The Serine/Threonine Kinase Nek6 Is Required for Cell Cycle Progression through Mitosis*

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The Aspergillus nidulans protein NIMA (never in mitosis, gene A) is a protein kinase required for the initiation of mitosis, whereas its inactivation is necessary for mitotic exit. Here, we demonstrate that human NIMA-related kinase 6 (Nek6) is required for mitotic progression of human cells. Nek6 is phosphorylated and activated during M phase. Inhibition of Nek6 function by either overexpression of an inactive Nek6 mutant or elimination of endogenous Nek6 by siRNA arrests cells in M phase and triggers apoptosis. Time-lapse recording of the cell cycle progression of cells expressing kinase-inactive Nek6 reveals mitotic arrest at the metaphase stage prior to cells entering apoptosis. In contrast to NIMA and the closely related mammalian Nek2 kinase, which regulate centrosome function and separation, our data demonstrate an important function for Nek6 during mitosis and suggest that Nek6 kinase is required for metaphase-anaphase transition.

NIMA1 (never in mitosis, gene A) was originally identified in Aspergillus nidulans as a serine/threonine kinase critical for cell cycle progression. Temperature-sensitive mutants of NIMA or overexpression of dominant-negative forms of NIMA cause cells to arrest in G2 with uncondensed DNA and interphase microtubules (1). In addition, overexpression of NIMA in fungus as well as in mammalian cells leads to the early onset of mitotic events including chromatin condensation and depolymerization of microtubules (2). Furthermore, the appropriate degradation of NIMA is required for mitotic exit (3). The ability of NIMA to functionally regulate mitosis in higher organisms suggests the existence of a conserved NIMA-like pathway in eukaryotes. However, to date, the mammalian functional counterpart has not been identified.

The sequencing of the human genome has revealed a relatively divergent family of 11 mammalian Neks. The Neks are most closely related to NIMA in their N-terminal catalytic domain sequences, but each diverges substantially from NIMA in its non-catalytic C-terminal tail. Nek1 is highly expressed in meiotic germ cells and was proposed to play roles in meiotic events (4). Position cloning studies revealed Nek1 as the gene altered in polycystic kidney disease, although the underlying mechanism remains unknown (5). Nek2 was the first human Nek shown to play a role in mitotic progression. Nek2 probably plays a role in centrosome duplication but is dispensable for cell cycle entry (6). Endogenous Nek2 associates with centrosomes, and overexpression of Nek2 causes a striking splitting of centrosomes but does not prevent cells from entering mitosis. The functions of other Neks are largely unknown. Recently Nek6 and the closely related Nek7 were shown to phosphorylate the protein kinase p70 S6 kinase on Thr-412 within a hydrophobic motif (7). However, studies to determine the substrate specificity of Nek6 using peptides derived from annotated phosphorylation sites in human proteins suggested that Nek6 does not phosphorylate p70 S6 kinase either in vitro or in vivo (8). More recently, Nercc1 (also termed Nek8 in Ref. 9) was identified by association with Nek6 and Nek7. Microinjection of anti-Nercc1 antibodies in prophase causes spindle abnormalities and/or chromosomal misalignment suggesting that Nercc1, and possibly also Nek6 and Nek7, may play a role in mitotic regulation (10).

In this study, we find that Nek6 kinase activity is up-regulated in M phase. Expression of an inactive Nek6 mutant arrested cells in M phase and interfered with chromosome segregation. Depletion of endogenous Nek6 protein using siRNA resulted in M phase arrest followed by apoptosis. In conclusion, our data suggest that Nek6 is required for cell cycle progression through mitosis and demonstrate that loss of Nek6 function leads to defective mitosis and apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Cell Cycle Synchronization—HeLa, HeLa Tet-On, and MDA-MB231 cell lines were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. For transfection, FuGENE 6 (Roche Applied Science) was used according to the manufacturer’s instructions. siRNA duplex transfection was performed using OligofectAMINE reagent (Invitrogen) according to the manufacturer’s instructions. A lamin siRNA was used as control. The following oligonucleotides were used. Lamin A/C, 5′-CUGGACUCCCAGAAGACACdTdT (11). Two oligonucleotides were used as siRNA specific for Nek6: 5′-GAUCGACAGCGUGACUACdTdT and 5′-GGCUCGGUGACCUUGUCdTdT. All siRNA duplexes were purchased from Dharmacon Research. To synchronize the cells for the cell cycle studies, HeLa cells were seeded at 60% confluency and cultured for 24 h, and then 2 mM thymidine was added for another 14 h. After washing the cells, the medium was replaced, and the cells were allowed to continue the cell cycle for 11 h. Aphidicolin (1 μg/ml) was added to the cells for 14 h. The cells were then washed, released to grow in fresh medium, and then harvested every 2 h to monitor the different cell cycle stages.

Plasmid Constructs and Establishment of Stable Tumor Cell Lines—Wild type Nek6 was obtained by PCR using an expressed sequence tag clone (accession number, NM_014397) as template. The resulting cDNA was subcloned into the EcoRI and SalI sites of the pCAN-HA vector (a pcDNA3 backbone plasmid with a HA tag N-terminal of the fusion protein). Two point mutations were made in the wild type Nek6 cDNA, K74M and K75M (KK/MM), to generate a kinase-inactive mutant. The KK/MM mutant was made with the following oligonucleotides using a QuikChange PCR mutagenesis kit (Stratagene): GACAGTGCCGTTGATGGAAGGTGCGAGTC and GACAGTGCCGTTGATGGAAGGTGCGAGTC.
Nek6 Regulation of Mitosis

TCTTTGAG. Nek6 wild type and mutant (KK/MM) cDNAs were subcloned into the p Babe-puro retroviral vector as a blunted HindIII and NotI fragment. pBabe-Nek6 constructs were co-transfected into 293 cells with amphotropic retroviral packaging vectors (12) using FuGENE 6. Viral supernatants were used to infect HeLa and MDA-MB231 cell lines. Puromycin-resistant pools were generated, and individual clones were grown out of the pools to screen for higher expression of the Nek6 constructs. Wild type and KK/MM Nek6 HindIII and NotI fragments were also cloned into a pRevTRE vector (Clontech). The expression of Nek6 wild type and the KK/MM mutant was under the control of the tetracycline-inducible promoter. Hygromycin B was used to select for stable clones. The experiments described in this study were done using at least three individual cell clone lines.

Immunoprecipitation and in Vitro Kinase Assays—Cells were lysed with cold phosphate-buffered saline three times and harvested. Cell pellets were incubated with lysis buffer (150 mM NaCl, 1.5 mM MgCl₂, 50 mM Hepes, pH 7.5, 10% glycerol, 1 mM EGTA, 1% Triton X-100, 0.5% Nonidet P-40, 0.1 mg/ml AEBSF, 2 μg/ml aprotinin, pepstatin, leupeptin, and E-64 as protease inhibitors) at 4 °C for 20 min and centrifuged at 12,000 × g for 20 min. The supernatant was collected as whole cell lysate. Anti-HA monoclonal antibody (12CA5 at 1:1000 dilution) or Nek6 N-serum (1:500) was incubated with cell lysate at 4 °C for 1 h. The expression of endogenous Nek6 protein from the lysate of HeLa cells that had been synchronized at the G₁/S boundary by thymidine and aphidicolin double blocks. At various time points after release from the block, samples were collected to obtain cells enriched in S, M, and G₂ phases of the cell cycle. To verify proper synchronization, samples were labeled with propidium iodide and analyzed by flow cytometry (Fig. 1B, bottom panel). The results of the in vitro kinase assay revealed that Nek6 autophosphorylation and kinase activity toward the peptide substrate are highest in the G₂/M phase. Nek6 protein levels did not change significantly during the cell cycle (Fig. 1B, panel 3). We did, however, observe reduced electrophoretic mobility of Nek6 from mitotic cells probably because of phosphorylation modification of Nek6 protein. To determine whether this slower mobility was because of phosphorylation, we used lysate from nocodazole-treated cells to arrest cells in mitotic phase to perform kinase assays and Western analysis. Lysates were prepared from the mitotic-arrested population as well as from the interphase cells, and endogenous Nek6 was isolated by immunoprecipitation and assayed for kinase activity. The immune complex was also treated with alkaline phosphatase (Fig. 1C, lane CLAP) to examine the phosphorylation state of endogenous Nek6 (Fig. 1C). Consistent with the results shown in Fig. 1B, Nek6 from mitotic cells had increased kinase activity. In addition, a fraction of the isolated Nek6 had reduced mobility on SDS-PAGE. Upon treatment with alkaline phosphatase, the slower mobility band disappeared indicating that the observed band shift is because of phosphorylation (Fig. 1C). Furthermore, phosphatase treatment abolished the kinase activity indicating that phosphorylated Nek6 behaves as an active form to contribute its kinase activity during the M phase. We noticed a different amount of modified Nek6 between nocodazole-treated cells and thymidine-aphidicolin double block synchronized cells; this is probably because of the different synchronization methods. HeLa cells were released to grow in fresh media after thymidine-aphidicolin double blocks, and most of the cells are in the same M phase stages at the 8-h time point. While using nocodazole to block cells in the M phase, a fraction of cells were expected to undergo apoptosis because of disruption of the microtubule organization; therefore a smaller fraction of total cell lysate contains active phosphorylated Nek6. Nocodazole is thought to target microtubules to arrest cells in M phase. It is also possible that the microtubule disorganization has caused less active Nek6 in M phase; however, whether microtubule structure plays a role in Nek6 activity still needs to be investigated more carefully. These results indicate that Nek6 kinase activity is cell cycle-regulated and suggest that Nek6 may play a role in the regulation of mitotic progression.

Nek6 Kinase Activity Is Required during Mitosis—Because we have shown that Nek6 kinase activity is cell cycle-regulated, we were interested in determining whether Nek6 activity is critical in entry, progression, or exit from mitosis. To this end, we overexpressed wild type Nek6 or inactive Nek6-KK/MM in MDA-MB231 cells. We first performed proliferation assays to determine whether Nek6 had a general effect on the cell cycle. Fig. 2A shows the results from colorimetric assays (left panel) as well as direct cell counting (right panel). Wild type Nek6 and vector control stable clones had similar proliferation curves, although stable clones expressing kinase-dead Nek6 exhibited a slower proliferation rate. Next, MDA-MB231 stable clone cells were seeded at 60% confluency, cultured for 2 days, and harvested to determine cell cycle profile by FACS analysis (Fig. 2B). The results showed that MDA-MB231 cells expressing wild type Nek6 had cell cycle profiles comparable to that of parental MDA-MB231 cells (3–5% apoptotic cells, 6–13% G₂/M phase cells, 5–11% S phase cells, and 75–85% G₁ cells). In contrast, MDA-MB231 cells expressing kinase-dead
Nek6 showed an accumulation in G2/M phase, together with a reduction of the G1 population (30–35% G2/M phase cells, 8–12% S phase cells, and 30–40% G1 cells). In addition, cells expressing inactive Nek6-KK/MM showed a significant increase in the sub-G1 population, indicative of apoptosis. In repeated experiments we typically observed 20–28% apoptotic cells as determined by propidium iodide staining. Three individual clones were used to repeat the results for each experiment. Similar results were also observed when HeLa stable clones were used (data not shown). These results demonstrate that expression of kinase-dead Nek6 arrests cells in G2/M phase and leads to apoptosis.

To determine whether cells expressing Nek6-KK/MM arrest in G2 or in M phase, we used time-lapse microscopy. HeLa Tet-On stable clones in which expression of wild type or Nek6-KK/MM can be induced by the addition of doxycycline were used in these experiments. Fig. 3A shows induction of both wild type and kinase-inactive Nek6 proteins in response to doxycycline. In vitro kinase assays confirmed that the induced wild type protein is active. As expected, no kinase activity was detected for Nek6-KK/MM protein isolated from HeLa Tet-On cells. To visualize the chromosomal DNA in live cells, HeLa Tet-On stable cell lines were transfected with an expression plasmid for green fluorescent protein-histone 2B, which incorporates into chromosomal DNA and allows observation of chromosome actions during cell cycle progression (15). Expression of wild type Nek6 in HeLa cells did not result in any obvious defects during mitosis (data not shown). However, expression of kinase-dead Nek6 caused multiple defects (Fig. 3B). Most of the mitotic cells showed normal chromosome condensation and alignment but were unable to complete mitosis. Time-lapse experiments showed that these cells accumulated at the metaphase stage and were unable to perform chromosome segregation. This failure during mitotic progression frequently leads to the induction of apoptosis. Fig. 3C shows the quantitation from multiple time-lapse experiments with cells overexpressing wild type Nek6 or kinase-dead Nek6-KK/MM. In contrast to wild type-expressing cells, which only showed occasional mitotic defects and apoptosis, almost half of the Nek6-KK/MM-expressing cells apoptosed during mitosis. Careful examination of the video recordings showed that most of the cells undergoing apoptosis had been arrested at the metaphase stage. A smaller fraction of cells initiated apoptosis during anaphase or telophase. Together, the results demonstrate that Nek6 activity is required for progression.
through mitosis and, in particular, for metaphase-anaphase transition.

Knockdown of Endogenous Nek6 by RNA Interference Arrests Cells in M Phase and Triggers Apoptosis in Human Tumor Cells—As the expression of kinase-dead Nek6 induced mitotic arrest and apoptosis, we wished to determine whether there were similar effects upon depletion of endogenous Nek6 protein in cells. We used RNAi as a means of reducing the endogenous level of Nek6 mRNA and protein (11). Small interfering RNAs corresponding to two distinct Nek6 coding sequences were chosen to target Nek6, and a lamin A/C RNAi was selected as a nonspecific control. Transfection of either of the two Nek6 siRNAs, but not the control siRNA in HeLa cells, resulted in a significant reduction in Nek6 protein levels as shown in Fig. 4 A. HeLa cells transfected with Nek6 siRNA or control siRNA were harvested at different time points and subjected to FACS analysis to assay for DNA contents. Similar to cells expressing kinase-dead Nek6, a knockdown of Nek6 protein by siRNA resulted in a G2/M phase arrest (Fig. 4B). In addition, we observed an increase in the sub-G1 population, indicative of enhanced apoptosis. To further confirm this observation, we assayed induction of apoptosis following transfection of siRNAs over a period of 11 days. Apoptotic cell populations were quantitated in parallel by annexin V-fluorescein isothiocyanate staining and by propidium iodide staining (Fig. 4C). The data clearly show that depleting Nek6 from the cells induces apoptosis.

To confirm that the observed G2/M phase accumulation is due to arrest in M phase, we determined the mitotic index of transfected cells. RNAi-transfected cells were harvested and fixed, then stained with Hoechst to visualize chromosomal DNA. Cells with different nuclear figures were scored and are summarized in Fig. 4, D and E. Consistent with the FACS profile (Fig. 4B) we observed a significant increase in apoptosis in cells transfected with Nek6 siRNA compared with the siRNA control. In addition, we found an increase in the mitotic index, suggesting that the increase in the G2/M cell population observed by propidium iodide staining and FACS analysis is largely because of an accumulation of mitotic cells. In cells transfected with control siRNA, we observed cells in all stages of mitosis (prometaphase, metaphase, anaphase, and telophase). In cells transfected with Nek6 siRNA, however, most of the mitotic cells were in prometa- or metaphase (Fig. 4D). In repeated experiments we rarely found mitotic cells that were in anaphase or telophase (Fig. 4D). Depletion of Nek6 by siRNA also resulted in a significant increase in cells with two or more nuclei, again, suggesting a defect in the proper completion of mitosis (Fig. 4E). Taken together, we conclude that similar to the effects seen with the kinase-dead allele, depletion of endogenous Nek6 induced mitotic arrest and apoptosis. These results further suggest that Nek6 is required for mitotic progression.

DISCUSSION

Mitosis is a critical step within the cell cycle to ensure the accurate distribution of the duplicated chromosomes to the daughter cells. Progression into and through mitosis is associated with distinct patterns of protein phosphorylation and is regulated by various protein kinases and phosphatases. Transition from G2 into M phase requires the activation of the CDK family member Cdc2 (16, 17). Other kinases with important functions during mitosis are Polo-, Aurora-, and NIMA-related kinases. The results presented here demonstrate that the NIMA-related kinase Nek6 is required for cell cycle progression.
HeLa tetracycline-inducible cells were induced by the addition of doxycycline and exhibit chromosome segregation during mitosis. Stable clones of doxycycline, either wild type or kinase-dead Nek6 in HeLa Tet-On cells were established. Green fluorescent protein-histone 2B DNA was transfected into these clones. 18 h after expression of Nek6 was induced with doxycycline, they were used for time-lapse recording for 30 h. A, HeLa tetracycline-inducible cells were induced by the addition of doxycycline. 40 μg of protein of whole cell lysates from stable clones of either wild type or kinase-dead was loaded into each well to perform SDS-PAGE. Anti-HA and anti-α-tubulin Western analysis showed successfully induced Nek6 proteins and a relative similar amount of proteins loaded in each well. Nek6 kinase assays showed Nek6 protein activity after induction. B, mitotic failure and apoptosis of a representative HeLa cell expressing kinase-dead Nek6. The sequence of images shows chromosome condensation and progression to metaphase, which is followed by apoptosis. C, expression of kinase-dead, but not of wild type (WT), Nek6 induces mitotic catastrophe. HeLa cells expressing wild type or kinase-dead Nek6 with or without doxycycline (Dox) induction were followed through mitosis and recorded by time-lapse microscopy. The results shown are the mean (± S.D.) of three independent experiments corresponding to 20 individual cell events in each experiment. Cells that exhibited anaphase, telophase, and metaphase blocks that were followed by apoptosis were scored as mitotic death events; cells that exhibited chromosome fragmentations and apoptosis were scored as other death events.

Nek6 Regulation of Mitosis

Although NIMA has been shown to affect mammalian cell cycle regulation by overexpression, it has not been clear whether functional homologs of NIMA exist in metazoans (18, 19). Among the previously characterized mammalian Nek kinases, only Nek2 has been shown to play a role during mitosis, although Nek2 seems mainly to be involved in centrosome separation and does not seem to be required for entry or progression through mitosis (6). The results presented here establish Nek6 as a mitotic kinase. Its activity is specifically up-regulated during the M phase. Increased activity is also associated with Nek6 phosphorylation, which could be auto-phosphorylation or activating phosphorylation by an upstream kinase. Furthermore, Nek6 kinase activity is required for progression through mitosis. Blocking Nek6 function, either by expression of an inactive mutant or by siRNA knockdown, resulted in mitotic arrest. The results further demonstrate that Nek6 activity is required after the G2/M phase transition, suggesting a function distinct from NIMA or Nek2, as overexpression of kinase-dead Nek6 did not interfere with chromosome condensation. In time-lapse experiments we observed that cells expressing Nek6-KK/MM were arrested at the metaphase stage. This suggests that Nek6 kinase activity is required for completion of metaphase or for the initiation of anaphase. To ensure the accurate distribution of the sister chromatids, the mitotic checkpoint is activated until all chromosome kinetochores are attached to spindle microtubules. Once this is accomplished, activation of the anaphase-promoting complex then mediates the separation of the sister chromatids, allowing anaphase to proceed (20, 21). It is likely that Nek6 kinase activity is required for the recruitment or phosphorylation of mitotic checkpoint components. Several mitotic checkpoint proteins have been shown to regulate mitosis by phosphorylation (22). At the present time, however, we do not yet know the identity of the critical Nek6 substrate(s) that is required to mediate its effects. It was reported that Nek6 can phosphorylate p70 S6 kinase, but more recent findings suggest that p70 S6 kinase is not an in vivo substrate (7, 8).

Recently, Roig et al. (10) demonstrated that Nek6 forms a complex with Nercc1 (Nek9). Inhibition of Nercc1 by antibody microinjection caused multiple mitotic defects. Dependent on the cell lines examined, inhibition of Nercc1 resulted in spindle abnormalities and mis-segregation of chromosomes or prolonged prometaphase arrest. It is possible that Nek6, as a component of the Nercc1 complex, is involved in some of the Nercc1-dependent effects. Here it should be noted that loss of the entire Nek6 protein, by siRNA transfection, arrested cells earlier than expression of kinase-dead Nek6. siRNA-mediated knockdown predominantly caused cells to be blocked in prometaphase, similar to the reported effect of anti-Nercc1 antibody injection. It is possible that the loss of Nek6 protein interferes with the proper function of the Nercc1 complex or Nercc1 itself. When bound to Nercc1, Nek6 may play a structural role, as expression of kinase-dead Nek6 did not lead to significant prometaphase arrest. As mentioned above, Nek6 kinase activity seems to be critical for metaphase-anaphase transition. It has not yet been established whether the entire pool of cellular Nek6 is in a complex with Nercc1, and the possibility remains that Nek6 also might have Nercc1-independent roles during mitosis. Immunofluorescence with anti-Nek6 or anti-Nercc1 antibodies did not reveal obvious localization of either protein to spindle microtubules or chromosomal structures such as kinetochores (10) (data not shown). These results could indicate that Nek6 and Nercc1 modulate the activity of mitotic regulators that do not directly associate with the spindle or the chromosomes.

The anti-cancer drug Taxol induces cell apoptosis by stabilizing the spindle microtubules and causing mitotic arrests at
the metaphase stage (23). Mitotic block of tumor cells leads to Bcl-2 hyperphosphorylation at multiple serines, and Bcl-2 phosphorylation also coincides with the onset of apoptosis by Taxol treatment (24). Several other serine/threonine kinases such as Raf1 and p34<sup>cdc2</sup> also have been reported to play a role in mitotic death regulation (25, 26). Interfering with Nek6 function not only resulted in mitotic arrest but also induced apoptosis in human tumor cell lines. This was the case for both siRNA transfection and overexpression of kinase-dead Nek6. In siRNA-transfected cells we also observed a significant increase in multinucleated cells indicating that the cells exited mitosis without cell division. These results further demonstrate the requirement for Nek6 in mitosis. Time-lapse microscopy demonstrated that apoptosis occurred as a consequence of the mitotic arrest. Cells arrested by overexpression of Nek6 KK/MM most frequently died after being arrested at the metaphase stage. These observations underscore the fact that human tumor cells cannot tolerate prolonged absence of Nek6 activity. It remains to be determined whether the mitotic arrest produced by interfering with Nek6 function coincides with Bcl-2 or other serine kinase phosphorylation to induce apoptosis.

In summary, our data demonstrate that Nek6 kinase activity is up-regulated during the M phase of cell cycle progression. Interference with Nek6 function blocks chromosome segregation at the metaphase-anaphase transition and triggers cell apoptosis. Our studies demonstrate for the first time a critical role for Nek6 in progression through mitosis.

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Nek6 Regulation of Mitosis

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