NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1

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The cell cycle-regulated protein serine/threonine NIMA-related kinase 2 (Nek2), which shows a predominant localization at centrosomes, is identified as a protein which interacts with protein phosphatase 1 (PP1) using the yeast two-hybrid system. Complex formation between Nek2 and PP1 is supported by co-precipitation of the two proteins using transfected expression constructs of Nek2 and the endogenous Nek2/PP1 proteins. The sequence KVHF in the C-terminal region of Nek2, which conforms to the consensus PP1-binding motif, is shown to be essential for the interaction of Nek2 with PP1. Nek2 activity increases with autophosphorylation and addition of phosphatase inhibitors and decreases in the presence of PP1. PP1 is a substrate

for Nek2 and phosphorylation of PP1 γ_1 on two C-terminal sites reduces its phosphatase activity. The presence of a ternary complex containing centrosomal Nek2-associated protein (C-Nap1), Nek2 and PP1 has also been demonstrated, and C-Nap1 is shown to be a substrate for both Nek2 and PP1 *in vitro* and in cell extracts. The implications of kinase–phosphatase complex formation involving Nek2 and PP1 are discussed in terms of the coordination of centrosome separation with cell cycle progression.

Key words: centrosome, cell signalling, NIMA related kinase, protein serine/threonine phosphatase.

INTRODUCTION

Most cellular functions are regulated by reversible protein phosphorylation catalysed by protein kinases and protein phosphatases. Protein phosphatase 1 (PP1) is a major eukaryotic protein phosphatase that regulates a diverse range of cellular processes by dephosphorylation of serine and threonine residues [1,2]. The pleiotropic function of PP1 resides in the ability of the catalytic subunit of PP1 (PP1c) to associate with many different regulatory subunits *in vivo* that may target PP1c to specific subcellular locations, alter its substrate specificity and allow its activity to be modified by extracellular signals. Over 20 different regulatory or targeting subunits of PP1c have now been identified. For example, glycogen-binding subunits target PP1c to regulate the enzymes of glycogen metabolism, and myosin-targeting subunits enable PP1c to regulate myosin contractility [3,4]. Several proteins that interact with PP1c permit this phosphatase to regulate specific nuclear processes. These include NIPP-1 (nuclear inhibitor of PP1) [5,6], p99/PNUTS (PP1 nuclear targeting subunit) [7,8] and the polypyrimidine tract-binding protein-associated splicing factor, PSF1 [9] (proteins that are likely to be involved in RNA processing and transport), and sds22 [suppressor 2 of dis2 (PP1) mutant], a protein required for exit from mitosis [10]. Binding of PP1c to scaffold proteins may modulate ion channel activity [11] and other neuronal functions [12]. Complex formation of PP1c with other proteins, such as p53-binding protein 53BP2 [13], the ribosomal-binding protein RIPP1 [14], and a protein of herpes simplex virus 1 [15] that influences protein synthesis, may direct PP1c to specific substrates. PP1c also binds to a number of small inhibitor proteins, including inhibitor-1 and inhibitor-2, which inhibit PP1c activity at nanomolar concentrations [1,16].

Interaction of regulatory subunits with PP1c is usually mutually exclusive, an observation recently explained by the discovery that a short motif -(R/K)(V/I)XF- present in the majority (but not all) of these subunits is sufficient for binding to PP1c [4,17]. In the present study, we identify the protein serine/threonine NIMA-related 2 kinase (Nek2) as a protein that interacts with PP1c via the PP1c consensus-binding motif.

Nek2 is a mammalian kinase structurally related to the protein kinase NIMA of *Aspergillus nidulans* that is required for entry into mitosis [18–20]. Temperature-sensitive mutations of NIMA reversibly arrest cells in late G2 [21], despite the presence of activated p34^{cdc2} kinase activity, indicating that NIMA function plays an essential role downstream or parallel to p34^{cdc2} for progression through mitosis [22]. Although phosphorylation of NIMA by p34^{cdc2}/cyclin B is not required for basal level NIMA kinase activity, it appears to be required for mitotic entry [23].

Evidence for a NIMA-like mitotic pathway in mammalian cells was suggested by the induction of G2 arrest in HeLa cells with dominant negative mutants of NIMA, although the arrest could be overcome by a constitutively active mutant of p34^{cdc2} [24]. The most closely related mammalian kinase to NIMA is Nek2 (47% amino acid identity in the catalytic domain), and both kinases are expressed in a cell cycle-regulated manner [25]. However, expression of the Nek2 protein is low during M and G₁, but high during S and G₂ phases of the cell cycle, its serine/threonine kinase activity paralleling its abundance [26]. This suggests that Nek2 may function earlier in the cell cycle than NIMA (which accumulates in G₂), its expression is high during M phase, and it is degraded at the end of M phase. Nek2 shows an intense localization at centrosomes, the principal microtubule-organizing centre of mammalian cells [27]. Overexpression of Nek2 induces centrosome splitting, while more prolonged over-

Abbreviations used: NIMA, never in mitosis in *Aspergillus nidulans*; Nek2, NIMA-related kinase 2; PP1, protein phosphatase 1; PP1c, protein phosphatase 1 catalytic subunit; C-Nap1, centrosomal Nek2-associated protein; GST, glutathione-S-transferase; MBP, maltose-binding protein; ATP[S], adenosine 5'-[γ -thio]triphosphate.

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expression of active or inactive Nek2 leads to dispersal of centrosomal material, suggesting that Nek2 is involved in centrosome separation at the G₂/M transition. A yeast two-hybrid screen with Nek2 as bait led to the isolation of centrosomal Nek2-associated protein (C-Nap1), a protein with extensive coiled-coil structure that was highly enriched in centrosome preparations. The C-terminal region of C-Nap1 was phosphorylated by Nek2 *in vitro*, as well as after coexpression of the two proteins *in vivo*. It was proposed that C-Nap1 could be a component connecting the proximal ends of the duplicated centrioles to each other that, upon phosphorylation by Nek2, would cause loss of centriole-centriole cohesion and permit centrosome splitting [28]. In the present article, we show that Nek2 and PP1 form a kinase-phosphatase complex that can influence the phosphorylation state of C-Nap1, and discuss the implications of the interactions for the regulation of centrosome separation and cell cycle progression.

MATERIALS AND METHODS

General methods and the yeast two-hybrid analysis

Microbial strains and methods for the yeast two-hybrid screen are described in Helps et al. [13,29]. In order to isolate proteins capable of interacting with PP1, the yeast strain Y190 containing a pAS2-PP1₁ plasmid was transformed with DNA from a human peripheral B lymphocyte cDNA library in pACT. Ten colonies were obtained on selective media from which the pACT plasmids were recovered into *Escherichia coli* and their cDNA inserts sequenced.

Oligonucleotides were synthesized by Miss Audrey Gough and Miss Kaera Maxwell (University of Dundee, Scotland, U.K.) on an Applied Biosystems model 394 DNA synthesizer. DNA sequencing was performed using Taq dye terminator cycle sequencing on PE Biosystems automated DNA sequencers.

Construction of glutathione-S-transferase (GST) fusion vectors for the expression of Nek2 variants in mammalian cells

Reverse transcription was used to obtain the coding region of Nek2 5' of PP1H5 (which encodes amino acid residues 227–445 of Nek2) using total RNA from a human breast adenocarcinoma cell line MCF7, mouse Moloney murine leukaemia virus reverse transcriptase (Promega) and oligo(dT) primers. The resulting cDNA served as template for a PCR using two specific primers, Nek2_{1–21} (5'-GCGCGGATCCCATATGCCTTCCCGGGCTG-AGGAC-3') and Nek2_{1105–1083} (5'-TCTCTTGCTTTGAGAG-CTCGCTC-3'), containing the restriction sites *Bam*HI and *Sac*I respectively (underlined). The PCR product of 973 bp was digested with *Bam*HI and *Sac*I, purified using QIAquick Nucleotide Removal Kit (Qiagen) and inserted into pACT-PP1H5 cleaved with the same enzymes. The resulting construct, containing the full-length cDNA of Nek2 in a *Bam*HI-*Bgl*II fragment, was verified by DNA sequencing. For expression of Nek2 as a GST fusion protein in mammalian cells, the Nek2 cDNA was subcloned into the *Bam*HI site of the vector pEBG-2T [30] to produce the expression plasmid, pEBG-Nek2.

The construct pEBG-Nek2KD (kinase dead) was produced by mutating the codon for lysine-37 (AAA) in Nek2 to the codon for arginine (AGA). The mutation was performed by a two-step recombinant PCR method [31,32], using pEBG-Nek2 as template DNA with primer Nek_{252–230} (5'-CAAGTTCTCTCCAACT-AATATC-3') containing the desired mutation (underlined) and its reverse complement. The outside primers were Nek_{1–21} and Nek_{625–606} (5'-CTAGCTAGCCCAAAGTCTCC-3'), downstream of a *Hinc*II site. The PCR product was subcloned into

pCR2.1-TOPO vector (Invitrogen, Leek, The Netherlands) and verified by DNA sequencing. A 0.4 kb *Bam*HI-*Hinc*II fragment containing the desired mutation was cleaved out and used to replace the corresponding fragment in pEBG-Nek2.

The construct pEBG-Nek2NB (non-binding) was produced by mutation of the codon for phenylalanine-386 (TTC) to alanine (GCC). The primer Nek2_{747–766} (5'-TTGCTGTATGAGTTA-TGTGC-3'), which lies upstream of an *Eco*RI site, and primer Nek2_{1337–1277} (5'-CTCAGAATTCTCACTCCTCATGATGTT-CTCTTTACTTTCCCACTGGCATGAACCTTTCTTC-3') containing an *Eco*RI site (double underlined) and the mutation (underlined), were employed using pEBG-Nek2 as a template in a PCR to obtain a fragment containing the desired mutation. The 0.5 kb PCR fragment was digested with *Eco*RI and this fragment was substituted for the corresponding *Eco*RI fragment of Nek2 in pFASTBACHTb (Life Technologies), a vector that has no *Eco*RI sites. The *Bam*HI-*Kpn*I fragment of pEBG-Nek2 was replaced by the corresponding fragment from pFASTBACHTb-Nek2 to give pEBG-Nek2NB, the mutation being confirmed by DNA sequencing.

Expression of proteins in bacteria

C-Nap1 fused to maltose-binding protein (MBP)

DNA encoding the C-terminal amino acid residues 1851–2442 of C-Nap1 [28] was PCR-amplified from human testis Marathon-Ready cDNA according to the manufacturer's instructions (Clontech, Palo Alto, CA, U.S.A.) using the oligonucleotide primers CCATATGACACTGAAGGAGCGTCATGG and TTCTAGATGAGAGGCAGTACATGTC, containing naturally occurring *Nde*I and *Xba*I sites (underlined) present within the cDNA. The PCR product was cloned into the pCR2.1 (TOPO) vector, its sequence was confirmed and the insert was subcloned into the MBP fusion vector, p-MAL-HA [pMAL-c2 (New England Biolabs) modified by Dr C. A. Armstrong to contain an HA tag and additional restriction enzyme sites including an *Nde*I site] using the *Nde*I site and a *Hind*III site (present in the pCR2.1 vector). The plasmid was transformed into *E. coli* BL21 (DE3) pLysS which were grown at 37 °C to *D*₆₀₀ of 0.3, then expression was induced with 100 µM isopropyl β-D-thiogalactoside for 3 h at 37 °C. The cells were harvested by centrifugation, resuspended in ice-cold lysis buffer [50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.02% (w/v) Brij 35, 5% (v/v) glycerol, 1 mM EDTA, 200 mM NaCl], containing 0.1 mM PMSF and 1 mM benzamide, and lysed by sonication. The lysate was centrifuged at 45000 *g* for 20 min and the supernatant was added to 1.5 ml of amylose resin (New England Biolabs) equilibrated in lysis buffer. After incubation with mixing at 4 °C for 1 h, the resin was washed three times with 20 ml of the same buffer, then placed in an Econpac (Bio-Rad) column. After further washing, the bound MBP-C-Nap1 protein was eluted from the column with buffer containing 10 mM maltose. The peak protein fractions were dialysed against 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol, 0.02% (w/v) Brij 35, 5% (v/v) glycerol and stored at –70 °C.

PP1H5 fused to GST and MBP

A *Bgl*II fragment encompassing the PP1H5 fragment of Nek2 was excised from the pACT plasmid and inserted into the *Bam*HI site of the expression plasmids pGEX-3X and pMAL-HA, which were expressed in *E. coli*. GST-PP1H5 was affinity purified on GSH-agarose [13] and MBP-PP1H5 was affinity-purified as described above for MBP-C-Nap1.

PP1 variants

Rabbit (identical sequence to human) PP1 α and human PP1 γ_1 [33] were expressed in *E. coli* and purified as described [34]. The PP1 γ_1 cDNA in the pCW expression vector was mutated at histidine-125 to alanine by using the Quikchange mutagenesis system (Stratagene) and the complimentary oligonucleotides CAGAGGGAACGCTGAATGTGCC and GGCACATTCAGCGTTCCCTCTG (mutated codons in **bold**). After verifying that the sequence was correct, the mutated PP1 γ_1 protein was expressed and purified as described for PP1 γ_1 . The construction and expression of PP1 γ_1 tagged with the epitope EFMPME(H)₆ is described in [13].

Production of antibodies

A 10 mg aliquot of GST–PP1H5 was cleaved with 0.1 mg of Factor Xa (New England Biolabs) in 50 mM Tris/HCl (pH 8.0)/100 mM NaCl/2 mM CaCl₂/1 mM dithiothreitol/0.5 mM EDTA/0.5% Triton for 1 h at 23 °C. The pH was then adjusted to 7.5, the solution was made 2 mM in EGTA and the Factor Xa was removed by adsorption to *p*-aminobenzamidine–agarose beads. Free GST was removed from the PP1H5 by adsorption to GSH–agarose beads. The purified PP1H5 (C-terminal 219 amino acids of Nek2) was used to raise anti-Nek2 antibodies in sheep at the Scottish Antibody Production Unit (SAPU, Carlisle, Ayrshire, Scotland U.K.), which were affinity purified on a column matrix of MBP–PP1H5 covalently coupled to CNBr–Sephacryl (Pharmacia). Affinity purified anti-Nek2 antibodies (1 mg) were coupled to Protein G–Sephacryl (1 ml) using dimethylpimelidate [35]. Anti-GST antibodies were obtained from Santa Cruz Biotechnology. Anti-PP1 antibodies were raised against the human PP1 γ_1 in sheep by SAPU, affinity purified on a column matrix of PP1 γ_1 coupled to CNBr–Sephacryl and coupled to Protein G–Sephacryl as described for Nek2 antibodies. Antibodies against the PP1 γ_1 epitope tag EFMPME were coupled to Sepharose as described in [13]. Anti-C-Nap1 antibodies were raised in sheep by SAPU to the peptides CPHSHKTSPMEEQS, CQAPATVLEAETRR and CLHPSPSTTQAASR, conjugated to keyhole limpet haemocyanin and affinity purified on peptide CH–Sephacryl columns.

Transfection of 293 cells and preparation of cell extracts

Human embryonic kidney 293 cells were cultured in 10 cm-diameter dishes and transfected with pEBG constructs using a modified calcium phosphate method [36]. Cell lysates were prepared 48 h post-transfection after one medium change 24 h after transfection. Each dish of cells was lysed in 0.5 ml of ice-cold lysis buffer [50 mM Hepes (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, 5 mM MnCl₂, 5 mM KCl, 5 mM EGTA, 2 mM EDTA, 0.1% Nonidet P40] containing Complete Protease Inhibitor Cocktail (Boehringer Mannheim) and supplemented with 2 μ M microcystin where stated. The lysate was centrifuged through a QIAshredder (Qiagen) for 10 min at 4 °C for homogenization. The cleared extract was snap-frozen in liquid nitrogen and stored at –80 °C.

Immunoprecipitation, glutathione- and amylose-affinity methods

Cell lysate containing 1 mg of protein in 0.5 ml of lysis buffer was used for each immunoprecipitation. The lysates were precleared at 4 °C for 1 h on a shaking platform with 10 μ l of Protein G–Sephacryl. The supernatants were incubated with 10 μ l of Protein G–Sephacryl coupled to 10 μ g of anti-PP1 antibodies for

1 h at 4 °C. The immunoprecipitates were washed four times with lysis buffer and subjected to 10% PAGE in SDS buffer. Glutathione-affinity and amylose-affinity sedimentations were performed in a similar manner from cell extracts and with purified proteins, except that anti-PP1–Sephacryl was replaced by GSH–Sephacryl (Pharmacia) and amylose resin respectively.

For immunoprecipitations using anti-Nek2 antibody, cell extracts at 1 mg/ml protein in lysis buffer were precleared for 30 min at 4 °C using pre-immune IgG covalently bound to Protein G–Sephacryl, then transferred to anti-Nek2–Sephacryl and incubated for 1 h at 4 °C. The immunoprecipitates were washed three times with lysis buffer and then used for SDS/PAGE or protein phosphatase assays.

In vitro kinase assays

Lysates from cells transfected with pEBG–Nek2 constructs were incubated at 4 °C for 1 h on a shaking platform with 10 μ l of GSH–Sephacryl. The beads were washed twice in lysis buffer containing 0.5 M NaCl, once with lysis buffer and once with kinase buffer [50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂]. The standard Nek2 kinase assay (50 μ l) contained washed GSH–Sephacryl precipitate, 50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM [γ -³²P]ATP (1000 c.p.m./pmol). Myelin basic protein (Gibco Life Sciences, Paisley, Renfrewshire, Scotland, U.K.), dephosphorylated β -casein (Sigma), PP1 or C-Nap1 were included as exogenous substrates at 18 μ M, 16 μ M, 2.7 μ M and 0.7 μ M respectively. The assays were carried out for 15 min at 30 °C, with continuous agitation. Reactions were stopped by the addition of 10 μ l of 6 $\times$ SDS/PAGE loading buffer and heated at 95 °C for 5 min, following by separation of the reaction products by SDS/PAGE and detection by autoradiography. Alternatively, for reactions containing myelin basic protein, a 40 μ l aliquot was removed, spotted onto a 2 $\times$ 2 cm square of phosphocellulose paper and immersed in 75 mM phosphoric acid. After washing four times in phosphoric acid and once in acetone, the papers were dried and the ³²P radioactivity determined by Cerenkov counting. One unit of kinase activity is that amount of enzyme that incorporates 1 nmol ATP into the substrate in 1 min.

Identification of Nek2 phosphorylation sites on PP1 γ

GST–Nek2 protein was purified from 400 μ g of protein from extracts of transfected 293 cells. A 1 μ g portion of PP1 γ (H125A) was then phosphorylated by this kinase preparation in 30 μ l of reaction mixture containing 50 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 0.1% (v/v) 2-mercaptoethanol and 100 μ M [³²P] γ ATP for 1 h at 30 °C. The reaction products were separated by SDS/PAGE in 10% gels and stained with Coomassie Brilliant Blue R250. The PP1 γ band was excised from the gel, digested with trypsin and the peptides were separated by HPLC. ³²P-labelled peptides were analysed by Dr N. Morrice (University of Dundee, Scotland, U.K.) using matrix-assisted laser desorption/ionization–time-of-flight MS on an Elite-STR mass spectrometer (PerSeptive Biosystems) in reflector mode with 4-hydroxy- α -cyanocinnamic acid (10 mg/ml in 50% acetonitrile/0.1% trifluoroacetic acid/49.9% water) as the matrix. The sequences were confirmed by Edman degradation on an Applied Biosystems 476A sequencer. The sites of phosphorylation were determined by measuring the release of radioactivity following solid-phase Edman degradation of the peptides coupled to Sequelon-AA membrane (Milligen, Bedford, M.A. U.S.A.).

Protein phosphatase assays

32 P-labelled rabbit skeletal muscle glycogen phosphorylase was prepared using phosphorylase kinase to a stoichiometry of 1 mol phosphate/mol subunit by Dr H. Y. L. Tung [37]. [32 P]-Labelled MBP-C-Nap1 was prepared as follows: GST-Nek2NB was purified from 1 mg of cell extract as described above and the washed GSH-Sepharose beads further washed once in reaction buffer [50 mM Tris/HCl (pH 7.5), 0.5 mM EGTA, 10% (v/v) glycerol, 0.5% (v/v) 2-mercaptoethanol]. The kinase (attached to the beads) was then used to label 1 mg of pure MBP-C-Nap1 in 1 ml of reaction buffer containing 10 mM MgCl_2 , 100 μM [32 P] γ -ATP, 150 mM NaCl and 20 μM microcystin YR. After incubation at 30 °C for 2 h, NaCl was added to 0.5 M and incubation continued for 10 min. The beads were then pelleted by centrifugation (6000 g, 1 min) and the supernatant loaded onto a 20 $\times$ 1 cm G50 superfine column equilibrated in reaction buffer. Fractions were collected and the peak of radiolabelled protein stored frozen at -20 °C. The specific activity of the [γ - 32 P]ATP used for phosphorylations was 1000 c.p.m./pmol. Protein phosphatase assays were performed as described in [37,38] using phosphorylase (10 μM or 2.7 μM) or C-Nap-1 (2.7 μM). Immunoprecipitation phosphatase assays were performed in a similar manner, except that a shaking incubator was used. One unit of phosphatase activity was that amount of enzyme that catalysed the release of 1 μmol [32 P]phosphate/min from [32 P]-labelled substrate in the standard assay.

Figure 1 Co-sedimentation of GST-Nek2 with PP1

(A) Soluble protein (10 μg) from lysates of 293 cells overexpressing GST (lane 1), GST-Nek2 (lane 2), GST-Nek2KD (lane 3) and GST-Nek2NB (lane 4) was separated by SDS/PAGE, transferred onto a nitrocellulose membrane and then probed with anti-GST antibody. (B–D) Cell extracts as in (A), containing 1 mg of soluble protein, were precleared with 10 μl of Protein G-Sepharose and then incubated for 1 h at 4 °C with 10 μl of GSH-Sepharose (B) or 10 μl of anti-PP1 antibody linked to Protein G-Sepharose (C, D). The Sepharose beads were washed four times with lysis buffer and the bound proteins eluted with SDS/PAGE loading buffer and separated by SDS/PAGE. (B) Immunoblotting was performed with anti-PP1 antibody (bottom panel) and then, after stripping, the filter was reprobed with anti-Nek2 antibody (top panel). Immunoblotting was performed with anti-Nek2 antibody (C) and then, after stripping, with anti-PP1 antibody (D). The positions of the standard (Amersham Rainbow) marker proteins, myosin (220 kDa), glycogen phosphorylase (97 kDa), BSA (66 kDa), ovalbumin (46 kDa) and carbonic anhydrase (30 kDa) are indicated. Arrows mark the positions of the relevant proteins.

RESULTS

Identification of Nek2 as a PP1-binding subunit

In order to identify proteins capable of interacting with PP1, a yeast two-hybrid screen was performed with PP1 γ_1 as bait [29,39]. One of ten positive clones identified from a human B lymphocyte library contained a cDNA, termed PP1H5, that was found to encode the C-terminal region of the protein kinase Nek2. PP1H5 comprised 1294 bp, which encoded amino acids 227–445 and encompassed the entire 3' untranslated region of Nek2. The sequence was identical with that reported previously [25]. The binding of the C-terminal region of Nek2 to PP1 was confirmed by co-immunoprecipitation of bacterially expressed GST-PP1H5 with bacterially expressed epitope-tagged PP1 γ_1 (results not shown).

Full-length Nek2 (amino acids 1–445) was transiently expressed in 293 cells as a GST fusion protein (Figure 1A). The binding of Nek2 to PP1 was shown by sedimentation of GST-Nek2 followed by immunodetection of PP1 in the pellet (Figure 1B). Immunoprecipitation of PP1 followed by immunodetection of the GST-Nek2 fusion protein in the PP1 immunoprecipitates using anti-Nek2 antibody (Figure 1C) or anti-GST antibody (results not shown) confirmed the specificity of the Nek2-PP1 interaction. The band detected in lane 2 of Figure 1 (A, B and C) has an apparent molecular mass of 73 kDa, as expected for the fusion of Nek2 (47 kDa) and GST (26 kDa). This band cannot be seen in lane 1 of the same Figures, which contain the extract of cells transfected with pEBG-2T vector expressing only GST. The results indicate that the binding of GST-Nek2 to PP1 is caused by the interaction of Nek2 with PP1 and not by an interaction of GST with PP1.

The phenylalanine in the -(R/K)(V/I)XF- motif of Nek2 is essential for binding to PP1

The kinase domain of Nek2 encompasses the N-terminal 271 amino acids of Nek2, while the C-terminal region is non-catalytic. This region contains the sequence KVHF, at position 383–386, that conforms to the consensus sequence -(R/K)(V/I)XF- for binding of many regulatory subunits to PP1 [17]. We therefore sought to determine whether the sequence KVHF was responsible for the binding of Nek2 to PP1 by mutating phenylalanine-386 to alanine. Transient transfection of the construct pEBG-Nek2NB (non-binding), which encodes the mutation F386A, resulted in the expression of GST-Nek2NB as detected by immunoblotting (Figure 1A, lane 4). In contrast to the results with wild-type Nek2 (Figure 1C, lane 2), no binding of GST-Nek2NB to PP1 could be detected on immunoprecipitation of PP1 (Figure 1C, lane 4). Figure 1(D) shows that an equal amount of PP1 was precipitated from all lysates with anti-PP1 antibodies. No binding of GST-Nek2NB to PP1 was detected by pelleting GST-Nek2NB with glutathione beads followed by immunoblotting with PP1 antibodies (Figure 1B, lane 4). Although it is possible that the lack of co-sedimentation of GST-Nek2NB with PP1 could be caused by a conformational change in GST-Nek2NB, this is unlikely because the latter still retains its protein kinase activity (see Figure 3C and Figure 4). A catalytically inactive Nek2 mutant, termed GST-Nek2KD (kinase dead), in which lysine-37 had been changed to arginine, resulted in loss of Nek2 activity [26]. As expected, loss of kinase activity did not result in loss of PP1 binding (Figure 1B, lane 3 and Figure 1C, lane 3). The slightly lower levels of GST-Nek2KD compared with GST-Nek2 sedimented with PP1 are explained by the lower expression level of GST-Nek2KD compared with GST-Nek2 after transfection of the cells with equal amounts of each construct (Figure 1A). In

Figure 2 Identification of endogenous Nek2 and Nek2-PP1 complex

(A) Aliquots (200 μ g) of protein from 293 cell extracts (non-transfected) were precleared with preimmune IgG beads, then incubated with either anti-Nek2 beads (lane 1) or pre-immune IgG beads (lane 2). The beads were washed with lysis buffer and the bound proteins separated by SDS/10% PAGE. After transfer to nitrocellulose, the Nek2 (47 kDa) was detected with biotinylated anti-Nek2 followed by avidin-horseradish peroxidase. Lane 3, Nek2 controls: 10 ng, 1 ng and 0.1 ng of GST-Nek2. An unknown protein of lower molecular mass is detected in both lanes 1 and 2. (B) Phosphatase assays on Nek2-immunoprecipitates. A 100 μ g aliquot of protein from 293 cell extracts (non-transfected) was precleared with preimmune IgG beads, then incubated with pre-immune IgG or anti-Nek2 beads. The beads were washed with lysis buffer, then the bound phosphatase activity assayed using [³²P]-labelled phosphorylase as substrate. 1, Pre-immune beads; 2, anti-Nek2 beads; 3, anti-Nek2 plus 100 nM inhibitor-2. The [³²P] released from the substrate was measured after extraction with molybdate [38] to ensure that the product of the reaction was P_i and not phosphopeptides released by proteinases present in the Nek2 immunoprecipitates.

addition, phosphatase activity was consistently associated with GST-Nek2 and GST-Nek2KD bound to GSH-Sepharose, but not with GST-Nek2NB or GST (results not shown).

Interaction of the endogenous Nek2 and PP1 proteins

In order to investigate whether a complex is formed between endogenous Nek2 and PP1, we immunoprecipitated Nek2 from 293 cell extracts using anti-Nek2. Since the Nek2 protein and

Figure 3 Protein kinase activities of GST fusion proteins towards casein (A), PP1 (B) and C-Nap1 (C)

Extracts containing 1 mg of soluble protein from 293 cells overexpressing GST, GST-Nek2KD (KD), GST-Nek2 (Nek2) and GST-Nek2NB (NB) were incubated with 10 μ l of GSH-Sepharose at 4 °C for 1 h. After sedimentation and washing of the GSH-Sepharose pellets, the associated kinase activity was assayed in the presence of casein, PP1 or MBP-C-Nap1 as described in the Materials and methods section. The reactions were stopped by boiling in 10 μ l of 6 × SDS/PAGE loading buffer. Proteins were separated on SDS/PAGE, the gels stained with Coomassie Blue, dried, and phosphorylated proteins detected by autoradiography. For (A) and (B), the positions of the standard marker proteins, indicated on the right, are as listed in Figure 1. For (C), the marker proteins are myosin (212 kDa), α -2-macroglobulin (170 kDa), β -galactosidase (116 kDa), transferrin (76 kDa) and glutamate dehydrogenase (53 kDa). Arrows mark the positions of the relevant proteins

IgG heavy chain migrate similarly on SDS/PAGE, detection of the precipitated protein required use of biotinylated anti-Nek2 followed by avidin-horseradish peroxidase. Figure 2(A) shows that the Nek2 protein is bound specifically by the anti-Nek2 antibody (lane 1) and not pre-immune IgG (lane 2), but only approx. 1 ng of Nek2 protein was recovered from 200 μ g of protein in 293 cell extracts. Lack of detection of Nek2 on immunoblots of whole cell lysates (results not shown) also

suggested the level of Nek2 in the cell may be very low and would preclude detection of any associated PP1 by immunoblotting. We therefore assayed the Nek2 immunoprecipitates to detect the PP1 bound to Nek2. Figure 2(B) shows that significant phosphatase activity was present within the anti-Nek2 immunoprecipitate. This activity was blocked by inhibitor-2, a specific inhibitor of protein phosphatase 1 that does not inhibit other protein serine/threonine phosphatases, demonstrating that the activity was PP1.

GST–Nek2 undergoes autophosphorylation and can phosphorylate casein, PP1, myelin basic protein and C-Nap1

For all assays of Nek2 most of the bound PP1 was removed by washing the GSH–Sepharose pellets in buffer containing 0.5 M NaCl. GST–Nek2 was able to phosphorylate casein, PP1, C-Nap1 (Figure 3) and myelin basic protein (Figure 4A). As expected no activity could be detected in the precipitates from cells expressing GST–Nek2KD or GST. Figure 3(A) shows the autoradiograph of the components of a protein kinase assay separated on SDS/PAGE when casein was used as substrate. The upper band (73 kDa) is the expected size for GST–Nek2, and is phosphorylated. No band of the same size can be seen where GST–Nek2KD was assayed, indicating that the phosphorylation of GST–Nek2 resulted from autophosphorylation, rather than phosphorylation by traces of a contaminating kinase present in the assay. The position of the lower phosphorylated band corresponded to casein. Lack of casein kinase activity in GSH–Sepharose pellets from extracts expressing GST or GST–Nek2KD indicated that the phosphorylation of casein was also catalysed by Nek2 kinase, and that no other kinases capable of phosphorylating casein are present in the GST–Nek2KD pellet. Figure 3(B) shows that in the presence of the phosphatase inhibitor okadaic acid, PP1 could also be phosphorylated by GST–Nek2, but not by GST–Nek2KD. Again, the 32 P-labelled GST–Nek2 was consistently seen only in the GST–Nek2 and not in the GST–Nek2KD or GST assays. No phosphorylated bands (Nek2 or PP1) were seen in the absence of okadaic acid when PP1 was the substrate (results not shown).

Since C-Nap1 has been reported to be a protein that not only binds to Nek2 but also may be an *in vivo* substrate for this kinase [28], we examined the phosphorylation of a C-terminal fragment of C-Nap1 (amino acids 1851–2442) by the Nek2 proteins. Figure 3(C) shows that in the absence of PP1 inhibitors, C-Nap1 can be phosphorylated by GST–Nek2, but not by GST–Nek2KD. GST–Nek2NB had an enhanced activity towards C-Nap1 compared with that of GST–Nek2, presumably because dephosphorylation by Nek2-bound PP1 was eliminated.

GST–Nek2 and GST–Nek2NB, but not GST–Nek2KD, were also capable of phosphorylating myelin basic protein (Figure 4A). The activities of both GST–Nek2 and GST–Nek2NB were substantially increased when the protein serine/threonine phosphatase inhibitor, microcystin, was included in the cell lysis buffer. One interpretation of these results is that serine/threonine autophosphorylation of Nek2 leads to an increase in the protein kinase activity of Nek2. The fact that there was an approx. 3-fold increase in GST–Nek2 activity and a less than 2-fold increase in GST–Nek2NB activity in the presence of microcystin suggests that PP1 bound to GST–Nek2 is responsible, at least in part, for the dephosphorylation and partial inactivation of GST–Nek2. Figure 4(B) shows that GST–Nek2NB (lane 5) and a component of GST–Nek2 (lane 1) have a reduced electrophoretic mobility compared with GST–Nek2KD (lane 3), consistent with autophosphorylation of GST–Nek2 and GST–Nek2NB. In the presence of microcystin the mobility of GST–Nek2 (Figure 4, lane 2)

Figure 4 Protein kinase activities of GST–Nek2 fusion proteins using myelin basic protein as substrate

(A) Cell extracts containing 10 μ g of soluble protein from 293 cells overexpressing GST–Nek2 (lanes 1 and 2), GST–Nek2KD (lanes 3 and 4) and GST–Nek2NB (lanes 5 and 6), prepared with lysis buffer without microcystin (MC) (lanes 1, 3 and 5) or containing microcystin (lanes 2, 4 and 6) were incubated with 10 μ l of GSH–Sepharose for 1 h at 4 °C. After sedimentation and washing of the GSH–Sepharose pellets, the associated kinase activity was assayed by counting [γ - 32 P]ATP incorporated into myelin basic protein. The data are presented as means $\pm$ S.E.M. for two separate experiments with each determination carried out in duplicate. (B) A 10 μ g aliquot of the same cell extracts was separated on 7.5% PAGE and immunoblotting was performed with anti-Nek2 antibody.

and GST–Nek2NB (lane 6) is further decreased, consistent with additional phosphorylation underlying the increase in activity. Addition of excess PP1 to the lysates resulted in shifting the mobility of GST–Nek2 in lanes 1, 2, 5 and 6 (Figure 4) to that seen in lanes 3 and 4, consistent with the mobility shift being due to phosphorylation (results not shown).

Incubation of Nek2 with PP1 causes a reduction of Nek2 activity

In order to examine whether Nek2 activity could be altered by PP1, GST–Nek2 was incubated with or without bacterially expressed PP1 or with microcystin-inactivated PP1, prior to being subjected to protein kinase assays. Incubation of Nek2 with an equimolar amount of PP1 caused approx. 65% inhibition of Nek2 activity in 2 min, but no further inhibition with longer incubation times (Figure 5). However, Nek2 inhibition could be further enhanced if the concentration of PP1 was increased (results not shown). Incubation of GST–Nek2 with microcystin-inactivated PP1 had no effect on Nek2 activity.

Figure 5 Effect of PP1 on the myelin basic protein kinase activity of Nek2

Assays were performed using GST–Nek2 eluted from GSH–Sepharose and myelin basic protein as substrate. Prior to the kinase assay, GST–Nek2 was incubated at 30 °C with active PP1 (□) or microcystin-inactivated PP1 (◇) for varying times. The incubation with active PP1 was then stopped by adding 5 μ M microcystin. The ratio of Nek2/PP1 was equimolar as judged by Coomassie-stained PAGE. Kinase assays were carried out as described in the Materials and methods section. Microcystin-inactivated PP1 was prepared by incubation of PP1 with microcystin for 10 min at 30 °C. The complete inactivation was confirmed by protein phosphatase assay using [32 P]phosphorylase as substrate.

Phosphorylation of PP1 γ on two threonine residues by Nek2 reduces its phosphatase activity *in vitro*

Since PP1 could be phosphorylated by Nek2, we sought to investigate whether the enzymic activity of PP1 was changed upon phosphorylation. Addressing this problem is not straightforward. First, if PP1 and Nek2 were added together, Nek2 activity was reduced (Figure 5). Secondly, PP1 could autodephosphorylate itself after being phosphorylated by Nek2. In order to partially resolve these problems, the molar ratio of Nek2/PP1 was maintained at a high level (> 3:1) and the assay was performed with adenosine 5'-[γ -thio]triphosphate (ATP[S]) instead of ATP, since ATP[S] can be used as a substrate by kinases, but the thiophosphate is resistant to dephosphorylation by protein phosphatases. Under these experimental conditions, the enzymic activity of PP1 was found to be reduced by approx. 30% after incubation with Nek2 and Mg^{2+} -ATP[S], compared with controls in which either Nek2 or ATP[S] were excluded (Figure 6). Since this result could be achieved only with ATP[S], autodephosphorylation of PP1 must occur after it is phosphorylated by Nek2 using ATP.

Due to autodephosphorylation, active PP1 could not be used to determine phosphorylation sites. Also, PP1 γ_1 inactivated by okadaic acid (Figure 3B) was not phosphorylated to a sufficient level to identify the phosphorylation sites. We therefore mutated the PP1 γ_1 catalytic subunit at histidine-125. This residue acts as a general acid in the proposed catalytic mechanism of PP1 [40] and its mutation to alanine would be expected to dramatically reduce catalytic activity. Soluble PP1 γ (H125A) protein was expressed, demonstrating that the mutation does not result in denaturation of the protein and, when assayed against 32 P-labelled phosphorylase in the standard phosphatase assay, showed essentially no activity (results not shown). Phosphorylation by Nek2 followed by matrix-assisted laser desorption

Figure 6 Effect of Nek2 on the phosphatase activity of PP1

GSH–Sepharose beads incubated with cell extracts (containing 2 μ M microcystin) from control 293 cells or 293 cells overexpressing GST–Nek2 were washed twice with lysis buffer containing 0.5 M NaCl, once with lysis buffer and once in 50 mM Tris/HCl, pH 7.5. Phosphorylation reactions were carried out for 60 min at 30 °C in 40 μ l containing 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM ATP[S], with PP1 being included at 3 μ g/ml. Phosphatase assays were then performed immediately using 20 μ l of [32 P]phosphorylase (final concentration 10 μ M). Protein phosphatase activity was plotted as a percentage of the activity observed when GSH–Sepharose beads were incubated with the extract from control 293 cells, PP1 being included at 3 μ g/ml. The data are presented as the means $\pm$ S.E.M. for two separate experiments with each determination carried out in duplicate.

ionization–time-of-flight MS and solid-phase sequencing revealed that this protein is phosphorylated on two C-terminal threonines. Threonine-307 and -318 appear to be equally well phosphorylated by Nek2 under the conditions used and a stoichiometry of approx. 2 moles phosphate/mol PP1 γ was obtained. The consensus sequence for these two Nek2 phosphorylation sites is (R/K)XX(A/I)(phosphoT)(R/K) and supports previous studies using peptide substrates, which suggested that basic residues may contribute to substrate recognition by Nek2 [26]. Although PP1 α and PP1 β do not contain homologous serine/threonine residues to threonine-307, PP1 α contains a serine residue and PP1 β contains a threonine residue homologous to PP1 γ threonine-318, with basic residues in the surrounding sequence. Nek2 was previously shown to phosphorylate serine residues in peptides and proteins [26]. We also found that MBP–C–Nap1 is an excellent substrate for Nek2. Approx. 13 moles of phosphate were incorporated/mol of C–Nap1 by Nek2 (results not shown).

Nek2, PP1 α and C–Nap1 form a ternary complex *in vitro*

In order to ascertain whether Nek2, PP1 α and C–Nap1 can form a ternary complex we performed amylose resin affinity binding experiments with GST–Nek2 variants, MBP–C–Nap1 and PP1 α . Amylose resin has a high affinity for MBP, but no affinity for GST. Hence any complex containing GST–Nek2, MBP–C–Nap1 and PP1 α must represent a ternary complex and not separate dimeric complexes. Figure 7 shows that all three proteins are present in the pellet when GST–Nek2 or GST–Nek2KD are used, confirming the presence of a ternary complex. However, the PP1 α is not present when either GST–Nek2NB or GST are used. All three versions of the GST–Nek2 protein are recovered in the pellet, demonstrating that they can all bind to C–Nap1 and

Figure 7 Co-sedimentation of Nek2, C-Nap1 and PP1.

293 cells were transfected with the Nek2 constructs indicated. A 200 μ g portion of protein from each cell extract was incubated with 2 μ g of MBP-C-Nap1, 2 μ g PP1 α and amylose resin. The beads were washed and bound proteins separated on either 7.5% acrylamide (for C-Nap1 and Nek2 blots) or 10% acrylamide (for PP1 blots) SDS/PAGE. After transfer to nitrocellulose, the blots were incubated with the indicated antibodies and proteins visualized by ECL[®]. (A) Anti-C-Nap1; (B) anti-Nek2; (C) anti-PP1 γ . In all panels: lane 1, GST-Nek2; lane 2, GST-Nek2KD; lane 3, GST-Nek2NB; lane 4, GST only. Size markers are in kDa.

Figure 8 Characterization of Nek2-PP1 phosphatase activity

A 100 μ g portion of 293 cell extract (non-transfected) was incubated with pre-immune IgG or anti-Nek2 beads. The beads were washed with lysis buffer, then the bound phosphatase activity measured. 1 and 5, pre-immune beads; 2 and 6, anti-Nek2 beads; 3 and 7, anti-Nek2 beads plus 1 μ M peptide 294 (GKRTNLRKTGSEIRIAMGMRVKFNPLALLDSC); 4 and 8, anti-Nek2 beads plus 1 μ M control peptide 575 (GKRTNLRKTGSEIRIAMGMRVKANPLALLDSC). The PP1-binding motif is underlined. Bars 1–4 were assayed using [³²P]phosphorylase as substrate (final concentration 2.7 μ M) and bars 5–8 used phosphorylated MBP-C-Nap1 as substrate (final concentration 2.7 μ M). Activity (after molybdate extraction) is stated as m-units $\times 10^{-3}$ /mg of protein in the extract.

that the PP1 α is recovered in the pellet only because of its ability to bind to Nek2. The slightly lower level of GST-Nek2KD seen in the pellet fraction compared with the other tracks probably explains the slightly reduced amount of PP1 α seen in this track. The mobilities of the GST-Nek2 variants are in accordance with

those in Figure 4. Consistent with being a multiply phosphorylated substrate of Nek2 (see earlier), MBP-C-Nap1 shows a large decrease in mobility (approx. 20 kDa) in the presence of GST-Nek2NB compared with unphosphorylated C-Nap1 in the GST-Nek2KD and GST incubations.

Nek2-PP1 dephosphorylates C-Nap1

Immunoprecipitation of the endogenous Nek2-PP1 from 293 cell lysates using the anti-Nek2 antibody allowed us to test whether C-Nap1 phosphorylated by Nek2 could be dephosphorylated by the Nek2-PP1 complex, as suggested by the results in Figure 7(A). Assays using C-Nap1 as substrate and Nek2-PP1 immunoprecipitates as the source of phosphatase were compared with parallel assays with phosphorylase as substrate. Figure 8 shows that the Nek2-PP1 complex has an approx. 2-fold higher activity towards C-Nap1 than phosphorylase. The influence of Nek2 on the phosphatase activity was assessed by prior incubation of the PP1-Nek2 complex with peptide-294 (amino acids 773–810 of 53BP2), which has previously been shown to disrupt targeting subunit-PP1 complexes and, in many cases, to relieve inhibition of phosphorylase phosphatase activity caused by the targeting subunit [4]. The activity towards phosphorylase was dramatically increased by prior incubation of PP1-Nek2 with peptide-294, while the control peptide-575, mutated in the PP1 binding site, had no effect. When C-Nap1 protein was used as substrate, addition of peptide-294 caused only a slight increase in phosphatase activity and the control peptide-575 again had no effect. These results indicate that binding of PP1 to Nek2 suppresses the phosphorylase phosphatase activity, resulting in a large increase in the C-Nap1/phosphorylase phosphatase activity ratio.

DISCUSSION

Although many proteins have been identified at centrosomes, the molecular mechanisms underlying the regulation of centrosome function are unclear. The processes by which centrosomes duplicate, nucleate microtubules and separate at mitosis to form the spindle poles all require elucidation. The protein serine/threonine kinase, Nek2, which exhibits predominant localization at centrosomes and causes splitting or dispersal of centrosomes when overexpressed [27], is shown here to form a complex with the protein serine/threonine phosphatase PP1. We demonstrate that the Nek2-PP1 complex occurs both *in vitro*, using bacterially expressed proteins, and in cell extracts through the motif KVHF at position 383–386 of Nek2, a sequence that conforms to the known PP1 consensus binding motif. Nek2 activity is not necessary for the formation of the Nek2-PP1 complex. We also demonstrate that at least two distinct isoforms of PP1 (PP1 α and PP1 γ_1) can interact with Nek2. Interestingly, PP1 α has recently been reported to show a marked localization at centrosomes during mitosis [41]. These data point to a phosphorylation-dephosphorylation cycle catalysed by the Nek2-PP1 complex being important in centrosome function and most probably in centrosome separation. Other isoforms of mammalian NIMA-related kinases, namely Nek1 and Nek3, do not possess a consensus PP1-binding motif and there is no PP1-binding motif in *A. nidulans* NIMA that conforms to the mammalian or yeast consensus PP1-binding motifs [17].

Several kinase-phosphatase complexes have been postulated to mediate cellular signals, such as calmodulin kinase IV-PP2A [42] and extracellular-signal-regulated kinase 2/mitogen-activated protein kinase phosphatase 3 [43]. Complex formation

localizes both enzymes to a specific substrate and tight regulation of the kinase by the phosphatase and vice versa can explain how a kinase can be activated transiently in response to an extracellular signal. However, there is no evidence for an upstream kinase activating Nek2. Fry et al. [26] showed that the levels of Nek2 protein changed during the cell cycle, being high throughout S and G₂ phases and low during other phases of the cell cycle. We have demonstrated here that Nek2 is activated by autophosphorylation and that this activation can be at least partially reversed by PP1. The kinase activity of Nek2 may only become effective on exogenous substrates when its activity outstrips that of PP1, at which point it may also partially inhibit PP1 by phosphorylation. Once the activity of Nek2 falls below that required to inhibit PP1, the rise in PP1 activity would very rapidly dephosphorylate and switch off Nek2 activity. Such rapid molecular switching, proposed for kinase–phosphatase complexes involved in signalling, may be relevant here to rapidly and transiently switch on centrosome separation.

One possible mechanism by which the Nek2–PP1 complex could function is that Nek2 may phosphorylate a substrate protein and this is then followed by dephosphorylation of the substrate protein by PP1. Successive cycles of phosphorylation and dephosphorylation could result in centrosome separation in a process similar to muscle contraction. A more likely alternative is that phosphorylation of a substrate protein by Nek2 may allow centrosomes to move apart, and that PP1 may prevent the newly formed centrioles from separating until the G₂/M transition. A putative *in vivo* substrate is C-Nap1, previously shown to bind to Nek2 and to undergo phosphorylation *in vitro* [28]. Whereas Nek2 [27] and PP1 α [41] localize to centrosomes in both interphase and mitosis, C-Nap1 was found to localize to centrosomes during interphase, but anti-C-Nap1 antibody interaction at centrosomes diminished at the onset of prophase and was lost during mitosis. C-Nap1, a protein with coiled coil structure, was therefore postulated to tether the proximal ends of centrioles together during interphase, and phosphorylation of C-Nap1 by Nek2 at G₂/M to cause depolymerization or degradation of C-Nap1 and thus centriole separation [28]. Our results support the idea that C-Nap1, Nek2 and PP1 could be involved in centrosome separation. We show that C-Nap1 is an *in vitro* substrate for both Nek2 and PP1 that is bound to Nek2. Additionally all three proteins can form a ternary complex *in vitro*. The very high level of phosphorylation on the C-terminal 592 amino acids of C-Nap1 could represent a method to facilitate centrosome separation through either a conformational change or a surface charge effect.

Assays comparing C-Nap1 and phosphorylase as substrates for the Nek2–PP1 complex showed that the phosphatase activity towards C-Nap1 was higher than towards phosphorylase. Addition of a peptide (294) that should disrupt the interaction of PP1 with Nek2, caused the phosphorylase phosphatase activity to increase approx. 7-fold, while there was only a marginal effect of the C-Nap1 phosphatase activity. These results indicate that PP1c has a much higher activity towards phosphorylase than C-Nap1. Binding of PP1c to Nek2 suppresses the phosphorylase phosphatase activity, while having little effect on the C-Nap1 phosphatase activity, an effect consistent with Nek2 being a regulatory subunit for PP1. Thus the interaction of PP1c with Nek2 increases PP1c specificity for C-Nap1 by enhancing the C-Nap1/phosphorylase phosphatase activity ratio. Combined with the highly specific location of Nek2 at the centrosome, this may minimize dephosphorylation of irrelevant substrates by the Nek2–PP1 complex.

Overexpression of active Nek2 in mammalian cells was shown to initially induce centrosome splitting, while more prolonged

overexpression of active Nek2 led to dispersal of centrosomal material, as did overexpression of inactive Nek2 at all time points [27]. We found that overexpression of Nek2 mutated in the PP1-binding site (GST–Nek2NB) in a human fibroblast cell line (MCF7) also caused centrosome dispersal at even the earliest time points we could examine (results not shown). However, the kinase activity of Nek2NB is likely to be higher than that of Nek2 due to the absence of bound PP1, which would inhibit autophosphorylation. Consequently, overexpression of Nek2NB might be expected to cause more rapid centrosome dispersal than the overexpression of Nek2. In the light of our finding that Nek2 binds PP1, overexpression of Nek2KD might cause centrosomal dispersal because it brings to the centrosome active PP1 that, in excess, might interfere with many phosphorylation reactions.

Since centrosome separation must be coordinated with other cell cycle events it is likely that proteins regulating the Nek2–PP1 switch will be involved in initiating other aspects of cell division. Although, p34^{cdc2} has been shown to phosphorylate further and activate *A. nidulans* NIMA subsequent to autophosphorylation [23], there is no evidence for p34^{cdc2} activation of mammalian Nek2. However, it has been shown that both p34^{cdc2}-cyclinA and p34^{cdc2}-cyclinB are able to phosphorylate and inactivate PP1 α on threonine-320 and the homologous threonine-311 in PP1 γ_1 [44]. This site is distinct from the sites (threonine-307 and threonine-318) on PP1 γ_1 that are phosphorylated by Nek2. Phosphorylation of PP1 by p34^{cdc2} would enhance the ability of Nek2 to autophosphorylate and, consequently, to phosphorylate its target substrates such as C-Nap1. Furthermore, it has been shown that peak PP1 phosphorylation occurs at the G₂/M phase of the cell cycle [45]. This coincides with centrosome separation and a peak in Nek2 kinase activity [26]. A possible mechanism whereby the initiation of mitosis is coupled to centrosome separation at G₂-M is that p34^{cdc2} phosphorylates and inactivates PP1, thereby allowing Nek2 kinase to phosphorylate the C-Nap1 protein thus causing centrosome separation. However, other kinases activated at G₂/M with similar substrate specificities, such as polo-like kinases [46], could have the same effect on PP1. We are currently attempting to test this hypothesis by studying the effect of phosphorylation by p34^{cdc2}-cyclinA and p34^{cdc2}-cyclinB and polo-like kinases on PP1 activity of the endogenous Nek2/PP1 complex.

While this paper was under review, Uto and Sagata [47] showed that *Xenopus* Nek2B, an alternatively spliced form of Nek2, is required for the assembly and/or maintenance of centrosomes in early *Xenopus* embryos in which centrosome cycles can occur autonomously. Interestingly, Nek2B does not contain the PP1-binding site that we identify here, an observation that may explain why Nek2B, in contrast with Nek2, does not cause centrosome splitting and functions at a time when the centrosome cycle is not coupled to nuclear events.

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