Alternative Splicing Controls Nuclear Translocation of the Cell Cycle-regulated Nek2 Kinase*

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Nek2 is a cell cycle-regulated serine/threonine protein kinase that is up-regulated in human cancers. Functionally, it is implicated in control of centrosome separation and bipolar spindle formation in mitotic cells and chromatin condensation in meiotic cells. Two major splice variants have been described in vertebrates, Nek2A and Nek2B, that differ in their non-catalytic C termini. Recently, a third splice variant, Nek2C, was identified that lacks an eight-amino acid internal sequence within the C-terminal domain of Nek2A. This excision occurs at the same position as the Nek2A/Nek2B splice point. As predicted from their high degree of similarity, we show here that Nek2C shares many properties with Nek2A including kinase activity, dimerization, protein phosphatase 1 interaction, mitotic degradation, microtubule binding, and centrosome localization. Unexpectedly, though, the non-centrosomal pool of protein exhibits a marked difference in distribution for the three splice variants. Nek2C is mainly nuclear, Nek2B is mainly cytoplasmic, and Nek2A is evenly distributed within nuclei and cytoplasm. Mutagenesis experiments revealed a functional bipartite nuclear localization sequence (NLS) that spans the splice site leading to Nek2C having a strong NLS, Nek2A having a weak NLS, and Nek2B having no NLS. Finally, we identified a 28-kDa protein in nuclear extracts as a potential novel substrate of Nek2. Thus, alternative splicing provides an unusual mechanism for modulating Nek2 localization, enabling it to have both nuclear and cytoplasmic functions.
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a protein of 384 amino acids (44 kDa) with the sequence diverging after amino acid 370. Downstream of the kinase domain, both Nek2A and Nek2B share a leucine zipper motif (amino acids 304–340) that promotes dimerization, autophosphorylation, and activation of the kinase (20, 21). This is followed by a short motif (amino acids 333–370) shown to be required for centrosome targeting and microtubule binding (22). However, the position of splicing means that a binding site for protein phosphatase 1 (PP1; amino acids 383–386) and two APC/C-dependent degradation motifs, a KEN box (amino acids 391–399), and an MR-tail (amino acids 444–445), are present in Nek2A but missing from Nek2B (23–25). This falls in line with observations that Nek2A is degraded upon mitotic entry, whereas Nek2B remains stable. It also suggests that Nek2B may not be subject to regulation by PP1, although this hypothesis is complicated by the fact that Nek2A and Nek2B can heterodimerize via their leucine zippers, at least in vitro (19).

Recently, a third splice variant of Nek2 was identified during a yeast two-hybrid screen using PP1γ1 and PP1γ2 as bait (26). This isoform is identical to Nek2A except that it lacks amino acids 371–378. This eight-amino-acid deletion starts at the splice donor position of the Nek2A-Nek2B splice site but uses a downstream splice acceptor sequence within exon 8. This variant was originally named Nek2A-T as it was isolated from testis mRNA. However, we prefer now to call it Nek2C because Nek2A is also expressed in the testis (26–29), whereas Nek2C is clearly not exclusively expressed in testis (see results). In this study we investigated the properties of Nek2C in comparison to those of Nek2A and Nek2B. As one would predict from the position of the regulatory motifs described above, Nek2C is an active kinase that can undergo autophosphorylation, bind microtubules, localize to the centrosome, and be degraded in early mitosis. However, upon overexpression, there is a significant increase in accumulation of Nek2C in the nucleus as compared with Nek2A or Nek2B, and through mutagenesis we show that this is the result of the eight-amino-acid deletion creating a strong nuclear localization sequence (NLS) in Nek2C that is weaker in Nek2A and absent in Nek2B. We speculate that the nuclear accumulation of Nek2C may be essential to catalyze the phosphorylation of nuclear substrates that promote mitotic or meiotic entry.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pCMV-mycNek2A, pCMV-mycNek2A-K37R, and pCMV-mycNek2B have been previously described (15, 19). To generate pCMV-mycNek2C, a C-terminal fragment was excised from pCMV-mycNek2A with BspEI and XbaI restriction enzymes and replaced with the corresponding fragment from pACT-Nek2C (26). Because BspEI is blocked by overlapping dam methylation, dam′ Escherichia coli JM110 were used for transformation. Mutations were introduced using the Gene Tailor™ site-directed mutagenesis system (Invitrogen). The GST-PP1α and His-Nek2A-CTD bacterial expression constructs were generated by subcloning human PP1α from BS-mycPP1α into the pGEX-4T1 vector and amino acids 255–445 of Nek2A into the pET22b vector, respectively. All constructs were confirmed by DNA sequencing by Lark Technologies, Inc. (Saffron Walden, UK).

Cell Culture, Transfection, and Extraction—All media and additives were obtained from Invitrogen. Human cervical epithelia HeLa cells were cultured in minimal essential media with 1% nonessential amino acids, 10% heat-inactivated fetal bovine serum, and 1% antibiotic/antimycotic mix. Human osteosarcoma U2OS cells and human embryonic kidney cells, HEK 293, were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% antibiotic/antimycotic. Cells were cultured at 37 °C in a 5% CO2 atmosphere. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cell extracts were prepared as previously described (30).

Reverse Transcriptase-PCR—Total RNA was isolated from cultured cells using TRI Reagent (Sigma) according to manufacturer’s instructions. Extracted RNA (5 μg) was used for first strand synthesis using SuperScript II reverse transcriptase (Invitrogen) and oligo-(dT)20 at 50 °C for 60 min. PCR amplification of Nek2A- and Nek2C-specific fragments of 153 and 129 bp, respectively, was performed in a 50-μl reaction using Taq polymerase (Invitrogen), 2 μl of first strand cDNA synthesis mixture as template, and the following primers: 1077F, 5′-GGAAACGGAAGTTCCTGTC-3′; 1229R, 5′-CATTGGACCTAGATTGTGA-3′. Amplified products were purified using a PCR purification kit (Qiagen Ltd., Crawley, UK), cloned into a pBlueScript vector, linearized with SmaI, and sequenced.

In Vitro Translation (IVT)—Nek2, Rab4, and lamin A proteins were generated by IVT from appropriate vectors using the TnT-coupled transcription/translation kit (Promega, Southampton, UK) according to manufacturer’s instructions. For radioactive detection of proteins, reactions were carried out in the presence of [35S]methionine, separated by SDS-PAGE, and exposed to x-ray film.

Immunoprecipitation (IP) Kinase Assays—For IP of proteins generated by IVT, 10 μl of pre-washed protein G-Sepharose beads (Sigma) were added to 490 μl of NeB buffer (30) containing 9 μl of nonradioactive IVT reaction and mixed on a rotating wheel for 30 min at 4 °C before centrifugation at room temperature for 10 s. The preclarified supernatant was transferred to a fresh tube, and 1 μl of mouse anti-Myc monoclonal antibody (Cell Signaling Technology, Beverly, Mass.) was added and incubated on ice for 60 min. Meanwhile, 40 μl of protein G-Sepharose beads were blocked with 5 μl of rabbit reticuloocyte lysate in 500 μl of NeB buffer on a rotating wheel for 30 min. The blocked beads were centrifuged at room temperature for 10 s, the supernatant was removed, and the beads were washed 3 times with 100 μl of NeB buffer. The blocked beads were resuspended in 50 μl of NeB buffer and added to the antibody-IVT mixtures. This bead slurry was placed on a rotating wheel for 60 min at 4 °C before centrifugation for 10 s at room temperature. The supernatant was removed, and the beads

The abbreviations used are: PP1, protein phosphatase 1; NLS, nuclear localization sequence; GST, glutathione S-transferase; CTD, C-terminal domain; IVT, in vitro translation; KESTREL, kinase substrate tracking and elucidation; IP, immunoprecipitation; HEK cells, human embryonic kidney cells; NES, nuclear export signal; APC/C, anaphase-promoting complex cyclosome.
were washed twice with 100 μl of Neb buffer and finally resuspended in 100 μl of Neb buffer. A 25-μl aliquot was transferred to a fresh tube for Western blot analysis of the efficiency of the IP. The remaining 75-μl aliquot was spun down, and the bead pellet was used for kinase assays. For IP of cell lysates, essentially the same protocol was followed except that to preclar the extract, 50 μl of pre-washed protein G-Sepharose beads were added to 40 μl cell lysate diluted in 500 μl of ice-cold Neb buffer, and then 30 μl of protein G-Sepharose beads were added to the antibody-extract mixtures. Immune complexes generated by IP were washed three times with 100 μl kinase assay buffer (30). The beads were resuspended in a final volume of 50 μl of kinase assay buffer (containing [γ-32P]ATP and β-casein) and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 50 μl of 2X Laemmli sample buffer. After separation by SDS-PAGE, the gel was stained with Coomassie Brilliant Blue, dried, and exposed to x-ray film.

**PP1 Binding, Dimerization, and Microtubule Binding Assays—GST, GST-PP1α, and His-Nek2A-CTD were expressed in BL21 E. coli and purified from bacterial lysates on glutathione-Sepharose (GE Healthcare) or Ni^{2+}-nitrilotriacetic acid-agarose beads (Invitrogen) according to standard protocols. Purified proteins were then dialyzed against phosphate-buffered saline for storage at −80 °C. For pulldown assays purified proteins were re-bound to glutathione-Sepharose or Ni^{2+}-nitrilotriacetic acid-agarose beads for 60 min followed by 3 washes with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8, 0.5% Nonidet-P40). The His-Nek2A-CTD bead complexes were blocked by first washing 3 times with 0.5% Marvel in phosphate-buffered saline and then incubating with rabbit reticulocyte lysate for 30 min before 3 washes in NETN buffer. Protein-bead complexes were then incubated with IVT reaction mixes for 60 min before washing 3 times in NETN buffer containing 450 mM NaCl. Bound proteins were analyzed by SDS-PAGE and autoradiography. Microtubule pulldown assays with proteins generated by IVT were performed as previously described (22).

**Degradation Assays—Preparation of Xenopus laevis cytostatic factor egg extracts, in vitro degradation assays, and the addition of MG132 were performed as previously described (24). Generation of S- and M-phase-arrested U2OS cells was also as described (24).

**Immunofluorescence Microscopy—Cells grown on glass coverslips were fixed at −20 °C for 30 min using 100% methanol (precooled at −20 °C). Antibody staining and epifluorescence microscopy was performed as previously described (11, 22). Primary antibodies used were rabbit anti-γ-tubulin (0.15 μg/ml; Sigma), mouse anti-Myc (1:2000; Cell Signaling Technology), rabbit anti-PP1α (31), rabbit anti-Nek2 (2 μg/ml; Invitrogen), mouse anti-phospho-H3 (1:400; Cell Signaling Technology), and mouse anti-nucleophosmin (1:500 (32)). Secondary antibodies were Alexa 488-conjugated goat anti-rabbit and Alexa 594-conjugated goat anti-mouse secondary antibodies (1 μg/ml; Invitrogen). DNA was stained with 1 μg/ml 4,6-diamidino-2-phenylindole or 0.2 μg/ml Hoechst 33258 dye.
Nek2C is an active protein kinase that binds PP1α and undergoes dimerization. A, Myc-tagged Nek2A, Nek2C, and Nek2A-K37R proteins were expressed in HEK 293 cells. After immunoprecipitation with an anti-Myc antibody, an in vitro kinase assay was performed using [γ-32P]ATP and β-casein as substrates. Phosphorylated products were detected by SDS-PAGE and autoradiography. B, the positions of β-casein and autophosphorylated Nek2 proteins are indicated. Equal levels of immunoprecipitated proteins were confirmed by Western blotting (WB) with an anti-Nek2 antibody (lower panel). C, 35S-labeled Nek2A, Nek2B, Nek2C, Nek2A-F386A, and lamin A proteins were generated by in vitro translation (top panel) before binding with either GST-PP1α (middle panel) or GST alone (bottom panel). Proteins were analyzed by SDS-PAGE and autoradiography. The position of the 45-kDa molecular mass marker is indicated. D, 35S-labeled Nek2A, Nek2B, Nek2C, and Rab4 proteins were generated by in vitro translation (top panel) before binding to His-Nek2A-CTD (bottom panel). Proteins were analyzed by SDS-PAGE and autoradiography.

Nek2C is degraded in mitotic egg extracts and mitotic cells. A, 35S-labeled Nek2A and Nek2C proteins were generated by in vitro translation and degradation assays performed in Xenopus cytosolic factor-arrested egg extracts without (−Ca2+) or with (+Ca2+) calcium. Samples were taken at the times indicated (min), separated by SDS-PAGE, and exposed to autoradiography. B, the x-ray films were analyzed by densitometry, and the amount of protein remaining at each time point was plotted. C, degradation assays were carried out in cytostatic factor extracts with +Ca2+ as described above in the absence (−MG132) or presence (+MG132) of the proteasome inhibitor, MG132. D, Myc-tagged Nek2A and Nek2C proteins were transfected into U2OS cells before arresting the cells either in S-phase with hydroxyurea (S) or in M-phase with nocodazole (M) for 16 h. Cell extracts were analyzed by Western blotting with Myc and α-tubulin antibodies, as indicated.

(China) were co-stained with the secondary antibody. Coverslips were mounted on slides in a drop of FluoroGuard anti-fade reagent (Bio-Rad). Images were captured on Nikon TE300 or Olympus IX81 fluorescence microscopes. To score whether transfected cells had predominantly nuclear accumulation of recombinant protein, cytoplasmic accumulation, or relatively equal distribution, pixel levels were measured in representative areas of the nucleus and cytoplasm using analysis software. If these values were >10% different, then the cell was judged to have the protein predominantly in one compartment or the other.

Nuclear-Cytoplasmic Fractionation—Nuclear and cytoplasmic fractions of HeLa cells transfected with Myc-tagged Nek2 constructs were performed using the Nuclear/Cytosol fractionation kit (Cambri Bridge BioScience Ltd., Cambridge, UK) according to manufacturer’s instructions. Western blots were performed as described (11) using anti-Myc (1:1000; Cell Signaling Technology), anti-lamin A/C (1:30; Chemicon, Chelmsford, UK), and anti-α-tubulin (1:3000; Sigma) antibodies.

Kinase Substrate Tracking and Elucidation (KESTREL) Analysis—HEK 293 cells were lysed in extraction buffer (EB; 50 mM Tris-HCl, pH 7.5, 5% glycerol, 1% Triton X-100, 14 mM β-mercaptoethanol, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, 1 mM Pefabloc), and the nuclear fraction was sedimented by centrifugation for 20 min at 15,000 × g. The nuclear pellet was incubated for 20 min with EB plus 0.5 mM NaCl, and protein was extracted by sedimenting insoluble material by centrifugation for 20 min at 28,000 × g. The supernatant was filtered, desalted on Sephadex G25, and chromatographed on heparin-Sepharose. Aliquots of the desalted extracts and eluted fractions were diluted 10-fold in 50 mM Tris-HCl, pH 7.5, 7.5 mM β-mercaptoethanol, 1 mM EGTA, 10 μg/ml leupeptin, 1 mM Pefabloc). These aliquots were incubated for 5 min with 3 mM MnCl2, 2 Kβq/ml [γ-32P]ATP in the absence or presence of Nek2, Nek6, Nek7, or Nek9 recombinant kinases (Invitrogen) and then separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and autoradiographed.
RESULTS

Expression of Nek2C in Human Cell Lines—Three splice variants of the cell cycle-regulated Nek2 protein kinase have been described, Nek2A, Nek2B, and Nek2C (originally called Nek2A-T; Fig. 1A). To confirm the expression of Nek2C, primers were designed for use in reverse transcription-PCR analysis that spanned the splice site and, when used on plasmids containing Nek2A and Nek2C cDNAs, amplified distinct bands of 153 and 129 bp, respectively (Fig. 1B). Using these primers, a major band of 153 bp, representing Nek2A, was amplified using mRNA isolated from HeLa, U2OS, and HEK 293 human cell lines. However, a 129-bp band at the expected size of Nek2C was also reproducibly detected, although this was ∼10-fold less abundant (Fig. 1, B and C). To confirm the identity of this band, it was excised from the gel, re-amplified using the same primers, purified, and subcloned into a pBlueScript vector. DNA sequencing confirmed that the 129-bp product contains the predicted sequence of Nek2C and lacks the 24 nucleotides present in Nek2A (Fig. 1D). Hence, Nek2C is a bona fide splice variant whose expression can be detected, albeit consistently more weakly than Nek2A, in different human cell lines.

Nek2C Is an Active Kinase That Binds PP1α and Undergoes Dimerization—Nek2C contains the complete catalytic kinase domain (Fig. 1A). It also contains the leucine zipper motif that is required for dimerization and autophosphorylation and the PP1 binding site (20, 23). However, the 8-amino acid deletion removes two serine residues (Ser-377 and -378) present in Nek2A that could act as sites for regulation (26). First, to compare Nek2A and Nek2C kinase activity, full-length Myc-tagged Nek2A, Nek2C, and Nek2A-K37R (catalytically inactive variant whose expression can be detected, albeit consistently more weakly than Nek2A, in different human cell lines.

Nek2C Is Degraded in Mitotic Egg Extracts and Prometaphase-arrested Cells—Total Nek2 exhibits cell cycle-dependent mRNA and protein expression with low levels in G1 and increased expression in S and G2 (33, 34). After mitotic entry, Nek2A is specifically targeted for proteasomal degradation as a result of APC/C-mediated ubiquitylation, whereas Nek2B remains stable as it lacks the degradation motifs present in the C terminus of Nek2A (24, 25). These degradation motifs, a KEN-box and the C-terminal MR dipeptide, are present in Nek2C, and we predicted that this isoform would also be degraded in mitosis. To test this we first generated Nek2A and

FIGURE 4. Nek2C binds microtubules in vitro and associates with centrosomes in cells. A, Nek2A and Nek2C proteins were produced by in vitro translation in the presence of [35S]methionine and analyzed by SDS-PAGE and autoradiography either before (Input) or after sedimenting at 35,000 rpm in the absence (−MT) or presence (+MT) of taxol-stabilized microtubules. Pellet (P) and supernatant (S) fractions were analyzed. The Coomassie Blue-stained gel (CB) is also shown indicating the presence of tubulin in the pellet fraction in the presence of microtubules. B and C, immunofluorescence microscopy of U2OS cells transfected with myc-tagged Nek2A or Nek2C and stained with anti-myc (red) and either anti-γ-tubulin (B) or anti-PP1α (C) antibodies (green). Merged images are shown, and centrosomes are indicated with arrowheads; enlargements of the centrosomes are also included. Scale bar, 5 μm.
Nek2C proteins by IVT and added them to mitotic extracts prepared from cytostatic factor-arrested *Xenopus* eggs. These extracts contain all the necessary machinery for APC/C-mediated ubiquitlation and proteasomal degradation (35). In untreated extracts, which represent metaphase II of meiosis, both proteins were slowly degraded, whereas upon the addition of calcium, which releases the extracts into anaphase, both proteins were rapidly degraded (Fig. 3, A and B). This degradation was proteasome-dependent as it was inhibited by MG132 (Fig. 3C). Moreover, both Nek2A and Nek2C were degraded in untreated extracts, which represent metaphase II of meiosis, Nek2C could indeed bind to microtubules in vitro (Fig. 4A), whereas transfection revealed colocalization of myc-tagged Nek2C with γ-tubulin at the centrosome (Fig. 4B). As has been previously described, Nek2 associates with proteins at the proximal ends of centrioles, whereas γ-tubulin is distributed throughout the pericentriolar material, thus explaining why the two signals only partially overlap at high resolution. Previous localization of PP1 isoforms had shown that PP1α and PP1γ1 are present at the centrosome in mitotic cells (36). Because Nek2C had been isolated in a yeast two-hybrid screen with PP1γ as bait and Nek2C retains the PP1 binding site (KVHF386), we stained cells transfected with Nek2C with antibodies against PP1α. Again, we observed colocalization of the signals at interphase centrosomes (Fig. 4C). These centrosome localizations patterns were identical to those of Nek2A (Figs. 4, B and C). Thus, as predicted from the known motifs, Nek2C can associate with microtubules and localize to the centrosome probably in a complex with PP1.

**Non-centrosomal Nek2C Accumulates in the Nucleus**—While examining U2OS cells transfected with Nek2C, we noticed that in most cells (82%) the non-centrosomal protein was predominantly present in the nucleus (Fig. 5, A and B). This was in contrast to U2OS cells transfected with Nek2A in which the protein showed a variable localization, sometimes mainly nuclear (35%), sometimes mainly cytoplasmic (29%), and sometimes distributed evenly throughout the cell (36%). Almost identical results were obtained in HeLa cells (Fig. 5, A and B). For comparison, we decided to determine the cytoplasmic-nuclear distribution of the third splice variant, Nek2B. Strikingly, Nek2B was rarely detected predominantly in the nucleus (7%) with the majority of cells having the transfected protein predominantly in the cytoplasm (52%). To corroborate these microscopy experiments, transfected HeLa cells were also subject to subcellular fractionation and analysis by Western blotting (Fig. 5C). Again, Nek2C was predominantly found in the nuclear fraction (70%), whereas Nek2A was present in equal amounts in the nuclear (49%) and cytoplasmic (51%) fractions. Using this approach, Nek2B also appeared to be equally distributed between nuclear (48%) and cytoplasmic (52%) fractions. This discrepancy with the microscopy data for Nek2B is most likely due to the microscopy being a measure of individual cells, whereas the Western blot is a measure of total transfected protein. It was clear from the microscopy that the most strongly...
expressing cells were the ones that tended to show equal staining throughout the cell (i.e., N/H11005 C). However, counting individual cells, very few showed accumulation of NekB in the nucleus, but there were clearly cells with protein accumulated in the cytoplasm (see Fig. 5A). Hence, we conclude that there is a preferential uptake of Nek2C into the nucleus, whereas Nek2A tends to be more evenly distributed, and Nek2B remains more in the cytoplasm, at least in the less strongly expressing cells. Thus, nuclear translocation of Nek2 is regulated by alternative splicing.

Nuclear Translocation of Nek2C Depends upon an NLS That Flanks the Splice Site—To explain the unexpected nuclear accumulation of Nek2C, but not Nek2A or Nek2B, we scanned the Nek2 protein sequence for potential NLSs and nuclear export signals (NESs) (37, 38). We identified in Nek2A a putative bipartite NLS at residues 361–383 and a weak NES at residues 372–375 (Fig. 6A). The NLS comprises two patches of basic residues, RK (361–362) and KKK (381–383). Intriguingly, these two patches span the 8 amino acids missing from Nek2C, resulting in a linker of 18 amino acids in Nek2A but a linker of only 10 amino acids in Nek2C. Because most bipartite NLSs have a linker of 10–12 amino acids (39), we hypothesized that, as a result of the eight amino acid deletion, the NLS may be better recognized by the nuclear transport machinery in Nek2C than in Nek2A. Nek2B lacks the second basic patch so does not contain a bipartite NLS. Meanwhile, the leucine-rich NES sequence LLNL (372–375) falls within the eight-amino acid stretch missing from Nek2C and so could act as a functional NES only in Nek2A.

To test whether either of these signals was functional, we generated a series of mutants in the predicted NES and NLS (Fig. 6A). All mutants localized correctly at the centrosome, suggesting that protein folding in the C-terminal domain was largely unaffected (data not shown). Mutations to abolish the putative NES (L372A/L373A/L375A) in the Nek2A protein had no effect with the protein again showing a fairly even mix of distributions. In contrast, mutation in the Nek2C protein of either the first basic patch (R361A/K362A) or the second basic patch (K373A/K374A/K375A) led to proteins that were rarely predominantly nuclear (<18%) (Fig. 6, B and C). A combined mutant with all five basic residues mutated was even more effective at keeping the protein from accumulating in the nucleus (<2%). Thus, mutation of the NES did not lead to an accumulation of Nek2A in the nucleus, but mutation of the NLS did substantially reduce uptake of Nek2C into the nucleus. We conclude that a functional bipartite NLS is present in Nek2C, is less effective in Nek2A, and is completely absent in Nek2B. Hence, as a result of the NLS spanning the splice site, the cell can regulate the overall abundance and the particular isoform distribution of Nek2 in the cytoplasm and nucleus.

Detection of a Novel Nek2 Nuclear Substrate—Aspergillus NIMA promotes chromatin condensation through phosphorylation of histone H3 (8). We, therefore, tested whether

TABLE 1

| Nek2 Variant | Amino Acid Sequence |
|--------------|---------------------|
| Nek2A        | 359-KERKFLASNPPELLNLPSVVIKKVKHV |
| Nek2B        | 359-KERKFLASNPGLRNLVNRSCYK      |
| Nek2C        | 359-KERKFLASNPVIIKKVKHV         |
| Nek2A-mNES   | 359-KERKFLASNPEnaPSSVVIKKVKHV  |
| Nek2C-mNLS-2A| 359-KeaFLASNPVIIKKVKHV         |
| Nek2C-mNLS-3A| 359-KERKFLASNPVlaaaVHF         |
| Nek2C-mNLS-5A| 359-KeaFLASNPVlaaaVHF          |

FIGURE 6. Mutation of the bipartite NLS leads to loss of Nek2C from the nucleus. A, the amino acid sequences of Nek2A, Nek2B, and Nek2C starting at Lys-359 and encompassing the proposed NLSs are indicated. The two basic patches of the bipartite NLS are in bold, and the eight-amino acid insertion is underlined. For comparison, the sequences within this region of the four mutants, Nek2A-mNES and Nek2C-mNLS-2A, -3A, and -5A, are shown with the mutated residues in lowercase. B, immunofluorescence microscope images of U2OS (a–f) and HeLa (m–x) cells transfected with the Myc-tagged Nek2 mutants as indicated stained with anti-myc antibodies (red) and with 4,6-diamidino-2-phenylindole (DAPI) to reveal the nucleus (blue). Merge images are shown. Scale bar, 20 μm. C, The subcellular distribution of mutant protein was scored as either predominantly nuclear (N>C), evenly distributed throughout the cell (N= C), or predominantly cytoplasmic (N<C) in 100 cells in three independent experiments. Error bars, S.D.
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**FIGURE 7. Detection of a 28 kDa Nek2 substrate in HEK 293 nuclear extracts.** A. U2OS cells that were either untransfected (a–c) or transfected with mycNek2C for 24 h (d–f) were processed for immunofluorescence microscopy with Nek2 (a and d) and phospho-H3 (b and e) antibodies, and DNA was stained with Hoechst 33342 (c and f). Scale bar, 5 μm. B, U2OS cells transfected for 24 h with mycNek2C were stained with anti-Nek2 (d) and anti-nucleophosmin (b) antibodies, and DNA was stained with Hoechst 33342 (c). A merged image is shown (d). C, nuclear extracts from HEK 293 cells were desalted and chromatographed on heparin-Sepharose. Aliquots of the desalted extract (load) and the fractions (numbered) were incubated with Mn-ATP in the absence (−) or presence of Nek2, Nek6, Nek7, or Nek9 kinases as indicated. The reactions were separated by SDS-PAGE, transferred to polyvinylidene difluoride, and analyzed by autoradiography. The positions of molecular mass markers (kDa) and autophosphorylated Nek kinases are indicated. The 28-kDa protein in fraction 3, phosphorylated only in the presence of Nek2, is indicated (*).

nuclear accumulation of Nek2C might similarly promote this event. However, although untransfected prophase cells had clearly condensed chromatin that stained with phospho-H3 antibodies, interphase cells with strong nuclear accumulation of transfected Nek2C exhibited neither condensed chromatin nor phospho-H3 staining (Fig. 7A). Nek2A has also been reported to colocalize with Nek11 in nucleoli and to interact with the nucleolar protein, nucleophosmin (40, 41). We, therefore, examined whether Nek2C was concentrated in nucleoli and colocalized with nucleophosmin. As expected, the bulk of transfected cells contained mycNek2C in the nucleus, but there was almost complete exclusion from the nucleoli and, hence, no colocalization with nucleophosmin (Fig. 7B). With a nuclear function for Nek2C still unclear, we, therefore, decided to perform an unbiased search for nuclear substrates of Nek2 using the KESTREL approach for substrate detection (42). This method has been developed for identifying bona fide substrates of kinases by making use of short incubation times, high concentrations of added kinase, and high specificity of radioactivity combined with extracts that have been fractionated to separate endogenous kinases from their substrates and concentrate the substrates. Cytoplasmic and nuclear extracts were prepared from HEK 293 cells and fractionated by heparin-Sepharose chromatography. These fractions were then incubated for 5 min with Mn-ATP, the preferred substrate of Nek2 (34), and either no kinase or recombinant Nek2A, Nek6, Nek7, or Nek9 before separation by SDS-PAGE and autoradiography (Fig. 7C). A 28-kDa protein in fraction 3 of the nuclear extract was specifically phosphorylated by the Nek2 kinase but not the closely related Nek6, Nek7, and Nek9 kinases. This protein was not detected in fraction 3 of the cytoplasmic extract. Although substantial further work will be required to validate this observation, the specificity of the phosphorylation and the stringent nature of the KESTREL approach leads us to propose that this 28-kDa protein is likely to represent a novel nuclear substrate of Nek2.

**DISCUSSION**

Nek2A was originally isolated in a degenerate PCR-based screen for human kinases related to Aspergillus NIMA (43). The first alternative splice variant, Nek2B, was identified in Xenopus oocytes (44) and subsequently in cultured human cells (19). Most recently, Nek2C (originally called Nek2A-γ) was described after a yeast two-hybrid screen performed with a testis library and PP1 as bait (26). These three splice variants differ only in their non-catalytic C-terminal domains, and hence, not surprisingly, all encode active protein kinases. Indeed, Nek2C differs from Nek2A only in so much as it lacks eight amino acids (371–378) in the middle of the non-catalytic domain. Nevertheless, our results suggest that this small change has significant consequences on subcellular localization, which in turn may allow the different splice variants to undertake distinct functions during cell division.

Although several independent clones representing Nek2C were identified in the two-hybrid screen with a testis library, the majority of clones isolated in that study were Nek2A (26). Likewise, Nek2A cDNAs were isolated from mouse testis libraries in three independent laboratories (27–29). Hence, both Nek2A and Nek2C are present in testis, although they may have distinct functions to play during meiotic cell division. By using common primers that span the splice site and amplify products of distinct sizes, we were able to detect Nek2C mRNA in three different human cell lines (U2OS, HeLa, and HEK 293), although at levels ~10-fold lower than Nek2A. Previous studies revealed elevated expression of Nek2 in cancer cell lines and primary tumors without distinguishing between Nek2A and Nek2C (45). It will be interesting to determine whether there is elevation of both splice variants.

Clearly, at the sequence level, there is very little difference between Nek2A and Nek2C, and one would predict that they share many of the same properties. With respect to regulation
of kinase activity, we confirmed that Nek2A and Nek2C phosphorylate the exogenous substrate β-casein equally, show similar levels of autophosphorylation, have the capacity to undergo dimerization, and can both bind PP1α. Moreover, the extreme C terminus, which contains two APC/C-dependent degradation motifs, is identical in the two proteins, and we found no difference in their destruction in mitotic extracts or prometaphase-arrested cells. Finally, Nek2A and Nek2C both retain the region encompassing amino acids 333–370 required for microtubule binding and centrosome localization, and experimentally, we were able to confirm that Nek2C retains these properties. Thus, our data not only confirm that Nek2A and Nek2C are biological very similar but provide further evidence for the correct mapping of these different functional motifs.

Despite the high degree of sequence identity, we were able to identify an important difference in the behavior of the two variants. After expression of recombinant proteins, it was clear that Nek2C was preferentially taken up into the nucleus, whereas Nek2A was more evenly distributed throughout the cell. Mechanistically, this can be explained by the loss of the eight amino acids from Nek2C that result from splicing. Nek2C contains an RKX_{10}KKK sequence, where X is an uncharged linker, that conforms perfectly to the well defined bipartite NLS motif (39). Mutation of the NLS led to loss of nuclear Nek2C, whereas mutation of a putative NES located within the eight amino acids missing from Nek2C had no effect. Furthermore, Nek2B, which only has the first basic patch and so lacks a functional NLS altogether, was present within the nucleus less than either Nek2A or Nek2C in single cell analysis. Because Nek2 proteins are generally around 45 kDa, they are close to the cut-off for proteins that can diffuse passively into the nucleus. This would explain why proteins were sometimes found in both compartments, particularly in the most strongly expressing cells. Localization of the endogenous protein, which is present at much lower levels, is likely to be more discretely controlled.

Structural analysis of NLS-containing peptides bound to the cargo binding domain of importin-α show that there is space to accommodate two lysine or arginine residues in the upstream basic patch and five lysine or arginines in the second basic patch; these are ideally separated by a linker of 10 residues (46, 47). Studies on the NLS sequence of nucleoplasmin revealed that linkers shorter than 10 amino acids are not tolerated, whereas longer linkers are tolerated but can have reduced activity (39). In Nek2A, the linker is extended to 18 amino acids and includes additional hydrophobic and proline residues that could alter the conformation of the linker and interfere with its ability to function as a bipartite NLS. Indeed, there is support for interaction of the linker backbone with importin-α residues (47). However, there are cells in which Nek2A is concentrated in the nucleus, indicating that the longer linker does not completely abrogate NLS activity (15). Indeed, there may be an additional level of regulation at which the distribution of Nek2A is controlled. It is intriguing that the second basic patch overlaps with the PP1 binding site (383–386) raising the possibility that nuclear uptake via importin-α interaction and PP1 binding are mutually exclusive. On the other hand, simultaneous binding to importin-α and PP1 would be possible for opposing polypeptides of dimeric Nek2.

The function of Nek2 in the nucleus remains to be determined. Conflicting data exists on whether endogenous Nek2 is localized to condensed chromatin in early stage meiotic cells (27, 28). However, persuasive studies on mouse spermatocytes suggest that Nek2 plays an active role in chromatin condensation during the first meiotic division through phosphorylation of the chromatin-associated protein, high mobility protein A2 (17, 18). Nuclear entry of Nek2C may be necessary for providing access not only to downstream substrates, such as high mobility protein A2, but also to upstream regulators, including activated extracellular signal-regulated kinase 1/p90Rsk2, before nuclear envelope breakdown. This, coupled with the fact that NIMA regulates chromatin condensation in Aspergillus (8) seems the most promising explanation for nuclear Nek2. However, in contrast to NIMA, we found that nuclear accumulation of Nek2C did not promote phosphorylation of histone H3, indicating that, alone, it is not sufficient to trigger premature chromatin condensation. Interactions have been described for Nek2 with nucleolar proteins, including nucleophosmin (also known as nunaturin or NPM/B23) and, interestingly, another NIMA-related kinase, Nek11 (40, 41). However, we found that nuclear Nek2C was excluded from nucleoli and did not co-localize with nucleophosmin, raising questions over the significance of these reported interactions. Because the nuclear function of Nek2C remains obscure, we opted instead to initiate a search for novel nuclear substrates of Nek2 using the unbiased KESTREL chromatographic approach. This led to identification of a 28-kDa protein as a likely physiological substrate of Nek2. Future efforts will be required to scale up this process to identify this protein by mass spectrometry. In conclusion, besides providing support for a nuclear role of Nek2, this work neatly illustrates how alternative splicing can modulate the structure, and thereby functionality, of a bipartite NLS motif.

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