NIMA-Related Kinases Defective in Murine Models of Polycystic Kidney Diseases Localize to Primary Cilia and Centrosomes

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A key feature of the polycystic kidney diseases is aberrant cell proliferation, a consequence of dysfunctional ciliary signaling. The NIMA-related kinases (Nek) Nek1 and Nek8 carry the causal mutations of two of the eight established mouse models of polycystic kidneys. Nek proteins have roles in cell cycle and may contribute to coordinate regulation of cilia and cell-cycle progression. Herein is reported that in a mouse kidney epithelial cell line, mNek1 localizes to centrosomes in interphase and remains associated with the mitotic spindle pole during mitosis. In contrast, mNek8 localizes to the proximal region of the primary cilium and is not observed in dividing cells. Knockdown of mNek8 by siRNA does not affect ciliary assembly. Taken together with the phenotypes of the mutant mice, these data suggest that mNek1 and mNek8 provide links between cilia, centrosomes, and cell-cycle regulation.

The roles of Nek in ciliary regulation have been studied in the unicellular bicuspidal Chlamydomonas reinhardtii and the ciliated protozoan Tetrahymena thermophila (4,8). The Chlamydomonas Nek Fa2p, which is essential for Ca^{2+}-mediated axonemal microtubule severing, is located at a specific region of the proximal cilium, the site of flagellar autotomy (SOFA) (9). Several Tetrahymena Nek and the Chlamydomonas Nek Cnk2p are localized to cilia and regulate ciliary length (4,10). In addition to their roles in the regulation of cilia, Fa2p and Cnk2p affect the cell cycle. Cells that lack Fa2p have a G2/M cell-cycle delay and, Cnk2p affects cell size control (8). The dual roles of the Fa2p and Cnk2p kinases, taken together with the murine PKD phenotypes of Nek1 and Nek8 mutations, suggest that Nek are a direct link between cilia and centrosomes and the aberrant cell proliferation of cystic kidneys.

Here we report the localization of endogenous murine Nek1 and Nek8 (mNEK1 and mNEK8) in an inner medullary collecting duct (IMCD-3) cell line. mNek1 was observed in multiple foci associated with the centrosomes during interphase and remained associated with the microtubule organizing center at the mitotic spindle pole. In contrast, the mNek8 signal was restricted to the proximal region of the primary cilium during interphase and was not observed during mitosis. siRNA knockdown of mNek8 resulted in loss of an immunofluorescence signal at the cilia but did not affect ciliary assembly. These data support the idea that mNek1 and mNek8 are involved in ciliary cell-cycle signaling.

Materials and Methods
Cell Culture, Synchrony, and Immunofluorescence
IMCD-3 cells were grown in a 1:1 mixture of DMEM and Ham’s F12 medium supplemented with 10% FBS (all from Life Technologies, BRL, Auckland, New Zealand). For synchrony, cells that were grown to approximately 50% confluence were incubated with 2 mM thymidine...
for 18 h and then rinsed briefly with PBS. Cells were incubated with regular growth medium for 14 h, and samples were taken at 2-h intervals. For indirect immunofluorescence, cells were fixed with ice-cold methanol and incubated at −20°C for 10 min, then rehydrated in PBS. The primary antibodies used include rabbit polyclonal anti-mNek1 (diluted 100-fold) and anti-mNek8 (diluted 100-fold [11]), mouse monoclonal anti-γ tubulin (clone GTU-88, diluted 1000-fold; Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti-acetylated tubulin (clone 6–11B-1, diluted 10,000-fold; Sigma-Aldrich), and human autoimmune serum M4491 (diluted 3000-fold [12]). The secondary antibodies used include Alexa Fluor 488–conjugated goat anti-rabbit IgG (diluted 1000-fold; Molecular Probes, Eugene, OR), Alexa Fluor 594–conjugated goat anti-mouse IgG (diluted 2000-fold; Molecular Probes), Alexa Fluor 594–conjugated goat anti-human IgG (diluted 500-fold; Molecular Probes), and Cy5-conjugated goat anti-mouse IgG (diluted 500-fold; Southern Biotech, Birmingham, AL). All antibody incubations were done at room temperature for 1 h, followed by a wash in PBS. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole for 10 min and coverslips were mounted using Mowiol (Calbiochem, San Diego, CA). Immunofluorescence microscopy was performed using the Delta Vision system (Applied Precision, Issaquah, WA) as described previously (9).

RNA Interference

The siGENOME SMARTpool reagent that contained four different siRNA duplexes that target mNek8 (Dharmacon catalog no. M-044403-00) was used to transfect IMCD-3 cells according to the manufacturer's instructions. Untransfected and mock-transfected cells were used as negative controls. Cells were transfected for 24, 48, 72, and 96 h; harvested; and processed as described above.

Western Analysis

IMCD-3 cells were grown to confluence, harvested, and resuspended 1× SDS sample buffer. Immunoblot analysis using rabbit anti-mNek1 (diluted 2000-fold) was performed as described previously (9).

Results and Discussion

**mNek1 Localizes to Centrosomes during Interphase and Mitosis**

IMCD-3 cells that were stained with polyclonal rabbit anti-mNek1 antibodies showed multiple puncta associated with centrosomes during interphase (Figure 1A). In general, we observed more mNek1 foci surrounding the daughter centriole than the mother centriole, which nucleates a cilium. Similar staining was observed in the mouse fibroblast line NIH 3T3 and in the human embryonic kidney cell line HEK 293 (data not shown). During mitosis, mNek1 staining remained associated with the centrosomes in metaphase, anaphase, and cytokinesis (Figure 1A).

The multiple foci of mNek1 suggest that it is not a component of the centriole itself. To resolve whether mNek1 localization was specific to pericentriolar material, IMCD-3 cells were stained with antibodies against γ- and acetylated tubulin (red) to mark the positions of the centrioles and the cilia, respectively. mNek1 (green) was detected using a rabbit polyclonal antibody, and DNA was stained with 4′,6-diamidino-2-phenylindole for 10 min and coverslips were mounted using Mowiol (Calbiochem, San Diego, CA). Immunofluorescence microscopy was performed using the Delta Vision system (Applied Precision, Issaquah, WA) as described previously (9).

**Figure 1.** Murine NIMA-related kinase 1 (mNek1) is localized to centrosomes throughout the cell cycle. (A) Synchronized inner medullary collecting duct-3 (IMCD-3) cells were stained with antibodies against γ- and acetylated tubulin (red) to mark the positions of the centrioles and the cilia, respectively. mNek1 (green) was detected using a rabbit polyclonal antibody, and DNA was stained with 4′,6-diamidino-2-phenylindole (blue). CB, cytoplasmic bridge. Bar = 5 μm. (B) To refine the centrosomal localization of mNek1, IMCD-3 cells were stained with the centrosome-reactive human autoimmune serum M4491 (red), which identifies the pericentriolar material (PCM) tube (12,13). Cells were co-stained with antibodies against γ-tubulin (blue) and mNek1 (green). Bar = 1 μm. (C) Western analysis of IMCD-3 cells showing anti-mNek1 antibody specificity. A single band of expected molecular weight (approximately 143 kD) is observed after incubation with the rabbit anti-mNek1 antibody.
ates from centrosomes during mitosis (14), whereas mNek1 remains associated.

**mNek8 Localizes to Primary Cilia during Interphase but Is not Observed during Mitosis**

Indirect immunofluorescence of endogenous mNek8 revealed a specific signal that usually is restricted to the proximal region of primary cilia in IMCD-3 cells during interphase (Figure 2). We observed the same localization in NIH 3T3 cells (data not shown). A quantitative analysis revealed that mNek8 was present in the cilia of 96% of ciliated cells. During mitosis, no specific mNek8 staining was observed in cells that were undergoing metaphase, anaphase, or cytokinesis (Figure 2).

The ciliary localization of mNek8 is reminiscent of other proteins that are implicated in PKD. Polycystin-1 and polycystin-2 are found within primary cilia (15,16), as well as fibrocystin-1 (17,18), polaris, and cystin (16). The localization of mNek8 is also similar to Fa2p, which localizes to the base of the cilia at the SOFA in Chlamydomonas and when exogenously expressed in IMCD-3 cells (9). However, the mNek8 signal labels a broader region of the cilium than the tightly focused Fa2p signal.

**Knockdown of mNek8 Does not Affect Cilia Formation**

Renal cyst formation as a result of failure to assemble cilia is often accompanied by pleiotropic pathologies. This is apparent in the orpk mouse model, in which a hypomorphic allele of polaris/IFT88, encoding a component of the intraflagellar transport machinery, is defective in ciliary assembly (2). Because mNek8 localized to the cilia, we hypothesized that it could play a role in ciliary assembly. siRNA that targeted Nek8 was transfected into IMCD-3 cells, and mNek8 knockdown was measured at 96 h after transfection. There was a dramatic decrease in the ciliary staining of mNek8 in cells that were treated by siRNA (Figure 3A), in which only 34% of ciliated cells contained mNek8 versus 96% of untransfected and 94% of mock-transfected cells (Figure 3B). However, the percentage of ciliated cells (>90%) in the population did not change when compared with the negative controls. Staining at the plasma membrane was still observed in knockdown cells, suggesting nonspecific staining by the polyclonal mNek8 antibody. Western blot analysis of cellular protein indicated knockdown of mNek8 protein below detectable levels (data not shown).

The mNek8 knockdown indicates that ciliogenesis is unaffected by loss of ciliary mNek8 and suggests that mNek8 func-

**Figure 2.** mNek8 is localized to the proximal region of primary cilia during interphase. Synchronized IMCD-3 cells were stained for γ- and acetylated tubulin (red), mNek8 (green), and DNA (blue). No mNek8 signal was detected during mitosis and appears only in ciliated cells in interphase (interphase panel and arrow in cytokinesis panel). Bar = 5 μm.

**Figure 3.** siRNA knockdown of mNek8 does not affect ciliogenesis. (A) IMCD-3 cells were transfected with siRNA targeting mNek8 and incubated for 96 h. Cells were stained with antibodies against γ- and acetylated tubulin (red) and mNek8 (green). Bar = 5 μm. (B) Quantification of mNek8 loss from cilia. Two hundred cells from each experimental sample were analyzed for the presence of mNek8 signal in the cilium.
tion is not essential for cilia assembly. The jck mutation is a single amino acid substitution in the C-terminus of mNek8 (11), and only the kidneys are affected (19), unlike other models, in which multiple organ systems are defective. Taken with our findings that mNek8 knockdown does not affect ciliogenesis strongly suggests that mNek8 is involved in a signaling role specific to the kidney.

Our discovery that mNek1 is centrosomal and mNek8 is ciliary completes the subcellular localization studies of the seven proteins identified in the mouse models of cystic kidney disease. It is interesting that mNek1 is the only gene product of these mouse models that does not localize to the primary cilia. Many of the proteins that are implicated in renal cyst formation are not members of conserved protein families; polycystin-1 and fibrocystin-1 are novel integral membrane proteins (20,21), polycystin-2 is a novel cation channel (22), cystin is a novel lipid-anchored membrane protein (23), and inversin is a novel protein that contains ankyrin repeats and calmodulin-binding motifs (24). PKD is a ciliopathy that results in aberrant cell proliferation; therefore, conserved ciliary and cell-cycle proteins are expected to contribute to the mechanism of disease. mNek1 and mNek8 are excellent candidates because they are members of a cell-cycle kinase family that is conserved throughout ciliated eukaryotes, and, as shown here, they localize to basal bodies and cilia. Considering Fa2p and Cnk2p and their roles in regulating ciliary function and cell-cycle progression, Nek may provide a common link between cilia and cell-cycle regulation, although the cellular mechanisms remain to be established.

An important aspect of elucidating the signal transduction pathway may lie in identification of modifying loci, as genetic background greatly influences the variable PKD phenotype. Although PKD1, PKD2, and PKHD1 are the genes that are responsible for PKD, the age of onset and disease severity are greatly influenced by additional germline and somatic mutations (25). It is possible that hNek1 and hNek8 are important modifiers of PKD.

Acknowledgments

This work was funded by an operating grant from the Canadian Institutes of Health Research (MOP 37861) to L.M.Q. M.R.M. and M.L.T are supported by graduate fellowships from the Michael Smith Foundation for Health Research and the Natural Sciences and Engineering Research Council of Canada.

We are deeply indebted to David Beier for the mNek8 antibody, Yumay Chen for the mNek1 antibody, and Jerome Rattner for the M4491 anti-serum. We also thank Michel Leroux and his laboratory members for the use of their facilities.

References


