Dynamic Recruitment of Nek2 Kinase to the Centrosome Involves Microtubules, PCM-1, and Localized Proteasomal Degradation[□] [▽]

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Centrosomes undergo dramatic changes in composition and activity during cell cycle progression. Yet mechanisms involved in recruiting centrosomal proteins are poorly understood. Nek2 is a cell cycle-regulated protein kinase required for regulation of centrosome structure at the G2/M transition. Here, we have addressed the processes involved in trafficking of Nek2 to the centrosome of human adult cells. We find that Nek2 exists in small, highly dynamic cytoplasmic particles that move to and from the centrosome. Many of these particles align along microtubules and a motif was identified in the Nek2 C-terminal noncatalytic domain that allows both microtubule binding and centrosome localization. FRAP experiments reveal that 70% of centrosomal Nek2 is rapidly turned over ($t_{1/2} \sim 3$ s). Microtubules facilitate Nek2 trafficking to the centrosome but only over long distances. Cytoplasmic Nek2 particles colocalize in part with PCM-1 containing centriolar satellites and depletion of PCM-1 interferes with centrosomal recruitment of Nek2 and its substrate C-Nap1. Finally, we show that proteasomal degradation is necessary to allow rapid recruitment of new Nek2 molecules to the centrosome. Together, these data highlight multiple processes involved in regulating the abundance of Nek2 kinase at the centrosome including microtubule binding, the centriolar satellite component PCM-1, and localized protein degradation.

INTRODUCTION

The microtubule cytoskeleton provides a highly dynamic intracellular framework upon which trafficking of individual proteins and macromolecular complexes can occur. It is particularly important during mitosis for segregation of the duplicated chromosome masses toward opposite poles of the dividing cell (McIntosh *et al.*, 2002). The majority of microtubules in animal cells are nucleated from the centrosome and have their minus ends at the centrosome and their plus ends extending toward the cell periphery (Bornens, 2002). Microtubule nucleation is catalyzed by γ -tubulin ring complexes, multisubunit protein assemblies that are concentrated at the centrosome and cap the minus ends of many

microtubules (Job *et al.*, 2003). The centrosome itself consists of two centrioles, short barrels of nine triplet microtubules, surrounded by a fibrous network of proteins that constitutes the pericentriolar material.

The use of autoimmune antibodies, genetic studies, interaction assays, and more recently, proteomic approaches has led to the identification of many centrosomal proteins (Doxsey, 2001; Andersen *et al.*, 2003). However, much less is known about how these proteins are assembled into a functional microtubule organizing center capable of duplicating once per cell cycle. Some proteins may dock with the centrosome through passive diffusion, particularly if they are present in the cell at high concentration. However, most centrosomal components are in relatively low abundance and it is likely that active mechanisms ensure their delivery to the centrosome at the correct rate and time. As the centrosome sits at the heart of the microtubule network, it is not surprising that dynein/dynactin-based transport has been implicated in delivery of proteins to the centrosome and spindle pole (Karki and Holzbaur, 1999; Zimmerman and Doxsey, 2000).

A number of studies have highlighted a role for the PCM-1 protein in centrosomal trafficking. PCM-1 exists in 70–100-nm cytoplasmic granules, alternatively called centriolar satellites, that move toward the centrosome along microtubules in a dynein-dependent manner (Kubo *et al.*, 1999). Surprisingly, PCM-1 itself is not concentrated at the centrosome, raising the possibility that it acts purely as a transporter or assembly factory for other centrosomal proteins. Centrin, ninein, and pericentrin have all been found to colocalize with PCM-1 and be dependent on PCM-1 for their

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Abbreviations used: APC/C, anaphase promoting complex/cyclosome; C-Nap1, centrosomal Nek2-associated protein 1; EGFP, enhanced green fluorescent protein; FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; Nek2, NIMA-related kinase 2; PCM-1, pericentriolar material protein 1.

centrosomal recruitment (Dammermann and Merdes, 2002). The precise nature of the molecular interactions between motors, cargoes, and PCM-1 in these granules remains unclear. PCM-1 can associate with specific isoforms of both centrin and pericentrin (Li *et al.*, 2001; Dammermann and Merdes, 2002), and direct interaction has been reported between pericentrin and dynein light IC (Purohit *et al.*, 1999). Meanwhile, PCM-1 was also isolated in a yeast two-hybrid screen with Huntingtin-associated protein 1, which binds the p150^{Glued} subunit of dynactin (Engelender *et al.*, 1997). Whether γ -tubulin is recruited to centrosomes by dynein-dependent microtubule transport remains an interesting controversy that may reflect the fact that more than one mechanism can exist for protein recruitment (Khodjakov and Rieder, 1999; Young *et al.*, 2000; Dammermann and Merdes, 2002; Quintyne and Schroer, 2002).

So far, studies on centrosome assembly have mostly concentrated on proteins involved in microtubule nucleation or anchoring. We were interested to know whether regulators of the centrosome duplication cycle, such as protein kinases, are also recruited to centrosomes via dynein-dependent microtubule-based transport. In support of this hypothesis, both dynein and dynactin have been implicated in centrosome duplication and separation (Gonczy *et al.*, 1999; Ma *et al.*, 1999; Quintyne and Schroer, 2002). We have previously investigated the role of the cell cycle-regulated protein kinase Nek2 in the centrosome duplication cycle (Fry, 2002). Nek2 is expressed in vertebrates as two splice variants, Nek2A and Nek2B (Uto *et al.*, 1999; Hames and Fry, 2002). In *Xenopus* early embryos, Nek2B, is the only isoform present and is required for assembly and maintenance of centrosomes (Fry *et al.*, 2000; Uto and Sagata, 2000). Nek2B is rapidly recruited to sperm basal bodies in a microtubule-independent manner during formation of the zygotic centrosome (Twomey *et al.*, 2004). In human adult somatic cells, Nek2A is the predominant isoform although Nek2B is still present (Hames and Fry, 2002). Nek2A is required for separation of duplicated centrosomes and formation of a bipolar mitotic spindle (Faragher and Fry, 2003). A number of important regulatory motifs have been identified within the Nek2A molecule. It has an N-terminal catalytic domain and a C-terminal regulatory domain within which are found a leucine zipper dimerization motif, a binding site for protein phosphatase 1, and two destruction motifs that are recognized by the anaphase promoting complex/cyclosome (APC/C; Fry, 2002). Because of the position of the splice site, Nek2B contains the kinase domain and leucine zipper motif, but lacks the protein phosphatase 1 binding site and destruction signals. Both Nek2A and Nek2B localize to centrosomes (Hames and Fry, 2002); however, the region required for centrosome localization has yet to be defined.

Here, we have addressed the mechanisms that contribute to recruitment of Nek2 to the adult cell centrosome. Our results indicate that Nek2 is present in small cytoplasmic granules. These undergo trafficking to the centrosome that is rapid but only partly dependent on microtubules. A short region of the noncatalytic C-terminal domain of Nek2 is identified as both a potential microtubule binding and centrosomal targeting motif. Finally, we show that PCM-1 and proteasomal degradation also play important roles in the assembly and turnover of Nek2 at the centrosome.

MATERIALS AND METHODS

Cell Culture and Transfection

U2OS osteosarcoma cells stably transformed with constructs expressing either EGFP-Nek2A or EGFP-Nek2A-K37R from tetracycline-inducible CMV pro-

motors had previously been generated in our laboratory (Faragher and Fry, 2003). These cell lines, together with standard U2OS and HeLa cells, were grown in DMEM (Invitrogen, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μ g/ml streptomycin, at 37°C in a 5% CO₂ atmosphere. To induce and maintain expression from tetracycline-responsive promoters, tetracycline or doxycycline (1 μ g/ml) was added to the culture medium every 24 h (Faragher and Fry, 2003). The EGFP-Nek2A-ND (nondegradable) cell line was generated by the introduction of pcDNA/TO/GFP-Nek2A-ND into U2OS T-Rex cells (Invitrogen) and subsequent clonal selection, as previously described (Faragher and Fry, 2003). The centrin3-GFP Chinese hamster ovary (CHO) cell line was also generated by the same method. CHO cells and the derived cell line were grown in Ham's F12 medium supplemented with 10% FCS, 100 IU/ml penicillin, and 100 μ g/ml streptomycin, at 37°C in a 5% CO₂ atmosphere. Transient transfections were performed with either Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche Diagnostics, Lewes, East Sussex, United Kingdom) according to manufacturer's instructions.

Plasmid Constructions

To generate pDsRed-Nek2A, Nek2A was excised from pEGFP-Nek2A (Faragher and Fry, 2003) on a *Xho*I-*Bam*HI fragment and subcloned into pDsRed2-C1 (BD Biosciences, Oxford, United Kingdom) cut with *Xho*I and *Bam*HI. To generate pCMV-mycNek2A- Δ C127, mycNek2A (amino acids 1–318) was excised from pBS-mycNek2A (Fry *et al.*, 1998a) on a *Sall*-*Sac*I fragment, blunted with T4 ligase and subcloned into pRCMV (Invitrogen) cut with *Sall*-*Xba*I, and then blunted. Generation of pCMV-mycNek2A-CTD has previously been described (Fry *et al.*, 1999). To make pCMV-mycNek2-CAT, the catalytic domain of Nek2 (residues 1–263) with an N-terminal myc-tag was excised from pBS-mycNek2 on a *Clal*-*Xmn*I fragment, blunted and subcloned into pRCMV (Invitrogen) cut with *Not*I and *Apa*I, and blunted. To generate pEGFP-Nek2A- Δ C112, a fragment containing amino acids 1–334 was amplified from pEGFP-Nek2A and cut with *Hind*III and *Sall* site using sites introduced on the PCR primers. This was subcloned into pEGFP-C1 (BD Biosciences) cut with the same enzymes. pcDNA/TO/GFP-Nek2A-ND was generated by PCR-based mutagenesis of the pcDNA/TO/GFP-Nek2A plasmid using the Gene Tailor Site-Directed Mutagenesis System (Invitrogen). These changes included amino acids 391–393 KEN to AAA and 399, N to A. Also, codon 421 was changed to the stop codon, UAG. All constructs were confirmed by DNA sequencing (Lark Technologies, Saffron Walden, United Kingdom).

Fixed Cell Microscopy

Immunofluorescence microscopy was performed as previously described (Faragher and Fry, 2003). Primary antibodies used were anti-Nek2 (1 μ g/ml; Zymed, South San Francisco, CA [Fry *et al.*, 1999]; or 2.5 μ g/ml; BD Biosciences), anti-PCM-1 (1/100; Dammermann and Merdes, 2002), anti-C-Nap1 (1 μ g/ml; Fry *et al.*, 1998), anti- γ -tubulin (0.15 μ g/ml; Sigma, Poole, Dorset, United Kingdom) and anti- α -tubulin (0.3 μ g/ml; Sigma). Secondary antibodies used were Alexa Fluor 488 and 596 goat anti-rabbit and goat anti-mouse IgGs (1 μ g/ml; Invitrogen). Fluorescence images were captured on a TE300 inverted microscope (Nikon, Kingston upon Thames, United Kingdom) using an ORCA ER CCD camera (Hamamatsu, Hamamatsu, Japan) using Openlab 3.5.1 software (Improvision, Coventry, United Kingdom) and processed using Adobe Photoshop (San Jose, CA). Centrosome intensity measurements were made as described (Faragher and Fry, 2003).

Time-lapse Imaging

For live cell microscopy, cells were grown on sterile coverslips and induced with doxycycline as indicated in the text. Coverslips were then washed once with 1 $\times$ phosphate-buffered saline before mounting in a steel coverslip holder (made in-house) in CO₂-independent medium without glutamine (Invitrogen) but with doxycycline. The surface of the medium was overlaid with mineral oil (Sigma) to prevent evaporation. The steel coverslip holder was mounted in a Patch Slice MicroIncubator (PSMI) regulated at 37°C by a TC-202A temperature controller (Harvard Apparatus, Holliston, MA, supplied by Digitimer, Welwyn Garden City, United Kingdom). The PSMI was placed on the microscope stage and images captured at 4-s intervals with the microscope system described above. Images were processed using Adobe Photoshop or converted to QuickTime movies.

FRAP/FLIP Analysis

For fluorescence recovery after photobleaching (FRAP) analysis, cells were cultured on the microscope using a POC-chamber-system (La Con, Staig, Germany) maintained at 37°C by a Tempcontrol-37 (La Con). FRAP was performed on an LSM 510 laser scanning confocal unit fitted on an Axiovert 100M inverted microscope (Zeiss, Welwyn Garden City, United Kingdom) using a 100 $\times$ oil objective (NA = 1.4) and scan zoom = 3. A small spot or square region of interest (ROI) of 50 $\times$ 50 pixels (small square) or 250 $\times$ 250 pixels (large square) centered on both centrosomes was bleached with 40 iterations and 100% laser power (488-nm argon laser). Two images were captured before bleaching with a 1-s interval. An image was taken every

second (488-nm argon laser at 4% power) after bleaching over a 30–90-s period. For each time point, the fluorescence intensity of the photobleached ROI (P1) was determined using LSM 510 software. The fluorescence intensity of an unbleached ROI in the cell was determined (U2) and a normalized intensity (In) was calculated using $P1/U2$. The background fluorescence intensity (Bk) was set as the first frame after photobleaching, and this value was subtracted from all frames such that the fluorescence intensity for the ROI at a given time is $In-Bk$. The amount of fluorescence recovery was then calculated as the fluorescence intensity value of a given frame divided by the fluorescence intensity value of the frame immediately before photobleaching and was expressed as a percentage (Thompson *et al.*, 2004). Images were processed with Adobe Photoshop 4.0. *p* values were calculated for $t_{1/2}$ or % recovery where indicated using the Student's *t* test.

Fluorescence loss in photobleaching (FLIP) experiments were also performed on the LSM 510 laser scanning confocal system. For each cell a large designated region of the cytoplasm was bleached with 40 iterations and 100% laser power. After the bleach time, images were taken every second for 10 s. This process was repeated 11 times to ensure complete loss of cytoplasmic fluorescence. For each time point, three ROIs were determined: an area within an adjacent cell to indicate the loss of fluorescence due to imaging, an area within the bleached region, and an area encompassing the centrosome that was not bleached. The fluorescence intensities of the latter two ROIs were determined as a percentage of the initial fluorescence intensity of the centrosome ROI. Fluorescence intensities are plotted with respect to bleach number.

Microtubule Pull-down Assays

In vitro translated (IVT) proteins were generated using the TnT-coupled transcription/translation kit in the presence of [35 S]methionine according to manufacturer's instructions (Promega, Southampton, United Kingdom). Plasmids used for IVT were pGEM-Nek2A (Schultz *et al.*, 1994), pGEM-Nek2B (Hames and Fry, 2002), pGEM-Nek2A- Δ LZ (Fry *et al.*, 1999), pCMV-mycNek2A- Δ C127 (this study), pBS- γ -tubulin and pcDNA3-Rab4 (gift from Dr. J. Norman, University of Leicester). 35 S-labeled IVT protein, 2 μ l, was added to microtubule stabilizing buffer (80 mM K.Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 25 mM NaCl, 20 μ M taxol, 100 mM GTP, 50 mM AMP-PNP) and centrifuged at 35,000 RPM at 20°C for 30 min. The supernatant was collected and purified bovine (Cytoskeleton, Denver, CO) or rat brain (gift from B. Edde, Montpellier) tubulin added to 0.7 μ g/ μ l. This was incubated for 30 min at 30°C before centrifugation at 35,000 RPM at 20°C for 30 min on a 20% sucrose cushion. The supernatant was carefully removed and added to one third volume of 3 $\times$ protein sample buffer. The pellet was then carefully washed with microtubule stabilizing buffer, before being resuspended in 3 $\times$ protein sample buffer. Equal volumes of the samples were incubated at 95°C for 3 min and loaded onto 12% SDS-polyacrylamide gels. Gels were stained with Coomassie Blue, destained, dried, and exposed to x-ray film. Density measurements were carried out using NIH image software (v1.62).

siRNA Experiments

Small interfering RNA oligonucleotides (siRNAs) were obtained from Dharmacon (Boulder, CO). Depletion of PCM-1 or lamin A/C using siRNAs and calculation of centrosome intensities after immunofluorescence staining was performed as described (Dammermann and Merdes, 2002).

Drug Treatments

To inhibit the proteasome, cells were incubated in 20 μ M MG132 (in dimethyl sulfoxide [DMSO]) for 4 h at 37°C. As a control 0.2% DMSO was used. To depolymerize the microtubule cytoskeleton, cells were incubated with 7.5 μ g/ml nocodazole for 1 h at 37°C followed by a 30-min incubation on ice. Lysates of drug-treated cells were prepared and analyzed by Western blot as previously described (Fry *et al.*, 1998b).

RESULTS

Cytoplasmic Nek2 Exists in Dynamic Particles That Colocalize with Microtubules

We recently described the generation of human U2OS cell lines expressing EGFP-tagged Nek2A proteins from tetracycline-inducible promoters (Faragher and Fry, 2003). Careful analysis of the distribution of EGFP-Nek2A in these cells revealed that, besides localizing to the centrosome, Nek2A was present in small particles distributed throughout the cytoplasm (Figure 1A, a and b). Immunofluorescence microscopy performed with two different Nek2 antibodies revealed similar cytoplasmic particles supporting the notion that this represents a bona fide distribution of Nek2 protein (Figure 1A, c–f). We then used time-lapse fluorescence microscopy to study the movements of EGFP-Nek2A particles

Figure 1. Dynamic behavior of cytoplasmic Nek2. (A) Fluorescence microscope images of EGFP-Nek2A U2OS cells induced with doxycycline for 24 h (EGFP fluorescence; a and b) and U2OS cells stained with a Zymed polyclonal (c and d), or BD Biosciences monoclonal (e and f) anti-Nek2 antibody. b, d, and f are magnified images of a, c and e, respectively. (B) EGFP-Nek2A U2OS cells were induced with doxycycline for 24 h before imaging by time-lapse fluorescence microscopy. b–g and i–n are magnified images of the indicated regions in a and h, respectively. Time in minutes is shown at the bottom of each panel. Closed arrowheads indicate moving particles; open arrowhead indicates a static particle. (C) U2OS cells transiently transfected with DsRed-Nek2A (red, a–c) or untransfected cells stained with the Zymed anti-Nek2 antibody (red, d–f) were costained with anti- α tubulin antibodies (green, a–f). b, c, e, and f are magnified images of the indicated areas in a and d, respectively; arrowheads indicate colocalization of Nek2A with individual microtubules. Scale bars, 10 μ m.

in live cells (Figure 1B). These exhibited two types of behavior with some particles remaining fairly static and others moving rapidly in linear directions (see Supplementary Figure 1 for movies). Close to the centrosome, some Nek2 particles moved away from the centrosome (Figure 1B, b–g), whereas others moved directly toward it sometimes fusing with the centrosome (Figure 1B, i–n). To determine whether there was any relationship between the cytoplasmic Nek2 particles and microtubule cytoskeleton, a DsRed-Nek2A construct was expressed in U2OS cells. Staining these cells with antibodies against α -tubulin revealed that many Nek2A particles were distributed along the lengths of microtubules (Figure 1C, a–c). In addition, Nek2 antibodies revealed colocalization of endogenous Nek2 particles with microtubules (Figure 1C, d–f). Thus, Nek2A protein is found both at the centrosome and in dynamic cytoplasmic particles that localize in part with microtubules.

Nek2 Binds Microtubules through a Motif in Its C-terminal Domain

To determine whether Nek2 can bind microtubules, pull-down experiments were performed using purified microtubules stabilized with taxol and Nek2A protein translated in rabbit reticulocyte lysate. As controls, the centrosomal protein γ -tubulin and the cytoplasmic protein Rab4 were used. As expected, γ -tubulin precipitated in the presence, but not absence, of microtubules and taxol, whereas Rab4 did not precipitate under either condition (Figure 2A). Nek2A also shifted from a predominantly soluble pool in the absence of microtubules to a predominantly insoluble pool in the presence of microtubules and taxol, indicating that Nek2A can interact with microtubules. This binding could be direct; however, as proteins were generated in complex reticulocyte lysates, it is equally likely that it is indirect. Indeed, the positive control, γ -tubulin, probably interacts with microtubules only when complexed with other members of the γ -tubulin ring complex. The interaction of both Nek2A and γ -tubulin was independent of AMP-PNP, suggesting that ATPase-dependent motor protein activity was not required for binding (Figure 2A). To identify which region of Nek2 is responsible for this interaction, further Nek2 proteins were tested in this assay (Figure 2B). In addition to Nek2A, Nek2B also bound indicating that the interaction region must be N-terminal to the splice site at amino acid position 370 (Figure 2C). A Nek2A deletion mutant lacking the leucine zipper motif (Nek2A- Δ LZ missing amino acids 306–334; Fry *et al.*, 1999) also precipitated in the presence of microtubules indicating that neither this region of the protein nor Nek2 dimerization is required for microtubule binding (Figure 2C). In contrast, a truncation mutant lacking the C-terminal 127 amino acids of Nek2A (Nek2A- Δ C127) did not precipitate with microtubules (Figure 2C). Together, these data suggest that a 36 amino acid region of Nek2 between residues 335 and 370 plays an important role in binding microtubules (Figure 2D).

The Nek2 Microtubule-binding Motif Is also Important for Centrosome Localization

We then asked whether the putative microtubule binding motif of Nek2 is also important for centrosome localization. Nek2 constructs were generated as either EGFP- or myc-tagged fusion proteins and transiently expressed in U2OS cells. As previously reported (Hames and Fry, 2002), full-length Nek2A and Nek2B localized to the centrosome (Figure 3, a–d), as did a construct expressing the complete C-terminal noncatalytic domain (Nek2A-CTD; amino acids 265–445; Figure 3, e and f). However, constructs expressing

the N-terminal catalytic domain alone (Nek2-CAT; amino acids 1–263), or the truncation mutant Nek2A- Δ C127 (amino acids 1–318), localized much less frequently to the centrosome (Figure 3, g–j). Likewise, a shorter truncation mutant that retained the complete leucine zipper but lacked the putative microtubule binding motif (Nek2A- Δ C112; amino acids 1–333) also failed to localize to the centrosome (Figure 3, k and l). These data indicate that the same C-terminal region important for microtubule binding is also likely to be required for efficient localization of Nek2 to the centrosome.

Nek2 Undergoes Rapid Recruitment to the Centrosome

To address the dynamics of Nek2 trafficking, FRAP was performed with the EGFP-Nek2A U2OS cell line. After doxycycline-induced expression of EGFP-Nek2A for 24 h, interphase cells containing paired centrosomes in the same plane of focus were selected for photobleaching and subsequent imaging according to the protocols described in *Materials and Methods*. Initially, either small points or slightly larger 50×50 -pixel squares surrounding the centrosome were bleached (bleach times, 2.5 or 5 s, respectively) and the recovery of centrosome fluorescence was imaged over time. For wild-type Nek2A, recovery of GFP fluorescence on centrosomes was extremely rapid, with a half-life ($t_{1/2}$) of ~ 3 s (Figure 4A, a–d, B, and D). This rapid recovery was not due to “fluorophore flickering” because fluorescence never recovered after formaldehyde fixation before photobleaching of cells (unpublished data). Nek2A recovery reached a plateau after ~ 30 s with a total intensity $\sim 70\%$ of the prebleaching value (Figure 4B). These data imply the presence of two populations of Nek2A at the centrosome: a rapidly exchanging pool ($\sim 70\%$) and a static pool ($\sim 30\%$). They also provide support to the previous calculation that only a fraction of total cellular Nek2 is at the centrosome (Fry *et al.*, 1998a). Similar dynamics were observed using the EGFP-Nek2A-K37R cell line ($t_{1/2} = 3.4$ s, 64% recovery), which expresses a catalytically inactive Nek2A mutant (Faragher and Fry, 2003), indicating that Nek2 kinase activity does not contribute to centrosomal trafficking (Table 1). To compare these rates of recovery with another centrosomal protein, a CHO cell line was generated expressing a centrin3-EGFP fusion protein. This exhibited a substantially slower rate of fluorescence recovery with a $t_{1/2} > 5$ min (Table 1).

Previously, we reported that induction of wild-type Nek2A stimulates premature centriole splitting in interphase cells (Fry *et al.*, 1998a; Faragher and Fry, 2003). We therefore applied FRAP to cells expressing EGFP-Nek2A in which centrioles were separated by $>5 \mu\text{m}$. However, no significant difference in the dynamics of Nek2A was observed between paired and split centrioles or between individual centrioles of a split pair (Figure 4, B and E, and unpublished data). The results of these FRAP studies are summarized in Table 1 and examples of individual FRAP experiments and the range of variation are shown in Supplementary Figure 2.

To obtain additional information on Nek2A dynamics, we performed FLIP experiments on cells expressing GFP-Nek2A. First, the entire cell, except a small region encompassing the centrosome, was continually bleached with intermittent imaging of fluorescence at the centrosome (Figure 5A). This provided a method to specifically assess the rate of Nek2A movement away from the centrosome, as well as a second method to calculate the % of centrosomal Nek2A that is static. FLIP confirmed that the majority of centrosomal Nek2A moves rapidly away from the cytoplasm (Figure 5B). Interestingly, addition of nocodazole had no effect on this rate of fluorescence loss, indicating that microtubules are not

Figure 2. Nek2 binds microtubules in vitro. (A) Nek2A and control proteins, Rab4 and γ -tubulin, were generated by in vitro translation in the presence of [35 S]methionine. These proteins (Input) were incubated without (–MT) or with (+MT) purified microtubules in the presence of Taxol and AMP-PNP, unless indicated. Proteins were then spun at 35,000 rpm on a sucrose cushion for 30 min. Pellet (P) and supernatant (S) fractions were collected and analyzed by SDS-PAGE, Coomassie Blue staining, and autoradiography. The Coomassie Blue-stained gel with Nek2A samples is shown, with tubulin being the predominant band. (B) Schematic representation of Nek2 constructs, indicating amino acid positions (CAT, catalytic domain; LZ, leucine zipper motif; CC, coiled-coil). The C-terminus of Nek2B (black) differs from Nek2A because of a splice site after residue 370. (C) These constructs were used in independent microtubule pull-down assays as detailed above. The presence of pelleted microtubules is shown by Coomassie Blue staining of the gels (right panels) before autoradiography (left panels). The histogram indicates the percentage of input protein found within the pellet fractions with (gray) and without (black) microtubules. (D) Amino acid sequence of the putative microtubule binding motif from human (Hs), mouse (Mm), and *Xenopus laevis* (Xl) Nek2 with amino acid positions indicated; complete match (*), conservative substitution in one sequence (:), divergent substitution in one sequence (·).

Figure 3. Identification of the Nek2 centrosomal targeting motif. Fluorescence microscope images of U2OS cells transiently transfected with pEGFP-Nek2A (a and b), pEGFP-Nek2B (c and d), pCMV-mycNek2A-CTD (e and f), pCMV-mycNek2-CAT (g and h), pCMV-mycNek2- Δ C127 (i and j), and pEGFP-Nek2- Δ C112 (k and l). In a, c, and k, the GFP fluorescence is shown, in e, g, and i cells are stained with anti-myc antibodies. To demonstrate centrosomal localization, cells were costained with anti- γ tubulin antibodies (b, d, f, h, j, and l). Centrosomes are indicated by arrows. Scale bar, 10 μ m.

required for ejection of Nek2A from the centrosome (Figure 5C). FLIP also confirmed that a minor population of Nek2A remains static at the centrosome. By monitoring the fluorescence in the cytoplasm at the same time as at the centrosome, we found that when the cytoplasmic fluorescence had decreased to zero there remained a small pool of fluorescence at the centrosome (Figure 5B). This fluorescence continued to decrease very slowly but only at the same rate as the decrease in fluorescence of the cytoplasm of a neighboring unbleached cell, i.e., as a result of imaging. To ensure that what remained was not auto-fluorescence of the organelle, the centrosome was subsequently bleached in a FRAP-type manner (Figure 5D). Now, the level of fluorescence dropped to zero, indicating that some GFP-Nek2A had remained at the centrosome. There was no subsequent recovery to the centrosome, indicating that the complete pool of Nek2 in the cell had now been bleached. The static centrosomal population calculated by FRAP was $\sim 30\%$; using FLIP it was slightly lower ($\sim 15\%$). However, the fluorescence of a neighboring cell also decreased ($\sim 10\%$) as a result of imaging over the time-course of the FLIP experiment (Figure 5B) and so the FLIP technique probably underestimates the static population. We believe a reasonable estimate of static Nek2 at the centrosome is in the order of 20–30%.

Microtubules Are Required for Long-range Transport of Nek2

The observation that Nek2 binds microtubules raised the possibility that its recruitment to the centrosome might be dependent on the microtubule network. To test this, EGFP-Nek2A protein was induced for 24 h before depolymerization of the microtubule network by incubation with nocodazole at 4°C. This treatment alone had no effect on the total cellular pool of GFP-Nek2A, nor on the abundance of GFP-Nek2A at the centrosome (see Supplementary Figure 3). After FRAP analysis, cells were fixed and stained for microtubules to confirm depolymerization (Figure 4Ae). Using our standard approach of bleaching a 50×50 -pixel square, no change in Nek2A dynamics was observed after microtubule depolymerization (Figure 4A, f–h, and B). With split centrioles, microtubule depolymerization had no effect on the half-life of Nek2A recruitment, although there was a reduction in % recovery (Figure 4E). We then argued that Nek2A trafficking might be independent of microtubules when the unbleached region was very close to the centrosome. We therefore bleached a larger square (250×250 pixels) centered on the centrosome. In the case of both paired and split centrioles, this led to a slower half-life and reduced % total recovery as the fluorescence in a substantial proportion of EGFP-Nek2A molecules in the cell had now been lost (Figure 4, C and F, Table 1). Interestingly, though, there was a significant increase ($\sim$ twofold) in the half-life of recovery after microtubule depolymerization with both paired and split centrioles (Figure 4, C and F). Hence, we conclude that short range recruitment of Nek2A to the centrosome does not require microtubules and is likely to be primarily a result of diffusion, but that movement to the centrosome over larger distances is facilitated by the presence of microtubules.

FLIP was used to determine whether microtubules were necessary for cytoplasmic motion of Nek2A. A small area of cytoplasm was bleached and subsequent imaging revealed that the rate of movement of GFP-Nek2A within the cytoplasm was rapid and not influenced by incubation with nocodazole, implying that diffusion occurs within the cytoplasm independently of microtubules (unpublished data).

Figure 4. FRAP analysis reveals rapid recruitment of Nek2A to the centrosome. (A) EGFP-Nek2A U2OS cells induced with doxycycline for 24 h were mounted onto sterile glass coverslips, and FRAP was performed using an LSM 510 confocal microscope. One image was taken before bleaching (b and f, prebleach) and then the area within the indicated box was photobleached (c and g, bleach). An image was captured every second for 33 s (d and h, final image). FRAP was carried out in the presence (a–d, untreated) or absence (e–h, nocodazole-treated) of an intact microtubule cytoskeleton. U2OS cells treated in parallel were fixed and analyzed by indirect immunofluorescence microscopy (a and e) using anti- α tubulin antibodies (green) and anti- γ -tubulin (red) antibodies. Scale bar, 10 μ m. (B–F) FRAP data were analyzed using LSM 510 software, and the relative GFP intensity within the bleached area was recorded as a measure of time; each data point represents the average intensity from 10 cells. A 50 $\times$ 50-pixel square was centered on paired centrosomes (B). A 250 $\times$ 250-pixel square was centered on paired centrosomes (C). A small spot was centered on one centriole of a split pair (D). A 50 $\times$ 50-pixel square was centered on one centriole of a split pair (E). A 250 $\times$ 250-pixel square was centered on both centrioles of a split pair (F). In each case, black diamonds represent untreated cells and open squares represent nocodazole treated cells. The centrosomal area bleached is shown in white in the top right corner. p values calculated using the Student's *t* test indicate the significance of the difference in half-life between untreated and nocodazole-treated samples in C and F.

Nek2 Colocalizes with PCM-1-containing Centriolar Satellites

Trafficking of certain proteins, e.g., pericentrin, centrin, and ninein, to the centrosome is dependent on the PCM-1 protein (Dammermann and Merdes, 2002). PCM-1 is a major component of centriolar satellites, 70–100-nm cytoplasmic granules that are found in the vicinity of the centrosome, although not at the centrosome itself (Balczon *et al.*, 1994; Kubo *et al.*, 1999). We decided to test whether the small

cytoplasmic particles of Nek2A bore any relationship to PCM-1-containing centriolar satellites. First, U2OS cells were immunostained for endogenous Nek2 and PCM-1 proteins. This revealed partial colocalization particularly in the vicinity of the centrosome, although there were also Nek2 particles that were not associated with PCM-1 (Figure 6A). Second, a C-terminal truncation of PCM-1 (amino acids 1–1468) known to generate large cytoplasmic aggregates was overexpressed in U2OS cells (Dammermann and Mer-

Table 1. Fluorescence recovery after photobleaching analysis of Nek2A

Protein	Treatment	Bleach area	Centrosome	Half-life ($t_{1/2}$)	% Recovery
GFP-Nek2A		50 × 50	Paired	3.2 s	73
GFP-Nek2A-K/R		50 × 50	Paired	3.4 s	64
GFP-Nek2A	+ Nocodazole (7.5 μ g/ml)	50 × 50	Paired	3.5 s	78
GFP-Nek2A		50 × 50	Split	3.0 s	65
GFP-Nek2A	+ Nocodazole (7.5 μ g/ml)	50 × 50	Split	3.2 s	49
GFP-Nek2A		250 × 250	Paired	12.5 s	25
GFP-Nek2A	+ Nocodazole (7.5 μ g/ml)	250 × 250	Paired	19.5 s	18
GFP-Nek2A		250 × 250	Split	12.0 s	35
GFP-Nek2A	+ nocodazole (7.5 μ g/ml)	250 × 250	Split	21.3 s	25
GFP-Nek2A		Spot	Split	2.8 s	63
GFP-Nek2A	+ Nocodazole (7.5 μ g/ml)	Spot	Split	3.0 s	62
Centrin-GFP		50 × 50	Paired	>5 min	nd

The half-life and % fluorescence recovery at the centrosome was determined for GFP-Nek2A, GFP-Nek2A-K37R, and centrin-GFP proteins with or without drug treatment and with small or large bleach area as indicated. The Nek2A constructs were induced for 24 h with tetracycline in the relevant U2OS cell line, whereas the centrin-GFP was constitutively expressed in a stable CHO cell line. Photobleaching and subsequent confocal imaging was performed as described in *Materials and Methods*. The rate of recovery of centrin-GFP was so slow that the final % recovery could not be determined (nd) within the time frame of the experiment.

des, 2002; Kubo and Tsukita, 2003). By immunostaining, it was clear that endogenous Nek2 was recruited into these aggregates (Figure 6B, d–f). Third, in cells expressing EGFP-Nek2A, endogenous PCM-1 granules again partially colocalized with recombinant Nek2A cytoplasmic particles (Figure 6C), although it did not colocalize with Nek2A at the centrosome (unpublished data). Taken together, these results indicate that there is a partial overlap of PCM-1 and Nek2 localization in the cytoplasm. In support of an interaction, either direct or indirect, increased levels of Nek2A overexpression led to loss of the PCM-1-containing centriolar satellites (Figure 6D, a–c, and E). This was unlikely to be a direct consequence of PCM-1 phosphorylation by Nek2 because similar results were obtained with the catalytically inactive Nek2A mutant (Figure 6D, d–f).

Recruitment of Nek2 and C-Nap1 to the Centrosome Is Dependent on PCM-1

To determine whether association with PCM-1 satellites is required for recruitment of Nek2 to the centrosome, PCM-1 was depleted using siRNA oligonucleotides as previously described (Dammermann and Merdes, 2002). After depletion of PCM-1, the pericentriolar satellites disappeared in the majority of cells (Figure 7A, b and c) and the intensity of PCM-1 staining in the vicinity of the centrosome was reduced by 91% ($n = 129$) compared with untreated cells (Figure 7B). By staining with Nek2 antibodies it was apparent that there was a concomitant decrease (38%, $n = 72$) in the intensity of Nek2 at the centrosome (Figure 7A, e and f, and B). Because Nek2 interacts with another centriolar component, C-Nap1, we also tested whether depletion of PCM-1 altered the abundance of C-Nap1 at the centrosome. Intensity measurements revealed a similar decrease (57%, $n = 82$) in the abundance of C-Nap1 at the centrosome (Figure 7B). It has previously been shown that other centrosomal proteins (e.g., γ -tubulin) are not affected by depletion of PCM-1 (Dammermann and Merdes, 2002). We then analyzed the rate of Nek2A recruitment by FRAP in cells treated either with PCM-1 or lamin siRNA oligonucleotides. In this case, there was no significant reduction in the rate of Nek2A recovery after PCM-1 depletion compared with lamin depletion (Figure 7C). These results indicate that PCM-1 is impor-

tant for the stable assembly of Nek2 at the centrosome, but not for the rate of its delivery.

Recruitment of Nek2 to the Centrosome Depends on Its Localized Degradation

Finally, we sought to address whether protein degradation is important for the dynamics of Nek2 at the centrosome. Nek2A is a target for proteasomal degradation as a result of two destruction motifs in its C-terminus that are recognized by the APC/C ubiquitin ligase (Hames *et al.*, 2001). We first used Nek2 antibodies to stain cells that had been treated with the proteasomal inhibitor MG132 (Figure 8A, a and c). Cells treated with MG132 had an approximate 50% increase ($n = 30$) in the centrosomal intensity of endogenous Nek2 compared with untreated cells, suggesting the presence of additional Nek2 protein (Figure 8B). A similar response was seen with EGFP-Nek2A (Figure 8A, b and d). The intensity of the cytoplasmic Nek2 particles was also increased (Figure 8Ad). These observations reflect an increase in total cellular Nek2 protein upon treatment with MG132 as revealed by Western blot (Figure 8C). We therefore decided to test the effect of MG132 on fluorescence recovery of EGFP-Nek2A and observed an almost complete inhibition of the rate of Nek2A recruitment (Figure 8D). This was not a nonspecific effect of drug treatment because the rapid recovery of another centrosomal protein we are currently studying was unaffected (Figure 8E). To confirm that localized degradation is important for Nek2A dynamics, a U2OS cell line was generated that expresses a nondegradable version of Nek2A lacking the C-terminal destruction motifs. This protein still localized at the centrosome because it retains the centrosomal targeting region (Figure 8F), but FRAP analysis revealed that it had a significantly reduced % total recovery compared with wild-type Nek2A (Figure 8G). Interestingly, transiently transfected EGFP-Nek2B exhibited recovery dynamics similar to that of wild-type Nek2A ($t_{1/2} = 3.3$ s, 68% recovery) despite the fact that Nek2B does not contain the proteasomal destruction motifs present in Nek2A (Figure 8G). We emphasize though that the half-life of total Nek2B in the cell has been estimated at 75 min (whereas Nek2A is ~ 45 min), and hence both proteins are relatively short-lived (Hames *et al.*, 2001). These FRAP data are summarized in

Figure 5. FLIP analysis of GFP-Nek2A dynamics. (A) EGFP-Nek2A U2OS cells induced with doxycycline for 24 h were mounted onto sterile glass coverslips and FLIP was performed using a LSM 510 confocal microscope. One image was taken before bleaching (a, phase contrast; b, fluorescence) and then the area within the region outlined was photobleached, followed by image capturing for 10 s. This was repeated 11 times; confocal images shown represent end points of the first, third, fifth, and tenth bleach cycles (c–f). (B) FLIP data were analyzed using LSM 510 software and the relative GFP intensity within the bleached area (■, cytoplasm), the centrosome (○) and control cell cytoplasm (▲) was recorded for each bleach cycle. (C) FLIP as detailed above was carried out in the presence (■, untreated) or absence (◇, nocodazole-treated) of an intact microtubule cytoskeleton; each data point represents the average centrosome intensity from 10 cells. (D) A 50 × 50-pixel square was centered on paired centrosomes after 11 FLIP bleach cycles. One image was taken before bleaching (a, 0 s), and then the area within the indicated box was photobleached. An image was captured every second for 33 s and the relative GFP intensity within the bleached area was recorded as a measure of time (graph). No fluorescence recovery is seen (b).

Table 2. Thus, localized degradation of Nek2A at the centrosome is necessary to maintain the observed dynamics and allow recruitment of new Nek2A molecules to occur.

DISCUSSION

The animal cell centrosome lacks a surrounding lipid bilayer and yet it maintains a highly complex structure capable of performing multiple tasks involved in cytoskeletal organization and cell cycle progression (Doxsey, 2001; Fry and Hames, 2004). Much therefore depends on centrosomal proteins being delivered and forming specific interactions in a cell cycle-dependent manner. Yet, FRAP studies reported both here and by others indicate that, in mammalian cells, many centrosomal proteins are in rapid flux with cytoplasmic populations (Kallio *et al.*, 2002; Stenoien *et al.*, 2003; Thompson *et al.*, 2004). The mechanisms involved in centrosome assembly and maintenance are therefore both complex and obscure. Here, we have analyzed the intracellular dy-

namics of a centrosomal enzyme, Nek2, and find that microtubule binding, the centriolar satellite protein PCM-1 and proteasomal degradation all play a role in dictating the distribution of Nek2 in the cell and its assimilation into the centrosome (Figure 9).

The Role of Microtubules in Nek2 Localization

Because the centrosome acts as the primary site of microtubule nucleation in cells, microtubules would seem to provide an obvious route by which centrosomal proteins may be delivered. This would most likely involve transport by minus-end-directed microtubule motors such as cytoplasmic dynein. However, in many cell types the majority of microtubules do not remain anchored to the centrosome and so this alone may be an inefficient means of recruiting centrosomal proteins (Keating and Borisy, 1999). Cytoplasmic Nek2 is found in small particles, some of which distribute along the lengths of microtubules. In live cell experiments, these particles appear either fairly static or exhibit rapid

Figure 6. Colocalisation of cytoplasmic Nek2 with PCM-1 granules. (A) U2OS cells were fixed and analyzed by indirect immunofluorescence microscopy. Cells were costained with anti-Nek2 (green; a and d) and anti-PCM-1 (red; b, e) antibodies. Merged images (c and f) are shown. d–f are magnified images of the indicated regions in a–c, respectively. Arrowheads indicate colocalisation of Nek2 cytoplasmic particles and PCM-1 granules, whereas the arrow indicates a Nek2 particle that does not colocalize with PCM-1. (B) A C-terminal truncation of chicken EGFP-PCM-1 (amino acids 1–1468) was transiently transfected into U2OS cells (GFP fluorescence; a and d), fixed, and stained with either secondary antibody alone (red; b) or with anti-Nek2 antibodies (red; e). DNA was stained with Hoechst 33258 (blue) and merged images are shown (c and f). (C) U2OS cells expressing EGFP-Nek2A (GFP fluorescence; a and d) were induced for 24 h with doxycycline, before fixing and staining with antibodies against PCM-1 (red; b and e); merged images are shown (c and f). d–f are magnified images of the indicated regions in a–c, respectively. The arrowhead indicates colocalization of cytoplasmic Nek2 particles and PCM-1 granules, whereas the arrow indicates a Nek2A particle without PCM-1 colocalization. (D) Fluorescence microscopy of GFP-Nek2A-WT (a–c) or GFP-Nek2A-KR (inactive; d–f) U2OS cells induced with doxycycline for 24 h (GFP fluorescence, a and d). Cells were stained with anti-PCM-1 antibodies (red; b and e); merged images are shown (c and f). Arrowheads indicate cells overexpressing EGFP-Nek2A that have lost PCM-1-containing centriolar satellites. Scale bars, 10 μ m. (E) Histogram displaying the percentage of cells in the presence (+) and absence (–) of EGFP-Nek2A-WT that have PCM-1 satellites.

movements in linear directions. These observations are consistent with particles that associate transiently with microtubules and are transported when attached. However, FRAP and FLIP experiments indicate that the recovery of Nek2 at the centrosome is very rapid, with a half-life in the order of 3 s. This is identical to another centrosome kinase, Aurora-A ($t_{1/2} \sim 3$ s; Stenoi *et al.*, 2003), but very much faster than the recovery of other proteins such as γ -tubulin (Khodjakov and Rieder, 1999; Stenoi *et al.*, 2003) and centrin (this work). The rapid recovery of Nek2 may in part be due to

diffusion because cytoplasmic microtubules are not essential for the recruitment or localization of Nek2 to the centrosome (Fry *et al.*, 1998a), nor for cytoplasmic diffusion. Microtubules are certainly not required for the release of Nek2 from centrosomes. However, FRAP experiments using increasing bleach areas do support the hypothesis that microtubules facilitate its delivery from more distant sites in the cell.

Microtubules may serve a second, and possibly more important, role in anchoring Nek2 at the centrosome. In vitro assays revealed a specific 36 amino acid motif in the region

Figure 7. Depletion of PCM-1 interferes with Nek2 recruitment to the centrosome. (A) Indirect immunofluorescence microscope images of HeLa cells treated with control (a and d) or PCM-1-specific (b, c, e, and f) siRNA oligonucleotides for 72 h stained with anti-PCM-1 (red; a–c) and anti-Nek2 (green; d–f) antibodies. Scale bar, 10 μ m. (B) Histogram displaying the centrosomal intensities of PCM-1, Nek2, and C-Nap1 in the presence (+) and absence (–) of PCM-1 siRNA oligonucleotides. Values are expressed relative to the mean intensity in control siRNA treated cells. (C) U2OS cells treated with PCM-1 siRNA ($\diamond$) or control lamin siRNA ($\blacksquare$) oligonucleotides for 72 h were mounted onto sterile glass coverslips for FRAP analysis. The data were analyzed using LSM 510 Zeiss software and the relative GFP intensity within the bleached area recorded as a measure of time; each data point represents the average intensity from 10 cells.

of the C-terminus that is conserved between the Nek2A and Nek2B splice variants that must be present for microtubule binding. This motif lies C-terminal to the leucine zipper dimerization domain in a region with no previously ascribed function. Importantly, the same region is essential for targeting Nek2 to the centrosome. The full Nek2A C-terminal domain alone clearly localizes to the centrosome, as does Nek2B and the deletion mutant lacking the leucine zipper. The mutant that terminates after the leucine zipper, though, does not. Thus, amino acids 333–370 are required, but may not be sufficient because this short motif did not direct localization to the centrosome nor did it confer robust microtubule binding when fused alone to Rab4 (unpublished data). Previous immunogold labeling revealed that Nek2, and its binding partner C-Nap1 localize to the proximal ends

of centrioles, which represent the minus ends of the centriolar microtubules (Fry *et al.*, 1998b). Hence, Nek2, and perhaps specifically the 30% nonmobile fraction at the centrosome, may be anchored to the centrioles via its microtubule binding domain. Three-dimensional structure information would allow the key residues within this motif to be identified and functionally analyzed by site-directed mutagenesis.

Nek2, PCM-1, and Centriolar Satellites

Previous calculations indicated that only ~10% of endogenous Nek2 is at the centrosome (Fry *et al.*, 1998a). Here, we show that Nek2 is also found in small cytoplasmic particles, some of which exhibit highly dynamic movements. Close to the centrosome, Nek2 particles either move toward and

Figure 8. Proteasomal degradation of Nek2 contributes to its dynamics. (A) Indirect immunofluorescence microscopy of U2OS cells stained with anti-Nek2 antibodies (a and c) or EGFP-Nek2A (b and d). U2OS cells induced for 24 h with doxycycline (GFP fluorescence; b and d), treated with 0.2% DMSO (a and b) or 20 μ M MG132 (c and d) for 4 h. Scale bar, 10 μ m. (B) Histogram displaying the percentage intensities (arbitrary units) of endogenous Nek2 at the centrosome after treatment with DMSO or MG132. Thirty cells were counted for each treatment; error bars, SD. (C) Induced EGFP-Nek2A U2OS (GFP-Nek2A) and U2OS cells (endogenous Nek2) were treated with DMSO or MG132. Extracts were prepared from cells and Western blotted with anti-Nek2 and anti- α -tubulin antibodies. Histograms display the Nek2 protein abundance in these samples (arbitrary units). (D) Induced EGFP-Nek2A U2OS cells treated with MG132 (black triangles), DMSO (gray diamonds), or left untreated (black squares) were mounted onto sterile glass coverslips, and FRAP was performed using a LSM 510 Zeiss confocal microscope. The data were analyzed using LSM 510 Zeiss software and the relative GFP intensity within the bleached area recorded as a measure of time; each data point represents the average intensity from 10 cells. (E) U2OS cells transiently transfected with a GFP-tagged novel centrosomal protein were treated with MG132 ($\blacktriangle$) or left untreated ($\blacksquare$) and FRAP analysis was undertaken as above. (F) EGFP-Nek2A-ND (nondegradable mutant) U2OS cells were induced with doxycycline for 24 h, fixed, and stained with anti-C-Nap1 antibodies. Arrowheads indicate centrosome localization. Scale bar, 10 μ m. (G) Induced EGFP-Nek2A-ND U2OS cells (black triangles) and cells expressing EGFP-Nek2A (black squares) and EGFP-Nek2B (gray diamonds) were subjected to FRAP analysis as above. The p value ($n = 10$) refers to the comparison of total % recovery for Nek2A-ND and Nek2A.

sometimes fuse with the centrosome or migrate in the opposite direction. The size and motion of these particles resemble the previously described centriolar satellites that contain the PCM-1 protein. Although cytoplasmic Nek2 does not exclusively localize to centriolar satellites, a number of pieces of evidence support the idea that PCM-1 has a role in Nek2 assembly. First, both endogenous and recombinant Nek2 partially colocalize with PCM-1 granules. Second, endogenous Nek2 is recruited into the large aggregates generated upon expression of PCM-1 lacking its C-terminal domain. Third, overexpression of Nek2 causes the loss of obvious PCM-1 containing centriolar satellites. Fourth, de-

pletion of PCM-1 by RNAi reduces the abundance of Nek2 at centrosomes. Because PCM-1 can move along microtubules in a dynein-dependent manner (Kubo *et al.*, 1999), it may therefore be involved in microtubule-based transport of Nek2. However, PCM-1 is also required for delivery of centrin to the centrosome (Dammernann and Merdes, 2002), yet centrin is recruited very slowly. Moreover, PCM-1 is not required for the rapid assembly of Nek2 at the centrosome. Thus, it seems unlikely that PCM-1 is simply serving a transport function. The reduction in Nek2 at the centrosome in PCM-1-depleted cells may indicate that Nek2 requires some of PCM-1's other cargoes for stable association with

Table 2. FRAP analysis of Nek2 protein stability

Protein	Treatment	$t_{1/2}$	% Recovery
GFP-Nek2A		3.2 s	72
GFP-Nek2B		3.3 s	68
GFP-Nek2A-ND		5.9 s	49
GFP-Nek2A	+ DMSO (0.2%)	3.2 s	72
GFP-Nek2A	+ MG132 (20 μ M)	nd	8

The half-life ($t_{1/2}$) and % fluorescence recovery at the centrosome were determined for GFP-Nek2A, GFP-Nek2B, and GFP-Nek2A-ND (non-degradable) proteins. The Nek2 constructs were transiently transfected into U2OS cells 24 h before photobleaching. Drug treatments were carried out for 4 h where indicated. Photobleaching and subsequent confocal imaging were performed as described in *Materials and Methods*. The % recovery of GFP-Nek2A after MG132 treatment was so low that the half-life could not be determined (nd).

the centrosome, such as centrin. However, we suggest that PCM-1, and by implication centriolar satellites, may have functions distinct from transport, for instance, as a chaperone helping proteins to fold and assemble into subcentrosomal complexes. Centriolar satellites are regulated in a cell cycle-dependent manner, dispersing during mitosis (Kubo and Tsukita, 2003). Overexpression of Nek2A also led to dispersal of PCM-1 granules, although this was independent of its kinase activity. However, it remains possible that Nek2 might have a physiological role in regulating localization of PCM-1 and/or centriolar satellites.

Proteasomal Turnover of Nek2 Occurs at the Centrosome

We previously reported that the Nek2A splice variant is subject to proteasomal degradation in a cell cycle-dependent manner (Hames *et al.*, 2001). This is due to destruction motifs present in the extreme C-terminus that are recognized by the APC/C ubiquitin ligase. However, to date, the location at which APC/C substrates are destroyed has not been known. Our results point strongly to proteasomal degradation of Nek2A occurring directly at the centrosome. Addition of a proteasome inhibitor increased the abundance of Nek2 at the centrosome while simultaneously reducing the amount of fluorescence recovery. This would be explained by the accumulation of stable protein at the centrosome. In agreement with this, the amount of fluorescence recovery was significantly reduced when a nondegradable mutant of Nek2A was studied. FRAP analysis on synchronized cells may provide further data on the importance of cell cycle position for Nek2 degradation at the centrosome.

The 26S proteasome is not only associated with the centrosome (Wigley *et al.*, 1999), but is present in an active conformation (Fabunmi *et al.*, 2000). Our data therefore indicate that localized proteasomal degradation is necessary to maintain the dynamic turnover of an APC/C substrate at the centrosome. It is worth speculating that the rapid recovery of Aurora-A at the centrosome may also be in part dependent on proteasomal degradation because it is another APC/C substrate (Littlepage and Ruderman, 2002). Previously, the destruction of cyclin B has been studied by live cell imaging, revealing that the protein disappears first at the centrosome (Clute and Pines, 1999; Huang and Raff, 1999; Yanagida *et al.*, 1999). This is also consistent with destruction occurring at the centrosome. Although subunits of the APC/C have been detected at the centrosome (Huang and Raff, 2002), it remains possible that ubiquitylation of its substrates occurs elsewhere in the cell before transport of the ubiquitylated molecules to the centrosome for destruction.

Figure 9. A model illustrating Nek2 dynamics in the cell. Immuno-EM studies suggest that Nek2 (red), together with its substrate C-Nap1 (orange), preferentially localize to the proximal ends of the two centrioles (blue cylinders), whereas neither protein requires an intact microtubule cytoskeleton to maintain their centrosomal localization (Fry *et al.*, 1998b). However, this current study reveals that a number of mechanisms are involved in regulating Nek2 trafficking to the centrosome. (1) FRAP and FLIP indicate that Nek2 can load on and off the centrosome independently of microtubules. (2) Time-lapse imaging and FRAP studies suggest a possible contribution of microtubules to long-range transport of Nek2. (3) Partial colocalization of Nek2 with PCM-1 highlights a possible role for PCM-1 in delivery of Nek2 to the centrosome along microtubules. (4) Alternatively, PCM-1 may have a chaperone function in facilitating assembly of protein complexes, in this case Nek2 and C-Nap1, before transport to the centrosome. (5) Proteasomal degradation of Nek2A is essential to allow turnover of Nek2 molecules at the centrosome. Together, these processes ensure the correct abundance of Nek2 kinase at the centrosome through the cell cycle.

Addition of proteasome inhibitors to cells has been reported to increase the concentration of proteasomal subunits, and other E3 ubiquitin ligases, at the centrosome, perhaps in response to accumulation of either proteasomal substrates or misfolded proteins (Wigley *et al.*, 1999; Fabunmi *et al.*, 2000; Zhao *et al.*, 2003). Our data, the first time that FRAP has been used to address the role of localization in protein degradation, lends support to this hypothesis.

The simple observation that centrosomal Nek2 is increased upon inhibition of the proteasome demonstrates that the normal amount of Nek2 at the centrosome is not saturating. This, together with our other results, indicates that the steady-state level of Nek2 at the centrosome is regulated by a careful balance of recruitment, release, and degradation. These findings are of clinical relevance as we have recently shown that Nek2 protein is frequently overexpressed in human cancer cells (Hayward *et al.*, 2004). Hence, excess centrosomal Nek2 may contribute to chromosomal instability and aneuploidy, classic hallmarks of most aggressive tumors.

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