Characterization of NIP2/centrobin, a novel substrate of Nek2, and its potential role in microtubule stabilization

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Summary

Nek2 is a mitotic kinase whose activity varies during the cell cycle. It is well known that Nek2 is involved in centrosome splitting, and a number of studies have indicated that Nek2 is crucial for maintaining the integrity of centrosomal structure and microtubule nucleation activity. In the present study, we report that NIP2, previously identified as centrobin, is a novel substrate of Nek2. NIP2 was daughter-centriole-specific, but was also found in association with a stable microtubule network of cytoplasm. Ectopic NIP2 formed aggregates but was dissolved by Nek2 into small pieces and eventually associated with microtubules. Knockdown of NIP2 showed significant reduction of microtubule organizing activity, cell shrinkage, defects in spindle assembly and abnormal nuclear morphology. Based on our results, we propose that NIP2 has a role in stabilizing the microtubule structure. Phosphorylation may be crucial for mobilization of the protein to a new microtubule and stabilizing it.

Key words: NIP2, Centrobin, Nek2, Centrosome, Mitosis, Microtubule

Introduction

Phosphorylation is one of major control mechanisms used during mitosis. A mitotic kinase phosphorylates a specific set of proteins in a cell-cycle-stage-specific manner for activation, interaction, translocation and degradation of the substrates. Nek2 has been considered as a mitotic kinase whose activity oscillates during the cell cycle (Fry et al., 1995). It is known that alternative splicing of Nek2 transcripts produces two isoforms with distinct C-terminal ends (Uto et al., 1999; Hames and Fry, 2002). In *Xenopus* embryos, *Nek2B* was expressed mostly in early stages and *Nek2A* began to be expressed after the neurula stage (Uto et al., 1999). In tissue culture cells, the Nek2A, but not Nek2B proteins, are destroyed in early mitosis by APC/C–Cdc20-dependent proteolysis, which results in abrupt reduction at early M phase (Hames et al., 2001). Functional differences between the two isoforms, however, remain to be investigated.

Immunocytochemical analysis revealed distinct localization of Nek2 at the centrosome (Fry et al., 1998b). Nek2 is transported into the centrosome in small, highly dynamic cytoplasmic particles, many of which align along microtubules (Hames et al., 2005). Centrosome splitting was induced rapidly in *Nek2*-overexpressing cells and monopolar spindle was formed in the kinase-defective *Nek2A*-mutant-expressing cells, which suggests that Nek2 regulates separation of centrosomes during the cell cycle (Fry et al., 1998b; Faragher and Fry, 2003). C-Nap1, a substrate of Nek2, may be a key player, since both microinjection of anti-C-Nap1 antibodies and knockdown of *C-Nap1* expression each induced centrosome splitting (Fry et al., 1998a; Mayor et al., 2000; Bahe et al., 2005). It has been proposed that Nek2 promotes the centrosome disjunction through the phosphorylation-induced displacement of C-Nap1 from the centriolar ends (reviewed in Fry, 2002).

A number of studies provide evidence that Nek2 is involved not only in centrosome splitting but also in other centrosomal functions. First, very few centrosomes were detected in cells with prolonged overexpression of *Nek2* (Fry et al., 1998b). Microinjection of kinase-defective *Nek2* mRNA or anti-Nek2 antibody into *Xenopus* embryos resulted in developmental arrest at the cleavage stage and impaired bipolar spindle formation due to fragmented centrosomes (Uto and Sagata, 2000). Aster formation activity was delayed significantly in Nek2 immuno-depleted *Xenopus* embryo extracts (Fry et al., 2000). Development of *Nek2*-suppressed mouse embryos was blocked specifically at M phase by defects in spindle assembly (Sonn et al., 2004). These results indicate that Nek2 is also required for maintaining the integrity of the centrosomal structure and microtubule nucleation activity (reviewed in Fry, 2002). However, the exact mechanisms by which Nek2 carried out such activities has remained unknown, largely due to the fact that the corresponding substrates have not yet been identified. A recent study reported that Nek2 and Plk1 phosphorylated ninein-like protein (Nlp) sequentially and induced displacement of the protein from centrosomes (Rapley et al., 2005). It was proposed that, like ninein, Nlp might be involved in microtubule nucleation at interphase cells (Casenghi et al., 2003). The exact mechanisms of action of Nlp and its modification by phosphorylation during the cell cycle, however, remain to be elucidated.

A recent proteomic analysis of the human centrosome identified a few hundred proteins as centrosomal components (Andersen et al., 2003). Centrobin was classified as a centrosome candidate protein according to the proteomic

analysis (Andersen et al., 2003), and was initially identified as an interacting protein of BRCA2 (Zou et al., 2005). Knockdown of centrobin expression inhibited centriole duplication and resulted in centrosomes with one or no centriole, which demonstrates that centrobin is required for centriole duplication (Zou et al., 2005).

In the present study, we identified NIP2/centrobin as a novel substrate of Nek2. Our knockdown experiments reveal that NIP2 is involved in the microtubule organizing in interphase cells and in spindle assembly in mitotic cells. Furthermore, we propose that mobilization of NIP2 may be controlled by phosphorylation.

Results

Identification of NIP2, a Nek2-interacting protein

We carried out a yeast two-hybrid screen with Nek2 as bait and isolated *NIP2* (Nek2-interacting protein 2) along with several other clones (Yoo et al., 2002). A human *NIP2* cDNA of 3573 bp in size contained a 903 amino acid-long putative ORF with characteristic coiled-coil domains in the middle (NCBI accession number, NM_053051). NIP2 was previously reported as Lyst-interacting protein 8 (Tchernev et al., 2002). The proteomic analysis of the human centrosome reported NIP2 as one of the candidate centrosome proteins (Andersen et al., 2003). Recently, the same gene was also identified as encoding a BRCA2-binding protein, and named centrobin (Zou et al., 2005).

A polyclonal antibody was raised and purified for detection of the NIP2 protein. The purified antibody specifically detected the endogenous NIP2 protein at the expected position of 101 kDa in the lysates of 293T cells (Fig. 1A). Specificity of the NIP2 antibody was also examined by immunoprecipitation followed by immunoblot analysis (Fig. 1B). The results confirmed that our antibody recognizes the endogenous NIP2 protein specifically.

Immunoblot analysis was carried out for detection of the NIP2 protein in several cell lines (Fig. 1C). The results showed that NIP2 was present in all cell lines tested, suggesting ubiquitous expression of *NIP2*. The cell cycle dependency of *NIP2* expression was examined. NIP2 protein levels were determined in U2OS cells in which cell cycle was arrested at G1/S phase with thymidine or hydroxyurea, or at G2/M phase with nocodazole or taxol. Immunoblot analysis revealed that NIP2 protein levels were constant more or less throughout the cell cycle, but slightly reduced at G2/M phase (Fig. 1D). Such an expression pattern was comparable with those of *Nek2* and *C-NAP1* (Fig. 1D) that had been reported previously (Mayor et al., 2002).

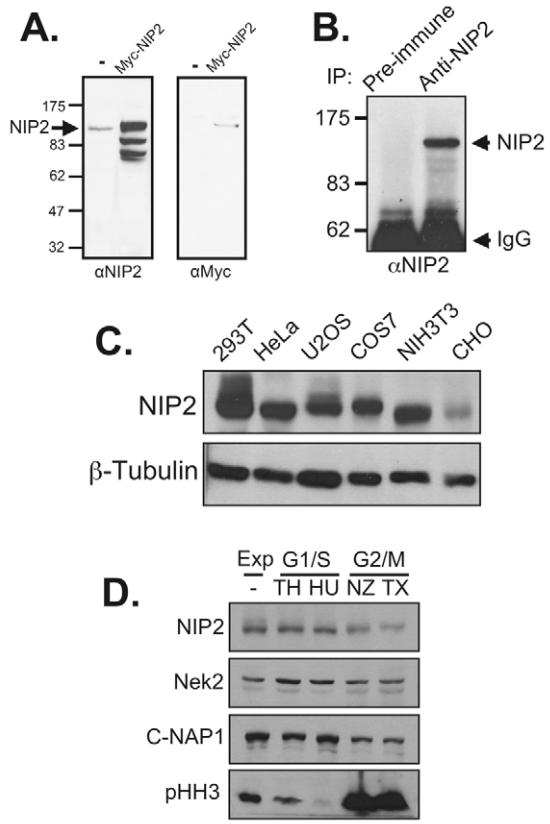

Fig. 1. Immunoblot analysis of NIP2. (A) Lysates of 293T cells transfected with Myc-NIP2 were used for immunoblot analysis with antibodies specific to NIP2 or a Myc tag. The arrow indicates endogenous NIP2. (B) Lysates of HeLa cells were immunoprecipitated (IP) with pre-immune serum or the NIP2 anti-serum, followed by immunoblotting with the NIP2 antibody. The arrows indicate specific bands for NIP2 and the IgG heavy chain. (C) Selected cell lines from human (293T, HeLa, U2OS), rhesus monkey (COS7), mouse (NIH3T3), and Chinese hamster (CHO) origins were subjected to immunoblot analysis with the NIP2 antibody. The same blot was re-used for detection of β-tubulin. (D) The cell cycle of HeLa cells was arrested at G1/S phase with either 2.5 mM thymidine (TH) or 4 mM hydroxyurea (HU), or at G2/M phase with either 100 μg/ml nocodazole (NZ) or 1 μM taxol (TX). An exponentially growing cell population without any treatment (Exp) was included as a control. The cell lysates were subjected to immunoblot analysis with antibodies against NIP2, Nek2, C-NAP1 and phospho-histone H3 (pHH3).

Centrosomal localization of NIP2

During the preparation of this manuscript, Zou et al. reported that centrobin/NIP2 was specifically located at daughter centrioles (Zou et al., 2005). We also observed that NIP2 was present at a centriole in which GFP-centrin 2 was not prominent (data not shown) (White et al., 2000). Furthermore, in NIH3T3 cells, NIP2 was detected at a centriole devoid of cilium, which is the daughter centriole (data not shown). Therefore, it is clear that NIP2 is a daughter-centriole-specific protein.

We examined the microtubule dependency of the centrosomal localization of NIP2 by placing the U2OS cells on ice to disrupt the cellular microtubule structure or treated them with taxol to stabilize it. The results showed that the NIP2 antibody stained centrosomes irrespective of the status of the cellular microtubule organization, indicating that NIP2 is a core centrosomal component (Fig. 2A).

In order to determine the NIP2 sequence critical for centrosomal localization, we prepared a series of NIP2 truncated mutants linked to GFP and expressed them in HeLa cells (Fig. 2B). When the GFP-NIP2 proteins were expressed at a moderate level, all the tested GFP-NIP2 mutant proteins located to the centrosome except GFP-NIP2$^{1\text{-}728}$ (Fig. 2C). These results suggest that the C-terminal end of NIP2 is crucial for its centrosomal localization.

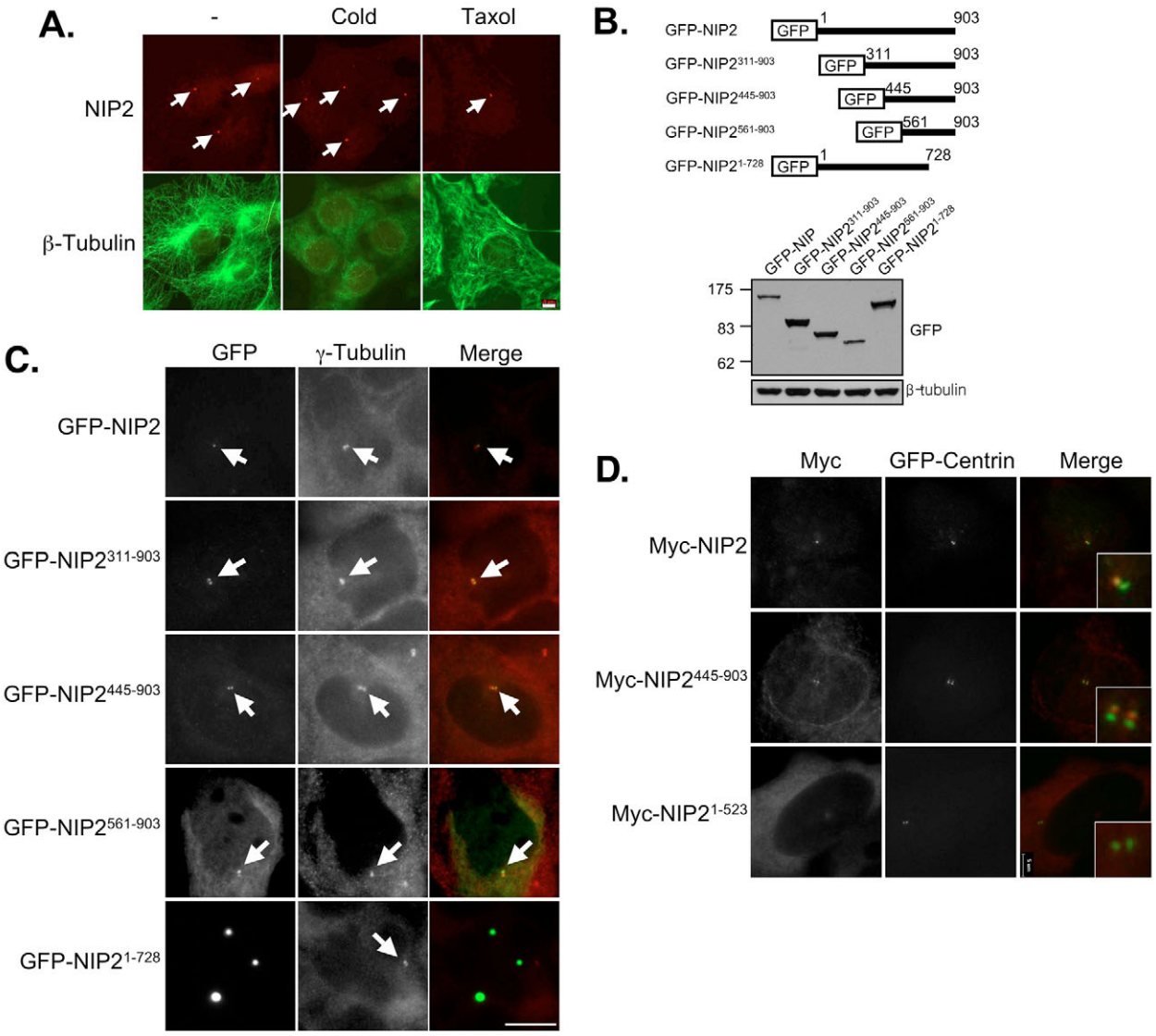

Fig. 2. The C-terminal end of NIP2 is critical for centriolar localization. (A) U2OS cells were placed on ice for 30 minutes to disrupt microtubules or treated with 1 μg/ml taxol for 2 hours to stabilize them, and were immunostained with antibodies against NIP2 and β-tubulin. Arrows indicate the endogenous NIP2 staining at the centrosomes. Bar, 5 μm. (B) Several NIP2-truncated mutants with the GFP tags were expressed in HeLa cells. The β-tubulin was detected as a control. (C) HeLa cells were transfected with the NIP2-truncated mutant genes and immunostained with antibodies against GFP (green) and γ-tubulin (red). Arrows indicate the centrosomes. Bar, 10 μm. (D) Myc-tagged NIP2 truncated mutant proteins (Myc-NIP2, Myc-NIP2$^{445-903}$, and Myc-NIP2$^{1-523}$) were co-expressed with GFP-centrin 2 (green) in HeLa cells and detected with the Myc antibody (red). The insets are magnified views of the centrosomes.

Some centriolar proteins were known to require two different domains for proper targeting to the centriole. For example, ninein was targeted to the centrosome by its C-terminus but stabilized at the mother centriole by its N-terminus (Delgehyr et al., 2005). In order to test whether NIP2 is also required for two different domains for proper targeting, we ectopically expressed the N-terminal- or C-terminal-truncated mutants and observed localization of the ectopic NIP2 proteins. The results showed that Myc-NIP2 and Myc-NIP2$^{445-903}$ were detected at the daughter centriole but Myc-NIP2$^{1-523}$ was absent from the centrosome (Fig. 2D). These results indicate that the C-terminal end of NIP2 is sufficient for targeting to the daughter centriole.

NIP2 proteins outside the centrosome

Results of the immunocytochemical analyses clearly indicated centrosomal localization of NIP2. However, it is possible that NIP2 is also present outside the centrosome. In fact, a number of centrosomal proteins, such as γ-tubulin, Nek2 and C-Nap1, are known to have cytoplasmic pools outside the centrosome (Moudjou et al., 1996; Fry et al., 1998a; Fry et al., 1998b). We determined the presence of cytoplasmic NIP2 by comparing it with centrosomal proteins that were known to have significant cytoplasmic pools (Fig. 3). First, we wished to detect centrosomal NIP2 with immunoblot analysis. We fractionated the cell lysates using sucrose gradient centrifugation for enrichment of the centrosomes, and detected NIP2 in the same

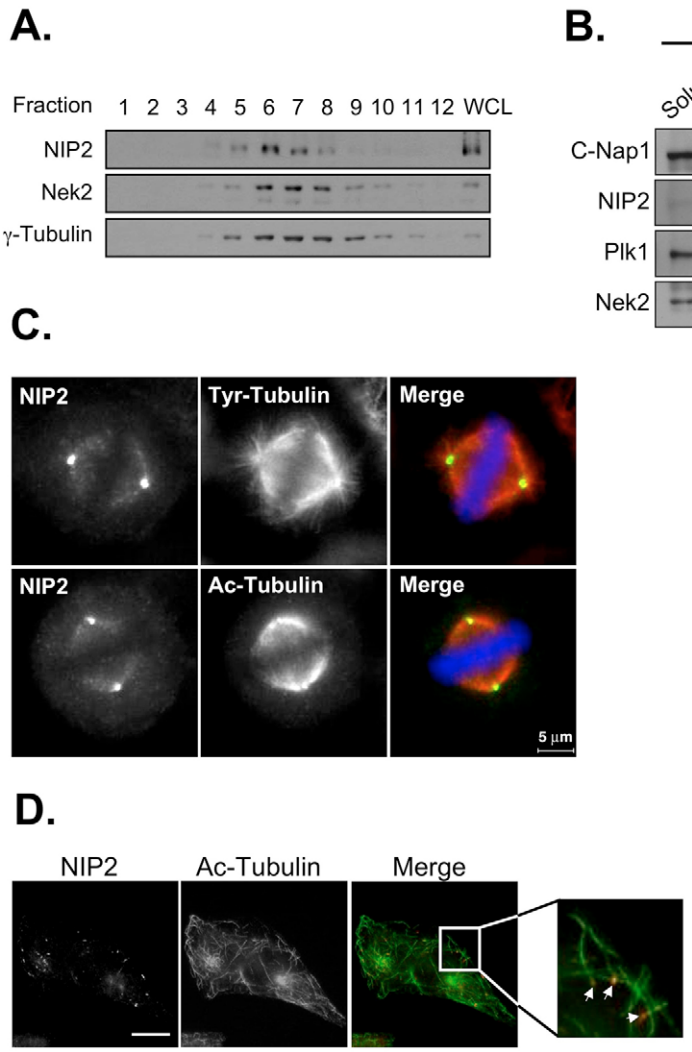

Fig. 3. NIP2 proteins outside the centrosome. (A) KE37 cell lysates were fractionated by sucrose gradient centrifugation for enrichment of the centrosome. Immunoblot analysis was carried out using antibodies against NIP2, Nek2 and γ-tubulin. Whole cell lysates (WCL) were used as a control. (B) Immunoblot analysis was carried out using soluble and insoluble fractions of whole cell lysates (WCL) and the centrosome fraction, and using antibodies against C-Nap1, NIP2, Plk1 and Nek2. (C) U2OS cells at mitosis were immunostained with NIP2 antibody (green). Antibodies against tyrosinated and acetylated tubulins (red) were used for staining dynamic and stable microtubules, respectively. (D) U2OS cells at interphase were immunostained with antibodies against NIP2 (red) and acetylated tubulin (green). Arrows indicate NIP2 signals in the cytoplasm. Note that centrosomal signals are out of focus in this figure. Bar, 10 μm.

fractions as Nek2 and γ-tubulin (Fig. 3A). Next, we compared relative amounts of NIP2 with other centrosomal proteins both in the centrosome fraction and in whole cell lysates. NIP2 in whole cell lysates was present both in soluble and insoluble forms (Fig. 3B). The relative amounts of NIP2 in whole cell lysates and in the centrosome-enriched fractions were comparable with those of C-Nap1, Plk1 and Nek2 (Fig. 3B). Since it had been estimated that over 90% of Nek2 was present outside the centrosome (Fry et al., 1998b), it is likely that NIP2 follows a similar subcellular distribution rate to that of Nek2. If NIP2 were solely centrosomal without any cytoplasmic pool, the NIP2-specific band would not be detected in whole cell lysate in the same quantities as C-NAP1, Plk1 and Nek2.

Cytoplasmic NIP2 was not detected readily by immunocytochemical analysis, which suggests that the majority of NIP2 proteins stay in a diffused form. However, careful examination revealed that a fraction of cytoplasmic NIP2 was associated with the microtubule network. In mitotic cells, NIP2 was associated with spindle as well as spindle poles (Fig. 3C). NIP2 was more abundant in chromosomal spindles, where acetylated tubulin is prominent, than in astral spindles, where tyrosinylated tubulin is prominent (Fig. 3C). In selected cell lines such as U2OS and MCF7, but not HeLa cells, NIP2 was detected in association with the roots or initiating points of microtubules that were stained with the acetylated-tubulin-specific antibody (Fig. 3D). These results indicate that a fraction of cytoplasmic NIP2 can bind to microtubules transiently. Furthermore, it is likely that NIP2 is associated with stabilized microtubules preferentially.

NIP2 was phosphorylated by Nek2 both in vitro and in vivo

Even if we identified NIP2 as a Nek2-interacting protein in yeast two-hybrid screening, we were not able to confirm any physical interactions between NIP2 and Nek2 by co-immunoprecipitation assay, implying that their interaction might be weak or transient. Therefore, we asked whether NIP2 is a substrate of Nek2. An in vitro kinase assay of Nek2 was carried out using NIP2 fragments as substrates. Nek2 could phosphorylate GST-NIP2$^{1\text{-}523}$ and Myc-NIP2$^{1\text{-}523}$ fusion proteins as well as casein, a known in vitro substrate of Nek2, along with autophosphorylation (Fig. 4A). The kinase-defective Nek2 did not show any kinase activity, which provides evidence that NIP2 is a specific substrate of Nek2 in vitro.

To define the Nek2 phosphorylation region within the NIP2 polypeptide, we carried out an in vitro kinase assay with truncated NIP2 mutant fusion proteins as substrates (Fig. 4B). The results showed that Nek2 phosphorylated GST-NIP2$^{1\text{-}193}$ most efficiently among the NIP2 truncated mutant proteins, which suggests that the N-terminal end of NIP2 is the main target of Nek2 kinase activity (Fig. 4B). No specific phosphorylation was observed with the kinase-defective Nek2.

An in vivo labeling assay was carried out with the HeLa cells that had been transfected with wild-type Nek2 (*Nek2RHA1*) or kinase-defective Nek2 (*Nek2KHA5*) constructs (Fig. 4C). The endogenous NIP2 protein was phosphorylated strongly in *Nek2RHA1*-transfected cells, in comparison with *Nek2KHA5*-

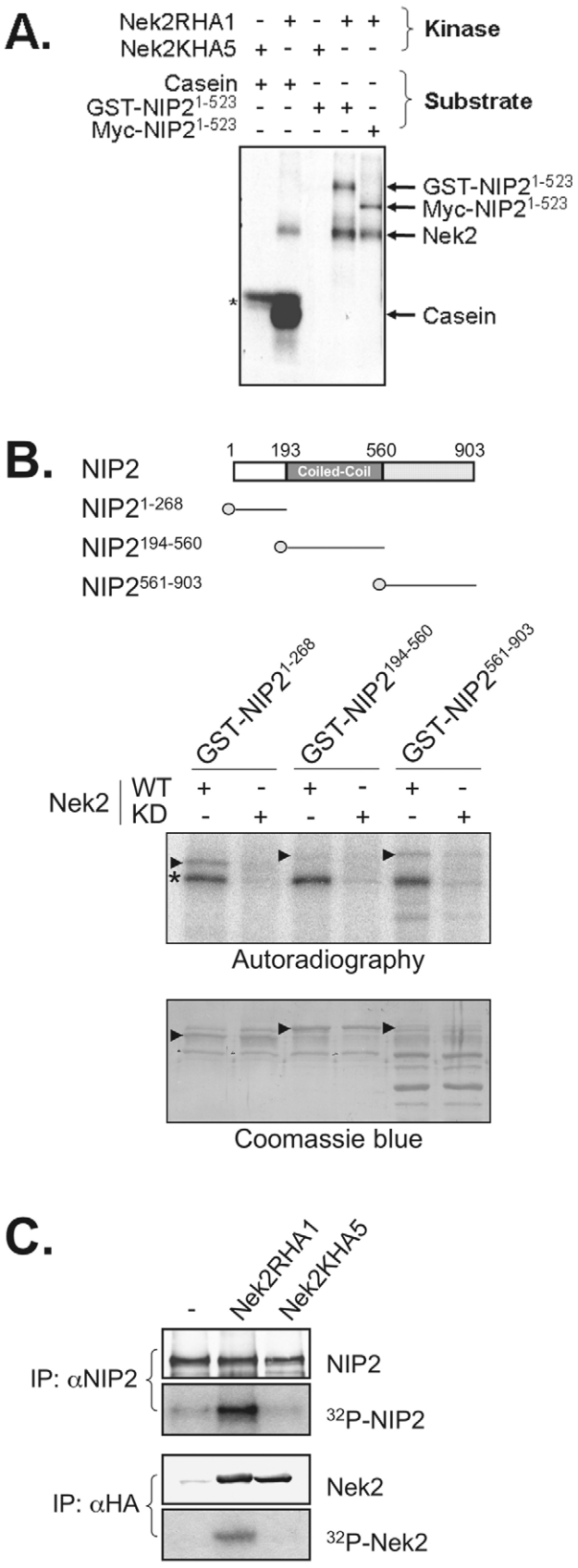

Fig. 4. NIP2 was phosphorylated by Nek2 in vitro and in vivo. (A) In vitro kinase assay. The wild-type (Nek2RHA1) and kinase-defective (Nek2KHA5) Nek2 proteins were prepared from transiently transfected 293T cells. As substrates, GST-NIP2$^{1-523}$ was purified from bacterial lysates and Myc-NIP2$^{1-523}$ was prepared by immunoprecipitation from transfected 293T cells. Casein was a control substrate for Nek2. Specific bands on the autoradiogram are marked. The bands marked with an asterisk are non-specific. (B) In vitro kinase assay carried out with wild-type (WT) or kinase-defective (KD) Nek2. The substrates were prepared from bacterially expressed GST fusion proteins linked to NIP2$^{1-193}$, NIP2$^{194-560}$ or NIP2$^{561-903}$. The separated gel was stained with Coomassie Blue and autoradiographed. The arrowheads indicate the substrate proteins and the asterisk indicates autophosphorylation of Nek2. (C) In vivo labeling of NIP2. HeLa cells transfected with *pNek2RHA1* or *pNek2KHA5* were incubated in the presence of ^{32}P-orthophosphate (250 μCi/ml) for 3 hours. Endogenous NIP2 was immunoprecipitated (IP) and subjected to immunoblot analysis as well as autoradiogram. Exogenous Nek2 was also immunoprecipitated with the HA antibody, followed by immunoblot analysis and autoradiogram. A faint band of HA immunoprecipitate from untransfected cells corresponds to the IgG heavy chain.

transfected and untransfected cells. Autophosphorylation was observed only in Nek2RHA1. These results indicate that NIP2 is an in vivo substrate of Nek2.

Effects of Nek2 on ectopic NIP2 proteins

We explored the effects of Nek2 phosphorylation on the NIP2 protein (Fig. 5). Like many other proteins with coiled-coil domains, the ectopic NIP2 protein was highly insoluble in a weak lysis buffer containing 1% NP40 (Andersen et al., 2003) (Fig. 5A). However, co-expression of NIP2 with Nek2 but not with kinase-defective Nek2 increased the solubility of NIP2 proteins in a dose-dependent manner (Fig. 5A). These results suggest that Nek2 phosphorylation induced conformational changes in NIP2 that made the protein soluble.

Previous results showed that ectopic NIP2 proteins located preferentially to the centrosome (Fig. 2C). When NIP2 was present in excess, it formed aggregates in the cytoplasm (Fig. 5B). Interestingly, when Nek2 was co-expressed, ectopic NIP2 was detected as tiny aggregates or a mesh-like network along with Nek2 (Fig. 5B). Co-expression of kinase-defective Nek2 did not affect the subcellular distribution of NIP2, which indicates that the enzymatic activity of Nek2 is crucial (Fig. 5B). The subcellular distribution of GFP-NIP2 was examined immunocytochemically. The results showed that both GFP-NIP2 and Nek2 associated with the microtubule network (Fig. 5C). These results and the previous results of the subcellular localization of endogenous NIP2 consistently suggest that NIP2 has an affinity with microtubules.

We observed that ectopic expression of *NIP2* frequently induced microtubule bundling. In particular, GFP-NIP2$^{445-903}$ showed the strongest bundling activity among the NIP2 mutants tested. In cells with GFP-NIP2$^{445-903}$, acetylated tubulin appeared well organized around the nucleus of cells along with the protein (Fig. 5D). Nocodazole degraded the microtubule network of untransfected cells, whereas it had limited effects on the *GFP-NIP2$^{445-903}$*-expressing cells, which suggests that ectopic NIP2 stabilizes the microtubule network significantly (Fig. 5D).

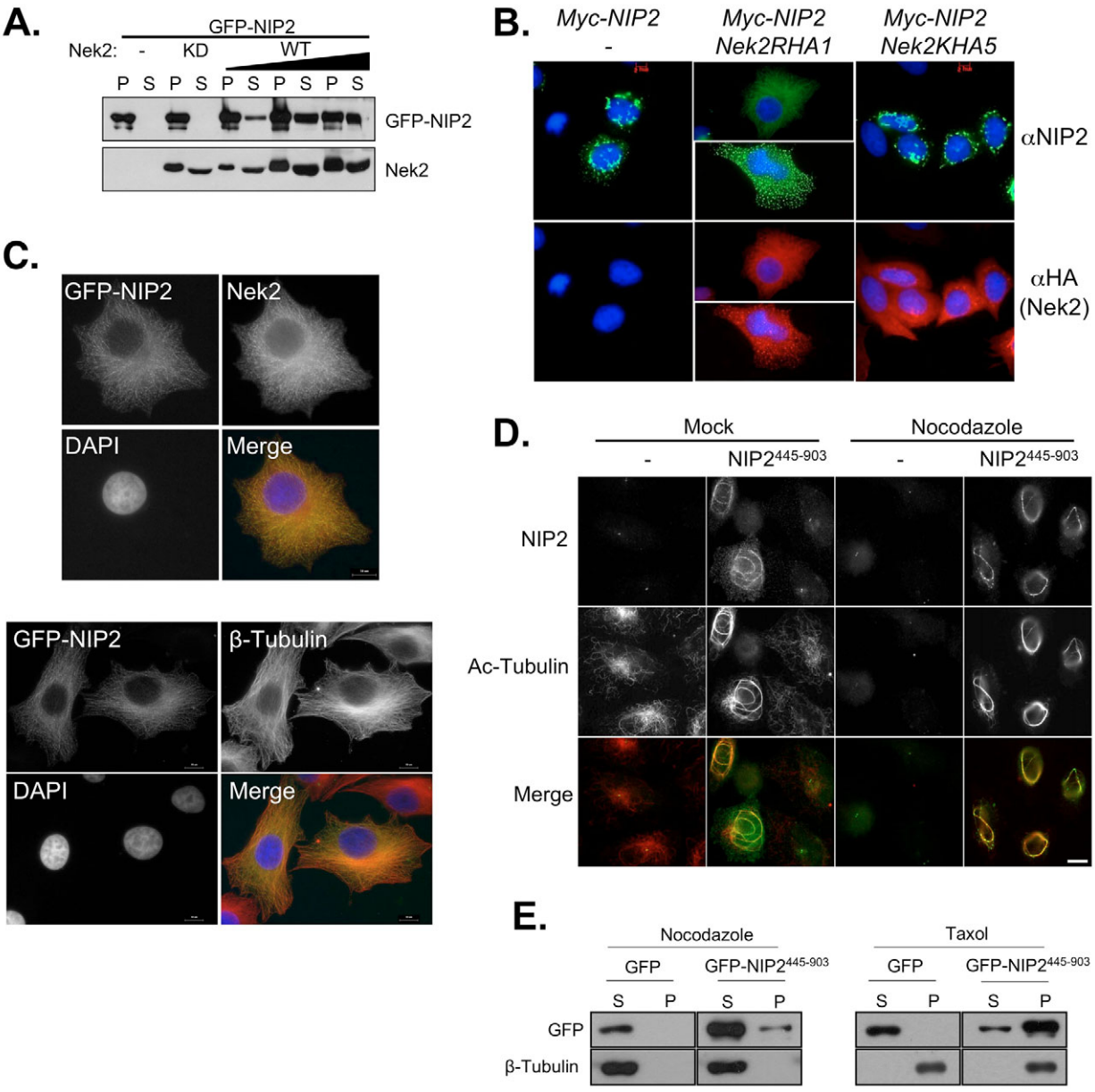

Fig. 5. Effects of Nek2 on ectopic NIP2 proteins. (A) 293T cells were co-transfected with *GFP-NIP2* and kinase-defective *Nek2KHA5* (KD) or varying amounts of wild-type *Nek2RHA1* (WT). Twenty-four hours later, cell lysates were prepared using a lysis buffer containing 1% NP40 and separated into the insoluble pellet (P) and soluble supernatant (S) by centrifugation. Equal amounts of pellet and supernatant proteins were subjected to immunoblot analysis using antibodies against GFP and the HA-tag for detection of ectopic NIP2 and Nek2 proteins, respectively. (B) *Myc-NIP2* was co-transfected into HeLa cells with either *Nek2RHA1* or *Nek2KHA5*. Co-immunostaining was carried out using NIP2 (green) and HA (red) antibodies. (C) HeLa cells that had been co-transfected with *GFP-NIP2* and *Nek2RHA1* were co-immunostained with antibodies against GFP (green) and Nek2 (red) or β-tubulin (red). DNA was stained with DAPI. Bars, 10 μm. (D) HeLa cells were transfected with *Myc-NIP2*$^{445\text{-}903}$. Twenty-four hours after transfection, nocodazole (100 μg/ml) was added for 2 hours and cells were immunostained with antibodies against NIP2 (green) and acetylated α-tubulin (Ac-tubulin, red). (E) HeLa cells with GFP or GFP-NIP2$^{445\text{-}903}$ proteins were lysed in the presence of nocodazole or taxol and then fractionated into supernatant (S) and pellet (P). β-tubulin was used as a marker for fractionation.

The association of NIP2 with microtubules was confirmed biochemically (Fig. 5E). The HeLa cells with GFP-NIP2$^{445\text{-}903}$ were lysed in the presence of nocodazole or taxol and centrifuged for fractionation into supernatant and pellet. As expected, β-tubulin was detected in the supernatant of nocodazole-treated cell lysates and in the pellet of taxol-treated cell lysates. In such conditions, most GFP-NIP2$^{445\text{-}903}$ followed β-tubulin, whereas the GFP protein stayed in the supernatant irrespective of the presence of nocodazole or taxol (Fig. 5E). Furthermore, a fraction of GFP-NIP2$^{445\text{-}903}$ in nocodazole-treated cells stayed in the pellet whereas most of the β-tubulin was detected in the supernatant. These results are consistent with the hypothesis that the microtubule network is stabilized by NIP2.

Suppression of NIP2 expression with RNAi

The knockdown experiments were carried out to provide insight into the biological functions of NIP2 (Fig. 6). When *siNIP2* or *siNek2* was transfected into HeLa cells, the cellular levels of NIP2 or Nek2 were suppressed effectively (Fig. 6A). The NIP2 signal at the centrosome was not detectable in the *NIP2*-suppressed cells, whereas the centrosome was still stained by the γ-tubulin antibody (Fig. 6B). Suppression of *NIP2* expression caused a number of changes in the cells. First, the microtubule organizing center (MTOC) of the *NIP2*-suppressed cell was not visible and the microtubule network appeared poorly organized (Fig. 6C). Second, the *NIP2*-suppresssed cells became significantly smaller as measured by the area covered by a single cell (Fig. 6C). Such reduction in cell size might be due to a poor cytoplasmic microtubule network in the *NIP2*-suppressed cells. The importance of NIP2 on the microtubule network formation was tested by the microtubule re-growth assay (Fig. 6D). When cells were placed on ice to disrupt the microtubule network and quickly transferred to a 37°C environment, the microtubule initiated growth from the MTOC (Fry et al., 1998b). Microtubule growth activity was impaired so significantly that only 10% of the *NIP2*-suppressed cells formed asters within 5 minutes (Fig. 6D). A similar impaired re-growth activity in *NIP2*-suppressed cells was also observed in cells that had been treated with nocodazole and recovered from it (data not shown). These results propose specific roles for NIP2 on microtubule nucleation.

When the *NIP2*-suppressed U2OS cells were cultured up to 48 hours after transfection, many of them were arrested at M phase with disorganized spindle (Fig. 6E). Even in interphase, a significant proportion of the *NIP2*-suppressed cells contained nuclei with abnormal morphology (Fig. 6F). These results indicate the importance of NIP2 on spindle assembly and chromosome segregation during mitosis. When the *NIP2*-suppressed cells were cultured for up to 72 hours, a significant proportion of the cells followed apoptosis, as indicated by an increase in the sub-G1 population (Fig. 6G). At the same time, an increase in proportion of the G2/M phase cells was also observed in the *NIP2*-suppressed population (Fig. 6G). The proportion of the live versus dead cells was determined by MTT assay. The results showed that only about half of the *NIP2*-suppressed cells were alive 96 hours after transfection (Fig. 6H).

Nek2 controls the amount of NIP2 in the centrosome

In previous experiments, we observed that Nek2 dissolved the cytoplasmic NIP2 aggregates into small pieces and eventually let NIP2 associate with cytoplasmic microtubules. Kinase activity was required for this process, which suggests that phosphorylation induced a conformational change in NIP2 that caused the proteins to dissociate from each other. In order to determine the effects of Nek2 on NIP2 behavior in a physiological condition, we suppressed *Nek2* expression and observed the endogenous NIP2 proteins immunocychochemically. The results showed that the NIP2 signal in the centrosome increased significantly in *Nek2*-suppressed cells (Fig. 7A). By contrast, any noticeable change in centrosomal Nek2 levels was not observed in *NIP2*-suppressed cells. These results suggest a possible role for Nek2 in the regulation of centrosomal NIP2 levels.

Augmentation of the centrosomal NIP2 signals in *Nek2*-suppressed cells could be achieved by an increase in centrosome number. To rule out this possibility, we arrested the cell cycle at the G1/S boundary with a double thymidine block and observed the relative intensity of centrosomal NIP2 in *Nek2*-suppressed cells (Fig. 7B). The results showed that the staining intensity of centrosomal NIP2 was still higher in *Nek2*-suppressed cells than in the control cells (Fig. 7B). Assuming that centrosomal duplication did not occur in the G1/S arrested cells, we concluded that there were more NIP2 proteins associated with centrosomes in *Nek2*-suppressed cells.

Since we observed an increase in centromal NIP2 levels in *Nek2*-suppressed cells, we questioned whether *Nek2* overexpression would reduce centrosomal NIP2 levels. As expected, the level of centrosomal NIP2 was reduced significantly in *Nek2*-overexpressing cells whereas it increased twofold in *Nek2*-suppressed cells (Fig. 7C). These results are consistent with the hypothesis that Nek2 activity suppresses centrosomal NIP2 levels.

Discussion

In the present study, we identified NIP2/centrobin as a novel substrate of Nek2. Immunocytochemical analyses revealed that endogenous NIP2 can associate with microtubules both inside and outside the centrosome. NIP2 binding to the microtubule was selective so that the daughter centriole was preferred for NIP2 within the centrosome (Zou et al., 2005) (Fig. 2). Transient association of NIP2 with cytoplasmic microtubules was observed only under special cellular conditions. Assuming that a pool of soluble NIP2 protein is present in the cytoplasm, it would be interesting to discover how NIP2 is targeted into a specific region of the microtubule and what functions NIP2 carries out at the site.

When centrosomal proteins are ectopically overproduced, the excess proteins frequently form cytoplasmic aggregates after saturating their own centrosomal positions. Co-expression of specific kinases sometimes dissolves the cytoplasmic aggregates into small pieces, which suggests that phosphorylation induces conformational changes in the aggregated proteins causing them to dissociate. For example, Nek2 dissolved the cytoplasmic aggregates of Nlp and C-Nap1 into small pieces (Mayor et al., 2002; Rapley et al., 2005), as well as the cytoplasmic aggregates of NIP2 (Fig. 6B). Unlike Nlp and C-Nap1, however, a significant proportion of the dissolved NIP2 associated with cytoplasmic microtubules and, furthermore, induced microtubule bundling. Induction of microtubule bundling was observed previously in cells overexpressing Cdc14B (Cho et al., 2005), EB1 (Ligon et al., 2003), dynactin (Ligon et al., 2003), Cep170 (Guarguaglini et al., 2005) and CLIP115 (Hoogenraad et al., 2000). The microtubule bundling was considered a result of microtubule stabilization as evidenced by resistance to the nocodazole treatment (Cho et al., 2005; Hoogenraad et al., 2000). Therefore, we propose that NIP2 binds to microtubules and stabilizes it. Consistent with this view, endogenous NIP2 associated with the acetylated microtubules, which is considered a marker for a stable microtubule structure. It is possible either that NIP2 itself has a microtubule-stabilizing ability or that NIP2 recruits other proteins for the stabilization.

Zou et al. observed that the knockdown of NIP2/centrobin expression resulted in centrosomes with one or no centriole, which demonstrates that NIP2/centrobin is required for

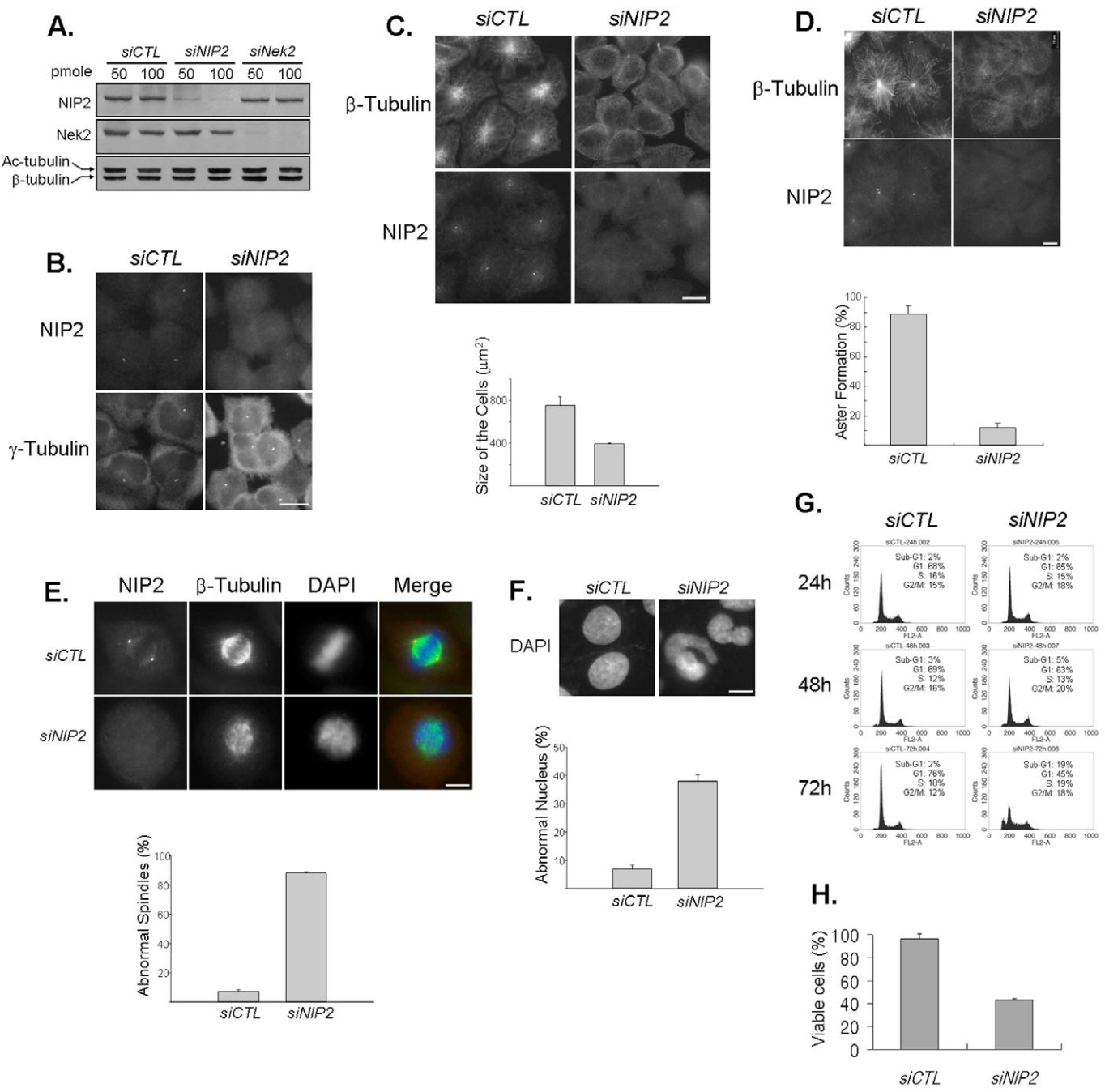

Fig. 6. Suppression of *NIP2* expression caused abnormality in spindle formation. (A) Two different amounts of siRNAs specific to *NIP2* (*siNIP2*), *Nek2* (*siNek2*) or non-specific control siRNA (*siCTL*) were transfected into HeLa cells. Forty-eight hours after transfection, immunoblot analysis was carried out to determine the cellular levels of NIP2 and Nek2 proteins. Acetylated tubulin and β-tubulin were detected as a control. (B) Twenty-four hours after transfection of *siCTL* or *siNIP2*, HeLa cells were co-immunostained with antibodies against NIP2 and γ-tubulin. (C) *NIP2*-suppressed HeLa cells were co-immunostained with antibodies against NIP2 and β-tubulin. The sizes of the cells were measured using Image-pro software and analyzed statistically. For statistical analysis of this and the following experiments, over 300 cells were counted for each group and the experiments were repeated three times. The results were presented as mean±s.e.m. (D) Microtubule re-growth assay. HeLa cells transfected with *siCTL* or *siNIP2* were placed on ice for 30 minutes to disrupt microtubules and transferred immediately to 37°C for microtubule re-growth. Five minutes after the temperature shift, cells were fixed and co-immunostained with antibodies against NIP2 and β-tubulin. (E) Forty-eight hours after transfection with *siCTL* or *siNIP2*, U2OS cells were co-immunostained with antibodies against NIP2 (red) and β-tubulin (green). The number of mitotic cells with abnormal spindles was counted. (F) The nuclei of *NIP2*-suppressed U2OS cells were stained with DAPI and the number of cells with abnormal nuclear morphology was counted. (G) HeLa cells transfected with *siCTL* or *siNIP2* were cultured for 24, 48 or 72 hours and subjected to FACS analyses to determine the DNA content of each cell population. (H) Ninety-six hours after transfection of *siCTL* or *siNIP2*, the MTT assay was carried out to determine the number of live cells. The proportion of the live cells was determined by counting the MTT-stained cells in the culture dishes.

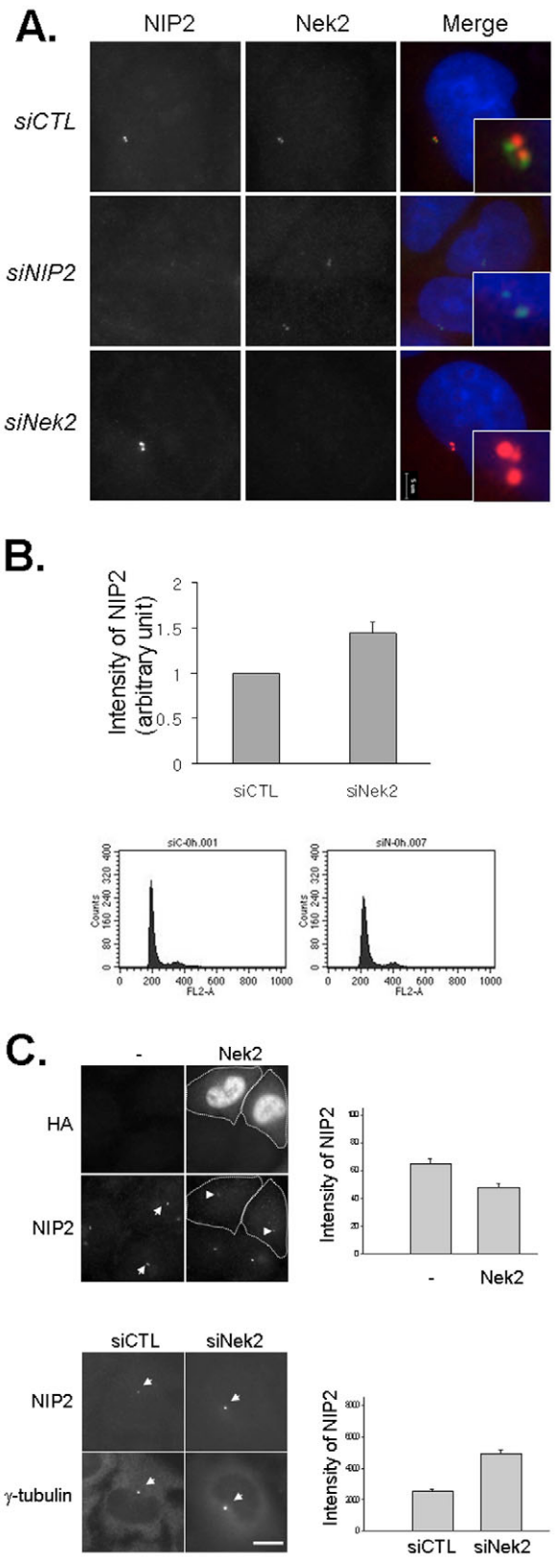

Fig. 7. Nek2 controls centrosomal NIP2 levels. (A) HeLa cells were transfected with *siCTL*, *siNIP2* or *siNek2*. Twenty-four hours after transfection, cells were co-immunostained with antibodies against NIP2 and Nek2. Insets are magnified views of the centrosomes. (B) HeLa cells were transfected with *siCTL* or *siNek2*, and their cell cycles were arrested at the G1/S boundary with a double-thymidine block. The cells were then immunostained with NIP2 antibody to determine centrosomal NIP2 levels. More than 300 cells were analyzed per group in three independent experiments. FACS histograms show synchronization of HeLa cells at the G1/S boundary. (C) The amount of cellular Nek2 increased due to overexpression of HA-tagged wild-type Nek2 (Nek2RHA1) or decreased due to transfection of *siNek2*. Twenty-four hours after transfection, cells were immunostained with antibodies against NIP2 and HA. The amount of the centrosomal NIP2 was determined by fluorescent intensity. More than 300 cells were counted in three independent experiments.

centrosome duplication (Zou et al., 2005). Considering that NIP2 stabilizes the microtubule structure, it might play a role in stabilizing the nascent centriole structure. If the cell lacks NIP2, the nascent centriole becomes unstable resulting in failure of centriole duplication. Once the centriole becomes stabilized, it may not need NIP2 activity anymore and therefore NIP2 may be released from the mother-to-be centriole. It remains to be determined whether NIP2 in the mother-to-be centriole is moved to a nascent centriole or is degraded while the newly synthesized proteins are associated with the nascent centriole.

Nucleation of microtubules is initiated by the γ-tubulin ring complex (γTuRC) probably by acting as structural templates for the minus ends of microtubules (reviewed in Job et al., 2003). The newly nucleated microtubules would be either released from the centrosome or anchored at the centrosome by binding to specific proteins such as ninein (Delgehyr et al., 2005) (reviewed in Dammermann et al., 2003). Molecular mechanisms that reside in microtubule anchoring are largely unknown but a growing list of proteins, including Nlp (Casenghi et al., 2003), GCP-WD (Luders et al., 2006), dynactin (Quintyne et al., 1999), pericentrin (Zimmerman et al., 2004), AKAP450 (Keryer et al., 2003), PCM-1 (Dammermann and Merdes, 2002), CEP135 (Ohta et al., 2002), BBS4 (Kim et al., 2004), FOP (Yan et al., 2006) and CAP350 (Yan et al., 2006), are known to be involved in this process. The centrosome in the *NIP2*-suppressed cell was visible but its biological activities appeared reduced. The knockdown phenotypes were observed both in interphase cells with a reduction in microtubule organizing activities and in mitotic cells with defects in spindle assembly. Such phenotypes have been observed typically in cells with reduced microtubule nucleation and/or anchoring activities. It remains to be investigated how NIP2 is involved in microtubule nucleation and/or anchoring. One attractive possibility is that NIP2 stabilizes the minus end structure of the newly synthesized microtubules both in interphase and mitotic cells.

Centrosomal proteins should be recruited and degraded properly, according to the physiological conditions. However, we know little of how centrosomal protein levels are controlled. Hames et al. showed that Nek2 could be transported into the centrosome in both microtubule-independent and -dependent manners (Hames et al., 2005). They revealed that PCM-1 is involved in Nek2 recruitment. In fact, PCM-1 is known to participate in the microtubule- and dynactin-dependent recruitment of proteins to the centrosome (Dammermann and Merdes, 2002). Proteasomal degradation is also considered an important regulatory mechanism in

controlling the centrosomal protein levels (Hames et al., 2005). In this study, we observed that Nek2 controls centrosomal NIP2 levels, which suggests that phosphorylated NIP2 escapes from the centrosome or is degraded preferentially. A similar regulatory mechanism has been reported, where Nlp1 is phosphorylated at G2/M phase when its centrosomal level is reduced (Casenghi et al., 2003; Rapley et al., 2005).

Involvement of Nek2 in diverse cellular functions has been reported previously (reviewed in Fry, 2002). In mouse embryos, specific suppression of *Nek2* expression resulted in mitotic arrest with defects in spindle formation and chromosome segregation, which suggests that Nek2 is crucial for spindle assembly (Sonn et al., 2004). We predict that NIP2 mediates such Nek2 functions in spindle assembly in the mouse embryo.

Materials and Methods

Antibodies

For generating polyclonal antibodies specific to NIP2, a partial coding sequence of the human NIP2 (residues 1-523) was subcloned into bacterial expression vectors, such as *pET21a* and *pGEX4T-1*. The fusion proteins were purified and injected into rabbits. The antisera were affinity-purified by incubation with a strip of nitrocellulose membrane blotted with the NIP2 fusion protein and eluted with 100 mM glycine, pH 2.5. The polyclonal C-NAP1 antibody was prepared by injection of the fusion protein with a partial human C-NAP1 sequence (residues 1984-2442) that had been used by Fry et al. (Fry et al., 1998b).

Antibodies against β-tubulin, γ-tubulin, and acetylated α-tubulin were purchased from Sigma. Antibodies against Myc, HA and GFP epitopes were purchased from Cell Signaling, Babco and Santa Cruz Biotechnology, respectively. The monoclonal Nek2 antibody was purchased from Transduction Laboratories. The polyclonal Nek2 antibody was described previously (Rhee and Wolgemuth, 1997).

Yeast two-hybrid screening

The yeast two-hybrid screening was carried out as described previously (Yoo et al., 2002). In brief, *pGBT9/Nek2*, in which the full-length mouse *Nek2* open reading frame (ORF) had been linked to the GAL4 DNA-binding domain sequence, was transformed into the HF7c yeast strain along with the human liver library. Positive clones were selected using the β-galactosidase activity assay among surviving colonies cultured in SD synthetic medium lacking leucine, tryptophan and histidine. False positive colonies were removed following a standard protocol. For identification of the isolated clones, nucleotide sequences were determined.

Cell culture and treatment

HeLa, U2OS and 293T cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Microtubule organizations in HeLa cells were disrupted with 10 μg/ml nocodazole or 1 μM taxol. The HeLa cells and U2OS cells were treated with 2.5 mM thymidine or 4 mM hydroxyurea for S phase arrest and with 100 ng/ml nocodazole or 1 μM taxol for G2/M phase arrest.

Transfection and immunocytochemistry

Transient transfection into 293T or HeLa cells was carried out using Lipofectamine Plus (Invitrogen) following the manufacturer's instruction. For immunocytochemistry, 4×10^4 cells were seeded into a 4-well dish and transfected 24 hours later. One or two days after transfection, cells were fixed either with cold methanol for 10 minutes or with 3.7% paraformaldehyde for 10 minutes. The paraformaldehyde-fixed cells were permeabilized with 0.5% PBST (phosphate-buffered saline with 0.5% Triton X-100), whereas the methanol-fixed cells skipped the permeabilization step. The fixed cells were blocked with 10% normal goat serum in 0.1% PBST (PBS with 0.1% Triton X-100) for 10 minutes, incubated with the primary antibodies for 1 hour, washed with 0.1% PBST three times, incubated with either FITC- or TRITC-conjugated secondary antibody (Jackson Immunoresearch) for 30 minutes, washed with 0.1% PBST three times, incubated with a DAPI solution, and observed using a fluorescence microscope with CCD (Qicam fast 1394, Qimaging) camera. The data were quantitated with Image-pro software and statistically analyzed with Sigma plot.

Immunoprecipitation and immunoblot

Immunoprecipitation and immunoblot analyses were carried out as described previously (Yoo et al., 2004). In brief, 2×10^6 cells were seeded onto a 60 mm dish and transfected a day later. Twenty-four hours after transfection, the cells were lysed with the RIPA (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 1% NP40, and 1 mM EDTA) or the NP40 buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1% NP40, 1 mM EDTA) with protease inhibitors for 20 minutes on ice and centrifuged with 15,000 ***g*** for 15 minutes at 4°C. For immunoprecipitation, the supernatant was incubated with specific antibodies for 2 hours, followed by protein A Sepharose (Amersham Pharmacia) for 1 hour at 4°C. The immunoprecipitates were immunoblotted with indicated antibodies as described.

In vitro kinase assay

The Nek2 kinase assay was carried out as described previously with a slight modification (Helps et al., 2000). In brief, 293T cells transfected with either wild-type Nek2 (*pNek2RHA1*) or kinase-defective Nek2 (*pNek2KHA5*) expression vectors were lysed with NP40 lysis buffer and subjected to immunoprecipitation with an antibody against the HA tag. The immunoprecipitates were washed three times with lysis buffer and once with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 1 g/ml heparin). Kinase reactions were carried out for 20 minutes at 30°C in kinase buffer supplemented with 5 μM ATP, 1 mM dithiothreitol and 5 μCi [γ-^{32}P]ATP in a total volume of 20 μl. The NIP2 substrates were prepared from bacterially expressed fusion proteins or from immunoprecipitates of the ectopically expressed 293T cells. The reactions were stopped by adding 2×SDS sample buffer and heating for 5 minutes at 95°C. Protein samples were resolved with 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was exposed to the X-ray film.

In vivo labeling assay

The in vivo labeling assay was carried out following the protocol described by Mayor et al. (Mayor et al., 2002). In brief, HeLa cells were incubated for 3 hours in a phosphate-free DMEM medium supplemented with 10% dialyzed FBS and 250 μCi/ml of [^{32}P] orthophosphate. Cells were collected and lysed with the RIPA buffer. The lysates were subjected to immunoprecipitation followed by immunoblotting and autoradiogram.

Microtubule fractionation

Microtubule fractionation was performed as described previously (Nadano et al., 2002). For the microtubule-depolymerizing conditions, microtubules were depolymerized using 10 μM nocodazole for 1 hour. The cells were then washed twice with PBS and lysed with TNE buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% NP40, 1 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin) for 3 minutes at 4°C. The cells were then centrifuged at 15,000 ***g*** for 5 minutes at 4°C. For the microtubule-stabilizing conditions, cells were incubated with 1 μM taxol for 1 hour, washed twice with 100 PEM (100 mM Pipes, pH 6.6, 1 mM EGTA, 1 mM Mg_2SO_4) and were lysed for 1 minute at room temperature with 100 PEM containing 1% NP40, 20 μM taxol, 1 mM GTP, 1 mM PMSF and 10 μg/ml aprotinin. The lysate was then centrifuged at 15,000 ***g*** for 5 minutes at room temperature. The supernatant thus obtained was clarified by centrifugation at 100,000 ***g*** for 60 minutes at 4°C. The precipitates were washed twice with the lysis buffer. These fractions were then subjected to immunoblot analysis.

RNA interference

NIP2 siRNA (AAGGATGGTTCTAAGCATATC) and control siRNA (AAGTAGCCGAGCTTCGATTGC) were transfected into HeLa or U2OS cells using Lipofectamine 2000 or oligofectamine (Invitrogen) following the manufacturer's instructions.

MTT assay

SiRNA-transfected HeLa cells were subjected to the MTT assay. 50 μl MTT solution (2 mg/ml 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide in PBS) was added to cells. Three hours later, media were discarded and reduced crystals of MTT were solubilized with 500 μl of isopropanol/0.04 N HCl. Optical density at 570 nm with background subtraction at 650 nm was read with a spectrophotometer.

Flow cytometry

Cells were trypsinized and suspended in 300 μl of PBS. 700 μl of cold ethanol were added dropwise and then removed by centrifugation. Pelleted cells were resuspended in PBS containing 100 μg/ml RNase A and incubated at 37°C for 30 minutes. Propidium iodide was added 10 μg/ml in final. Stained cells were loaded to FACScalibur (Beckton Dickinson) and analyzed with Cell Quest pro software.

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