Centrosome cohesion is regulated by a balance of kinase and phosphatase activities

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SUMMARY

Centrosome cohesion and separation are regulated throughout the cell cycle, but the underlying mechanisms are not well understood. Since overexpression of a protein kinase, Nek2, is able to trigger centrosome splitting (the separation of parental centrioles), we have surveyed a panel of centrosome-associated kinases for their ability to induce a similar phenotype. Cdk2, in association with either cyclin A or E, was as effective as Nek2, but several other kinases tested did not significantly interfere with centrosome cohesion. Centrosome splitting could also be triggered by inhibition of phosphatases, and protein phosphatase 1α (PP1α) was identified as a likely physiological antagonist of Nek2. Furthermore, we have revisited the role of the microtubule network in the control of centrosome cohesion. We could confirm that microtubule depolymerization by nocodazole causes centrosome splitting. Surprisingly, however, this drug-induced splitting also required kinase activity and could specifically be suppressed by a dominant-negative mutant of Nek2. These studies highlight the importance of protein phosphorylation in the control of centrosome cohesion, and they point to Nek2 and PP1α as critical regulators of centrosome structure.

Key words: Cell cycle, Centriole, Nek2, PP1α, Phosphorylation

INTRODUCTION

The centrosome, the major microtubule (MT) organizing center (MTOC) of animal cells, contributes to most MT-dependent processes, including organelle transport, cell shape and motility, cell polarity, as well as cell division and cytokinesis (Andersen, 1999; Kellogg et al., 1994). Because centrosomes are associated with the two spindle poles of a dividing cell, each progeny cell inherits one centrosome. Subsequently, the centrosome is duplicated once in every cell cycle, much like the genome. Although both meiotic and mitotic spindles can form in the absence of centrosomes (Bobinnec et al., 1998; Fry et al., 1998a; Heald et al., 1996; Khodjakov et al., 2000), these organelles exert a dominant effect on MT organization whenever present (Heald et al., 1997). Thus, centrosomes play an important role in determining both the fidelity of chromosome transmission and tissue architecture, and it has long been proposed that errors in the centrosome duplication cycle may contribute to tumor formation (Brinkley, 2001; Doxsey, 1998; Mayor et al., 1999; Sluder and Hinchcliffe, 1999; Urbani and Stearns, 1999; Zimmerman et al., 1999). In a typical animal cell in G_1 phase of the cell cycle, the centrosome is composed of two barrel-shaped centrioles (made of triplet MTs), and a surrounding pericentriolar matrix (PCM). During S phase, a procentriole forms adjacent to, and at right angles with, each of the two parental centrioles. Procentrioles then continue elongating throughout G_2 phase, and by late G_2 the cell harbors two doublets of centrioles. Shortly before the onset of mitosis the duplicated centrosomes finally separate, migrate apart and contribute to form the poles of the bipolar spindle apparatus. From the perspective of MT organization, it is critical to distinguish centrosome *duplication* from centrosome *separation*. While the former event occurs during S phase, the latter occurs typically at the G_2/M transition (Kochanski and Borisy, 1990; Vorobjev and Chentsov, 1982). The fact that two distinct MTOCs are generated only at the onset of mitosis, although centrioles duplicate already during S phase, implies that duplicated centrosomes are held together to form a single functional unit for a considerable part of the cell cycle. The mechanism underlying this centrosome cohesion is the subject of this study.

For the sake of clarity, we will use the term ‘centrosome splitting’ to describe any separation of parental centrioles, regardless of the presence or absence of procentrioles (Fig. 1). By contrast, the term ‘centrosome separation’ will be used only to describe the cell cycle-regulated separation of MTOCs at the onset of mitosis. Several lines of evidence indicate that centrosome splitting can be induced from any stage of the cell cycle. In particular, centrosome splitting has been described in response to treatment of cells with chemotactic stimuli, mitogenic growth factors and the tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) (Schliwa et al., 1982; Schliwa et al., 1983; Sherline and Mascardo, 1982). Moreover, centrosome splitting and repositioning of the mature centriole to the intercellular bridge during late mitosis has recently been implicated in the completion of cell division (Piel et al., 2001). These provocative studies indicate that centrosome cohesion is regulated by a variety of cues, and that the dynamic repositioning of centrioles may play an important role in the control of cell behavior.

It has been proposed that the mammalian interphase centrosome consists of two independent units held together primarily as a result of the dynamic properties of the MT

cytoskeleton (Jean et al., 1999). In support of this model, MT networks display self-centering activity, provided that they are confined to a limited space (Holy et al., 1997; Rodionov and Borisy, 1997). Moreover, drugs that affect the dynamics of either the MT network or the actin microfilament system clearly modulate centrosome cohesion (Buendia et al., 1990; Euteneuer and Schliwa, 1985; Jean et al., 1999). However, there is a substantial body of evidence to support the idea that centrosomes are connected through a proteinaceous structure linking the two parental centrioles to each other (Fig. 1). In particular, electron microscopic examination of isolated centrosomes reveals electron-dense material connecting the parental centrioles (Fuller et al., 1995; Paintrand et al., 1992). Furthermore, purified centrosomes usually display closely paired parental centrioles even though the cytoskeleton has been disrupted in the course of their isolation (Bornens et al., 1987; Chretien et al., 1997).

Most recently, we have proposed a specific model for the regulation of centrosome cohesion during the cell cycle. At the heart of this model are the protein kinase Nek2, a member of the NIMA kinase family implicated in regulating centrosome assembly and cohesion (Fry et al., 1998a; Fry et al., 2000a; Uto and Sagata, 2000), and the centrosomal coiled-coil protein C-Nap1 (also known as Cep2/Cep250; Mack et al., 1998; Fry et al., 1998b; Mayor et al., 2000). We envision that C-Nap1 provides a docking site for a dynamic linker structure that tethers parental centrioles until phosphorylation causes its disassembly. In support of this hypothesis, we have shown that splitting of centrosomes can be triggered by overexpression of active Nek2, as well as by microinjection of anti-C-Nap1 antibodies.

Here, we have further investigated the role of protein phosphorylation in regulating centrosome cohesion. Our results lead us to conclude that Nek2 is not the only kinase able to regulate centrosome cohesion, but that a balance between Nek2 and the counteracting protein phosphatase 1 α (PP1α) is particularly critical. Furthermore, we provide evidence that even cytoskeletal effects on centrosome cohesion depend on protein kinase activity. Taken together, our results identify protein phosphorylation as a major mechanism regulating centrosome cohesion during the cell cycle.

MATERIAL AND METHODS

Preparation of expression plasmids

A full length cDNA for aurora-A in pBlueScript KS+ was obtained from ATCC (GenBank accession no. AA305070) and its sequenced verified (Bischoff et al., 1998). To introduce an *Nco*I site at the initiator ATG and destroy a single *Eco*RI site upstream of the start codon, a 5′ fragment of aurora-A was amplified by PCR with the high fidelity Taq polymerase (Roche Diagnostics), using the two oligonucleotides CTGAATTCCATGGACCGATCTAAAG and ACACTGGTTGCCTGCAATTGC. The amplified fragment was cut with *Eco*RI (blunted)-*Hin*dIII and fused to the *Hin*dIII-*Xho*I aurora-A fragment in pBlueScriptII KS+. A catalytically inactive version of aurora-A (K162R) was produced by site-directed mutagenesis on an *Eco*RI-*Hin*dIII fragment, introducing a Lys→Arg mutation in position 162 (Bischoff et al., 1998). For fusion with the Myc-tag, both wild-type and K162R mutant cDNAs were cut with *Nco*I (blunted)-*Xba*I and ligated into pBlueScriptII KS-myc cut with *Sma*I-*Not*I. For expression in mammalian cells, myc-tagged cDNAs were also subcloned into the pBK-CMV vector using *Sal*I and *Xba*I sites. A full length cDNA for human PP1α was obtained from the IMAGE consortium (Est no. 650691) and its sequence verified (Song et al., 1993). The cDNA was cloned in-frame into the pBlueScriptII KS-myc cut with *Eco*RI-*Bam*HI to obtain pBS-myc-PP1α, and myc-PP1α was further subcloned into the pBK-CMV vector using *Sal*I and *Xba*I sites. All other plasmids used in this study were described previously (Fry et al., 1998a; Golsteyn et al., 1994; Meraldi et al., 1999).

Cell culture

U20S cells were grown at 37°C in a 7% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and penicillin-streptomycin (100 IU/ml and 100 μg/ml, respectively) (all Gibco-BRL).

Transfection experiments and analysis of centrosome splitting

U2OS cells were seeded onto HCl-treated 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 described previously (Krek and Nigg, 1991). Cells were fixed 24 hours after transfection and analyzed by indirect immunofluorescence microscopy. They were stained with an anti-γ-tubulin antibody (Fry et al., 1998a) to visualize the centrosome and with 9E10 anti-myc or 12CA5 anti-haemagglutinin (HA) tag monoclonal antibodies (Mabs) to identify transfected cells. Myc-tags were used for detection of cyclins A, E and D1, Nek2, Plk1, aurora-A and PP1α, whereas HA tags were used for detection of Cdks. As judged by fluorescence intensity, all kinases were expressed at comparable levels, but their exact in vivo activities are not known. Where indicated, cells were treated for 1 hour before fixation with nocodazole (5 μg/ml), taxol (5 μM), cytochalasin D (1.25 μg/ml), staurosporine (500 μM), 6-dimethylaminopurine (6-DMAP) (2.5 mM), okadaic acid (1 μM or 5 μM) or calyculin A (10 nM) or placed on ice. Quantitative analyses were performed on 400-600 cells, depending on the protein combinations being expressed, and at least three independent experiments were performed for each transfection or drug treatment. Centrosome splitting was quantified as follows: in untreated U2OS cells, anti-γ-tubulin antibodies usually stained two closely spaced dots (or, occasionally, a single dot, depending on the orientation of the centrosome) and in 90-95% of all cells, the distance between these γ-tubulin-positive dots was less than 2 μm. The remaining 5-10% of cells showed a separation of dots by more than 2 μm, and these were considered as harboring split centrosomes. Similarly, in all experiments described throughout this study, centrosomes were scored as split if the distance between the two γ-tubulin-positive structures was larger than 2 μm. Within a population of cells, the distances between parental centrioles varied widely, but they rarely exceeded 2-4 μm in untreated cells, whereas they ranged from 2 to about 20 μm (average 5-10 μm) in treated cells. As reported previously, centrosomes were difficult to detect in about 10-15% of Nek2-overexpressing cells (Fry et al., 1998a); these were not counted.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed using a Zeiss Axioplan microscope and a 63× 1.4 oil immersion objective. Pictures were taken using Quantix 1400 (Photometrics Inc) or Micromax (Princeton, Instruments) CCD cameras and IP-Lab or Metaview (Universal Imaging Corp.) software, and images were processed using Adobe PhotoShop (Adobe Systems, Mountain View, CA). For antibody staining U2OS cells were grown on acid-treated coverslips and fixed with methanol at −20°C for 6 minutes. Then, coverslips were washed three times in phosphate-buffered-saline (PBS), blocked with 1% bovine serum albumin (BSA) in PBS for 10 minutes, and again washed three times with PBS. All subsequent antibody incubations were carried out in PBS containing 3% BSA. Antibody reagents were anti-γ-tubulin (purified IgG; 1 μg/ml; Fry et al., 1998b), anti-Nek2

(R40) (affinity-purified IgG; 2.5 μg/ml; Fry et al., 1998b), 9E10 anti-myc Mabs (undiluted tissue culture supernatant), 12CA5 anti-haemagglutinin Mabs (1:50 tissue culture supernatant) and anti-α-tubulin Mabs (2 μg/ml, Amersham). Incubations with primary antibodies were carried out for 1 hour at room temperature, followed by three washes with PBS. For detection of primary antibodies, the following secondary reagents were used: biotinylated donkey anti-rabbit or goat anti-mouse antibodies (1:200, Amersham) followed by Texas red-conjugated streptavidin (1:100, Amersham) and Alexa Fluor 488-conjugated goat-anti-rabbit or goat-anti-mouse IgG (1:1000, Molecular Probes). Following three final washes with PBS, coverslips were mounted in 80% glycerol, 3% DABCO (in PBS) mounting medium.

Microtubule-regrowth assays

To assess the effects of centrosome splitting on centrosomal MT-nucleation capacity, U2OS cells were transiently transfected with active Nek2. After 24 hours, MTs were depolymerized by placing the culture dishes on ice for 30 minutes. Then, MT regrowth was allowed to proceed in the presence of warm (37°C) medium and after 45 seconds the cells were fixed with cold methanol (–20°C). Cells were stained with anti-Nek2 antibodies to detect both transfected cells and split centrosomes and with anti-α-tubulin antibodies to visualize MTs.

RESULTS

Centrosome splitting by protein kinases

This study was performed with a view to better understand the mechanisms governing centrosome cohesion and separation. Relevant to this problem, we have previously shown that overexpression of an active protein kinase, Nek2, can trigger centrosome splitting (Fry et al., 1998a). To examine whether this centrosomal response was specific for Nek2, we first asked whether other protein kinases implicated in the regulation of centrosome function might also produce centrosome splitting. In particular, we examined the consequences of overexpressing polo-like kinase 1 (Plk1), aurora-A, Cdk1/cyclin B, Cdk4/cyclin D1, as well as Cdk2/cyclin A and Cdk2/cyclin E. Plk1 has previously been implicated in centrosome maturation (Lane and Nigg, 1996), aurora-A and Cdk1/cyclin B1 in centrosome separation (Blangy et al., 1997; Giet et al., 1999; Glover et al., 1995; Sawin and Mitchison, 1995), and both Cdk2/cyclin A and Cdk2/cyclin E in centrosome duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999).

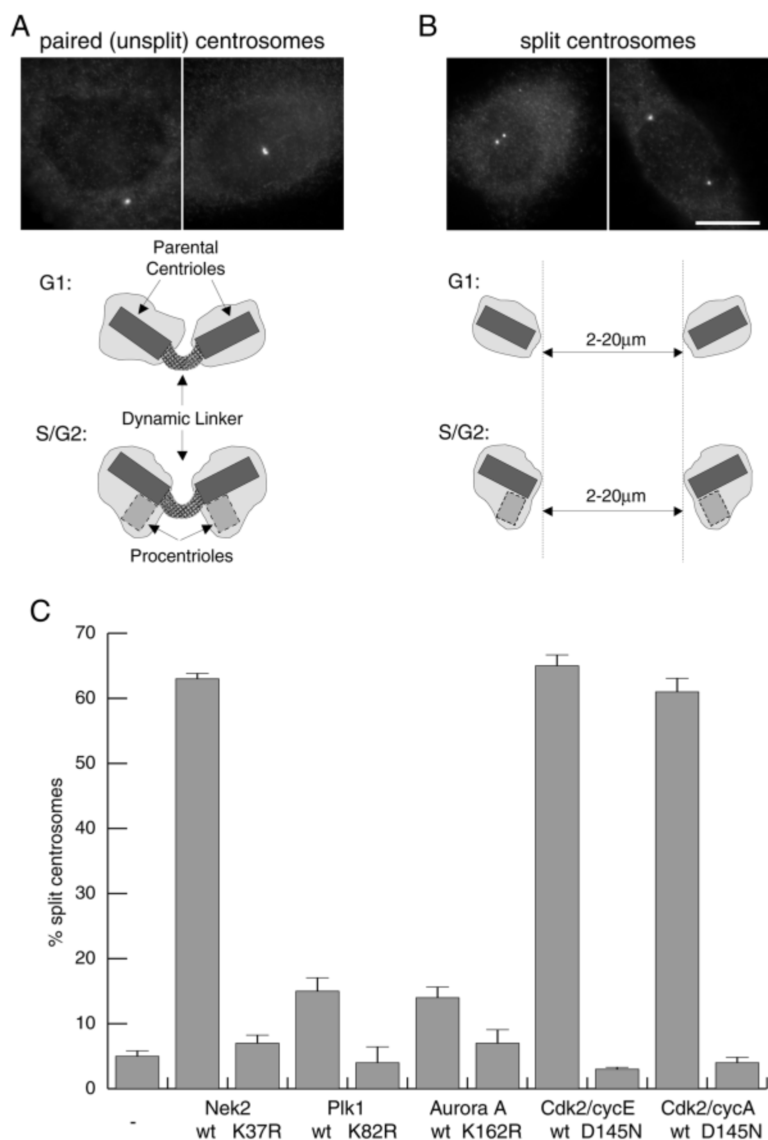

Fig. 1. Survey of protein kinases for their ability to induce centrosome splitting. U2OS cells were transfected with constructs encoding either wild-type or catalytically inactive protein kinases, as indicated. After 24 hours, centrosomes were analyzed by immunofluorescent staining with antibodies against γ-tubulin; transfected cells were identified using antibodies against the myc- or HA-tag, as appropriate. (A,B) Cells were classified as harboring either paired ('un-split') (A) or split (B) centrosomes, using the criteria described in Materials and Methods. Bar, 10 μm. As illustrated in schematic form, centrosome splitting occurs between the two parental centrioles, each with its associated PCM. Thus, splitting of an S or G_2 phase centrosome produces two products that each comprises one parental centriole and one tightly associated procentriole. The histogram (C) indicates the percentages of cells showing split centrosomes in response to expression of each kinase construct.

U2OS cells were transiently transfected with wild-type or catalytically inactive versions of the above kinases, and 24 hours later the extent of centrosome splitting was monitored using antibodies against γ-tubulin. Examples of transfected cells harboring split centrosomes are shown in Fig. 1B; for comparison, cells with typically paired ('non-split') centrosomes are shown in Fig. 1A. (For clarity, Fig. 1A,B also illustrate centrosome splitting in schematic form; the criteria used for quantitative analyses are described in Materials and Methods). Active Nek2 induced centrosome splitting in about 60-65% of the transfected cells, whereas the catalytically inactive kinase was unable to prompt such a response (Fig. 1C), consistent with previous results (Fry et al., 1998a). Interestingly, both Cdk2/cyclin A and Cdk2/cyclin E caused centrosome splitting to an extent similar to that of Nek2, and again, no splitting was produced by the catalytically inactive mutant, demonstrating that the observed phenotype was dependent on kinase activity (Fig. 1C). By contrast, Plk1,

aurora-A and Cdk4/cyclin D1 displayed at most marginal activities, producing split centrosomes in only 10-15% of cells, whereas Cdk1/cyclin B1 triggered splitting in up to 25% of cells (Fig. 1C; and data not shown). The results obtained with Cdk2 show clearly that Nek2 is not the only kinase able to induce centrosome splitting. This result falls in line with recent studies showing that Cdk2 induces centrosome splitting in *Xenopus* egg extracts, possibly in preparation of centrosome duplication (Lacey et al., 1999). By contrast, overexpression of most of the protein kinases tested produced little or no centrosome splitting, although they were expressed at comparable levels.

To determine whether Cdk2 and Nek2 function in a common pathway or, alternatively, act through distinct mechanisms, we examined the consequences of co-expressing reciprocal combinations of wild-type and catalytically inactive (i.e. putative dominant-negative) versions of Cdk2 and Nek2 (Fig. 2). Co-expression of catalytically inactive Nek2 (Nek2 K37R) with Cdk2 complexes did not significantly reduce the extent of centrosome splitting (Fig. 2A) and, conversely, Cdk2 D145N did not block the action of Nek2 (Fig. 2B). Instead, co-transfection of wild-type Nek2 with either Cdk2/cyclin A or Cdk2/cyclin E produced a cumulative effect, with over 90% of transfected cells showing split centrosomes (Fig. 2B). These results demonstrate that Nek2 and Cdk2 disrupt centrosome cohesion through at least partly distinct pathways.

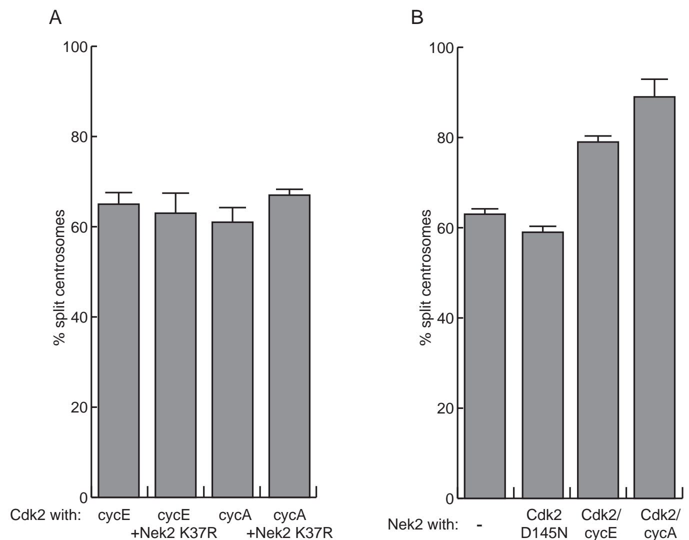

Fig. 2. Nek2 and Cdk2 induce centrosome splitting through independent pathways. U2OS cells were co-transfected with different combinations of wild-type Cdk2 and Nek2, or catalytically inactive Cdk2 (D145N) and Nek2 (K37R), together with cyclins A or E as indicated. Centrosome splitting was then analyzed as described in Fig. 1 legend.

Protein phosphatase 1, a physiological antagonist of Nek2

Because the most prominent form of centrosome splitting (i.e. centrosome separation) occurs shortly before mitosis, and since Nek2 is active throughout S and G_2 phase, we considered it likely that Nek2 activity is opposed by a regulated phosphatase (Fry et al., 1995; Fry et al., 1998a). To explore this hypothesis, we examined the influence of phosphatase inhibitors on centrosome cohesion. We found that a 1 hour treatment with 10 nM calyculin A triggered centrosome splitting in about 65% of U2OS cells (Fig. 3A). As calyculin A acts with comparable potency on both type 1 (PP1) and type 2A (PP2A) phosphatases (Favre et al., 1997), we also tested okadaic acid, an inhibitor that is considered specific for PP2A at low concentration, although it will block both PP1 and PP2A when used at higher concentration (Cohen et al., 1990; Favre et al., 1997). As shown in Fig. 3A, okadaic acid caused no centrosome splitting when used at 1 μM, but about 35% of cells showed split centrosomes in response to 5 μM okadaic acid, consistent with previous results (Matsumoto et al., 1999). Taken together, these date point to a type 1 phosphatase as a critical regulator of centrosome cohesion.

In strong support of this possibility, recent in vitro studies showed that the α-isoform of PP1 (PP1α) is able to form a complex with both Nek2 and C-Nap1 and can downregulate Nek2 activity (Helps et al., 2000). We therefore tested whether PP1α could also counteract active Nek2 in vivo. Whereas

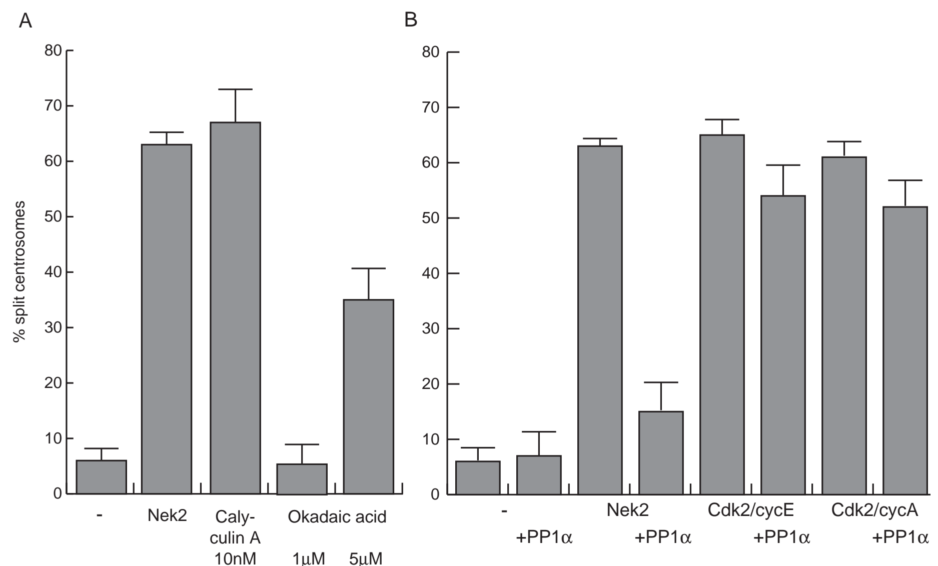

Fig. 3. Identification of PP1α as a likely Nek2 antagonist. (A) U2OS cells were treated for 1 hour with the indicated concentrations of okadaic acid or caliculin A. Then, centrosome splitting was analyzed as described. As a positive control, Nek2-transfected cells were examined in parallel. (B) U2OS cells were transfected with PP1α, either alone or in combination with Nek2, Cdk2/cyclin E or Cdk2/cyclin A, and the extent of centrosome splitting was determined.

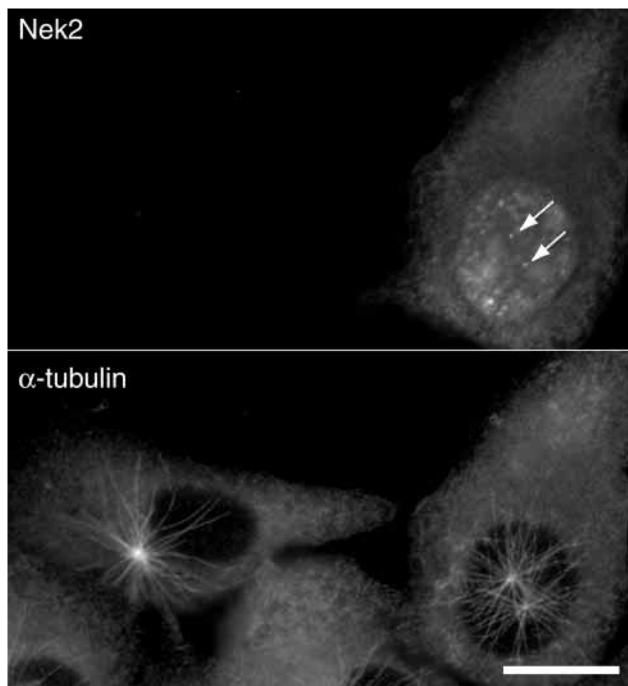

Fig. 4. MT-regrowth assay on Nek2-transfected cells. U2OS cells were transfected for 24 hours with myc-Nek2. Then, MTs were depolymerized by cold treatment for 30 minutes and allowed to re-grow for 45 seconds by addition of pre-warmed medium. Cells were immediately fixed for immunofluorescence microscopy and double-stained with antibodies against Nek2, to identify transfected cells (upper panel; arrows point to split centrosomes), and α-tubulin, to reveal the MTs (lower panel). Bar, 10 μm.

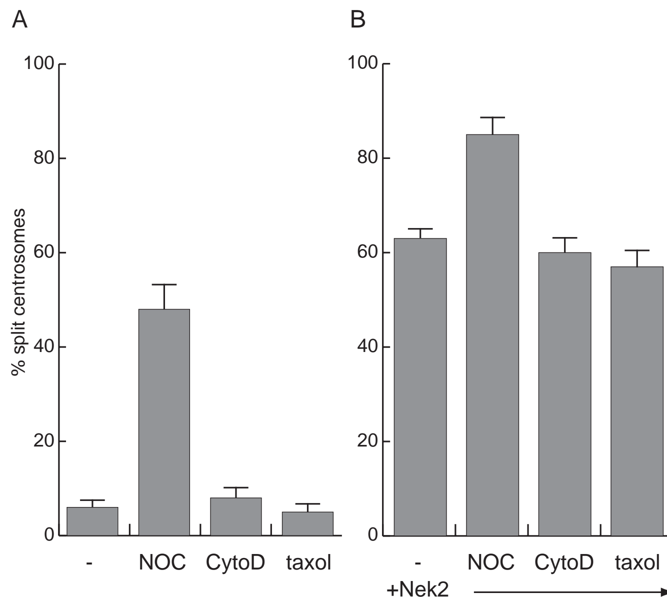

Fig. 5. Effect of anti-cytoskeletal drugs on centrosome cohesion. (A) U2OS cells were treated for 1 hour with nocodazole (5 μg/ml), taxol (5 μM) or cytochalasin D (1.25 μg/ml) and then analyzed for centrosome splitting. (B) U2OS cells were transfected for 24 hours with myc-Nek2. One hour prior to fixation, they were treated with the above concentrations of nocodazole, taxol or cytochalasin D, and the extent of centrosome splitting was determined.

overexpression of PP1α alone produced virtually no effect on the centrosome, co-expression of this phosphatase with Nek2 strongly suppressed centrosome splitting (Fig. 3B). Remarkably, this effect was specific for Nek2 since PP1α was unable to efficiently counteract the centrosome splitting produced by Cdk2 complexes (Fig. 3B). These results provide direct support for the hypothesis that PP1α and Nek2 regulate centrosome cohesion by counteracting each other (Helps et al., 2000). They are also consistent with the conclusion that Nek2 and Cdk2/cyclin complexes produce centrosome splitting through distinct pathways.

Centrosome splitting and the cytoskeleton

Because centrosome splitting can be induced by nocodazole, an MT-destabilizing drug, one could argue that kinases such as Nek2 might promote centrosome splitting via modulation of centrosome-MT interactions. To assess the capacity of centrosomes to nucleate and retain MTs after being split by Nek2, we therefore performed MT regrowth assays on Nek2-transfected cells. We found that both products of a split centrosome were able to nucleate MTs to a similar extent (Fig. 4). Moreover, the overall appearance of radial MT arrays was very similar in untransfected cells and in Nek2-tranfected cells with either paired or split centrosomes (Fig. 4; and data not shown). This indicates that Nek2 affects centrosome cohesion by a mechanism that does not bear on the ability of centrosomes to either nucleate or bind MTs.

We next examined whether the cytoskeleton was required for Nek2-induced centrosome splitting. Consistent with previous reports (Jean et al., 1999), a one hour treatment of U2OS cells with nocodazole caused not only depolymerisation of MTs but also centrosome splitting in about 50% of interphase cells (Fig. 5A). By contrast, a similar treatment with taxol, which stabilizes and bundles MTs, or with cytochalasin D, which depolymerizes filamentous actin, produced no significant effect on centrosome cohesion (Fig. 5A). None of these drugs was able to reduce the extent of centrosome splitting caused by active Nek2 (Fig. 5B), demonstrating that the persistence of split centrosomes does not require an intact cytoskeleton (Mayor et al., 2000). Interestingly, the combined action of Nek2 and nocodazole increased the percentage of cells bearing split centrosomes to about 85% (Fig. 5B), indicating that Nek2-dependent phosphorylation and MT-depolymerizing drugs produce a cumulative effect.

Having shown that Nek2-induced centrosome splitting is not a consequence of MT destabilization, we asked the reciprocal question: could it be that the drug-induced disruption of MTs induces centrosome splitting by altering centrosome-associated kinase and/or phosphatase activities? To explore this possibility, we first analyzed centrosome splitting after combined treatment of cells with nocodazole and staurosporine or 6-dimethylaminopurine (6-DMAP), two broad-specificity inhibitors of serine/threonine kinases. We found that the addition of either inhibitor almost completely eliminated the nocodazole-induced centrosome splitting, although it did not interfere with MT depolymerization (Fig. 6A,B; and data not shown). These results imply that MT depolymerisation per se is not sufficient to trigger centrosome splitting. In support of this conclusion, we emphasize that cold-induced MT

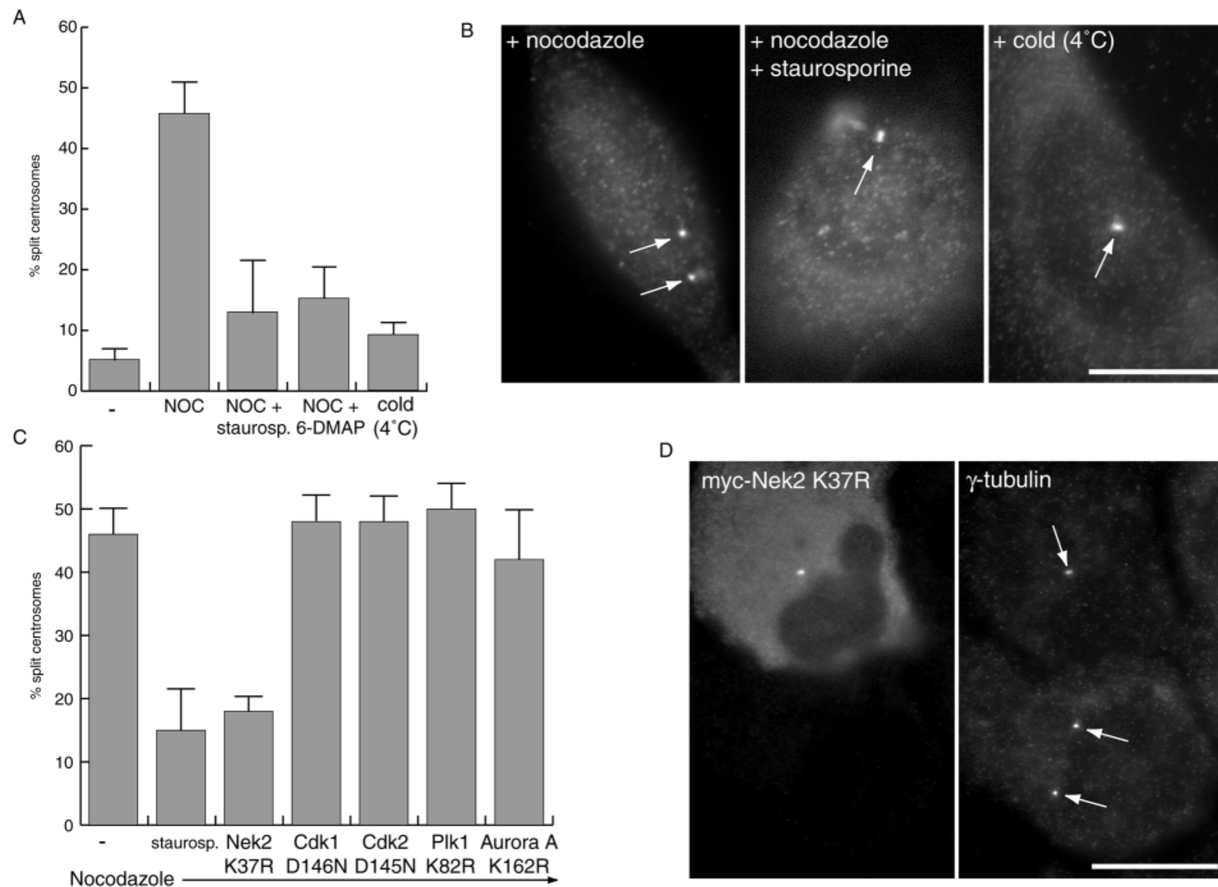

Fig. 6. Nocodazole-induced centrosome splitting depends on protein kinase activity. (A,B) U2OS cells either treated for 1 hour with nocodazole, either alone or in combination with staurosporine (500 μM) or 6-DMAP (2.5 mM), before centrosome splitting was analyzed. Alternatively, MT disassembly was induced by incubating cells for 1 hour on ice. As assessed by immunofluorescent staining with antibodies against α-tubulin, all treatments caused complete disassembly of MTs (not shown). (A) Histogram indicating the percentages of cells with split centrosomes; (B) γ-tubulin stained centrosomes, illustrating the suppression of nocodazole-induced centrosome splitting by staurosporine. Bar, 10 μm. (C,D) U2OS cells were transfected for 24 hours with catalytically inactive mutants of the kinases indicated, and 1 hour prior to fixation they were treated with nocodazole. For control, untransfected cells were treated with nocodazole alone or with nocodazole plus staurosporine. Then, all samples were scored for centrosome splitting (C). (D) γ-tubulin stained centrosomes, illustrating the suppression of nocodazole-induced centrosome splitting by the catalytically inactive Nek2 K37R mutant. Bar, 10 μm.

depolymerization did not trigger centrosome splitting (Fig. 6A,B). Instead, even the nocodazole-induced centrosome splitting requires the activity of a staurosporine/6-DMAP-responsive kinase.

This provocative finding prompted us to test whether overexpression of catalytically inactive (i.e. dominant-negative) mutants of selected protein kinases could mimic the effects of staurosporine and similarly inhibit nocodazole-induced centrosome splitting. U2OS cells were transfected with kinase mutant constructs and 23 hours later centrosome splitting was induced by a 1 hour treatment with nocodazole. As shown in Fig. 6C,D, the catalytically inactive Nek2 K37R was able to block nocodazole-induced centrosome splitting to an extent similar to that of staurosporine. None of the other kinase mutants analyzed showed such an effect (Fig. 6C), although all kinases were expressed to comparable levels (data not shown). Taken together, these results strongly support the view that centrosome cohesion is regulated through protein phosphorylation, and they point to Nek2 as a major regulator of this process.

DISCUSSION

The present study was aimed at exploring the mechanisms regulating centrosome cohesion and separation during the cell cycle. In particular, we have investigated the relative contribution of protein phosphorylation and cytoskeletal dynamics to the control of centrosome behavior. Our results lead us to conclude that cohesion between parental centrioles is regulated primarily through a balance of protein kinase and phosphatase activities. The available evidence identifies the centrosome-associated kinase Nek2 and the phosphatase PP1α as particularly important regulators of centrosome dynamics. With C-Nap1, one critical substrate of these enzymes has been identified (Fry et al., 1998b; Mayor et al., 2000; Helps et al., 2000), but the precise molecular nature of a purported centriolar linker remains to be uncovered.

A survey of kinases for their ability to trigger centrosome splitting

Focusing mainly on protein kinases that have previously been

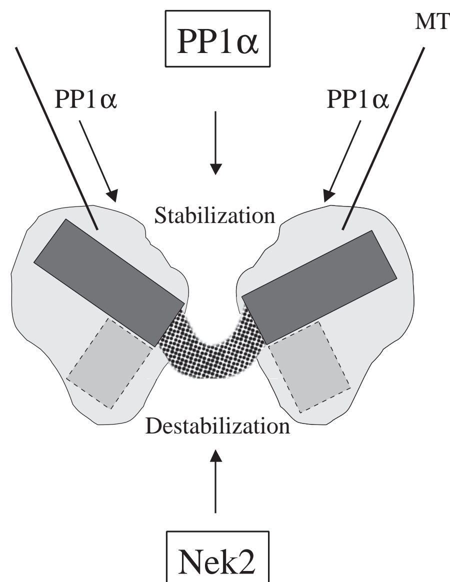

Fig. 7. A model illustrating the contribution of MT dynamics to the regulation of centrosome cohesion. We propose that parental centrioles are held together by a flexible proteinaceous linker whose assembly and disassembly is controlled by phosphorylation. If some of the regulatory kinases and/or phosphatases display affinity for MTs, their relative concentrations at the centrosome will depend on the MT dynamics typical of an intact MT network (particularly a constant flux of tubulin subunits towards the centrosome-associated MT minus ends). Whereas phosphatase activity will tend to stabilize the linker, any excess of kinase activity will cause its disruption.

implicated in the regulation of centrosome function, we have asked whether Nek2 is unique in its ability to split centrosomes. Our survey has clearly shown that this is not the case. However, of the six kinases tested, only Cdk2 was as efficient in triggering centrosome splitting as Nek2. Cdk2 is known to be required for centrosome duplication during S phase (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999), and it plausible that transient centriole separation represents a precondition for procentriole formation (Lacey et al., 1999). We have also examined whether Cdk2 and Nek2 function in a common pathway. Our results indicate that the two kinases most probably induce centrosome splitting through independent routes, although it remains possible that they act on common substrates. With C-Nap1, one likely centrosomal substrate of Nek2 has been characterized (Fry et al., 1998b), but other relevant substrates probably await identification. Likewise, the list of kinases acting at the centrosome will almost certainly grow (Fry et al., 2000b; Mayor et al., 1999). Indeed, recent studies point to a role of centrin phosphorylation, by protein kinase A (PKA) and perhaps other kinases, in the regulation of centrosome splitting (Lutz et al., 2001). In view of the striking centrosomal responses to mitogenic and chemotactic stimulation (Schliwa et al., 1982; Schliwa et al., 1983; Sherline and Mascardo, 1982; Sherline and Mascardo, 1984), it will be of particular interest to examine kinases involved in the corresponding signaling pathways.

A type 1 phosphatase opposes Nek2 activity

In principle, a net increase in the phosphorylation state of C-Nap1 (or any other substrate) may result from either the activation of a kinase or the inhibition of a phosphatase. Thus, the disruption of a centrosomal linker might be triggered by either the activation of a kinase or the inhibition of a phosphatase (or both). Because the onset of centrosome separation in G_2 is not accompanied by a detectable increase in Nek2 activity, we had previously proposed that Nek2 activity could be opposed by a centrosome-associated phosphatase until shortly before mitosis (Fry et al., 1998a). This view received strong support from the recent identification of a complex comprising PP1α, Nek2 and C-Nap1 (Helps et al., 2000). In a series of careful in vitro experiments, Cohen and co-workers showed that PP1α is able to suppress Nek2 activity as well as to dephosphorylate C-Nap1. As PP1α localizes to the centrosome (Andreassen et al., 1998) and is know to be inhibited at the onset of mitosis (Puntoni and Villa-Moruzzi, 1997), this phosphatase appears to constitute a prime candidate for opposing Nek2. Our finding that PP1α can suppress Nek2-induced centrosome splitting, but cannot counteract Cdk2-cyclin complexes, strongly supports this conclusion. The importance of phosphatases in regulating centrosome cohesion is further demonstrated by the fact that centrosome splitting can be caused by phosphatase inhibitors even in the absence of exogenous Nek2. Thus, centrosome cohesion appears to be regulated by a balance of centrosomal kinase and phosphatase activities.

On the role of microtubules in centrosome cohesion

In a final series of experiments, we have further examined the role of the cytoskeleton, particularly the MT network, in modulating centrosome cohesion. We could confirm that the nocodazole-induced depolymerization of MTs causes centrosome splitting (Jean et al., 1999). Most interestingly, however, we discovered that inhibitors of protein kinases block the centrosomal response to microtubule disruption. This unexpected finding indicates that even the centrosome splitting induced by nocodazole requires a phosphorylation event. Moreover, Nek2 appears to be particularly critical in this process because a dominant-negative mutant of Nek2 could mimic the effects of staurosporine and 6-DMAP. This result strengthens the view that Nek2 represents a prominent regulator of centrosome cohesion. Our favorite interpretation of the available data is that MT-depolymerization disrupts centrosome cohesion by causing an imbalance in the activities of centrosome-associated kinases and phosphatases (Fig. 7). This model is based on the fact that the steady-state concentration at the centrosome of any protein with affinity for MTs will depend on a constant flux of MT subunits towards the centrosome-associated MT minus ends. Thus, the MT network is expected to determine not only the local concentration of PCM components, but also that of kinases and phosphatases that are critical for centrosome structure. When MTs are disrupted, the activities of kinases (e.g. Nek2) apparently prevail over those of phosphatases (e.g. PP1α) and centrosome splitting ensues. According to this model, the balance of centrosome-associated kinase and phosphatase activities thus constitutes a major mechanism for determining centrosome dynamics during the cell cycle.

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