in the cytosol, as previously reported. This antibody also recognizes ciliary Nek8 in IMCD cells (Y.L. and J.Z., unpublished observations).

Immunoprecipitation analysis revealed that PC2 but not PC1 is in the same protein complex with Nek8. Although Nek8 could not co-immunoprecipitate PC1, PC1 was able to co-immunoprecipitate PC2 in both the wild-type kidney and the jck mouse kidney. PC2 (green) was not detected in cilia of WT mouse kidney but strongly expressed in cilia of jck mouse kidney. PC2 was not detected in cilia of WT mouse kidney, but clearly expressed in cilia of jck mouse kidney. PC1 in plasma membrane of cyst-lining epithelia in jck mouse kidney. Acetylated-α-tubulin was used as a cilia marker (red).
on its trafficking. In Caenorhabditis elegans, the phosphodefective mutant Pkd2 (S534A) localized to cilia, whereas a phospho-mimicking Pkd2 (S534D) mutant was largely absent from cilia.29 On the contrary, in mammalian cells both wild-type PC2 and phosphodefective mutant S812A localized to cilia, indicating phosphorylation of Serine at position 812 does not affect the trafficking of PC2 to cilia.19 Recent study has revealed that another phosphorylation site at Serine 76 in PC2 regulated cell-surface localization of PC2 in mammalian cells but not ciliary localization.20 The increased phosphorylation and ciliary localization of PC2 in jck mice suggest that protein phosphorylation may serve as a mechanism for the control of ciliary targeting of proteins. However, we cannot exclude the possibility that this ciliary localization of polycystins in jck mice is simply caused by an overflow resulting from an increased amount of polycystins. Further investigation, especially on fine characterization of phosphorylation site(s) of PC2 in jck mice, is required in future.

An accumulation of polycystins in cilia has been reported in other PKD animal models. A study of osm-5, an ortholog of the mouse ARPKD gene Tg737, revealed that the Caenorhabditis elegans ADPKD gene products accumulated in stunted cilia in the absence of osm-5.21 The study of the orpk mouse model revealed that the ciliary expression of PC2 is elevated in Tg737 mutant polycystic mouse kidneys.21 However, increased phosphorylation of polycystins has not been reported in these animal models, indicating that the aberrant phosphorylation of PC2 in jck mice may be caused by specific loss of Nek8 function.

PC1 and PC2 were clearly localized to the cilia of cyst-lining epithelia in the jck mouse kidneys, consistent with the recent report on cultured kidney cells.27 To our surprise, we could not detect ciliary expressions of PC1 and PC2 in wild-type kidneys. This may be a result of the age of mice we analyzed and a tightly regulated entry of PC1 and PC2 to cilia by proteins such as Nek8. It is known that PC1 expression is developmentally regulated, its expression level significantly decreases in wild-type mouse kidneys below the limit of immunohistochemical detection.28 Because we did observe very clear ciliary PC1 and PC2 expression in cultured cells with the same antibody,17,20 we cannot exclude the possibility that the amount of ciliary PC1 and PC2 in the wild-type kidney is below the limit of immunohistochemical detection.

We found the ciliary Nek8 to be restricted to the proximal segment of cilia in the kidney. In jck mouse, Nek8 expression was seen throughout the length of primary cilia. This finding is different from the recent report in which the authors found the loss of Nek8 in primary cilia of cultured kidney cells from jck mouse.27 This difference may be the result of differences in cell type and cell differentiation status between tissues and cultured cells. The characteristics of the proximal segment of cilia are not well investigated. It has been reported, however, that the proximal segment of olfactory cilia appears to express a small fraction of cyclic nucleotide-gated channels, whereas the ciliary distal segment contains the majority,33 indicating the existence of different characteristics between proximal and distal segments of cilia. Fa2p, a NIMA-related kinase important for cilia disassembly, localized to proximal segment of the cilia in Chlamydomonas.5 Our data showed that Nek 8 regulates cilium length and restricts polycystins entering cilia. Together with the specific localization of Nek8 to the proximal segment of primary cilia, it is tempting to hypothesize that the proximal segment may be an important site for gating the entry of proteins to the shaft of cilia. Our previous work showed that PC1 and PC2 cooperate in cilia as the machinery for sensing fluid flow, and regulating a signaling cascade induced by calcium influx.17 In the jck mouse kidney, PC1 and PC2 accumulated in cilia of renal epithelia. Although we do not know at present whether the cilia are functional in the jck mice, the longer than normal length of cilia and the identification of abnormal levels and phosphorylation of the ciliary sensory machinery in cystic kidney suggest that ciliary defects are responsible for the uncontrolled tube lumen size and abnormal kidney development in the jck mice.

CONCISE METHODS

Mice

Three-month-old wild-type or jck mice in a C57 background were used for immunostaining, Western blot analysis, and real-time RT-PCR of PC1 and PC2. Fifteen-day-old mice were used for immunostaining.

Antibodies

Affinity-purified polyclonal antibody was raised against the fusion protein of the C-terminal RCC domain (285 to 698 amino acids) of mouse Nek8 (1:200 dilution for immunofluorescence and 1:1000 for Western blotting).10 Affinity-purified polyclonal antibody MR3 raised against 2938 to 2956 amino acids of PC1 (1:200 dilution)14 was used for immunohistochemistry. For Western blotting of PC1, monoclonal antibody 7e12 to the N-terminus region of PC1 (1:600 dilution)33 and polyclonal PC1 antibody to the C-terminus region of PC1 (1:200 dilution) (sc-25570, Santa Cruz Biotechnology, Santa Cruz, CA) were used. Affinity-purified polyclonal antibodies 96525 were raised against 44 to 62 amino acids of mouse PC2 (1:200 dilution for immunofluorescence and 1:1000 for western blotting).17,20 Commercial antibodies anti-α-acetylated-tubulin antibody (Sigma-Aldrich, St. Louis, MO) was used as marker for immunofluorescence (1:5000 dilution). Fluorescent DBA and LTL were purchased from Vector Laboratories (Burlingame, CA) and used as markers for immunofluorescence (1:500 dilution).

Immunofluorescence of Tissue Sections

Formalin-fixed tissue sections were deparaffinized and rehydrated, and antigen retrieval was performed with Antigen Unmasking Solution (Vector Laboratories) according to the manufacturer’s protocol, except that we substituted 30-min boiling without high pressure for the prescribed 1-min boiling under high pressure. After antigen retrieval, the sections were preincubated with 10% goat serum in phos-
phosphate-buffered saline (PBS) and incubated with specific primary antibody in 10% goat serum in PBS for 2 h at 37°C. After washing, secondary antibodies were incubated for 1 h. Vectashield mounting medium with DAPI (Vector Laboratories) was used for DAPI staining and protection of immunofluorescence signals from fading. A Nikon TE2000-U microscope (Nikon Inc., Melville, NY) and a Hamamatsu ORCA-ER camera (Hamamatsu, Shizuoka, Japan) were used to capture images when noted.

Co-Immunoprecipitation and Western Blotting
Fresh mouse kidneys were homogenized in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). IMCD cells were homogenized in M-PER Tissue Protein Extraction Reagent (Pierce) containing protease inhibitor cocktail (Roche Diagnostics). The lysate was centrifuged at 10,000 × g at 4°C for 15 min, and the supernatant was collected. Co-immunoprecipitation and Western blotting were performed as described previously.20

For immunoblotting, we used 7e12 antibody for PC1 (1:600 dilution), C-terminus antibody for PC1 (1:200 dilution), and 96525 antibody for PC2 (1:1000 dilution). Next, filters were washed and incubated for 1 h in secondary antibodies (anti-mouse or anti-rabbit IgG horseradish peroxidase conjugated; Amersham Bioscience, Buckinghamshire, UK). Proteins were visualized with the ECL system (Hyperfilm, Amersham Bioscience). Dephosphorylation of kidney proteins was performed using a protein phosphatase 1 as recommended by the manufacturer (New England Biolabs, Beverly, MA). Kidney homogenate from jck mouse kidney were incubated with or without protein phosphatase 1 at 30°C for 2 h before denaturing. For quantitative analysis of Western blotting band, we used ImageJ software.

Real-Time RT-PCR
Primers for real-time RT-PCR of mouse Pkd1, Pkd2, and GAPDH were prepared as follows:

- mouse Pkd1: 5’ TAT CTG CAG TAC CGA CTG TGT TAC C 3’
- mouse Pkd2: 5’ TTC ACG TCC GTG AGT ACC TG 3’
- mouse Pkd1: 5’ TGT GTG GTC AGG TTA TTG GCG 3’
- mouse Pkd2: 5’ GCC AGG AGG AAA TCA AAG GC 3’

This primer set was designed to amplify cDNA from 3368 to 3500 of Pkd1 (U70209) and 1303 to 1415 of Pkd2 (NM008861). Total RNA isolated from whole kidneys of 3-mo-old mice was reverse transcribed with M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) and Random Hexamers (QIAGEN, Valencia, CA). Real-time PCR was carried out in a LightCycler (Roche Diagnostics) using QuantiTect SYBR Green PCR Kit (QIAGEN). Quantitative data were corrected with GAPDH mRNA expression level.

Short Hairpin RNA for PC2
pLKO.1-based lentiviral vectors that contain stem-loop cassettes encoding shRNAs targeted to the coding sequence (TRCN88790) of the mouse Pkd2 were obtained from the MISSION TRC shRNA library through Sigma-Aldrich. The oligonucleotide sequences of the shRNAs were as follows (21-nt stem sequences matching the target transcript are underlined; noncomplementary 6-nt loop sequences are italicized): MISSION shRNA TRCN88790, 5’-CCGCGCCTGATTGT-

REFERENCES


Supplemental information for this article is available online at http://www.jasn.org/.
Supplementary Figure 1