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A centrosomal function for the human Nek2 protein kinase, a member of the NIMA family of cell cycle regulators

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Nek2, a mammalian protein kinase of unknown function, is closely related to the mitotic regulator NIMA of *Aspergillus nidulans*. Here we show by both immunofluorescence microscopy and biochemical fractionation that human Nek2 localizes to the centrosome. Centrosome association occurs throughout the cell cycle, including all stages of mitosis, and is independent of microtubules. Overexpression of active Nek2 induces a striking splitting of centrosomes, whereas prolonged expression of either active or inactive Nek2 leads to dispersal of centrosomal material and loss of a focused microtubule-nucleating activity. Surprisingly, this does not prevent entry into mitosis, as judged by the accumulation of mitotically arrested cells induced by co-expression of a non-destructible B-type cyclin. These results bear on the dynamic function of centrosomes at the onset of mitosis. Moreover, they indicate that one function of mammalian Nek2 relates to the centrosome cycle and thus provide a new perspective on the role of NIMA-related kinases.

Keywords: cell cycle/centrosome/mitosis/Nek2/NIMA

Introduction

In the filamentous fungus *Aspergillus nidulans*, entry into mitosis requires the activation not only of the cyclin-dependent protein kinase Cdc2 (Cdk1), but also of a second, structurally distinct serine/threonine kinase, known as NIMA (Osmani *et al.*, 1991a). Mutation of the *nimA* gene causes cells to arrest in G_2—hence ‘nim’ for ‘never in mitosis’ (Morris, 1976; Osmani *et al.*, 1991a), whereas massive overexpression of the wild-type gene induces certain aspects of a premature mitosis, including condensation of chromosomes and, in some cases, formation of a mitotic spindle (Osmani *et al.*, 1988). The precise relationship between the NIMA and Cdc2 pathways at mitosis remains to be determined, but recent data suggest that they are interlinked, with NIMA possibly being a substrate of the Cdc2 kinase (Ye *et al.*, 1995; for reviews see Fry and Nigg, 1995; Osmani and Ye, 1996). In analogy to the evolutionary conservation of the Cdk cell cycle regulators, it seems plausible that NIMA-related kinases may also be required for regulating the cell cycle of eukaryotic organisms other than *Aspergillus*. Indeed, overexpression of wild-type NIMA or expression of dominant-negative mutants of NIMA disrupts the cell cycle of yeast, *Xenopus* and human cells, providing some support to the view that NIMA-related pathways might exist in these species (O’Connell *et al.*, 1994; Lu and Hunter, 1995a; reviewed in Lu and Hunter, 1995b).

The only organism for which a genuine functional homologue of *Aspergillus* NIMA has so far been cloned is another filamentous fungus, *Neurospora crassa* (NIM-1; Pu *et al.*, 1995). However, genes encoding protein kinases structurally related to NIMA have been isolated from several organisms, including *Saccharomyces cerevisiae* (KIN3/NPK1; Jones and Rosamond, 1990; Barton *et al.*, 1992; Schweitzer and Philippsen, 1992), trypanosomes (Nrk; Gale and Parsons, 1993) and mammals (Letwin *et al.*, 1992; Schultz and Nigg, 1993; Levedakou *et al.*, 1994; Schultz *et al.*, 1994). The mammalian kinases have been designated ‘Nek’s for NIMA-related kinases. Interestingly, both Nek1 and Nek2 are highly expressed in male and female germ cells, suggesting that they might be important during meiosis (Letwin *et al.*, 1992; Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997). Among the mammalian kinases known to date, human Nek2 is most closely related to NIMA, falling into the same subfamily as fungal NIMA, NIM-1 and KIN3 (Fry and Nigg, 1997). In addition, the *in vitro* substrate specificity and some biochemical properties of Nek2 are reminiscent of NIMA, and both Nek2 protein levels and activity are regulated through the cell cycle (Fry *et al.*, 1995). However, significant differences between NIMA and Nek2 have also been observed. In particular, NIMA activity peaks in mitosis and remains high during mitotic arrest (Osmani *et al.*, 1991b), whereas Nek2 activity is high during S and G_2 phases but low during M phase arrest (Fry *et al.*, 1995).

Although the Nek2 protein kinase is suspected to play some role in cell cycle progression towards mitosis, direct evidence implicating Nek2 in any process related to cell division is lacking. Here, we have investigated the subcellular localization of the human Nek2 kinase by both immunofluorescence microscopy and subcellular fractionation. We found that endogenous Nek2 is highly enriched at the centrosome, the microtubule organizing centre (MTOC) of animal cells. This organelle plays a crucial role in cell division, as it gives rise to the poles of the mitotic spindle apparatus. In turn, correct assembly of a bipolar spindle is important for the faithful segregation of sister chromatids during mitosis and homologous chromosomes during meiosis (Fuller *et al.*, 1992; Kellogg *et al.*, 1994). The centrosome consists of a pair of centrioles surrounded by an amorphous structure known as the pericentriolar material (PCM) from within which microtubules are nucleated. A key role in microtubule nucleation has been attributed to γ-tubulin (Oakley *et al.*, 1990; Horio *et al.*, 1991; Joshi *et al.*, 1992; Felix *et al.*, 1994; Stearns and Kirschner, 1994), which forms part of a γ-tubulin-

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containing ring complex, or γ-TuRC (Moritz *et al.*, 1995; Zheng *et al.*, 1995).

During the cell cycle, the centrosome undergoes a series of morphological and functional changes. These include the semi-conservative replication of centrioles in late G_1/S phase, the enlargement or 'maturation' of the centrosome through the recruitment of additional PCM proteins in S and G_2, and the physical separation of the duplicated centrosomes and their migration to opposite sides of the nucleus at the G_2 to M transition (Kochanski and Borisy, 1990; Kimble and Kuriyama, 1992; Kellogg *et al.*, 1994). In addition, the onset of mitosis is accompanied by an abrupt increase in the microtubule-nucleating capacity of centrosomes (Karsenti, 1991). Many of these events are thought to be brought about, at least in part, through changes in the phosphorylation state of critical centrosomal components. In particular, protein kinases have been implicated in centrosome duplication, maturation and separation (Blangy *et al.*, 1995; Glover *et al.*, 1995; Lauze *et al.*, 1995; Lane and Nigg, 1996), as well as in the regulation of the centrosomal microtubule nucleation capacity (Ohta *et al.*, 1990; Verde *et al.*, 1990, 1992; Buendia *et al.*, 1992). The identification of Nek2 as a novel component of the centrosome has prompted us to investigate in which aspects of centrosome function this protein kinase might be involved. Our results argue that Nek2 protein is important for centrosome integrity and, moreover, may play a role in the regulation of centrosome separation.

Results

Nek2 localizes to the centrosome

For the purposes of the present study, two new polyclonal anti-Nek2 antibodies, termed R40 and R50, were generated in rabbits. As shown by Western blotting, both antibodies recognized a major protein of 48 kDa in whole HeLa cell extracts, and this protein co-migrated exactly with Nek2 overexpressed from a recombinant baculovirus in Sf9 insect cells (Figure 1A). The two antibodies also precipitated a strong β-casein kinase activity from both HeLa and Nek2 baculovirus-infected insect cells (Figure 1B). β-Casein had previously been shown to be a good *in vitro* substrate for human Nek2 (Fry *et al.*, 1995). Consistent with earlier data (Fry *et al.*, 1995), autophosphorylation of recombinant Nek2 could also be observed (Figure 1B).

By immunofluorescence microscopy on cultured cells, both anti-Nek2 antibodies consistently stained one or two closely spaced dots which were usually located close to the nuclear envelope (shown for R40 in Figure 2c, not shown for R50). No such staining was produced by the pre-immune serum (Figure 2a). Co-staining with an anti-α-tubulin antibody revealed that the stained dots lay at the centre of the interphase microtubule array, suggesting that they might represent centrosomes (Figure 2d). This was confirmed by double-labelling of cells with anti-Nek2 antibody and CTR453, a monoclonal antibody (MAb) specific for a pericentriolar antigen (Bailly *et al.*, 1989; Moudjou *et al.*, 1991; data not shown). Staining of centrosomes was seen in all cell types analysed, and was independent of fixation method, as it was observed following procedures based on either aldehyde or organic solvent fixation. Finally, pre-incubation of the anti-Nek2

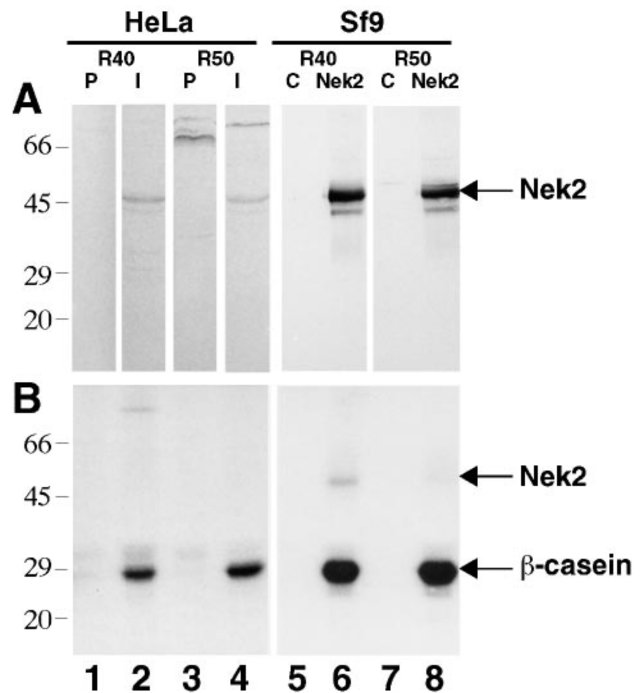

Fig. 1. Characterization of R40 and R50 anti-Nek2 antibodies. (**A**) Western blots on total cell lysates. (**B**) Immunoprecipitations, followed by Nek2 kinase assays using casein as a substrate, and analysis of phosphoproteins by SDS–PAGE and autoradiography. Anti-Nek2 antibody reactivities were tested on total HeLa cell extracts (lanes 2 and 4) and lysates prepared from Sf9 insect cells infected with recombinant Nek2 baculovirus (lanes 6 and 8). For control, the corresponding pre-immune sera were used under identical conditions (lanes 1 and 3), and lysates prepared from wild-type baculovirus-infected insect cells were analysed in parallel (lanes 5 and 7). P, pre-immune serum; I, immune serum; C, control lysate. The migrations of Nek2 and β-casein are marked on the right. Molecular weight markers are indicated on the left. Equivalent Western blots performed on lower percentage SDS–polyacrylamide gels confirmed that there were no high-molecular weight (>80 kDa) immunoreactive bands with either anti-Nek2 antibody (data not shown).

antibody with baculovirus-expressed recombinant Nek2 completely abolished centrosome staining (Figure 2f), whereas mock competition had no effect (Figure 2e).

Enrichment of Nek2 in purified human centrosomes

To corroborate and extend the above findings, the association of Nek2 with centrosomes was explored using a biochemical approach. Centrosomes were isolated from either CCRF-CEM or KE37 human leukaemic cell lines (Bornens *et al.*, 1987; Moudjou and Bornens, 1994) and then examined by both immunofluorescence microscopy and Western blotting. Immunofluorescent staining of these preparations with MAb CTR453, a marker for the PCM, revealed a sharp, punctate staining with the frequent occurrence of two closely spaced dots, characteristic of intact centrosomes (Figure 3A, panels b and d). Similar results were obtained with antibodies against α-tubulin, a reporter of centrioles (data not shown). Double staining with anti-Nek2 antibodies revealed positive staining of all centrosomes detected by CTR453 (Figure 3A, panel c), suggesting that Nek2 was associated with centrosomes in all cells, regardless of their position in the cell cycle (see below). No centrosome staining was produced by the

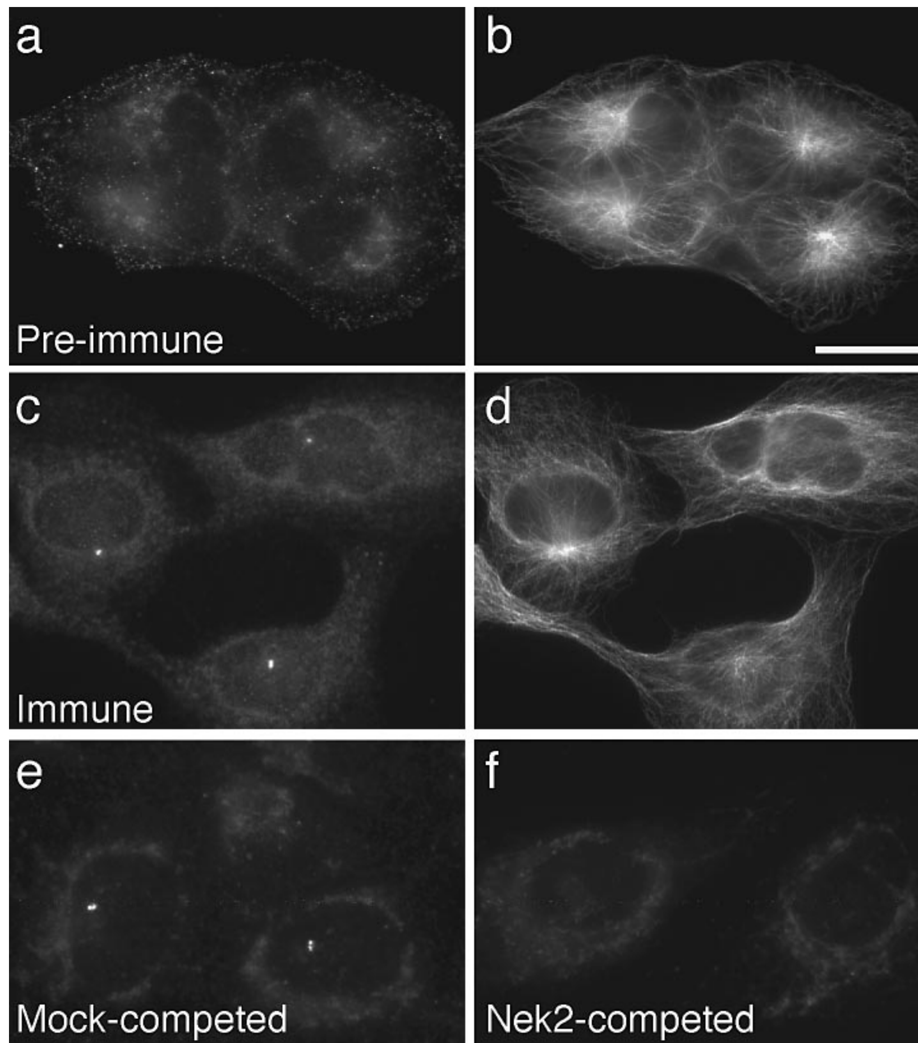

Fig. 2. Localization of endogenous Nek2 to the centrosome in U2OS osteosarcoma cells. Asynchronous U2OS cells, growing on coverslips, were fixed with methanol and double stained for Nek2 (**a**, pre-immune; **c**, immune serum) and α-tubulin (**b** and **d**). Note that the immune R40 antibody stains two closely opposed points which co-localize with the centre of the interphase microtubule array, suggesting an association of Nek2 with the centrosomes. No such staining is produced by the corresponding pre-immune serum. Competition with recombinant Nek2 abolished centrosome staining (**f**), whereas mock-competition did not (**e**). Scale bar, 10 μm.

corresponding pre-immune antibodies (Figure 3A, panel a).

The final step of the centrosome preparation procedure involves fractionation on a sucrose gradient. Analysis of several neighbouring fractions by SDS–PAGE and silver staining revealed similarly complex protein profiles (data not shown, but see Bornens *et al.*, 1987). Yet, as judged by immunofluorescent staining with MAb CTR453, only one or two of these fractions contained large numbers of centrosomes. To determine whether the bulk of Nek2 protein co-fractionated with centrosomes, aliquots of the final sucrose gradient fractions were stained with MAb CTR453, and centrosomes were counted using a fluorescence microscope. In parallel, each fraction was subjected to Western blotting with anti-Nek2 antibodies. As shown in Figure 3B, the highest levels of Nek2 protein were indeed seen in the very same fractions that contained the vast majority of centrosomes. To provide an approximate measure for the enrichment of Nek2 in centrosome preparations, Nek2 protein levels in total KE37 cell extracts and centrosome preparations were compared by semi-quantitative Western blotting (Figure 3C). Although total cellular protein was loaded in a 20-fold excess over centrosomal protein, ~5-fold more Nek2 was found in the centrosomal fraction (Figure 3C, lanes 1 and 2), suggesting a 100-fold enrichment in the centrosomal preparation. In comparison, γ-tubulin was found to be 2- to 3-fold more abundant in centrosome preparations than in total cell extracts (Figure 3C, lanes 3 and 4), indicating a 50-fold enrichment. As negative controls, the fractionation of two nuclear proteins was examined under identical conditions. Nuclear lamins as well as a nucleoplasmic cyclin-dependent kinase, Cdk7, were virtually absent from the centrosomal fraction (Figure 3C, lanes 5–8), arguing that the centrosomes analysed here were not significantly contaminated with either structural or soluble nuclear material.

Next, we asked what proportion of the total cellular Nek2 is associated with the centrosome. By comparing the amount of Nek2 protein present in a known number of KE37 cells with that in a known number of centrosomes isolated from the same cells, we estimate that only ~10% of endogenous Nek2 protein is associated with the

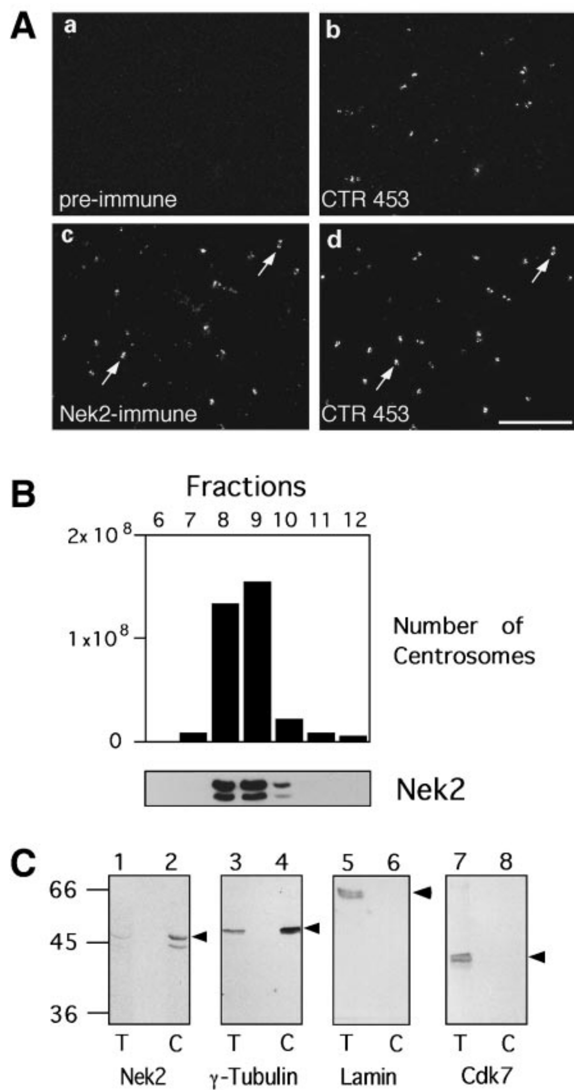

Fig. 3. Enrichment of Nek2 protein in isolated centrosome preparations. (**A**) Isolated centrosomes from KE37 cells were spun onto coverslips, fixed with methanol and then double stained with anti-Nek2 serum (panel a, pre-immune; panel c, immune serum) and MAb CTR453, marker for centrosomes (panels b and d). Co-localization of Nek2 and the CTR453 antigen on isolated centrosomes can be seen with the immune R40 antibody (arrows) but not with the pre-immune serum. Scale bar, 10 μm. (**B**) Centrosomes were isolated from exponentially growing KE37 human leukaemic cells as described in Materials and methods. The fractions eluting from the bottom of the final sucrose gradient were analysed for number of centrosomes by immunofluorescence observation, following staining with the anti-centrosomal marker CTR453, and for the abundance of Nek2 protein by Western blotting with anti-Nek2 antibody. The doublet seen in the Nek2 blot most likely represents full-length Nek2 and a proteolytically truncated form of Nek2 produced during isolation of centrosomes; a similarly truncated product can also be seen when overexpressed Nek2 is isolated from insect cells (Figure 1A, lanes 6 and 8). (**C**) Western blot analysis of total KE37 cell extracts (T) and isolated KE37 centrosome preparations (C), with antibodies against human Nek2 (lanes 1 and 2), γ-tubulin (lanes 3 and 4), lamin B2 (lanes 5 and 6) and Cdk7 (lanes 7 and 8). Antibodies against Nek2 and γ-tubulin are those described in this study; anti-lamin and anti-Cdk7 antibodies are E6 (Lehner *et al.*, 1986) and MO-1.1 (Tassan *et al.*, 1994), respectively. Approximately 20 times more protein was loaded in the total extract than in the centrosome fraction, as determined by a comparison of the two samples on a silver-stained gel. The positions of molecular weight markers (kDa) are indicated on the left and arrowheads mark the positions of the respective proteins on the right of each panel.

centrosome (data based on Western blotting; not shown). Although this number may appear low, we emphasize that similarly low numbers have recently been determined for other bona fide centrosomal components, notably γ-tubulin and centrin (Moudjou *et al.*, 1996; Paoletti *et al.*, 1996). Furthermore, we have used semi-quantitative Western blotting to determine the approximate number of Nek2 molecules present in cultured human cells. Using recombinant Nek2 expressed in *Escherichia coli* for standardization, we estimate that Nek2 is present at ~10^4 copies per (HeLa) cell (data not shown). Assuming that the non-centrosomal pool of Nek2 is diffusely distributed throughout the cytoplasm and/or the nucleus, it is not surprising that immunofluorescence microscopy preferentially reveals the Nek2 protein that is concentrated at the centrosome.

Centrosome association of Nek2 is independent of microtubules

To determine whether Nek2 association with the centrosome was dependent upon microtubules, U2OS cells were treated with either nocodazole or taxol, and the distributions of Nek2 and α-tubulin were examined by immunofluorescence microscopy (Figure 4). Nocodazole leads to a complete depolymerization of the cytoplasmic microtubule network, whereas taxol treatment has a stabilizing effect, resulting in long microtubule bundles and the loss of centrosome-nucleated microtubules (De Brabander *et al.*, 1986). In cells treated with nocodazole for 4 h, interphase microtubules were completely absent (Figure 4f). Yet, in comparison with untreated cells, there was no observable decrease in the concentration of Nek2 at the centrosome (Figure 4, compare b and a). In cells incubated with taxol, on the other hand, the microtubules appeared as dense bundles, which in many interphase cells were visibly detached from the centrosome (Figure 4g). In such cells, Nek2 was clearly still associated with the centrosome and not with microtubule bundles (Figure 4c). Furthermore, when taxol-treated cells entered mitosis, the long microtubule bundles were replaced by numerous small microtubule asters (Figure 4h). In these cells, Nek2 was invariably restricted to only two asters (Figure 4d), in line with previous data showing that only two of the many taxol-induced asters contain the duplicated centrosomes (De Brabander *et al.*, 1986). These data indicate that the centrosomal localization of Nek2 results from interactions with bona fide components of the centrosome rather than with microtubules, although a weak interaction of this kinase with microtubules cannot be rigorously excluded.

Nek2 localizes to the centrosome throughout the cell cycle

Considering the cell cycle-dependent expression of Nek2 and its suspected role in cell cycle control (Fry *et al.*, 1995), it was important to determine at which stage(s) of the cell cycle Nek2 associates with the centrosome. Following immunostaining of exponentially growing U2OS cells, Nek2 could unequivocally be identified at the centrosome in >90% of all cells. Moreover, Nek2 could be seen at centrosomes in paired cells that were still connected by post-mitotic bridges (early G_1 cells), in cells arrested at the G_1–S phase transition with aphidicolin, and in cells that were positive for bromodeoxyuridine

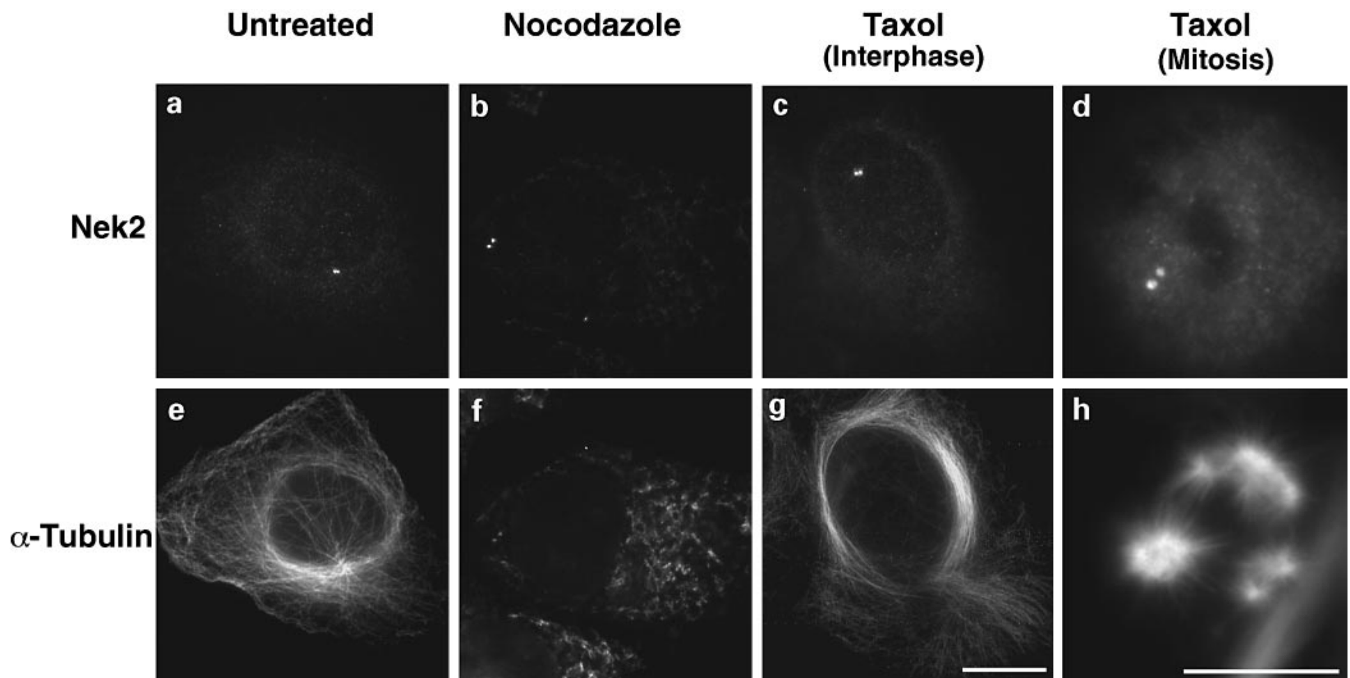

Fig. 4. Nek2 remains associated with the centrosome in cells treated with nocodazole or taxol. Asynchronous U2OS cells, growing on coverslips, were either untreated (**a** and **e**) or treated for 4 h with either nocodazole (6 μg/ml; **b** and **f**), or taxol (5 μM; **c**, **d**, **g** and **h**). Cells were then fixed with methanol and double stained for Nek2 (a–d) and α-tubulin (e–h). Nek2 can still be seen associated with the centrosome despite complete depolymerization of microtubules by nocodazole (b) or taxol-induced detachment of microtubule bundles from the centrosome (c). Taxol-treated mitotic cells form numerous MTOCs (h), but Nek2 remains associated with only two of these centres (d). Scale bars: (g), 10 μm, also for a–c and e–g; scale bar in (h), 10 μm, also for d and h.

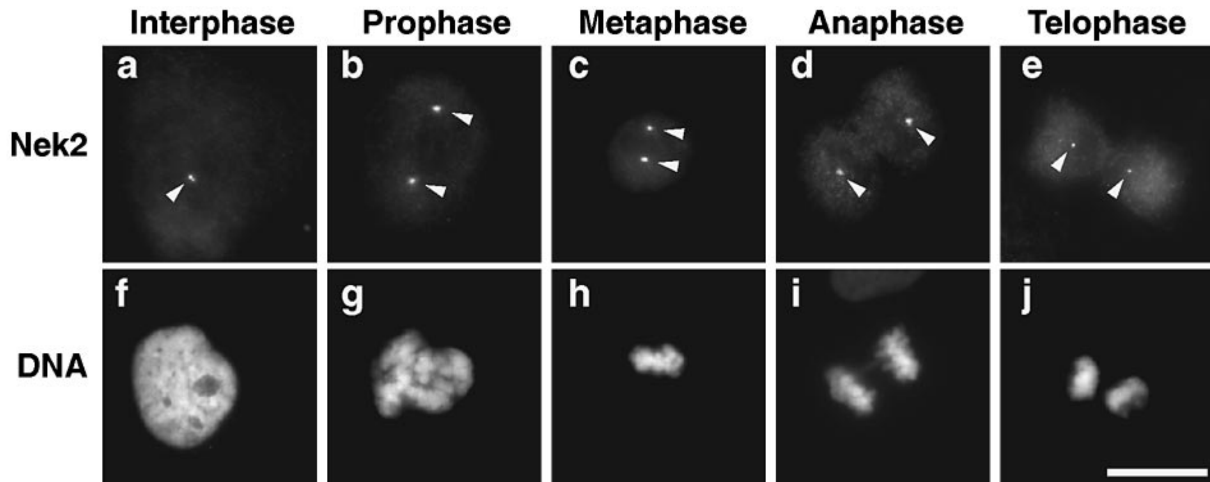

Fig. 5. Nek2 is localized to the centrosome throughout the cell cycle. Asynchronous U2OS cells, growing on coverslips, were fixed with paraformaldehyde and stained for Nek2 (top panels; a–e) and DNA (bottom panels; f–j). Representative cells are shown from different stages of the cell cycle: interphase (**a** and **f**); prophase (**b** and **g**); metaphase (**c** and **h**); late anaphase (**d** and **i**); telophase (**e** and **j**). Arrowheads indicate the association of Nek2 with the centrosome at all stages of interphase and mitosis. Scale bar, 10 μm.

after a 2 h pulse labelling (S or G_2 cells) (data not shown). As summarized in Figure 5, Nek2 was also visible at the spindle poles throughout mitosis, from early prophase (Figure 5b), through metaphase (Figure 5c) and anaphase (Figure 5d), to telophase (Figure 5e). These data clearly show that Nek2 is associated with centrosomes at all stages of the cell cycle. However, although a rigorous quantitation of such data is difficult, we note that Nek2 antibody staining of mitotic spindle poles was at most as intense as that of interphase centrosomes, and frequently appeared to be reduced, particularly in telophase. This is in striking contrast to several other centrosomal proteins, including γ-tubulin, which accumulate at the centrosome during G_2, and, as a consequence, are stained much more intensely at spindle poles than at interphase centrosomes (e.g. Zheng *et al.*, 1991; Lane and Nigg, 1996).

Overexpression of active Nek2 leads to centrosome splitting and dispersal

To gain insights into the possible function of Nek2 at the centrosome, myc epitope-tagged wild-type and catalytically inactive Nek2 were introduced into cells by transient transfection. After 24 or 48 h, transfected cells were identified by staining with anti-myc antibodies and

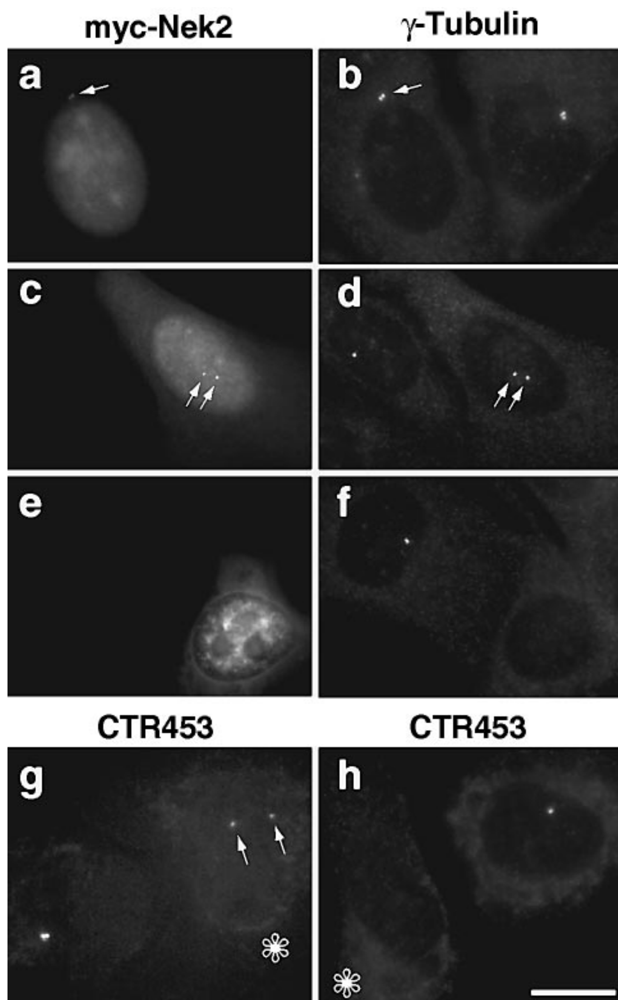

Fig. 6. Nek2 overexpression causes centrosome splitting and disappearance. U2OS cells were transfected with myc-tagged, wild-type Nek2 and double stained for the myc-epitope to identify transfectants (myc-Nek2; **a**, **c** and **e**) and γ-tubulin to reveal the centrosomes (γ-Tubulin; **b**, **d** and **f**). Some cells overexpressing Nek2 (a and b; left cell) display a normal pattern of centrosomes, as seen in untransfected cells (a and b; right cell). However, in other transfected cells, the two centrosomes show a clear splitting (c and d; right cell), and in yet others, the centrosomes are undetectable, regardless of the plane of focus (e and f; right cell). As long as centrosomes are present, the exogenous myc-Nek2 protein can be seen to co-localize with γ-tubulin (a–d, arrows). The same phenotypes could be seen using the anti-PCM monoclonal antibody, CTR453 to stain the centrosomes (g and h); in these two panels the transfected cells are indicated with a star. Scale bar, 10 µm.

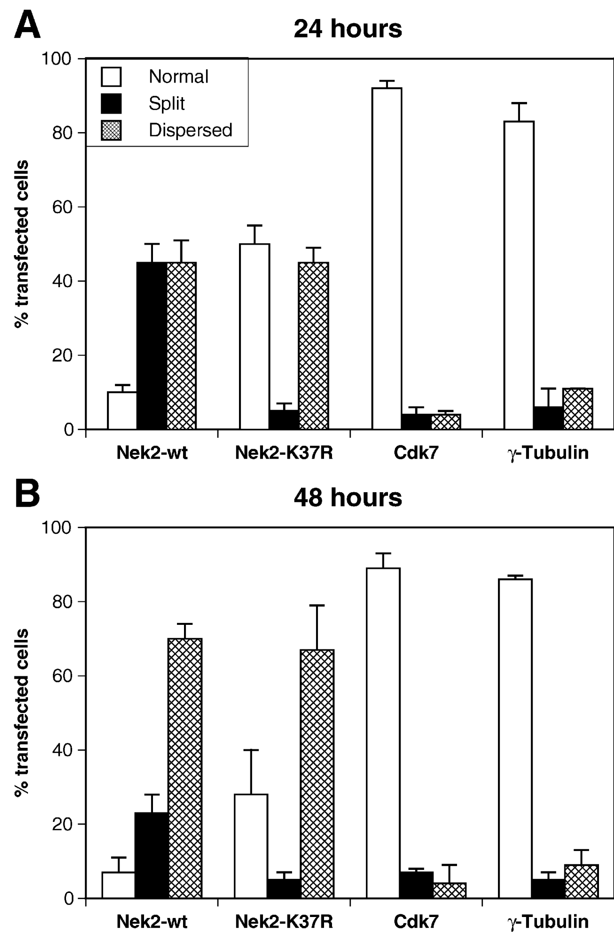

Fig. 7. Quantitation of centrosomal phenotypes caused by overexpression of wild-type and catalytically inactive Nek2. U2OS cells were transfected with cDNAs as indicated and analysed by indirect immunofluorescence microscopy at 24 h (**A**) or 48 h (**B**) after transfection. Cells were double stained with anti-myc antibodies to identify transfected cells and anti-γ-tubulin antibodies to observe the appearance of the centrosomes, except in the case of γ-tubulin transfections where transfected cells were identified with anti-γ-tubulin antibodies and centrosomes with the CTR453 monoclonal antibody. Centrosomes were classified as either normal (two closely opposed sharp points; open bars), split (a gap of at least 3–4 µm between the two centrosomes; filled bars) or dispersed (severely diminished, fragmented or undetectable; hatched bars). Results are the mean of at least five independent experiments for Nek2 plasmids and two independent experiments for Cdk7 and γ-tubulin plasmids; at least 100 transfected cells were counted for each coverslip. Error bars represent standard deviations.

the fate of centrosomes monitored by staining with anti-γ-tubulin antibodies. In some transfected cells, both anti-myc and anti-γ-tubulin antibodies could readily be seen to stain the same dots, indicating that ectopically expressed Nek2 kinase can localize to the centrosome (Figure 6a and b). This was particularly clear at early times after transfection and in cells expressing low levels of exogenous Nek2. In cells expressing higher levels, exogenous Nek2 was also seen in the nucleus (Figure 6a) or, in some cells, in the cytoplasm (see Figure 8A). Most interestingly, Nek2 overexpression caused two dramatic effects on centrosome structure. As visualized by γ-tubulin staining, the centrosome of many transfected cells had split into two foci, which were usually several microns apart (Figure 6c and d), and sometimes separated by more than half a cell diameter (data not shown). In other transfected cells, centrosomes were undetectable altogether (Figure 6e and f). Very similar effects could also be observed using antibodies against other centrosomal components, including the PCM marker CTR453 (Figure 6g and h), indicating that Nek2 overexpression profoundly affected centrosome structure and not merely the localization of γ-tubulin.

To analyse these phenotypes in quantitative terms, cells were transfected with either Nek2 or control plasmids, and then classified depending on whether they had apparently normal centrosomes, distinctly split centrosomes or dispersed centrosomes (Figure 7). Cells were counted as having dispersed centrosomes when γ-tubulin staining was virtually undetectable, or, in rare cases, confined to clusters of several tiny dots (not shown). By 24 h after transfection

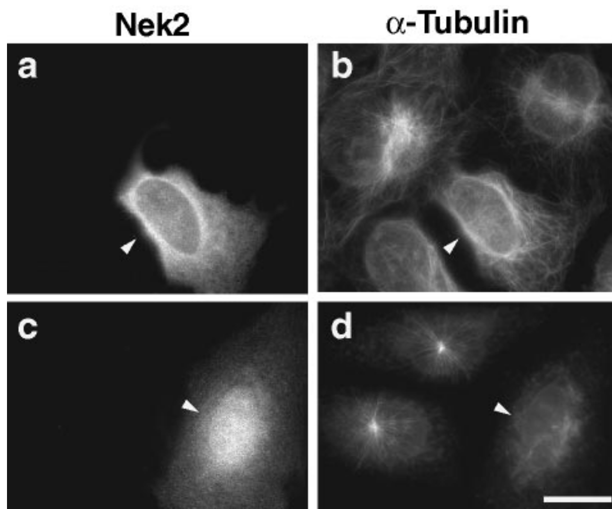

Fig. 8. Loss of focused microtubules in cells overexpressing Nek2. In cells transfected for 48 h with wild-type Nek2, microtubules were either untreated (a and b) or depolymerized by cold treatment and then allowed to regrow by the addition of warm medium (c and d). Cells were fixed with methanol and double stained with anti-Nek2 antibodies to identify transfected cells (**a** and **c**), and anti-α-tubulin antibodies to visualize the microtubules (**b** and **d**). In (b), the transfected cell can be seen to have a microtubule array emanating primarily from the perinuclear region, while in the regrowth assay (d) the transfected cell has failed to organize a focused microtubule regrowth. Arrowheads indicate transfected cells. Scale bar, 10 µm.

(Figure 7A), only 10% of the Nek2-transfected cells showed a normal centrosome pattern (i.e. one or two closely opposed sharp dots). Instead, in 45% of cells centrosomes had split over several microns, and in the remaining 45% they were almost or completely invisible. By 48 h (Figure 7B), split centrosomes could still be detected in 23% of cells, but >70% of cells lacked detectable centrosomes. Interestingly, transfection of a catalytically inactive Nek2 mutant (K37R; Fry *et al.*, 1995), also caused dispersal of centrosomal material, but did not trigger any centrosome splitting (Figure 7). This demonstrates that centrosome splitting depends on kinase activity, and suggests that centrosome splitting and dispersal are brought about by different mechanisms. Also, no centrosome splitting or dispersal could be observed in response to overexpression of either the protein kinase Cdk7 or the centrosomal component γ-tubulin (Figure 7), indicating that the observed effects were brought about specifically by Nek2 protein.

Loss of functional MTOCs in cells overexpressing Nek2

The above data clearly show that overexpression of Nek2 produces very drastic effects on centrosome structure. To extend our studies beyond a structural description, we asked to what extent overexpression of Nek2 interfered with the microtubule organizing function of centrosomes. When Nek2-transfected cells were stained for α-tubulin, it was frequently difficult to find a focal point of microtubule organization; instead, a dense concentration of microtubules could often be seen at the nuclear periphery (Figure 8a and b). This suggested that microtubules might be nucleated at the nuclear surface rather than from a centrosomal MTOC (see Spiegelman *et al.*, 1979). To corroborate this finding, microtubule regrowth assays were carried out. Following transfection of cells with a Nek2 plasmid, microtubules were depolymerized by cold treatment before microtubule regrowth was induced by the addition of warm medium. Cells were then fixed and double-stained with anti-Nek2 (Figure 8c) and anti-α-tubulin antibodies Figure 8d). Cells expressing either wild-type or catalytically inactive Nek2 showed no signs of organized centres of microtubule regrowth (Figure 8c and d, right cell). In contrast, non-transfected neighbouring cells consistently showed a highly focused centre of microtubule nucleation, with many long, thin microtubules emanating from the centrosome (Figure 8c and d, left cells). Entirely normal, focused centres of microtubule nucleation were also seen in cells transfected with a Cdk7 plasmid for control (not shown). Prolonged incubation of Nek2-transfected cells allowed regrowth of a large number of microtubules, presumably from both the perinuclear region and from diffusely distributed sites within the cytoplasm. This is in agreement with previous findings that mammalian cells contain other sites for microtubule initiation besides the centrosome (Spiegelman *et al.*, 1979). Virtually identical results were obtained using nocodazole treatment to depolymerize the microtubule network and then observing regrowth upon removal of the drug (data not shown).

Nek2-induced centrosome dispersal does not prevent entry into mitosis

To examine whether overexpression of Nek2 caused cells to arrest at a specific stage of the cell cycle, co-transfection experiments with a CD20 surface marker were performed, and the cell cycle distribution of Nek2-expressing cells was analysed by flow cytometry. No striking effects on cell cycle profiles could be observed (data not shown), suggesting that cells overexpressing Nek2 may be able to enter mitosis in spite of lacking functional MTOCs. To test this hypothesis directly, cells were co-transfected with Nek2 and a plasmid encoding a non-destructible mutant of cyclin B2 (Gallant and Nigg, 1992). If disruption of centrosome structure were to block entry into mitosis, we reasoned that the expression of Nek2 should suppress the accumulation of mitotic cells that is induced when the non-destructible cyclin is expressed alone (Gallant and Nigg, 1992). As negative and positive controls, similar co-transfections were performed using CD20 and a dominant-negative mutant of Cdc2, respectively, in place of Nek2. The CD20 surface marker is not expected to cause any effect on cell cycle progression, whereas the dominant-negative mutant of Cdc2 is known to block cells in G_2 (van den Heuvel and Harlow, 1993). To exclude from these analyses any cells that might have entered mitosis before Nek2 (or the Cdc2 mutant) could possibly produce a cell cycle block, cells that were already in mitosis by 30 h after transfection were discarded by mechanical shake-off. Then, 48 h after transfection, the remaining cells were fixed and those expressing the non-destructible cyclin B2 were classified depending on whether they displayed an interphasic or a mitotic phenotype (Figure 9). After co-transfection of the non-destructible cyclin B2 with the CD20 cell-surface marker, ~80% of the transfected cells were blocked in mitosis (Figure 9A), consistent with previous results (Gallant and Nigg, 1992). In contrast, co-expression of the dominant-negative Cdc2 mutant severely suppressed the accumulation of mitotic cells (Figure 9A,

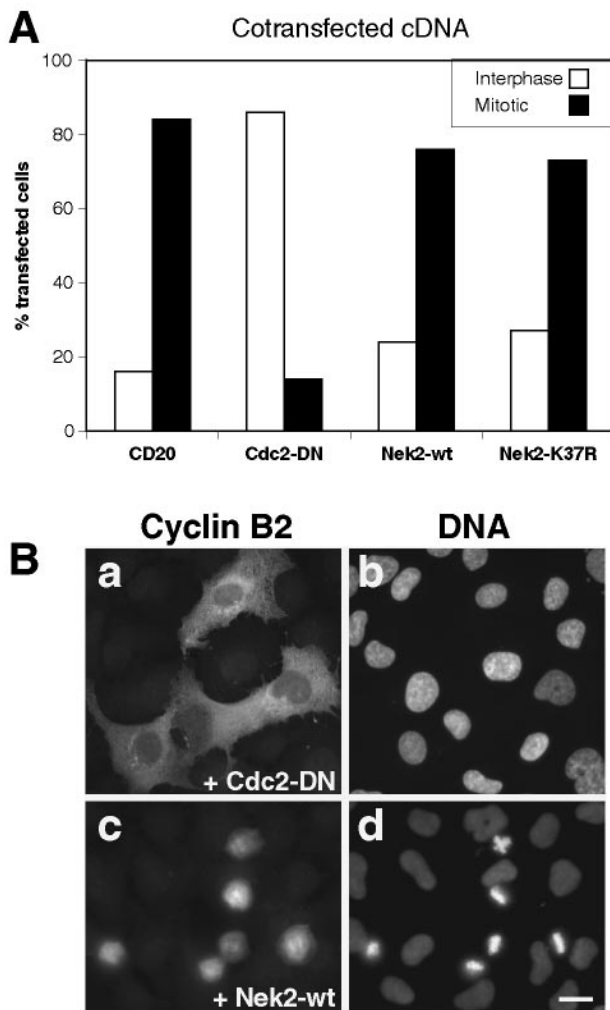

Fig. 9. Nek2 overexpression does not block the mitotic accumulation of cells co-transfected with non-destructible cyclin B2. (**A**) U2OS cells were co-transfected with a plasmid encoding a non-destructible cyclin B2 mutant and a second cDNA as indicated. Greater than 90% co-expression of both plasmids was confirmed by double antibody labelling after 24 h. At 30 h, mitotic cells were removed by mechanical shake-off, and after a further 18 h cells were fixed and stained with anti-cyclin B2 antibodies. The percentage of transfected cells in interphase and mitosis was determined. (**B**) Cells were co-transfected with plasmids encoding a non-destructible cyclin B2 mutant and either a dominant-negative mutant of Cdc2 (panels a and b) or wild-type Nek2 (panels c and d), as described for (A). Immunofluorescent staining was performed using antibodies specific for cyclin B2 (a and c); in parallel, the condensation state of DNA was visualized using Hoechst dye 33258 (b and d). Note that the dominant-negative mutant of Cdc2 caused mutant cyclin B2-expressing cells to remain in interphase, whereas Nek2 did prevent such cells from passing through G_2/M and arresting in mitosis. Scale bar, 10 μm.

and Figure 9B, panels a and b), as expected for a mutant causing cells to arrest in G_2 (van den Heuvel and Harlow, 1993). Co-transfection of either wild-type or inactive Nek2 did not prevent the accumulation of mitotic cells caused by the non-destructible cyclin B2 mutant (Figure 9A, and Figure 9B, panels c and d). This result is remarkable, considering that the bulk of these cells is expected to have entered mitosis with severely disrupted centrosomes (see Figure 7), and it suggests that a focused centrosomal structure is not indispensable for mammalian cells to enter mitosis.

Discussion

Among all presently known mammalian protein kinases, Nek2 is most closely related to the mitotic regulator NIMA of *A.nidulans*. In this study, we show that the human Nek2 protein localizes to the centrosome. Overexpression of Nek2 profoundly influences centrosome structure and activity, but, remarkably, does not prevent cells from entering mitosis. These findings provide the first direct evidence in support of a cell cycle role for human Nek2. Furthermore, they have implications for the dynamic function of centrosomes and open a new perspective on the range of possible functions performed by protein kinases of the NIMA family.

Nek2 is a novel centrosome-associated kinase

Immunofluorescence microscopy and subcellular fractionation concur to demonstrate that Nek2 is a bona fide centrosomal protein. Nek2 association with centrosomes is independent of microtubules, indicating that Nek2 should be classified as a 'core' component of the centrosome, along with proteins such as γ-tubulin, pericentrin and *Drosophila* CP190 and CP60 (Stearns *et al.*, 1991; Raff *et al.*, 1993; Doxsey *et al.*, 1994; Oegema *et al.*, 1995). Our data further show that Nek2 is present at the centrosome throughout the cell cycle, including all stages of mitosis. At first glance, this conclusion appears to conflict with previous data showing that the levels of Nek2 fluctuate during the cell cycle, being low during M and G_1 phase and high during S and G_2 phase (Fry *et al.*, 1995). However, we emphasize that the centrosome-associated pool amounts to only ~10% of the total Nek2 protein present in the cell types analysed here, and we presume that the bulk of Nek2 is distributed throughout the cytoplasm and/or the nucleus. In support of this view, we found that ectopically expressed myc-tagged Nek2 localized not only to the centrosome, but also to either the cytoplasm or the nucleus, depending on the cell cycle stage (A.M.Fry and E.A.Nigg, unpublished results). It appears likely, therefore, that cell cycle-regulated changes in Nek2 abundance may concern primarily the non-centrosomal pool. Also, the existence of a non-centrosomal population of Nek2 clearly leaves the possibility that this kinase may perform multiple functions.

Overexpression of Nek2 produced two striking consequences on centrosomes, namely centrosome splitting and the gradual disappearance of centrosomes. Whereas centrosome splitting strictly required Nek2 activity, centrosome dispersal was triggered by both active and inactive Nek2. This strongly suggests that the two phenotypes are endpoints of two independent processes (as opposed to splitting being an intermediate stage in a pathway leading to centrosome dispersal). Centrosome dispersal may result from the titration or competitive displacement of some Nek2-interacting protein, and is thus unlikely to directly reflect a physiological event. Centrosome splitting, on the other hand, most probably results from the phosphorylation of as yet unidentified centrosomal proteins by Nek2. We are encouraged to believe that the centrosome splitting observed here does reflect a physiologically relevant activity of Nek2, particularly since similar phenomena have been observed previously in cells treated with

mitogenic growth factors and chemoattractants (Schliwa *et al.*, 1982; Sherline and Mascardo, 1982).

Somatic mammalian cells are able to enter mitosis without focused MTOCs

Intrigued by the Nek2-induced dispersal of centrosomes, we asked whether cells overexpressing Nek2 would be able to reform a focused MTOC after the depolymerization of microtubules. We found that in Nek2-transfected cells, microtubule regrowth was disorganized and severely retarded, demonstrating that these cells had lost a focused MTOC. We then asked whether such cells would be able to enter mitosis, in spite of lacking focused MTOCs. Remarkably, Nek2 overexpression did not appear to cause a significant block to progression towards mitosis, as flow cytometric analyses of DNA profiles revealed no changes in the cell cycle distribution of Nek2-transfected cells. Furthermore, there was no reduction in the accumulation of mitotic cells when Nek2 was co-transfected with a non-destructible B-type cyclin, indicating that exogenous Nek2 was unable to impose a G_2 arrest. Taken together, these data suggest strongly that Nek2-transfected cells entered mitosis in spite of having mostly dispersed centrosomes.

Our observations fall in line with the fact that spindles form in the absence of centrioles during mitosis in plant cells, as well as during female meiosis in certain animal species (Kellogg *et al.*, 1994). Furthermore, recent studies using *Xenopus* egg extracts demonstrate that spindle formation *in vitro* can occur without the addition of centrosomes (Heald *et al.*, 1996), although centrosomes act dominantly when present (Heald *et al.*, 1997). All these observations concur to indicate that MTOCs (i.e. centrosomes) need not necessarily be focused for cells to progress into mitosis, and that bipolarity of the mitotic spindle may result from the coordinated action of plus-end- and minus-end-directed microtubule-dependent motors. However, whether somatic mammalian cells can assemble fully functional spindles in the absence of centrosomes, and whether such spindles are able to segregate sister chromatids accurately remains to be investigated. All our attempts at generating stable cell lines expressing Nek2 have failed, indicating that overexpression of this kinase is lethal in the long term (A.M.Fry and E.A.Nigg, unpublished results). Lack of efficient and faithful sister chromatid separation could be one explanation for this lethality.

A role for Nek2 in centrosome separation?

Immunostaining of interphase cells for centrosomal antigens generally reveals two closely associated but distinct dots. Likewise, immunostaining of centrosomes isolated from tissue culture cells commonly yields closely associated pairs of dots, reminiscent of what is seen in the intact cell. Depending on cell cycle position and spatial disposition of the object under the microscope, each visible dot may correspond to either a single centriole or a pair of centrioles, or centrioles in the process of duplication, each embedded in a cloud of PCM. Interestingly, centrioles (along with associated PCM) remain tightly associated even in the absence of microtubules. What holds these organelles together is presently unknown. Whatever the molecular nature of this connection, however, our data suggest that Nek2 activity can cause its disruption. We consider it attractive to propose, therefore, that Nek2 may play a role in preparing centrosomes for separation prior to the onset of mitosis. Specifically, we envisage that centrosome separation during G_2 may be a two-step process, as illustrated in Figure 10: in a first step, the connection between the two duplicated centrosomes may be severed in a Nek2-dependent reaction. In a second step, which is dependent on Cdc2 activity, the separated centrosomes may then be moved apart by microtubule-dependent motor proteins such as Eg5 or Xklp2 (Sawin *et al.*, 1992; Blangy *et al.*, 1995; Boleti *et al.*, 1996). In cells transfected by active Nek2, unscheduled splitting of centrosomes may occur, and these may drift apart even in the absence of active Cdc2. Consistent with this interpretation, we note that immunolabelling of Nek2-transfected cells with anti-Eg5 antibodies produced no staining of the split centrosomes (A.M.Fry and E.A.Nigg, unpublished results), indicating that Nek2 acts on centrosomes prior to the activation of Cdc2/cyclin B.

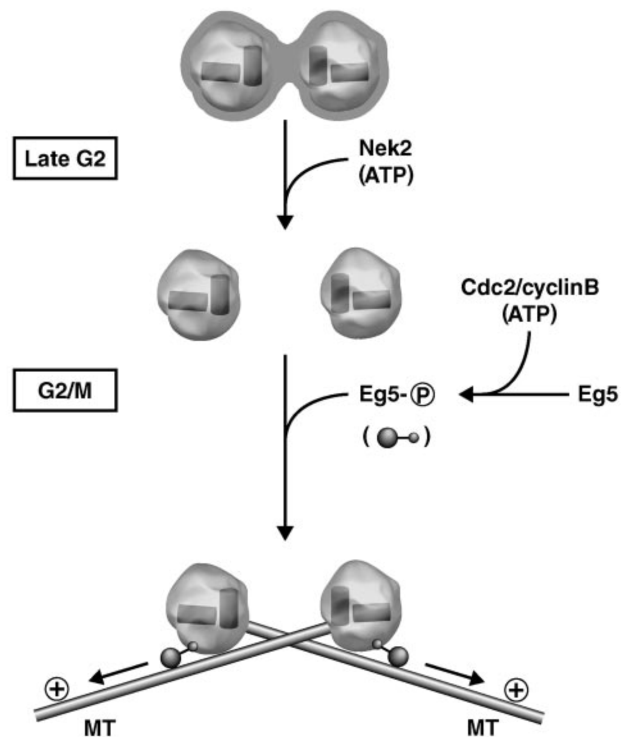

Fig. 10. A two-step model for centrosome separation. In this model, the process of centrosome separation is described in terms of two independent steps which must occur at the onset of mitosis. The first step involves the severing of a connection (e.g. the dissolution of a hypothetical cage) which holds the centrosomes in close proximity throughout interphase. Under physiological conditions, this step is proposed to be triggered in late G_2 by the activation of Nek2 (and/or the inactivation of a counteracting phosphatase). Under the experimental conditions described here, premature separation (i.e. splitting) of centrosomes results from ectopic expression of active Nek2. In both cases, phosphorylation of as yet unidentified centrosomal 'glue' proteins may cause their depolymerization or target them for proteolytic degradation. The second step involves several microtubule-based motor proteins, only one of which is depicted here. This kinesin-related motor, Eg5 is recruited to the centrosome in response to phosphorylation by Cdc2/cyclin B, and its activity contributes to the separation of centrosomes along microtubules. Note that objects are not drawn to scale, and the precise mode of action of Eg5 remains to be determined (for discussion, see Walczak and Mitchison, 1996).

Implications for the functions of NIMA family members

Previous studies have emphasized a potential role for NIMA kinases in chromosome condensation (O'Connell *et al.*, 1994; Lu and Hunter 1995a; Lu *et al.*, 1996; Osmani and Ye, 1996; Rhee and Wolgemuth, 1997), but alternative or additional roles have not been excluded. On the basis of structural criteria, Nek2 is presently the most closely related human protein to fungal NIMA (Fry and Nigg, 1997). Yet, our present data provide no evidence of a role for Nek2 in chromosome condensation and instead clearly indicate that one function of this kinase relates to the centrosome cycle. To reconcile these findings, one could postulate that NIMA might perform multiple functions in *Aspergillus*, whereas distinct members of the 'Nek' family might have evolved to assume specialized functions in higher eukaryotes. According to this scenario, Nek2 may primarily be concerned with centrosome function, while another mammalian Nek family member might be required for inducing chromosome condensation. Alternatively, our findings raise the possibility that NIMA (as well as related kinases such as Kin3p of *S.cerevisiae*) may also regulate the properties of the MTOC. In support of this possibility, overexpression of NIMA in *Aspergillus* cells was reported to cause mitotic spindle abnormalities in addition to chromosome condensation (Osmani *et al.*, 1988), something not seen when NIMA is introduced into human cells. In fungal species like yeast and *Aspergillus*, the microtubule-organizing role is performed by the spindle pole body (SPB). Although mammalian centrosomes and fungal SPBs exhibit gross functional similarities, they are structurally quite distinct (Winey and Byers, 1993; Kellogg *et al.*, 1994). Thus, if both *Aspergillus* NIMA and human Nek2 were to play a role in regulating MTOC function, then significant differences in the structures of these kinases would be expected to have arisen during evolution. In particular, it is attractive to speculate that the carboxy-termini of functionally homologous NIMA family members might be involved in interactions with structurally distinct components of the MTOCs in their respective species. This might explain why human Nek2, NIMA of *A.nidulans* and Kin3p of *S.cerevisiae* show little sequence conservation outside the catalytic domain. In the light of the results reported here, it may be rewarding to examine whether NIMA shows structural or genetic interactions with SPB components in *Aspergillus*.

Materials and methods

Preparation of expression plasmids

Nek2 constructs were first prepared as in-frame myc-tag fusions in a pBlueScript vector carrying the myc epitope tag, pBlueScript-myc (Schmidt-Zachmann and Nigg, 1993). These myc-tagged fusions were then transferred into the eukaryotic expression vector, pRcCMV (Invitrogen Corp.). Specifically, the complete coding sequence of Nek2 was excised from the pGEM–Nek2 plasmid (Schultz *et al.*, 1994) as a *Nae*I–*Xba*I fragment and subcloned into pBlueScript–myc cut with *Sma*I and *Xba*I, to create pBS–mycNek2. The mycNek2 fusion was then excised as a *Sal*I(blunted)–*Xba*I fragment from pBS–mycNek2 and subcloned into pRcCMV, using the *Hin*dIII(blunted) and *Xba*I sites, to create pCMV–mycNek2. The same cloning strategy was used to create pBS–mycNek2–K37R and pCMV–mycNek2–K37R from the catalytically inactive Nek2 mutant construct pGEM–Nek2–K37R (Fry *et al.*, 1995).

For construction of pCMV–γ-tubulin, the full-length human γ-tubulin cDNA (kindly provided by B.Oakley, Ohio State Univ., Columbus, OH) was amplified by PCR, checked by sequencing and inserted into a modified pBlueScript vector (Hadjeb and Berkowitz, 1996). From this plasmid, the entire γ-tubulin cDNA was excised as a *Hin*dIII–*Not*I fragment and inserted between the *Hin*dIII and *Not*I sites of the pRcCMV vector, to give pCMV–γ-tubulin.

Antibody production

A polyhistidine-tagged fragment of Nek2 (SPnek2) was isolated on a Ni^{2+}-agarose column as previously described (Schultz *et al.*, 1994), and purified further by elution from an SDS–polyacrylamide gel: the protein was run on a 12% gel and visualized by incubation of the gel with KCl (0.25 M KCl, 1 mM β-mercaptoethanol) at 4°C. The SPnek2 band was cut out and electroeluted into 0.5× protein electrophoresis running buffer. New Zealand White rabbits were injected with 500 μg of the purified protein at 2- to 3-week intervals, and immunoreactivity for Nek2 protein was monitored by immunoblotting analysis. Affinity purification of anti-Nek2 antibodies was carried out as described in Schultz *et al.* (1994). For antibody competition, Sf9 insect cell lysates were prepared following infection with either wild-type or Nek2 baculoviruses. These were separated by 12% SDS–PAGE and blotted onto nitrocellulose. Nitrocellulose strips corresponding to the 40–50 kDa region were cut out and incubated with the R40 antibody, to give a 'mock'-competed antibody (using the filter carrying the control lysate) and a Nek2-competed antibody (using the filter carrying the Nek2-containing lysate). Western blots on recombinant Nek2 confirmed the complete depletion of α-Nek2 antibodies from the Nek2-competed sample but not from the mock control.

For generation of anti-γ-tubulin antibodies, the synthetic peptide CAATRPDYISWGTQEQ, corresponding to the C-terminal 15 residues of human γ-tubulin plus an N-terminal cysteine, was coupled to keyhole limpet haemocyanin (at a peptide:carrier ratio of 1:1, w/w), using either glutaraldehyde or 3-maleimidobenzoic acid-*N*-hydroxysuccinimide ester (MBS), according to Harlow and Lane (1988). Both coupling products were mixed in equal amounts and injected intramuscularly into New Zealand White rabbits at intervals of 28 days (~100 μg of peptide per injection), and immunoreactivity of the sera for γ-tubulin protein was monitored by immunoblotting and immunofluorescence analysis. IgGs were purified from reactive sera on a protein A–Sepharose column (Harlow and Lane, 1988).

Cell culture and transfections

Human cells were grown at 37°C in a 7% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated serum and penicillin–streptomycin (100 IU/ml and 100 μg/ml, respectively). HeLa epithelial cells were grown in 5% fetal calf serum (FCS) and U2OS osteosarcoma cells in 10% FCS. The T-lymphoblastoid cell lines, CCRF-CEM (obtained from S.Eriksson, Uppsala, Sweden) and KE37 (Bornens *et al.*, 1987), were grown in suspension in the above medium supplemented with 10% heat-inactivated horse serum or 10% heat-inactivated FCS, respectively. Sf9 insect cells were grown in TC100 medium (Gibco-BRL), supplemented with 10% heat-inactivated FCS and penicillin–streptomycin at 27°C. For transient transfections, U2OS cells were seeded onto HCl-treated glass coverslips at a density of 1×10^5 cells per 35 mm dish and transfected with 10 μg of plasmid DNA using calcium phosphate precipitates as previously described (Krek and Nigg, 1991). Cells were fixed at the times indicated and analysed by indirect immunofluorescence microscopy. For co-transfections with the non-destructible cyclin B mutant, 5 μg of each plasmid DNA were used. At 30 h post-transfection, mitotic cells were removed by mechanical shake-off and pipetting vigorously over the coverslip before replacing into fresh medium. These coverslips were then fixed at 48 h post-transfection with paraformaldehyde and analysed as described below.

Cell extracts, immunoblotting and kinase assays

To characterize the specificity of Nek2 antibodies, cell extracts were prepared from exponentially growing HeLa cells, or from Sf9 insect cells which had been infected with either wild-type baculovirus or Nek2 recombinant baculovirus (Fry and Nigg, 1997). Proteins were resolved by electrophoresis on 12% SDS–polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes, using a semi-dry blotting apparatus (Biorad). Proteins were visualized by Ponceau S staining, before blocking the membranes with blocking buffer (5% low-fat dried milk in 1× PBS + 0.1% Tween-20). All antibody incubations were carried out in blocking buffer and bound immunoglobulins (IgGs) were visualized using alkaline phosphatase-conjugated anti-rabbit IgG secondary antibodies (Promega). Nek2 immunoprecipitations and *in vitro* kinase assays with β-casein as a substrate were performed according to

Fry and Nigg (1997). For immunoblot analysis of centrosomal fractions, proteins were resolved on 10% SDS–polyacrylamide gels and then processed as above. Total KE37 cell extracts were prepared by directly resuspending cells in boiling gel sample buffer and heating to 95°C for 10 min.

Isolation of human centrosomes

Centrosomes were isolated from the human lymphoblastic cell lines CCRF-CEM and KE37 according to the method of Moudjou and Bornens (1994). From 10^9 cells, ~3×10^8 centrosomes were recovered in the fractions eluting from the final sucrose gradient.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed using a Zeiss Axioplan II microscope and 40× or 63× oil immersion objectives. Photographs were taken using a Quantix 1400 CCD camera (Photometrics, Inc.) and IP-Lab software, and the images processed for presentation using Adobe Photoshop (Adobe Systems, Mountain View, CA). For antibody staining of whole cells, U2OS cells were grown on acid-treated coverslips and fixed with –20°C methanol for 6 min (for all data shown) or 3% paraformaldehyde/2% sucrose followed by 0.5% Triton X-100 in phosphate-buffered saline (PBS). Following fixation, coverslips were washed three times with PBS, blocked with 1% BSA in PBS for 10 min, and washed again three times with PBS. All subsequent antibody incubations were carried out in PBS containing 3% BSA. Reagents used for immunofluorescence in this study were anti-Nek2 (R40) affinity-purified antibodies (2.5 µg/ml), anti-γ-tubulin purified IgGs (3.6 µg/ml), 9E10 anti-myc monoclonal antibody (undiluted tissue culture supernatant), CTR453 monoclonal antibody (1:5 tissue culture supernatant), anti-Eg5 affinity-purified antibodies (2.0 µg/ml; Blangy *et al.*, 1995), anti-cyclin B2 serum (R18, 1:500; Gallant and Nigg, 1992) and anti-α-tubulin monoclonal antibody (3 µg/ml; Amersham). Primary antibody incubations were carried out separately for 45 min each, followed by three washes with PBS. Detection of primary antibodies was carried out using the following secondary reagents: biotinylated donkey anti-rabbit or goat anti-mouse antibodies (1:50; Amersham) followed by Texas red-conjugated streptavidin (1:200; Amersham), fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Fab fragment (1:100; Sigma) or dichlorotriazinyl amino fluorescein (DTAF)-conjugated goat anti-rabbit antibody (1:500; Pierce). All secondary antibodies had been tested and found to be negative for cross-reactivity against human centrosomes. DNA was stained with Hoechst 33258 (0.2 µg/ml). Following three final washes in PBS, coverslips were mounted in 80% glycerol, 3% *n*-propylgallate (in PBS) mounting medium. Isolated centrosomes were stained exactly as described by Moudjou and Bornens (1994), using the antibodies described in the text.

Drug treatments and microtubule regrowth assays

Asynchronously growing U2OS cells were treated for 4 h with either 6 µg/ml nocodazole or 5 µM taxol and then processed immediately for immunofluorescent staining with anti-Nek2 and anti-α-tubulin antibodies, as described above. To assess the effects of exogenously expressed Nek2 on microtubule regrowth, U2OS cells were transiently transfected with various Nek2 constructs and, 48 h later, microtubules were depolymerized by placing the tissue culture dishes on ice for 30 min. Cells were then either fixed immediately or microtubule regrowth was allowed to proceed for 2 min following the re-addition of pre-warmed (37°C) medium. Alternatively, microtubules were depolymerized in transfected cells by a 4 h treatment with 6 µg/ml nocodazole, and then regrowth was induced by washing cells three times with PBS and placing them into fresh medium for 2 min.

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