Alternative splice variants of the human centrosome kinase Nek2 exhibit distinct patterns of expression in mitosis

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Nek2 is a cell-cycle-regulated protein kinase that localizes to the centrosome and is likely to be involved in regulating centrosome structure at the G₂/M transition. Here, we localize the functional human *Nek2* gene to chromosome 1 and show that alternative polyadenylation signals provide a mechanism for generating two distinct isoforms. Sequencing of products generated by reverse transcriptase PCR, immunoblotting of cell extracts and transfection of antisense oligonucleotides together demonstrate that human Nek2 is expressed as two splice variants. These isoforms, designated Nek2A and Nek2B, are detected in primary blood lymphocytes as well as adult transformed cells. Nek2A and Nek2B, which can form homo- and hetero-dimers, both localize to the centrosome, although only Nek2A can induce centrosome

splitting upon overexpression. Importantly, Nek2A and Nek2B exhibit distinct patterns of cell-cycle-dependent expression. Both are present in low amounts in the G₁ phase and exhibit increased abundance in the S and G₂ phases. However, Nek2A disappears in prometaphase-arrested cells, whereas Nek2B remains elevated. These results demonstrate that two alternative splice variants of the human centrosomal kinase Nek2 exist that differ in their expression patterns during mitosis. This has important implications for our understanding of both Nek2 protein kinase regulation and the control of centrosome structure during mitosis.

Key words: cell cycle, centriole, phosphorylation.

INTRODUCTION

The centrosome is the primary organizer of the microtubule (MT) network in animal cells [1–3]. It is composed of two stable cylindrical structures, called centrioles, each of which is associated with a proteinaceous mass, known as pericentriolar material (PCM). Whereas centrioles are relatively simple in composition, comprised mostly of post-translationally modified α - and β -tubulin, the PCM is more complex. Experimentally, the PCM can be separated into a salt-insoluble meshwork of fibres, termed the centromatrix, and a soluble fraction of proteins that are held in place through association with either centrioles or the centromatrix [4,5].

Progression through the cell cycle is accompanied by gross changes in structural organization as well as the MT nucleation capacity of the centrosome. Centrosomes duplicate once per cell cycle to ensure bipolarity of the mitotic spindle as well as stable inheritance of one centrosome at each division [6]. Duplication is characterized by the appearance and gradual elongation of two new centrioles that grow perpendicular to and in close association with parental centrioles. In late G₂ phase of the cell cycle, centrosomes gain an increased capacity for MT nucleation through recruitment of additional PCM [7]. As mitosis begins, duplicated centrosomes move apart towards opposite ends of the cell, and forces generated by MT- and chromosome-associated motor proteins lead to establishment of the bipolar mitotic spindle [8,9]. In telophase, the new centrioles lose their tight association with parental centrioles, but appear to remain connected through establishment of a structure that has been referred to as the intercentriolar linkage [10–12].

Many of the changes that occur during the centrosome cycle are likely to result from protein phosphorylation [13,14]. Cyclin-

dependent kinases, polo kinases, aurora kinases and NIMA (never in mitosis A)-related kinases (Neks), as well as protein phosphatase (PP) 1 α and PP4, all associate with the centrosome at some point in the cell cycle. At the present time, however, little is known about how phosphorylation events modify centrosome structure or MT nucleation activity. Phosphorylation may act through regulating the association of proteins with the centrosome. Equally, phosphorylation could target centrosomal proteins for degradation and, as components of ubiquitin ligase enzymes and the proteasome have been detected at the centrosome, this could take place without need for prior dissociation [15–17].

Nek2 is a serine/threonine kinase that localizes to the centrosome throughout the cell cycle [18]. Its expression and activity are regulated by the cell cycle, with peak levels in the S and G₂ phases [19,20]. However, Nek2 interacts with the catalytic subunit of PP1, which is capable of dephosphorylating both Nek2 itself and other Nek2 substrates [21]. This direct competition between kinase and phosphatase means that centrosomal targets of Nek2 may only become significantly phosphorylated when PP1 activity is reduced at the G₂/M transition [22,23]. Overexpression of wild-type, but not catalytically dead, Nek2 to a level that is likely to be in excess of endogenous PP1 induces premature centrosome splitting [18]. This raises the possibility that Nek2 normally functions to promote centrosome separation. In support of this hypothesis, a Nek2 substrate has been identified, called centrosomal Nek2-associated protein 1 (C-Nap1), which has properties consistent with it being a component of the intercentriolar linkage [24,25]. C-Nap1 localizes to proximal ends of centrioles during interphase, but is absent from mitotic spindle poles. Furthermore, interference with C-Nap1 by antibody micro-injection or expression of dominant-negative constructs also

Abbreviations used: MT, microtubule; PCM, pericentriolar material; NIMA, never in mitosis A; Nek, NIMA-related kinase; PP, protein phosphatase; C-Nap1, centrosomal Nek2-associated protein 1; RT-PCR, reverse transcriptase PCR; GFP, green fluorescent protein.

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leads to premature centrosome splitting [26]. The role of Nek2 may therefore be to phosphorylate C-Nap1 and, perhaps, other structural proteins of the centrosome, triggering their dissociation or degradation and, in so doing, cause the intercentriolar linkage to be broken.

Recent studies on the *Xenopus laevis* homologue of Nek2 suggest additional functions in assembly and/or maintenance of centrosome structure. In *Xenopus* egg extracts, depletion of Nek2 interferes with centrosome assembly and MT aster formation from the sperm basal body [27]. Meanwhile, injection of neutralizing antibodies or kinase-dead Nek2 constructs into embryos causes fragmentation of spindle poles, aberrant spindle formation and abortive cleavage [28]. This function for Nek2 may not be restricted to early embryos, as long-term overexpression of wild-type or catalytically inactive Nek2 also leads to centrosome disintegration in adult human cells [18]. Two alternative cDNAs have been isolated for *Xenopus* Nek2 that encode products with distinct C-termini. These products could arise through allelic differences in the duplicate copies of the *Nek2* gene in the tetraploid *X. laevis* genome [29]. Equally, they could arise through alternative splicing. The two isoforms display distinct patterns of developmentally regulated expression, with the shorter form, X-Nek2B, present in oocytes, eggs and early embryos, and the longer form, X-Nek2A, present in embryos after the gastrula–neurula transition as well as in adult testis [30]. Murine Nek2 is also highly expressed in adult testis, although the possible existence of multiple Nek2 isoforms in mammals has not been addressed [31–33]. We therefore decided to examine whether alternative splicing represents a mechanism for regulating Nek2 kinase in vertebrates and, more specifically, in human cells. Our results reveal the existence of two alternatively spliced variants of the human Nek2 kinase. Both isoforms can localize to the centrosome but, importantly, they are subject to distinct patterns of cell-cycle-regulated expression with specific differences in mitosis.

EXPERIMENTAL

Genomic sequence analysis

For somatic cell hybrid analysis, a human monochromosomal somatic cell hybrid DNA panel was obtained from the U.K. HGMP Resource Centre (Hinxton, Cambridge, U.K.) [34]. PCR reactions were performed with the Expand high-fidelity PCR system (Roche Biochemicals) on 1 µl of each chromosomal hybrid in a total reaction volume of 20 µl, using 5' and 3' oligonucleotide primers complementary to the original *Nek2* cDNA sequence. PCR products were either separated on 1% agarose gels or purified using a PCR purification kit (Qiagen) for sequencing. To determine the genomic structure of human *Nek2*, a human single chromosome library representing chromosome 1 (LL01NC01) produced at the Lawrence Livermore National Laboratory was obtained as a high-density gridded filter from the U.K. HGMP Resource Centre. To generate a probe, *Nek2* cDNA was excised as a *NaeI*–*XbaI* fragment from pGEM-Nek2 and radiolabelled with [α^{32} P]dCTP using the Prime-It random priming kit (Stratagene). Positive clones were identified by autoradiography of the filter following overnight hybridization with the probe at 65 °C. Representative cosmids were ordered from the U.K. HGMP Resource Centre and sequenced using a range of oligonucleotide primers in the Protein and Nucleic Acid Chemistry Laboratory (University of Leicester, Leicester, U.K.) on an ABI 377 Automated Sequencer (PerkinElmer) using Big Dye technology.

Reverse transcriptase PCR (RT-PCR) and isolation of human *Nek2B* cDNA

RNA was isolated from HeLa and U2OS cells using TRI reagent (Sigma) according to manufacturer's instructions. RNAs were treated with amplification-grade DNase I (Gibco) for 15 min at room temperature before stopping the reaction with a one-tenth vol. of 25 mM EDTA and heating to 65 °C for 10 min. DNase I-treated RNAs were reverse transcribed at 42 °C for 50 min using oligo(dT) and Moloney-murine-leukaemia virus reverse transcriptase (Promega) according to manufacturer's instructions, and PCR reactions performed for 35 cycles (94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s) with oligonucleotide AF008 (5'-GGCGAATTCATACCGTTAC-3') as a 5' primer and AF007 (5'-GCCAGAGACAGGAACCTCCG-3'; specific for *Nek2A*) or AF017 (5'-GCTACCTAAGCCACTTTGGGAG-3'; specific for *Nek2B*) as a 3' primer. To generate plasmid pGEM-Nek2B, carrying the complete open reading frame of *Nek2B*, the 496 bp RT-PCR product from AF008/AF017 was first subcloned into the pCR2.1 TA cloning vector (Invitrogen) to create pCR2.1-Nek2B. A partial *SacI* fragment, encompassing the C-terminus of Nek2A, was then excised from pGEM-Nek2 [19] and replaced with the equivalent *SacI* fragment from pCR2.1-Nek2B, encoding the C-terminus of Nek2B. As these are splice variants, the sequences 5' to the splice site are identical.

Plasmid constructs

The following constructs were generated for protein expression in eukaryotic cells: pEGFP-Nek2A, pEGFP-Nek2B and pCMV-myc:Nek2B (pCMV-myc:Nek2A has been described in [18]). To create pEGFP-Nek2A and pEGFP-Nek2B, the coding region was excised on a *NaeI*–*BamHI* fragment from pGEM-Nek2A [19] and pGEM-Nek2B, respectively, and subcloned into the *SalI* (blunted with Klenow)–*BamHI* sites of the pEGFP(C1) vector (Invitrogen) that had been modified by the introduction of a T7 promoter upstream of the green fluorescent protein (GFP) coding region (a kind gift from Dr K. Tanaka, Paterson Institute for Cancer Research, Manchester, U.K.). For pCMV-myc:Nek2B, the coding sequence was excised from pGEM-Nek2B on a *NaeI*–*XbaI* fragment and subcloned into the pBlueScript:myc vector cut with *SmaI* and *XbaI*, to create pBS-myc:Nek2B. The in-frame fusion of Nek2B with a Myc-epitope tag was then excised from pBS-myc:Nek2B on a *SalI* (blunted with Klenow)–*XbaI* fragment and subcloned into the pRcCMV vector (Invitrogen) cut with *HindIII* (blunted with Klenow) and *XbaI*.

Cell culture and transfections

HeLa epithelial, U2OS osteosarcoma, HEK-293 epithelial and KE37 T-lymphoblastoid cells were grown at 37 °C in a 5%–CO₂ atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (100 i.u./ml and 100 µg/ml, respectively). Human T-lymphocytes were obtained from peripheral blood donated by healthy volunteers as described previously [35]. Transient transfection of expression plasmids was carried out using either Lipofectamine 2000 reagent according to manufacturer's instructions (Life Technologies), or calcium phosphate, as described elsewhere [36]. For oligonucleotide transfections, the following phosphorothioate-modified oligodeoxynucleotides were synthesized by Interactiva (Hybaid, Ulm, Germany): 5'-GAGCCTGTGCCAATGGTG-3' (antisense) and 5'-CACCATTGGCACAGGCTC-3' (sense). These were introduced into cells using Lipofectamine 2000 at a final concentration of 200 nM in cell-culture medium. The transfection mix was left on the cells for

24 h prior to preparation of cell extracts or processing for immunofluorescence microscopy. Cell extractions were as described [37,38].

Cell synchronization and flow cytometry

To obtain cells synchronized at different stages of the cell cycle, U2OS cells were first presynchronized in the S phase by the addition of 2 mM thymidine for 12 h and released by transfer into fresh medium for 8 h. Cells were then resynchronized, either at the G₁/S boundary or in the M phase, by incubation for 16 h with 1 mM hydroxyurea or 1 µg/ml nocodazole, respectively. S- and G₂-phase populations were obtained by releasing from the block induced by hydroxyurea for 3 and 9 h, respectively. The M-phase population was obtained by gently pipetting the mitotically arrested cells from the nocodazole block, while the G₁-phase population was obtained by releasing from the nocodazole block for 6 h. Release of cells from drug-induced blocks was performed by three washes in 1 × PBS and replacement in fresh medium. Cell-cycle distributions were confirmed by flow cytometry. For this purpose, 1 × 10⁶ cells were washed in ice-cold PBS and slowly resuspended while vortex-mixing in 1 ml of 70 % ethanol (−20 °C) before storage at −20 °C. Prior to flow-cytometric analysis, fixed cells were washed gently in ice-cold PBS, resuspended in 1 ml of DNA stain (0.02 mg/ml propidium iodide/0.2 mg/ml RNase A in PBS) and incubated at room temperature for 30 min. DNA content was measured using a FACScan II instrument (Becton Dickinson) and analysed using CellQuest.

Miscellaneous techniques

In vitro translation of Nek2 constructs was done using the TnT-coupled transcription/translation kit in the absence or presence of [³⁵S]methionine/cysteine (Expre³⁵S³⁵S; NEN Life Science Products) according to manufacturer's instructions (Promega). For co-precipitation experiments, reticulocyte lysates were first incubated separately at 30 °C for 90 min and then together for 20 min with 250 µg/ml cycloheximide to allow interactions without further translation. Immunological techniques were performed as described previously [18,36,37]. Primary antibodies used in this study were: α-Nek2 (R31, 1:500; R40, 1 µg/ml; anti-peptide antibody, 1 µg/ml [18,19,38]); α-C-Nap1 (R63, 1 µg/ml [24]); α-Myc (9E10, undiluted tissue-culture supernatant); α-cyclin A (1 µg/ml; Upstate Biotechnology); α-cyclin B1 (1 µg/ml; Upstate Biotechnology) and α-tubulin (0.3 µg/ml; Amersham Pharmacia Biotech). Fluorescence images were captured using a Quantix 1400 CCD camera (Photometrics) using SmartCapture software and processed using Adobe Photoshop (San Jose, CA, U.S.A.).

RESULTS

Structure of the human Nek2 gene

In the original characterization of the human Nek2 cDNA, two gene loci were identified by fluorescence *in situ* hybridization, at 1q32.2–1q41 and 14q12 [19]. Genomic clones containing the Nek2 sequence have also been deposited in the GenBank nucleotide sequence database from chromosome 22-specific libraries. Meanwhile, a number of partial cDNA sequences have been identified by PCR amplification that are highly related, but non-identical, to regions within the Nek2 catalytic domain [39]. To address the possible existence of multiple human Nek2 genes, PCR reactions were performed on a panel of somatic cell hybrids using non-degenerate oligonucleotide primers complementary to

Figure 1 The functional human Nek2 gene is located on chromosome 1

(A) Somatic cell hybrid analysis. Oligonucleotide primers complementary to regions within the Nek2 3'-untranslated region were used in PCR reactions on DNA isolated from a panel of mouse and hamster somatic cell hybrids carrying individual human chromosomes as indicated. Controls were no DNA (—), Nek2 cDNA (cD), and total mouse (M), hamster (Ha) or human (H) DNA. Fragments of the expected size were identified in panels containing chromosomes 1, 2, 14 and 22. (B) Partial protein sequences (amino acids 114–161) of hypothetical products from the different Nek2 genes based on DNA sequencing of PCR products generated from the somatic cell hybrids using primers within the catalytic domain. Note that Nek2 genes on chromosomes 2, 14 and 22 would encode proteins with a termination codon (●) instead of Glu-126 (arrowhead) and lack the conserved DFG (amino acids 159–161) of classical serine/threonine kinases. Asterisks indicate positions where the chromosome 2, 14 and 22 sequences differ from that of chromosome 1.

the published Nek2 cDNA. Products were amplified from chromosomes 1, 2, 14 and 22, suggesting that highly related copies do exist on at least four different chromosomes (Figure 1A). Investigation of the human genome sequence confirmed the existence of a single Nek2 sequence on each of these chromosomes, but no others. Sequencing of the PCR products (confirmed later by analysis of the human genome sequence) revealed that only the sequence on chromosome 1 bears complete identity with the published Nek2 cDNA and hence must encode the *bona fide* Nek2 protein. Multiple single-nucleotide substitutions are present in the Nek2 sequences on chromosomes 2, 14 and 22. Some of these cause amino acid substitutions in residues that are known to be critical for protein kinase activity. More importantly, a termination codon replaces glutamate-126 within the N-terminal catalytic domain of the chromosome 2, 14 and 22 sequences. Hence, any protein expression would lead to truncated polypeptides with a predicted molecular mass of 14.6 kDa. Scrutiny of the human genome database indicated that these other Nek2 sequences lack introns, which implies strongly that they are pseudogenes that have arisen from the integration of fully processed Nek2 mRNAs (Figure 1B).

Two alternative cDNAs have been isolated for the *X. laevis* homologue of human Nek2 [27,30]. These could have arisen from duplicate copies of the Nek2 gene in this tetraploid organism or, as the cDNAs diverge at a putative splice junction, they may have been generated through alternative splicing. To test whether human Nek2 is alternatively spliced, a cosmid encompassing the full-length Nek2 gene from chromosome 1 was isolated to first determine its gene structure. Sequencing of the cosmid indicated that Nek2 mRNA is encoded on eight exons, with the initiation codon in exon 1 and the UAG stop codon in exon 8 (Figure 2A and Table 1). Exon 7 terminates at a position that is equivalent to the point of divergence of the two *Xenopus* Nek2 cDNAs. Sequencing of the seventh intron revealed the presence of a

Figure 2 Structure of the human *Nek2* gene

(A) Intron–exon structure of the human *Nek2* gene on chromosome 1. Exons (black boxes) are drawn to scale. However, as not all introns have been fully sequenced these are not to scale. The positions of the single AUG initiation codon, the two alternative protein stop sites and polyadenylation signals (AAUAAA), and the primers used for RT-PCR reactions (horizontal arrows) are indicated. (B) Partial protein sequences of human (Hs; from amino acid 364) and *Xenopus* (Xi; from amino acid 365) Nek2A and Nek2B are shown with the position of the splice site indicated (arrowhead). In both species, Nek2B terminates soon after the splice site, whereas the C-termini of the Nek2As are somewhat longer. The conserved KEN box destruction motif is shaded and the PP1-binding site is underlined. Human Nek2B has the GenBank accession number AY045701.

termination codon starting 43 nucleotides after the splice site and an alternative polyadenylation signal after a further 625 nucleotides (Figure 2A). Based on the position of the termination codon in the seventh intron, use of this alternative polyadenylation signal would generate a human Nek2B protein of 384 amino acids, with a calculated molecular mass of 44.9 kDa and a pI value of 8.64. The predicted Nek2A and Nek2B proteins would diverge in sequence after amino acid 370, causing Nek2B to lack a number of important regulatory motifs that are present in the extreme C-terminus of Nek2A (Figure 2B).

Expression of Nek2A and Nek2B in primary and transformed cells

To determine whether alternative splice variants of Nek2 are expressed in human cells, RT-PCR reactions were performed on RNA isolated from cultured human cells. Forward and reverse primers were synthesized that would allow amplification of a fragment from the known Nek2A mRNA or from a product generated from the hypothetical Nek2B mRNA. RT-PCR products representing both Nek2A and Nek2B were generated readily using RNA isolated from either U2OS or HeLa cells and sequencing confirmed these to be the predicted Nek2 products

(Figure 3A). Amplification was dependent upon the presence of reverse transcriptase and gave rise to products of the expected sizes, ruling out the possibility that they were generated from either genomic DNA or unspliced primary transcripts.

To determine whether Nek2A and Nek2B are expressed as proteins in cultured cells, we re-examined the proteins recognized by Nek2 antibodies on Western blots. The anti-peptide Nek2 antibody described previously [38] was capable of recognizing both splice variants, as indicated by detection of recombinant Nek2A and Nek2B proteins translated *in vitro* (Figure 3B, lanes 1 and 2). The full-length recombinant Nek2B protein was generated using the product from the RT-PCR reaction. In extracts of HeLa cells, the Nek2 antibody recognized proteins of 48 and 44 kDa, which co-migrated with the *in vitro*-translated Nek2A and Nek2B proteins respectively (Figure 3B, lane 3). Two other previously described Nek2 antisera raised against bacterially expressed Nek2 fragments (R31 and R40) also detected both the 48 and 44 kDa proteins (results not shown). Transfection of HeLa cells with recombinant Nek2A led to a specific increase in abundance of the 48 kDa product; likewise, transfection with Nek2B caused only the 44 kDa product to become more abundant (Figure 3B, lanes 4 and 5). This argues strongly against the 44 kDa band being a degradation product of Nek2A, as there is no reason to suspect that the transfected protein would be processed differently from the endogenous protein. Although one additional cross-reacting band was detected with the anti-peptide antibody, this was not recognized by the other Nek2 antisera, leading us to conclude that HeLa cells specifically express two splice variants of the Nek2 kinase.

To determine whether both Nek2 isoforms are expressed in primary as well as transformed cells, extracts were prepared from a variety of transformed cell lines and from freshly collected peripheral T-lymphocytes. Western blotting revealed the presence of Nek2A and Nek2B in all cell lines tested and peripheral T-lymphocytes, emphasizing that Nek2B expression is not confined to either transformed cells or early stages of development (Figure 3C). A slower-migrating form of Nek2 was also detected in peripheral T-lymphocytes. This was not detected in transformed cell lines, implying that a substantial fraction of Nek2 might exist in a post-translationally modified state in primary cells.

Unfortunately, attempts to generate Nek2A- and Nek2B-specific antisera using peptides based on the predicted C-termini have so far proved unsuccessful. To confirm that the two bands detected by Nek2 antibodies at 48 and 44 kDa represent Nek2 proteins rather than unrelated cross-reacting antigens, we therefore used an antisense oligonucleotide approach. An antisense *Nek2* sequence was identified that, when introduced into human cells by liposome-mediated transfection, specifically caused down-regulation of both the 48 and 44 kDa proteins, but not α -

Table 1 Intron–exon boundaries of the human *Nek2* gene

Nucleotide sequences before and after each splice junction in the human *Nek2* gene sequence are indicated. Exon and intron sequences around the splice sites are shown in upper and lower case, respectively, and the conserved gt–ag of splice-site donor and acceptor are in bold. Position numbers relate to those used in the original isolation of the *Nek2* cDNA [19].

Exon no.	Exon sequence	Intron sequence	Exon sequence	Exon no.
1	TGATGGCAAG ¹⁷⁸	gtgagcctgggacctaccct...cttgaaattcttattcag	A ¹⁷⁹ TATTAGTTT	2
2	CCAAGGAAAG ³⁹⁶	gtaagcataatcttttaaaa...tataactgcttggttttag	G ³⁹⁷ CAATACTTA	3
3	CATGTCTCCT ⁶³⁷	gtaagtatttcagaatttag...tacccttttccctgtatag	G ⁶³⁸ AACAAATGA	4
4	GTGCATTAAT ⁷²⁰	gtaagtatgatgaagacaga...ttctctttttaaccctag	G ⁷²¹ CCTCCATTG	5
5	AAACTTAAAG ⁸⁴⁷	gtaagatgataagatgata...actgagtatttttcccccag	G ⁸⁴⁸ ATTACCATC	6
6	AGATTGGAGC ¹⁰⁶⁷	gtaagtctgaatgggggtca...tccattctctttttaagag	A ¹⁰⁶⁸ GAAAGAACAA	7
7	AGTAATCCAG ¹¹⁹³	gtatgagaatacaacttggtc...ttttcttttctcttaag	A ¹¹⁹⁴ ACTTCTTAA	8

Figure 3 Expression of human Nek2A and Nek2B

(A) RT-PCR demonstrates the synthesis of processed mRNAs representing both human Nek2A and Nek2B. RNA isolated from HeLa (lanes 2, 3, 7 and 8) or U2OS (lanes 4, 5, 9 and 10) cells was used in RT-PCR reactions with or without the addition of reverse transcriptase (RT), as indicated. No RNA was added to the reactions shown in lanes 1 and 6. PCR primers were designed (see Figure 2A) that would specifically amplify products from mRNA encoding either Nek2A (lanes 1–5) or Nek2B (lanes 6–10). As the 5' primer was made to sequence in exon 5, products generated from either genomic or unspliced primary transcripts would still contain intron sequence and so be much larger than those amplified. PCR fragment sizes are indicated (bp). (B) The migration of *in vitro*-translated (IVT) Nek2A (lane 1) and Nek2B (lane 2) was compared on Western blots with proteins recognized in extracts of untransfected HeLa cells (lane 3) and HeLa cells transfected with untagged Nek2A (lane 4) or Nek2B (lane 5). The anti-peptide anti-Nek2 antibody recognizes proteins at 48 and 44 kDa that co-migrate with recombinant Nek2A and Nek2B, respectively. A third band recognized by this antibody in HeLa cell extracts (*) was not recognized by other Nek2 antisera tested. (C) Western blotting with the anti-peptide Nek2 antibody indicates that Nek2A and Nek2B are present in extracts of the transformed cell lines shown in lanes 1–4 and T-lymphocytes collected from peripheral blood (lane 5).

tubulin or the lower-molecular-mass protein recognized by the Nek2 antisera (Figure 4A). The corresponding sense sequence (as well as scrambled and mismatch sequences; results not shown) did not cause suppression of either Nek2 isoform. Indirect immunofluorescence microscopy of cells stained with Nek2 antibodies revealed that Nek2 was detected at the centrosome in

Figure 4 Antisense oligonucleotides inhibit expression of Nek2A and Nek2B

(A) No DNA (lane 1), or 200 nM antisense (lane 2) or sense (lane 3) *Nek2* oligonucleotides was introduced into HeLa cells by liposome-mediated transfection. After 24 h, cells were lysed and extracts processed for Western blotting with anti-peptide Nek2 (top panel) or α-tubulin (bottom panel) antibodies. The positions of Nek2A, Nek2B and α-tubulin proteins are indicated together with the low-molecular-mass protein that cross-reacts with the anti-peptide Nek2 antibody (*). Molecular masses (kDa) are indicated on the left of the Nek2 blot. (B) HeLa cells were treated with 250 nM sense (a, b) or antisense (c, d) *Nek2* oligonucleotides and processed for immunofluorescence microscopy using anti-peptide Nek2 (a, c) and anti-γ-tubulin (b, d) antibodies. Nek2 was detected at centrosomes in the presence of sense, but not antisense, oligonucleotides (arrowheads). Scale bar, 10 μm.

the presence of sense, but not antisense, oligonucleotides (Figure 4B). Taken together, the RT-PCR, immunoblot and antisense data provide compelling evidence that human Nek2 is expressed as two distinct isoforms from a single gene on chromosome 1.

Dimerization of Nek2 proteins

Due to its alternative C-terminus, Nek2B protein lacks the PP1-binding site that is present in Nek2A. This implies that PP1 would not be able to counteract phosphorylation events catalysed by Nek2B. However, Nek2A has been shown previously to form homodimers via its leucine zipper dimerization motif [38]. As Nek2B retains the leucine zipper, it may be possible for PP1 to regulate Nek2B if the latter is able to heterodimerize with Nek2A. To test whether Nek2A and Nek2B can form heterodimers, recombinant Nek2 proteins were mixed *in vitro* and immunoprecipitated with antibodies against an N-terminal Myc-epitope tag that was present on either one or the other protein

Figure 5 Nek2A and Nek2B can form homodimers and heterodimers

The Nek2 proteins indicated were prepared by coupled *in vitro* transcription/translation reactions in either the absence (Myc-Nek2A and Myc-Nek2B) or presence (³⁵S-Nek2A and ³⁵S-Nek2B) of [³⁵S]methionine/cysteine. Samples were mixed as shown in the presence of cycloheximide to prevent further translation and an aliquot taken for direct analysis by SDS/PAGE, followed by either Western blotting with Nek2 antibodies (top panel) or autoradiography (middle panel). The remainder was immunoprecipitated with anti-Myc antibodies, processed by SDS/PAGE and exposed for autoradiography (bottom panel). The positions of Myc-Nek2A (mA), Myc-Nek2B (mB), ³⁵S-Nek2A (A) and ³⁵S-Nek2B (B) are indicated on the right.

(Figure 5). Myc-tagged Nek2A was capable of precipitating untagged versions of both Nek2A and Nek2B. Myc-tagged Nek2B could also precipitate untagged versions of Nek2A and Nek2B. Small amounts of ³⁵S-labelled Nek2 proteins were brought down in control precipitations, but significantly less than in the presence of the Myc-tagged Nek2 proteins. The observation that similar amounts of each isoform were co-precipitated irrespective of which protein was used as the bait indicates that Nek2A and Nek2B can form both homodimers and heterodimers with similar efficiency.

Localization of human Nek2B to centrosomes

Indirect immunofluorescence microscopy and biochemical fractionation have been used to show that endogenous Nek2 is a core component of the human centrosome. Whereas the antibodies used would not distinguish between the two isoforms in microscopy, both the 48 and 44 kDa Nek2 products were present on Western blots of enriched centrosome preparations [18]. *Xenopus* X-Nek2B is also rapidly recruited from egg cytoplasm to the sperm basal body during centrosome assembly and localizes to centrosomes when transfected into *Xenopus* cultured cells [27,28]. Based on these data, we would expect both Nek2 isoforms to be present at the centrosome of adult human cells. To verify this prediction, recombinant versions of Nek2A and Nek2B were introduced into U2OS and HeLa cells by transient transfection and analysed by immunofluorescence microscopy. Using either enhanced GFP or Myc-epitope tags for detection, Nek2A and Nek2B were clearly detected at the centrosome, as indicated by co-localization with antibodies against C-Nap1 (Figure 6A) and γ -tubulin (Figure 6B). As reported previously, overexpression of active Nek2A kinase induced centrosome splitting in a high proportion of cells [18]. Strikingly, however, overexpression of Nek2B did not cause any significant change in the percentage of cells with split centrosomes compared with non-transfected

cells (Figure 6C). These results imply that Nek2B is not capable of stimulating centrosome splitting with the same efficiency as Nek2A. This may reflect a difference in the affinity of Nek2B for certain centrosomal substrates.

Cell-cycle-dependent expression of Nek2A and Nek2B

Human Nek2 was reported previously to be cell-cycle-regulated, with peak levels in the S and G₂ phases and low levels in the M and G₁ phases [19,20]. These studies focused on the abundance of the 48 kDa Nek2A protein. However, during the first zygotic cell cycle of *Xenopus laevis*, the abundance of X-Nek2B remains constant [27]. It was important therefore to examine the relative expression of human Nek2A and Nek2B at different stages of the adult cell cycle. HeLa cells were synchronized carefully using drug arrest/release protocols, as described in the Experimental section, and the stage of the cell cycle confirmed by flow cytometry (Figure 7A). Immunoblotting of cell extracts with anti-Nek2 antisera confirmed that the expression pattern of Nek2A matched previous reports for full-length Nek2. On the other hand, the expression of Nek2B, although still cell-cycle-regulated, showed an important difference from Nek2A. Whereas the abundance of both Nek2A and Nek2B was low in the G₁ phase and increased in the S and G₂ phases, in cells arrested in mitosis with nocodazole Nek2B remained at a high level when Nek2A levels were reduced significantly (Figure 7). These results suggest strongly that the decrease in abundance of Nek2A in a prometaphase block is the result of a post-translational mechanism that targets motifs present only in the extended C-termini of the Nek2A isoform. Investigation of the mechanism of Nek2A destruction in mitosis is currently in progress.

DISCUSSION

It is now clear that bipolar spindles can form independently of centrosomes both *in vitro* and *in vivo* [40–45]. However, when centrosomes are present, as they are in the vast majority of animal cells, they play a dominant role in organizing the mitotic spindle [46]. For this reason, failure to correctly regulate either the structure or function of the centrosome would be expected to increase the possibility of misformed spindles and chromosome mis-segregation. Indeed, the prevalence of aneuploidy in tumour cells correlates well with the high incidence of centrosome defects, although the cause and effect of this relationship remains open to question [47,48]. Nek2 is one of only a handful of protein kinases that have so far been shown to (i) localize to the centrosome, (ii) alter centrosome structure upon overexpression or inhibition and (iii) have a *bona fide* substrate at the centrosome. Understanding its regulation is therefore of considerable importance in interpreting its contribution to centrosome regulation through the cell cycle.

Previously, it has been shown that Nek2 is regulated through a number of mechanisms that include homodimerization, *trans* auto-phosphorylation and association with the protein phosphatase PP1 [21,38]. Here, using several lines of evidence, we show that Nek2 is also regulated through the generation of alternative splice variants. First, two previously identified cDNAs for *Xenopus laevis* diverge at a position equivalent to an exon–intron boundary in the human *Nek2* gene located on chromosome 1. Secondly, distinct Nek2 products are amplified from human RNA by RT-PCR using primers that are specific for the two isoforms. Thirdly, several different anti-Nek2 antisera recognize common bands at the predicted molecular masses of Nek2A (48 kDa) and Nek2B (44 kDa). Fourthly, the 48 and 44 kDa proteins co-migrate precisely with *in vitro*-translated and -overexpressed versions of recombinant Nek2A and Nek2B.

Figure 6 Nek2A and Nek2B localize to the centrosome

(A) U2OS osteosarcoma cells were transiently transfected with GFP-Nek2A (a, e), GFP-Nek2B (b, f), Myc-Nek2A (c, g) or Myc-Nek2B (d, h). Cells were fixed 24 h after transfection and analysed by indirect immunofluorescence microscopy. Arrowheads indicate the co-localization of GFP signal (a, b) and anti-Myc antibodies (c, d) with centrosomes, as revealed by antibodies against the centrosomal protein C-Nap1 (e–h). (B) HeLa cells were transiently transfected with Myc-Nek2A (a, c) or Myc-Nek2B (b, d), fixed after 24 h and analysed by indirect immunofluorescence microscopy using anti-Myc antibodies to detect transfected protein (a, b) and anti- γ -tubulin antibodies to reveal centrosomes (c, d). Scale bars, 10 μ m. (C) The percentage of cells in which the two centrosomes were separated by more than 2 μ m was calculated for HeLa cells transfected with Myc-tagged Nek2A and Nek2B proteins and compared with non-transfected cells (control).

Finally, antisense oligonucleotides, complementary to a region of the *Nek2* cDNA common to both isoforms, inhibit expression of the 48 and 44 kDa products, but not other control proteins. The presence of alternative splice products in humans, and most likely *Xenopus*, suggests strongly that this form of regulation is conserved amongst vertebrate Nek2 kinases.

So what is the purpose of generating alternative splice variants? It seems unlikely that they are required in different subcellular compartments, as evidence presented here and elsewhere indicates that both Nek2 proteins are present at the centrosome. For instance, recombinant Nek2A and Nek2B can localize to the centrosome, proteins of 48 and 44 kDa are recognized by anti-Nek2 antibodies in preparations of isolated human centrosomes, and the *Xenopus* Nek2B protein is recruited to sperm basal bodies from egg extracts *in vitro* [18,27]. The fact that Nek2A and Nek2B can form heterodimers further supports the notion that, where their expression levels are similar, they are likely to be found in the same subcellular compartment. Differences would

only become apparent if one isoform was present in considerable excess, as X-Nek2B is in early *Xenopus* embryos. In this respect, it is interesting that the non-centrosomal pool of recombinant Nek2B protein was predominantly cytoplasmic, whereas that of Nek2A was frequently nuclear (R. S. Hames and A. M. Fry, unpublished work). The selective uptake of Nek2A into the nucleus may be through use of a putative bipartite nuclear localization sequence in the C-terminus of Nek2A (amino acids 361–383) that is missing from Nek2B, although this remains to be tested.

One reason for generating alternative splice variants may lie in the need for different levels of centrosome regulation at different stages of development. In fertilized eggs and early embryos of *Xenopus*, Nek2B is the only isoform detected, whereas a switch in expression to Nek2A occurs at the time of the gastrula–neurula transition [30]. Although we could detect Nek2B in both primary and transformed adult cells, Nek2A was always more abundant. The presence of an alternative polyadenylation site within intron

Figure 7 Distinct expression of Nek2A and Nek2B in mitosis

(A) Extracts were prepared from U2OS cells synchronized at different stages of the cell cycle, as described in the Experimental section. Samples were processed by SDS/PAGE and Western blotted with antibodies against Nek2, cyclin A, cyclin B or α -tubulin, as indicated. Flow cytometry was carried out on each cell population and cell-cycle profiles are shown below each lane, where the open and closed arrows indicate diploid and tetraploid DNA, respectively. (B) Histogram comparing Nek2A and Nek2B abundance at different cell-cycle stages as obtained by quantitative densitometric scanning (arbitrary units) of Western blots shown in (A). Black bars, Nek2A; white bars, Nek2B. Exp, cells in the exponential growth phase.

7 implies that Nek2A and Nek2B may be generated through recognition of different polyadenylation signals. The 3'-UTR of the predicted human Nek2B cDNA sequence also contains a cytoplasmic element, UUUUUAU, known to bind factors that regulate mRNA translation in oocytes via a complex cytoplasmic polyadenylation mechanism [49]. *Xenopus* Nek2B protein was reported to increase in abundance during oocyte maturation [30]. Hence, it is possible that vertebrate Nek2 is a general target for this highly specific form of developmentally regulated translational control.

Differential expression of the two Nek2 isoforms during development would imply a biological reason for switching from one isoform with a short C-terminus to another with an extended C-terminus. Importantly, we have found that human Nek2A and Nek2B show distinct patterns of expression during the cell cycle, specifically in mitosis. Whereas expression of both isoforms is low in the G₁ phase and increases in the S/G₂ phases, Nek2A is absent from cells arrested in the prometaphase with nocodazole, whereas Nek2B remains present. We have evidence that human

Nek2A is targeted for degradation in prometaphase and that this occurs as a result of motifs present in its distinct C-terminus (R. S. Hames and A. M. Fry, unpublished work). These results fall in line with the observation that recombinant *Xenopus* Nek2A is also unstable during mitosis of early embryos, whereas Nek2B is not [28]. Maintaining a stable version of the Nek2 protein may therefore facilitate the rapid cell cycles that take place during early embryogenesis.

Functions that have been proposed for Nek2 at the centrosome include triggering loss of centrosome cohesion and maintenance and/or assembly of the centrosome structure. In mammalian cells, centrosome separation is first detected in the prophase and precedes nuclear-envelope breakdown and spindle formation. A model for Nek2 function proposes that phosphorylation in the late G₂ phase of substrates such as C-Nap1 leads directly or indirectly to dissolution of a linkage that holds interphase centrosomes together [50]. This would facilitate the establishment of the bipolar mitotic spindle by MT- and chromosome-based motor proteins. In adult human cells, both Nek2A and Nek2B show cell-cycle-dependent expression, in line with a role late in the cell cycle. The association of Nek2A with active PP1 in the S and early G₂ phases, but with inactive PP1 in the prophase, could explain how Nek2A achieves a peak of substrate phosphorylation at the G₂/M transition. Although Nek2B lacks the PP1-binding motif, heterodimerization of Nek2B with Nek2A could allow phosphorylation by both kinases to be kept in check by PP1 until it is inactivated at the onset of mitosis. However, as Nek2B persists beyond the prometaphase, when Nek2A disappears, this may provide an alternative mechanism for overcoming PP1 inhibition and ensuring that Nek2 function is completed in early mitosis. Importantly, in contrast with Nek2A, overexpression of Nek2B does not stimulate centrosome splitting, implying that the prime function of Nek2B may not relate to centrosome separation. One speculation that is consistent with studies in *Xenopus* embryos is that Nek2B helps to maintain the integrity of spindle poles throughout mitosis. Determining whether the two Nek2 isoforms have separate functions in adult cells will require new approaches to selectively inhibit the function of one or the other protein.

We thank Erich Nigg (Max Planck Institute, Martinsreid, Germany) in whose laboratory the initial chromosome analysis was started, Roger Snowden for help with flow cytometry, Raj Patel for preparation of peripheral T lymphocytes and Kayoko Tanaka (Paterson Institute for Cancer Research, Manchester, U.K.) for the T7-modified pEGFP vector. We also thank the MRC-funded UK HGMP Resource Centre (Hinxton, Cambridge, U.K.) for the somatic cell hybrid panel, the chromosome 1 gridded filter LL01NC01 and the Nek2-containing cosmid clones ah39-h3, ah419-d2, ah60-d11, ah91-a12 and ah94-g7. Finally, we thank Sue Shackleton for critical reading of the manuscript and all members of the laboratory for technical help and useful discussion. This work was supported by grants to A.M.F. from The Wellcome Trust (056335) and the Cancer Research Campaign (SP2486/0101). A.M.F. is a Lister Institute Research Fellow.

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