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Advances in Brief

NIMA-Related Protein Kinase 1 Is Involved Early in the Ionizing Radiation-Induced DNA Damage Response

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Abstract

Cellular functions of the NimA-related mammalian kinase Nek1 have not been demonstrated to date. Here we show that Nek1 is involved early in the DNA damage response induced by ionizing radiation (IR) and that Nek1 is important for cells to repair and recover from DNA damage. When primary or transformed cells are exposed to IR, Nek1 kinase activity is increased within 4 minutes, and Nek1 expression is up-regulated shortly thereafter and sustained for hours. At the same early time frame after IR that its kinase activity is highest, a portion of Nek1 redistributes in cells from cytoplasm to discrete nuclear foci at sites of DNA double-strand breaks. There it colocalizes with γ-H2AX and NFBD1/MDC1, two key proteins involved very early in the response to IR-induced DNA double-strand breaks. Finally, Nek1-deficient fibroblasts are much more sensitive to the effects of IR-induced DNA damage than otherwise identical fibroblasts expressing Nek1. These results suggest that Nek1 may function as a kinase early in the DNA damage response pathway.

Introduction

Nek1 is a mammalian ortholog of NIMA (never in mitosis A) in the multicellular filamentous fungus *Aspergillus nidulans*. NIMA responds to DNA damage, regulates G_2-M–phase progression, and prevents aberrant chromosome transmission to daughter cells (1, 2). Nek1 was partially cloned after screening an expression library with antityrosine antibodies and identified as the first mammalian NIMA-related kinase (3). The kinase domain of Nek1, when expressed from bacteria, has dual serine-threonine and tyrosine kinase activity *in vitro*. Because *Nek1* mRNA is abundantly expressed in mouse gonads and in specific neurons, previous studies have suggested that Nek1 may play a direct and unique role in meiosis or in regulating the cell cycle (3, 4).

When Nek1 is mutated in either of two spontaneous mutant strains of mice, the so-called kidneys-anemia-testis (kat and kat2J) strains, pleiotropic and ultimately fatal defects develop. These defects include growth retardation, facial dysmorphism, chorioid plexus and neurologic abnormalities, male sterility, and progressive polycystic kidney disease (5, 6). Potential binding partners and substrates have been suggested through their interaction with coiled-coil domains of Nek1 in a yeast two-hybrid assay (7). Among those reported were proteins such as ZBRK1 (8), MRE11 (9), and 53BP1 (10), which have known roles in the DNA damage response. None of these proteins has yet been shown to be a *bona fide* substrate or interacting partner of Nek1, however, so the functions of Nek1 in DNA damage response and intracellular signaling have only been suggested indirectly to date.

In this report, we show that Nek1 responds to DNA damage by quickly up-regulating its kinase activity and that cells without functional Nek1 fail to repair their DNA properly and therefore survive poorly after ionizing radiation (IR)-induced DNA damage.

Materials and Methods

Cell Lines. Human HK2 proximal renal tubular epithelial cells and HeLa cervical carcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and cultured at 37°C/10% CO_2 in recommended media containing fetal bovine serum and antibiotics. Primary tail fibroblasts were generated by mincing tail tips from 3-week–old mice and plating the fragments in 60-mm dishes. The fibroblasts that grew from these fragments were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics for 3 weeks until they were nearly confluent; then they were removed from the plates with diluted trypsin-EDTA. Passage 3 to 5 cells derived from each littermate mouse were used for subsequent experiments.

Ionizing Radiation. Cells grown in log phase were irradiated with measured doses of γ-rays in a cesium-137 source (Mark I, Model 68A Irradiator; J. L. Shepherd & Associates, San Fernando, CA). Medium was replaced for all cells immediately after irradiation. Percentages of cells still surviving 24 hours after different doses of IR were determined by counting in triplicate the numbers of cells excluding trypan blue vital dye, divided by total number of cells per plate.

Antibodies. Murine and rabbit polyclonal anti-Nek1 antibodies were generated by immunizing mice and rabbits with a glutathione *S*-transferase–Nek1 (amino acid 490–774) fusion protein.[5] Monoclonal 5E10 anti-p84 antibodies (11) were a gift from W-H. Lee. Anti–γ-H2AX and anti-NFBD1 antibodies have been described previously (12).

Immune Kinase Assays. Cells (1×10^6) were lysed and immunoprecipitated with either preimmune serum, normal mouse IgG, or anti-Nek1 antibodies. After extensive washing with lysis buffer, the immunocomplexes were washed twice with Tris-buffered saline [10 mmol/L NaCl and 10 mmol/L Tris-HCl (pH 7.5)], twice with double distilled H_2O, and twice with kinase buffer [100 mmol/L Tris-HCl (pH 7.5), 5 mmol/L $MnCl_2$, 2 mmol/L dithiothreitol, and 1 μmol/L ATP]. Immune complexes were stored in 100 μL of kinase buffer at −80°C. The kinase reaction was carried out in a total volume of 30 μL, with 20 μL of immunocomplexes and 5 μg of β-casein in the presence of 3 μCi of [γ-^{32}P]ATP. After incubation for 30 minutes at 37°C, equal volumes of SDS sample buffer with EDTA to final concentration of 2 mmol/L were added to the reactions. Phosphorylated β-casein was electro-

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[5] Y. Chen, R. Polci, P. Litchfield, M. Gaczynska, D. J. Riley. NIMA-related kinase 1 regulates apoptosis through phosphorylation of the mitochondrial pore channel VDAC, submitted for publication.

phoresed on 12% SDS-polyacrylamide gels; the gels were stained with Coomassie Blue, destained, dried, and autoradiographed.

Western Blotting. Cells were suspended in Lysis 250 buffer. Lysates were subjected to three freeze/thaw cycles (liquid N_2/37°C) and then centrifuged at 14,000 rpm for 2 minutes at room temperature. The supernatants were used for Western blotting as described previously (13).

Immunocytochemistry. HK2 cells grown on coverslips to ~70% confluence were fixed in 4% formaldehyde with 0.1% Triton X-100. Fixed cells were permeabilized with 0.05% saponin and blocked with 10% normal goat serum. Primary antibodies were used at a dilution of 1:100 to 1:1,000 (3 to 0.3 μg/mL) in 10% goat serum. Secondary antibodies, including antirabbit or antimouse IgG-Alexa 555 (green) and antirabbit or antimouse IgG-Alexa 488 (red; Molecular Probes, Eugene, OR) were used at a dilution of 1:3,000. Cells were mounted in Permafluor (Lipshaw-Immunon, Pittsburgh, PA). Images were captured with a Ziess AxioPlan2 fluorescence microscope and digitally merged where appropriate.

Kat2J Mice and Genotyping. C57BL/6J-*Nek1*$^{kat2J+/-}$ founder mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Genomic DNA was extracted from 1×10^4 cultured cells, tail fragments, or blood according to a protocol available at The Jackson Laboratory.[6] Genotyping for the *Nek1*^{kat2J} mutation, a single guanine insertion at nucleotide +996, was performed with single-strand conformational polymorphism analysis. The primer pair (1633-5′ and 1633-3′) and conditions for detecting the 79-bp (wild-type) *versus* 80-bp (*Nek1*^{kat2J}) polymerase chain reaction (PCR) fragments amplified from genomic DNA were modified from those described previously (6). Seven microliters of genomic DNA, from a total of 100 μL per condition, were used with ^{32}P end-labeled 5′ primer and unlabeled 3′ primer in a final PCR volume of 27 μL. PCRs were performed with 35 cycles of 93°C for 1 minute, 54°C for 2 minutes, and 72°C for 30 seconds. Five microliters of each PCR product were incubated with 5 μL of 95% formamide for 5 minutes at 90°C and chilled on ice before loading onto a 9% acrylamide, 10% glycerol, 1× Tris-HCl borate EDTA buffer gel and subjected to electrophoresis for 2.5 hours at 40 W in 0.5× Tris-HCl borate EDTA buffer. The resulting gels were fixed and analyzed by phosphorimaging.

Densitometry, Graphing, and Statistics. Densitometry was performed on ^{32}P-labeled, dried gels using a phosphorimager and associated ImageQuant software (Amersham Biosciences, Piscataway, NJ) and on Western blots using Alpha Innotech software (Catalina St. San Leandro, CA). Histograms with error bars representing SEs of the means and other graphs were generated and analyzed using Prism 4.0 software (San Diego, CA).

Results and Discussion

Nek1 Kinase Activity Is Rapidly Up-Regulated in Response to Ionizing Radiation. We have recently shown that Nek1 expression is regulated during mouse kidney development and up-regulated after ischemia-reperfusion injury.[7] Up-regulation after ischemic injury occurred quickly, before cells underwent apoptosis or recovered from the ischemia-reperfusion injury, which is characterized by oxidative injury to DNA (14). These results and the known functions of the Nek1 homologs mammalian Nek2 (13) and fungal NIMA (1, 2) in G_2-M checkpoint control suggested that Nek1 might be involved in the DNA damage response signaling pathway. To further understand how Nek1 may be involved in the DNA damage response, we used a cell culture system. We examined the kinase activity of Nek1 in human cells treated with a sublethal dose (10 Gy) of γ-IR. Nek1 kinase activity was measured using Nek1 immunoprecipitated from cells 1 hour after IR, and β-casein was used as a substrate (3). Kinase activity increased 2- to 3-fold after IR compared with activity in cells that were not irradiated (Fig. 1*A* and *B*). This IR-induced Nek1 activation was not specific only to kidney cells; similar observations were made in HK2 human kidney epithelial cells, HeLa cells, and others (Fig. 1*A*; data not shown). To further understand whether

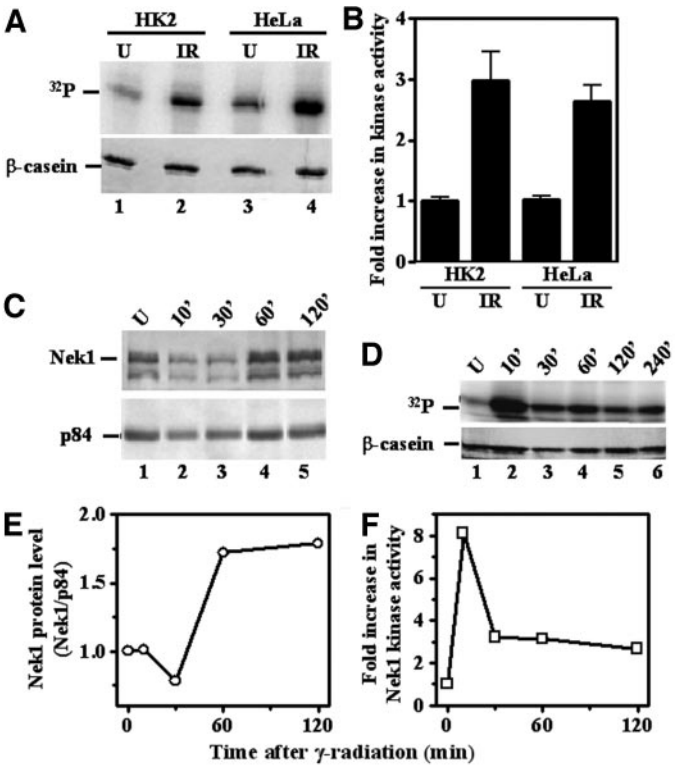

Fig. 1. IR increases Nek1 kinase activity before increasing Nek1 protein abundance. *A*, Nek1 was immunoprecipitated from equivalent numbers of HK2 and HeLa cells, 1 hour after treatment with a sublethal dose (10 Gy) of IR or after mock irradiation (*U*). Immunoprecipitated Nek1 was then used in an immune kinase reaction with equivalent amounts of [^{32}P]ATP and β-casein as an *in vitro* substrate. *B*, quantification of relative kinase activity from three separate experiments (means ± SE). *C*, Nek1 protein abundance in HK2 cells after the same dose of IR. Expression of the unregulated nuclear matrix protein p84 served as a loading control. *D*, time course of Nek1 immune kinase activity. *E* and *F*, graphic displays comparing time courses of increased Nek1 protein abundance and kinase activity after sublethal dose of IR.

activation of Nek1 is due to up-regulation at the protein level, we examined the expression and kinase activity of Nek1 at different time points after IR. Nek1 protein abundance actually decreased within 30 minutes after IR, but only transiently; it increased 1.8-fold compared with the baseline amount by 1 hour (Fig. 1*C* and *E*). In contrast, Nek1 kinase activity was highest (8-fold increase compared with basal activity) very quickly after IR-induced damage (within 10 minutes); it then declined but remained 2- to 3-fold greater than basal activity at 30 minutes and remained high for hours thereafter (Fig. 1*D* and *F*). Thus Nek1 seems to be activated as a kinase very early after IR-induced damage, somewhat independent of its expression amount. Only later after IR did Nek1 protein expression increase and remain increased for an extended period of time.

Nek1 Relocalizes from Cytoplasm to Nuclear Foci after DNA Damage. To determine whether Nek1 could function in nuclei at sites of DNA damage, we treated HK2 cells with low-dose IR (0.5 Gy) and examined the subcellular localization of Nek1 in individual cells at different time points thereafter. A portion of cellular Nek1 redistributed from cytoplasm to nuclei after IR. Discrete Nek1 nuclear foci were detected in <10% of the untreated cells, but >75% of cells had Nek1 IR-induced immunofluorescent foci (IRIF) as early as 4 minutes after irradiation (Fig. 2). Within 10 minutes after IR, >98% of treated cells contained Nek1 IRIF. The intensity of Nek1 nuclear foci also increased with time after induction of DNA damage. The time course of the appearance of Nek1 IRIF at presumed sites of DNA double-strand breaks (DSBs) paralleled almost precisely the early increase in Nek1 kinase activity observed after similar DNA damage. These

[6] http://www.jax.org/imr/tail_nonorg.html.

[7] D. J. Riley, S. G. Achinger, R. Polci, P. Litchfield, Y. Chen. Regulation of Nek1 protein kinase expression during development and after tubular injury, submitted for publication.

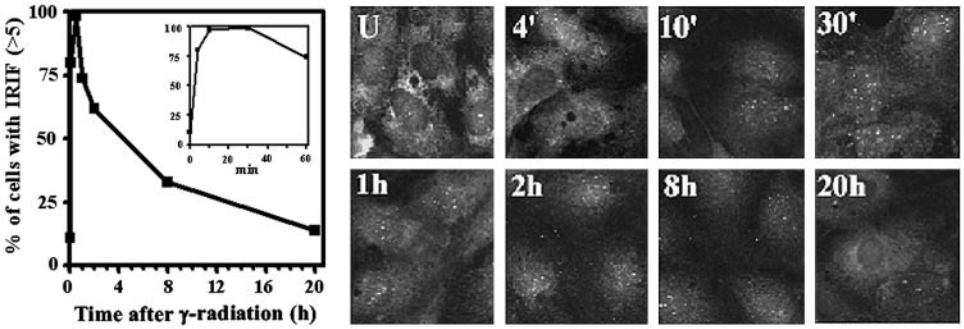

Fig. 2. Nek1 relocalizes to nuclear immunofluorescent foci very quickly after DNA damage by IR. Time course of redistribution of Nek1 to nuclear IRIF after a minimal, sublethal dose (0.5 Gy) in HK2 cells. At least 200 cell nuclei were scored for IRIF at each time point. Representative photomicrographs of at different time points are shown.

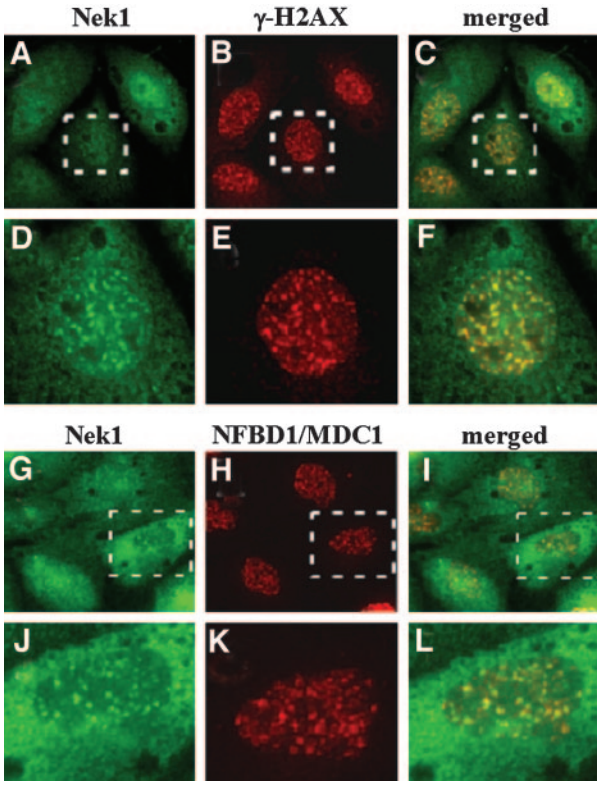

Fig. 3. Nek1 colocalizes in nuclear immunofluorescent foci with γ-H2AX and NFBD1/MDC1. HK2 cells were treated with minimal, sublethal doses of IR (0.5 Gy). One hour later, they were fixed and stained with murine anti-Nek1 and rabbit anti–γ-H2AX primary antibodies, followed by incubation with Alexa 555 (*green*)-labeled antimouse IgG and Alexa 488 (*red*)-labeled antirabbit IgG (*A*−*F*). Cells viewed by fluorescence microscopy with different excitation wavelengths are shown, as are the merged images. Similarly treated cells were immunostained with rabbit anti-Nek1 and murine anti-NFBD1 primary antibodies, followed by incubation with green-labeled antirabbit IgG and red-labeled antimouse IgG (*G*−*L*). *Bottom panels* in each group (*D*−*F* and *J*−*L*) represent higher-power views of individual nuclei *boxed* in the *top panels*.

consistent results suggest that Nek1 is involved very early in the DNA damage response, as a kinase. Other phosphatidylinositol 3-kinase–like kinases known to function proximally in DNA damage repair pathways, such as ATM and ATR, are similarly activated very early after induction of DNA DSBs. They also similarly localize to nuclear foci at sites of DNA damage, where they have specific substrates involved in cell cycle checkpoint control, in direct interaction with damaged DNA, and with effector pathways of DNA repair (15, 16).

Nek1 Colocalizes with Other DNA Damage Response Proteins in Ionizing Radiation-Induced Nuclear Foci. As the next step in determining whether Nek1 may interact with other proteins in the DNA damage response, we examined the localization of Nek1 and other proteins that function in DNA repair. In the same cells after IR, Nek1 colocalized with γ-H2AX and NFBD1/MDC1 (Fig. 3), two proteins known to be present at DNA damage sites early after DNA damage. γ-H2AX is an activated form of histone specifically phosphorylated at serine residue 139 by ATM (16); it interacts directly with DNA DSBs (17). NFBD1 is another chromatin-associated molecule thought to function as a DNA damage sensor (12). Taken together, the data showing redistribution of Nek1 to nuclear foci after IR-induced DNA damage and colocalization of Nek1 with γ-H2AX and NFBD1 at these same sites suggest that Nek1 is truly involved in the DNA damage response.

Nek1-Null Cells Are Hypersensitive to the Lethal Effects of Ionizing Radiation. If Nek1 is important in the pathway that senses, signals, and repairs DNA damage, then Nek1-deficient cells should be more sensitive than normal cells to the lethal effects of IR. To test this hypothesis, we examined the survival of cells with or without functional Nek1 after they were treated with equivalent doses of IR. Primary fibroblasts were prepared from kat2J mice and their phenotypically normal littermates. Genotyping of the mice themselves and of six primary fibroblast lines derived from the same litter identified three lines homozygous for the $Nek1^{kat2J}$ mutation and three lines heterozygous for the same mutation (Fig. 4*A*). We used Western blotting to confirm the lack of Nek1 expression in the $Nek1^{kat2J-/-}$ cells and the presence of normal Nek1 protein in the $Nek1^{kat2J+/-}$ cells (Fig. 4*B*). Equivalent numbers of subconfluent cells were then exposed to different doses of IR and assayed 24 hours later after staining with vital dye for viable and nonviable cells. Normal wild-type fibroblasts derived from a different litter of mice were also represented. Cells without functional Nek1 were significantly more sensitive to low doses of IR than were otherwise equivalent cells expressing Nek1. These data indicate that cells without Nek1 were

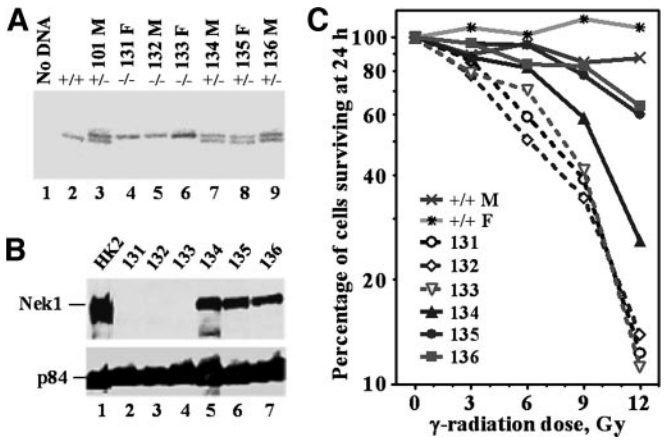

Fig. 4. Nek1-deficient fibroblasts are hypersensitive to IR. *A*, genotype of wild-type and heterozygous controls and six pups from a single litter of mice from $Nek1^{kat2J+/-} \times Nek1^{kat2J+/-}$ mating. *B*, Nek1 protein expression in tail fibroblasts from the same mice. *C*, dose-response: survival of fibroblasts with and without Nek1 expression 24 hours after IR.

unable to repair the same amount of DNA damage sufficiently to avoid IR-induced apoptosis.

NIMA in *A. nidulans* has known functions in G_2-M checkpoint control, as does at least one of its mammalian orthologs, Nek2. Nek2 phosphorylates the kinetochore-associated mitotic regulator protein Hec1 and is therefore crucial for faithful chromosome segregation to daughter cells (13). Our data suggest that Nek1 may be important for sensing, responding to, or repairing DNA damage and thereby for regulating apoptosis and cellular proliferation after injury. When Nek1 is mutated or when its up-regulation is otherwise insufficient, cells are more likely to die aberrantly or fail to proliferate when they normally should. Some cells surviving the insult would also be more likely to pass subtle mutations on to their daughters, which could then accumulate activating oncogenic or inactivating tumor suppressor mutations. Up-regulation of Nek1 may be a defensive response to cellular and DNA injury, a response to prevent excessive or unscheduled apoptosis. If so, we predict that Nek1 might be overexpressed in certain tumors and that its overexpression could make them relatively resistant to standard radiation and chemotherapeutic agents, which work by damaging DNA and causing apoptosis in rapidly proliferating tumor cells. To our knowledge, Nek1 is the first mammalian NIMA-related protein to be involved directly in the DNA repair pathway. It is also the only protein identified to date that has a significant function in the DNA damage response pathway despite being expressed primarily in the cytoplasm of uninjured cells. The human and murine Nek1 primary sequences have both putative nuclear localization and nuclear export signals (7). It is possible that DNA damage triggers a rapid modifying event such as phosphorylation that results in exposure of Nek1's nuclear localization signal or masking of its nuclear export signal, such that a portion of Nek1 moves to or stays in the nucleus.

Exactly where does Nek1 fit into pathways of DNA damage response? That question has not yet been answered. The use of RNA interference and/or cell lines deficient in individual DNA damage pathway proteins, however, should allow us to determine whether Nek1 lies upstream, downstream, or parallel to ATM, ATM-like kinases, and other molecules involved in the DNA damage repair pathway. It is possible that Nek1 serves a proximal, cascade-initiating kinase similar to ATM, but perhaps only in a unique subset of cells such as renal tubular epithelial cells, spermatogonia, and certain neurons. These tissues or cells have been shown to have either abundant expression of Nek1[7] (3, 4) or specific pathological consequences when Nek1 is inactivated (5, 6).

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