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# Nek2 Localizes to Multiple Sites in Mitotic Cells, Suggesting Its Involvement in Multiple Cellular Functions during the Cell Cycle

Yong Ha Kim,* Jun Yeol Choi,* Yeontae Jeong,* Debra J. Wolgemuth,† and Kunsoo Rhee*,[1]

**School of Biological Sciences, Seoul National University, Seoul 151-742, Korea; and †Department of Genetics and Development and Department of Obstetrics and Gynecology, Columbia University College of Physicians and Surgeons, New York, New York 10032*

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**Nek2 is a mammalian protein kinase that is structurally homologous to NIMA, a mitotic regulator in *Aspergillus nidulans.* To understand the possible cellular processes in which Nek2 participates during the cell cycle, we investigated the expression and subcellular localization of Nek2 in mitotic cells. The Nek2 protein levels were observed to be regulated in a cell cycle stage-specific manner in cultured cells. The cell cycle stage specificity of Nek2 expression was also confirmed in cells undergoing mitosis *in vivo.* Nek2 proteins were localized in both the nucleus and cytoplasm throughout the cell cycle, but exhibited dynamic changes in distribution, depending on the cell cycle stage. Nek2 was associated with chromosomes from prophase to metaphase and then was dissociated upon entering into anaphase. Nek2 then appeared at the midbody of the cytoplasmic bridge at telophase. Nek2 was also associated with the centrosome throughout the cell cycle as observed previously by others. Additionally, the nuclear localization of Nek2 was increased during S phase. Such dynamic behavior of Nek2 suggests that Nek2 may be a mitotic regulator that is involved in diverse cell cycle events.** 

***Key Words:*** **Nek2; cell cycle; centrosome; chromosome; mid-body; mitotic exit.**

A number of protein kinases are known to play critical roles in executing cell cycle events, in addition to the cyclin-dependent kinases. NIMA is a serine/threonine kinase that functions as a mitotic regulator in *Aspergillus nidulans* (1). Loss-of-function mutations in the *nimA* gene arrested the cell cycle at G2 phase, while overexpression of *nimA* induced a premature mitosis like chromosome condensation and bipolar spindle formation (2, 3). Based on these observations, it was proposed that NIMA plays a critical role in progression of M phase by controlling chromosome condensation/decondensation. Recently, it was reported that NIMA might help promote chromosome condensation through histone H3 phosphorylation (4). Ectopic expression of *nimA* can induce premature mitotic events in yeasts, frog or human cells as like in *Aspergillus*, suggesting that NIMA-like regulatory mechanisms may be present in other eukaryotes (5, 6).

So far, 7 mammalian genes have been known to share structural similarity with *Aspergillus nimA*, and named *Nek*s (7–12). Among them, Nek2 has been studied most extensively. Similarity between Nek2 and NIMA was not limited to their structures but it extended to their cell cycle stage-specific expression and substrate specificity in kinase assays *in vitro* (13). In the mouse testis, *Nek2* was expressed predominantly in spermatocytes that are essentially at the G2/M phase of meiosis (14). Moreover, Nek2 was associated with meiotic chromosome and its activity increased with induction of chromosome condensation (14). These results suggested that, like *Aspergillus* NIMA, Nek2 might be involved in chromosome condensation in mammalian meiotic cells.

A somewhat unexpected role for Nek2 in centrosome function has been demonstrated by Fry *et al.* (15), who showed that the human Nek2 protein was associated with centrosomes in cultured cells. Overexpression of *Nek2* induced a splitting of centrosomes, whereas prolonged expression of *Nek2* led to dispersal of centrosomal material and loss of a focused microtubule-nucleating activity (15). Centrosomal localization of Nek2 was also observed in *Xenopus* early embryos (16, 17). Specific inhibition of Nek2 expression did not interfere with the mitotic cell cycle; however, it did cause abortive cleavage of early embryos, in which bipolar spindle formation was severely impaired due to fragmentation or dispersal of the centrosomes (16, 17). Based on these

[1] To whom correspondence and reprint requests should be addressed. Fax: 82-2-872-1993. E-mail: rheek@snu.ac.kr.

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results, it was proposed that Nek2 is specifically required for centrosome assembly and/or maintenance (16, 17). A centrosomal protein has also been identified as a Nek2-associated protein (C-Nap1) (18). When the function of C-Nap1 was interfered with by antibody injection, centrosome splitting was induced (19). These results support the notion that C-Nap1 is a key component of a dynamic, cell cycle-regulated structure that mediates centriole–centriole cohesion and further implicates a role in centrosome function for Nek2 (19).

In the present study, we asked if Nek2 is involved in other cell cycle events in mitotic cells, in addition to its function in the centrosome, and whether it may associate with mitotic chromosomes as well as the previously demonstrated meiotic chromosomes. To address this question, we investigated the expression and subcellular localization of Nek2 in cultured mitotic cells and in actively dividing cells *in vivo*. Our observations revealed that the Nek2 protein was localized in multiple sites within a cell in a cell cycle stage-specific manner, suggesting that Nek2 may be involved in diverse cell cycle events, in addition to the centrosomal cycle.

## EXPERIMENTAL PROCEDURES

*Eukaryote expression vectors, cell culture, and transfection.* The open reading frame of the mouse Nek2 cDNA was tagged with the hemagglutinin (HA) epitope at the C-terminal end (*Nek2RHA1*) and subcloned into the *pLNCX* vector. We also generated a fusion gene in which the *Nek2* cDNA was linked to the *CMV* promoter (*pCMV/Nek2*).

Cells were grown at 37°C in a 10% $CO_2$ atmosphere in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (293T and BOSC cells) or 10% calf serum (Ratb1a and NIH3T3 cells).

To make stably transfected cell lines, we followed the viral infection method (20). In brief, the *pLNCX* viral vectors were transfected into the BOSC cells with the calcium phosphate method. Two days after transfection, the media that contained packaged viral particles were collected and added onto Ratb1a cells or NIH3T3 cells. Stably transfected cell populations were selected with G418 (500 $\mu$g/ml) for 2 weeks.

*Cell cycle synchronization.* The cell cycle was synchronized with two sequential thymidine blocks. In brief, cells were seeded in a concentration of $2 \times 10^5$ cells/100-mm dish. Twelve hours later, thymidine was added to give a final concentration of 2 mM and then was incubated for 16 h. The cells were washed with medium twice and incubated in fresh medium for 6–8 h to allow the cells to exit from the S phase. The second addition of thymidine had been prolonged for 12 h until a fresh medium was added to resume the cell cycle in a synchronized manner. The cells were harvested or fixed at indicated time periods after the release and used for specific experiments. The synchronization of the cell cycle was assessed with FACS (fluorescein-activated cell sorting) analysis.

*Nuclear and cytosolic fractionation.* For fractionation of the nuclear and cytosolic parts, cells were washed with PBS, suspended in 500 $\mu$l of a lysis buffer (10 mM Tris–HCl, pH 7.8, 1% NP-40, 10 mM $\beta$-mercaptoethanol, 0.5 mM PMSF, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, and 4 $\mu$g/ml calpain inhibitor), and incubated on ice for 3–5 min. The cell lysates were diluted with the equal volume of $H_2O$, incubated on ice for 3 min, passaged into the 22-gauge needle for 10 times to isolate nuclei, and centrifuged at 400*g* for 6 min. The nuclear pellet was suspended in 500 $\mu$l of a washing buffer (10 mM Tris–HCl, pH 7.4, 2 mM $MgCl_2$, protease inhibitors), centrifuged at 400*g* for 6 min, and suspended in the 2× SDS–PAGE sample buffer (100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, and 20% glycerol). The cytosolic supernatant was mixed with the 5× SDS–PAGE sample buffer (250 mM Tris–HCl, pH 6.8, 500 mM dithiothreitol, 10% SDS, and 50% glycerol).

*Immunoblot analysis.* The immunoblot analysis was carried out as described previously (14). In brief, cell lysates were separated by 10% SDS–PAGE and transferred onto PVDF membranes. The membrane was blocked with Blotto (5% skim milk in 0.1% TBST) at room temperature for 1 h. After blocking, the membrane was incubated with a primary antibody at room temperature for 1–3 h, washed with 0.1% TBST 3 times for 10 min each, incubated with a secondary antibody (alkaline phosphatase-conjugated IgG or HRP-conjugated IgG, 1:5,000) for 30 min, and washed again with 0.1% TBST 3 times for 10 min each. Specific signals were detected with NBT/BCIP color reaction or with ECL reaction. The affinity-purified anti-Nek2 (14) was diluted to 1:10 or 1:50, the HA antibody (Berkeley Antibody Company) was diluted to 1:100, the $\beta$-tubulin antibody (Sigma) was diluted to 1:200, and the lamin B antibody (Santa Cruz Biotechnology) was diluted to 1:100.

*Immunocytochemistry.* NIH3T3 cells were rinsed with PBS, fixed with a −20°C cold methanol or with 3.5% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.5% Triton X-100 for 10 min, and incubated with the primary antibodies in PBS with 3% BSA for 1 h. The samples were washed 3 times with PBS, incubated with secondary antibodies that had been conjugated with FITC or TRITC, mounted with a mounting solution that contained DAPI, and observed with a fluorescence microscope (Zeiss). The affinity-purified Nek2 antibody was diluted to 1:50, the monoclonal $\gamma$-tubulin antibody (Sigma) was diluted to 1:100 and the FITC- or TRITC-conjugated secondary antibodies were diluted to 1:100. For detection of BrdU-incorporated nuclei, the samples were permeabilized, treated with 2 M HCl for 20 min, and then neutralized with borate buffer (pH 8.5) for 10 min. The anti-BrdU antibody (Boehringer Mannheim) was diluted to 1:33.

*Immunohistochemical analysis of the ovarian tissues.* Immature female mice were injected with PMSG (5 IU/mouse, Sigma). The next day, the mice were injected with BrdU (50 mg/kg, Sigma) for the indicated time periods and the ovaries were collected. The ovarian tissues were fixed, embedded in paraffin, sectioned and analyzed immunohistochemically as described previously (14). Specific signals of antibody localization were detected with DAB staining method or with indirect immunofluorescence detection method.

## RESULTS

### *Regulation of the Cell Cycle Stage-Specific Expression of Nek2*

In previous studies, a cell cycle stage-specific expression of the endogenous *Nek2* gene was reported in HeLa cells synchronized by a drug treatment or by elutriation (8, 13). In the present study, we report the S/G2 phase-specific expression of an exogenous *Nek2* gene under control of the *CMV* promoter. The cell cycle of the Ratb1a fibroblasts stably transfected with the *pCMV/Nek2* vector was synchronized with the double thymidine block method. The FACS analysis revealed that majority of the cells reached at the G2 phase in 6 h and a new round of the G1 phase in 10 h after release from the second thymidine block (Fig. 1). The results showed that the Nek2 protein levels were higher in cell

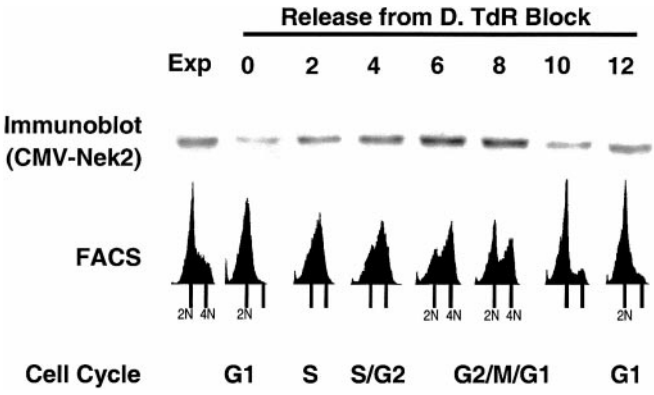


**FIG. 1.** Cell cycle stage-specific expression of the *pCMV/Nek2* vector. Nek2 protein levels were determined in Ratb1a fibroblastic cells that had been transfected stably with the *pCMV/Nek2* vector. The cell cycle was synchronized with the double thymidine block method and determined by the FACS analysis. Immunoblot analysis was carried out to determine Nek2 protein levels at indicated time points after the release of the cell cycle block.

populations at S/G2 phase than those at G1 phase (Fig. 1), indicating the S/G2 phase-specific expression of *Nek2*. It was believed that the most of the Nek2 proteins detected were contributed from the transfected vector rather than from the endogenous gene, because the levels of the endogenous Nek2 protein in the Ratb1a cells was much lower than those of the exogenous Nek2 protein (data not shown). Therefore, it is likely that the cell cycle-specific expression of *Nek2* may involve more than regulation at the transcriptional level.

### *Localization of Nek2 in Cultured Cells*

We then investigated the subcellular localization of Nek2 protein in mitotically dividing cells in culture. Immunoblot analyses were carried out with nuclear and cytosolic fractions of NIH3T3 cells stably transfected with the *Nek2* constructs. Antibodies against lamin B and $\beta$-tubulin were used to confirm the nuclear and cytosolic fractions, respectively. The exogenous Nek2 proteins were detected in both nuclear and cytosolic fractions (Fig. 2A). Nuclear and cytoplasmic localization of exogenous Nek2 was also confirmed with the immunostaining method. When we immunostained the NIH3T3 cells transfected with the *Nek2RHA1* construct (wild type) with antibodies against Nek2 and HA epitope, we observed both nuclear and cytoplasmic localization of the exogenous Nek2 protein (Nek2RHA1) in most cells (Fig. 2B).

Even if we failed to detect the endogenous Nek2 protein in NIH3T3 with the immunoblot method, we were sure that the Nek2 antibody could detect the endogenous protein specifically in the immunostaining method. First, the Nek2 antibody detected the exogenous Nek2 proteins specifically as in Fig. 2B. Second, the Nek2 fusion protein blocked the immunostaining with the Nek2 antibody (Fig. 3B). Third, when we stained the cells with the pre-immune serum or with secondary antibody only as controls, we observed no specific signal as with Nek2 antibody, suggesting that the staining with the Nek2 antibody was specific (data not shown). When subcellular localization of the endogenous Nek2 protein was investigated immunocyto-

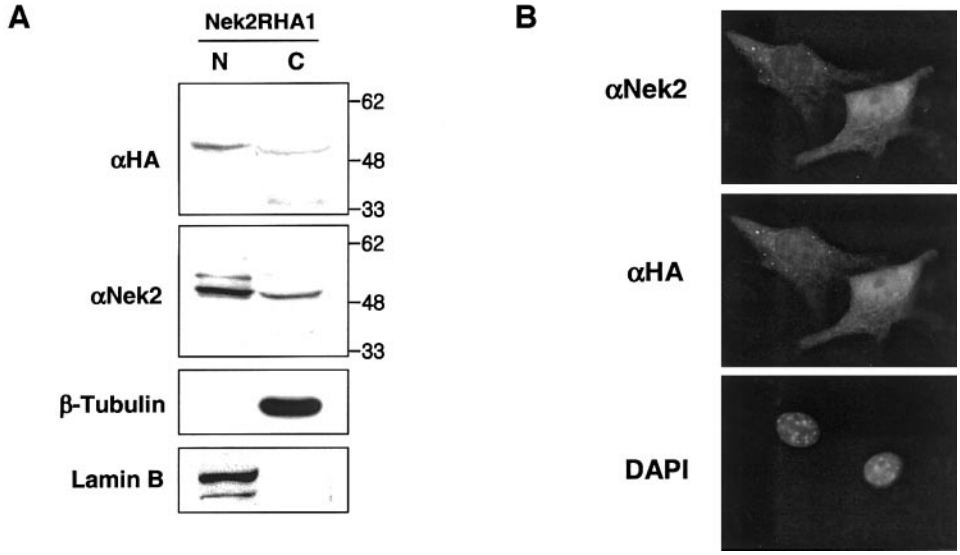


**FIG. 2.** Localization of overexpressed Nek2 protein in NIH3T3 cells. (A) NIH3T3 cells that had been transfected stably with the *Nek2RHA1* construct (wild type) were fractionated into the nuclear and cytosolic parts. Localization of the Nek2 proteins was determined by immunoblot analysis with antibodies against Nek2 and HA epitope. Lamin B and $\beta$-tubulin were measured to confirm nuclear and cytosolic fractions, respectively. (B) The NIH3T3 cells were transfected transiently with the *Nek2RHA1* construct and immunostained with antibodies against Nek2 and HA epitope. The nucleus was stained with DAPI.

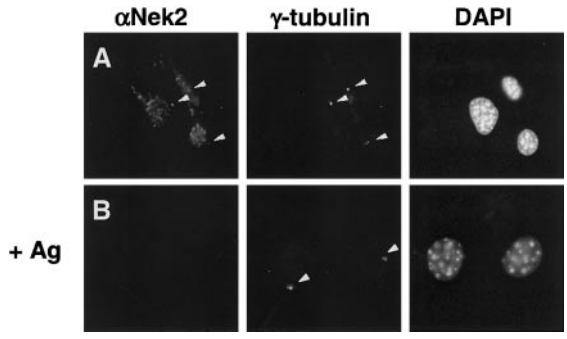


**FIG. 3.** Immunocytochemical analysis of the endogenous Nek2 protein in NIH3T3 cells. NIH3T3 cells were immunostained with Nek2 antibody in the absence (A) or presence (B) of the specific antigen in the incubation mixture. Centrosome was immunostained with the γ-tubulin antibody (arrowheads). The nucleus was stained with DAPI.

chemically, we observed that the Nek2 antibody stained centrosomes (Fig. 3A), in agreement with previous reports (15). However, in addition to centrosomes, the Nek2 antibody also stained nuclei with a punctuate pattern (Fig. 3A). This nuclear staining pattern was comparable to that observed previously in mouse germ cells (14).

### *Dynamic Changes of Subcellular Localization of Nek2 Protein during the Cell Cycle*

In the experiments shown in Fig. 3A, we observed that the immunostaining intensity and pattern with the Nek2 antibody looked different in unsynchronized population of cultured cells. These observations allowed us to postulate that the subcellular localization of the Nek2 protein might change during the cell cycle. To test this possibility, we carried out immunocytochemical analysis with NIH3T3 cells synchronized with the double thymidine block method (Fig. 4). BrdU incorporation was measured simultaneously to determine the cell cycle stages of the synchronized cell population. Two hours after release, most cells entered S phase and five hours later, most cells exited from the S phase and entered into G2 phase. As shown in Fig. 4, Nek2 protein was localized both in the nucleus and cytoplasm, irrespective of the cell cycle stage. However, we observed repeatedly that the nuclear Nek2 protein levels in S phase cells (4 h after the release) appeared higher compared to cells in G2 phase (5 h after the release). These results showed that the predominant subcellular localization of Nek2 protein varied according to the stage of the cell cycle, and suggested that Nek2 may have a specific role in the nucleus during S phase.

The subcellular localization of Nek2 protein was observed in cells at mitosis (Fig. 5). As reported previously (15), Nek2 was detected in the centrosome throughout mitosis. However, using our Nek2 antibody, we also observed Nek2 localization to condensed chromosomes in cells at the prophase and metaphase. Once cells entered anaphase, the immunostaining at the chromosome dispersed. At telophase, Nek2 antibody characteristically stained the midbody within the cytokinetic bridge of two daughter cells.

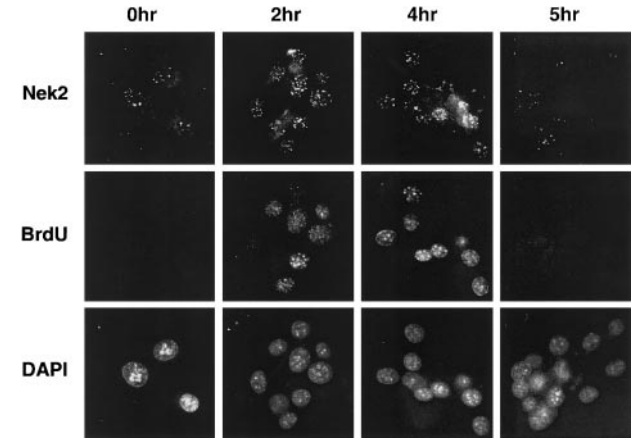


**FIG. 4.** Subcellular localization of Nek2 protein at S/G2 phase of the cell cycle. NIH3T3 cells were synchronized with the double thymidine block method. BrdU was added into the culture medium 1 h ahead of the indicated time points. The cells were fixed with the cold methanol and co-immunostained with the Nek2 and BrdU antibodies.

### *Cell Cycle Stage-Specific Expression of Nek2 in the Mouse Ovarian Follicle Cells*

To ask if the cell cycle stage-specific expression of *Nek2* was a general characteristic of most dividing cells beyond cultured cell systems, we turned to a physio-

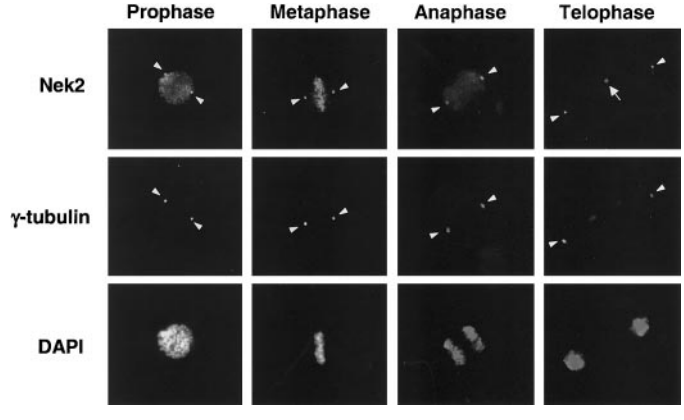


**FIG. 5.** Dynamic localization of Nek2 protein during mitosis. The NIH3T3 cells were fixed with paraformaldehyde and immunostained with antibodies against Nek2 and γ-tubulin. The nucleus was stained with DAPI. Representative cells at each stage of the mitosis were shown: prophase, metaphase, anaphase, and telophase. The Nek2 protein was localized in selected sites at specific stages of the mitosis, such as centrosomes (arrowheads), chromosomes, and the midbody (arrow).

logically dynamic *in vivo* system. We chose the mouse ovarian follicle cells as a model system, for the following reasons. First, *Nek2* was expressed abundantly in ovarian follicle cells. When ovarian sections were analyzed immunohistochemically, a subset of ovarian follicle cells were intensely stained with the Nek2 antibody (Fig. 6C). Second, it is well documented that ovarian follicle cells divide very actively in response to proper hormonal signals, such as pregnant mare serum gonadotropin (PMSG) (21).

The expression and localization of Nek2 proteins in the mouse ovarian follicle cells was determined with immunoblot analysis. The Nek2 protein was detected in both the nuclear and cytosolic fractions of the follicle cell (Fig. 6A). To confirm the proliferative activity of the ovarian follicle cells, we measured the proportion of follicle cells in which BrdU was incorporated after hormonal stimulation (Fig. 6B). About 20% of the ovarian follicle cells were labeled within 2 h after injection of BrdU and most of the cells were positive for BrdU within 24 h after injection (Fig. 6B), indicating that the ovarian follicle cells proliferate actively.

We then asked if *Nek2* was expressed at a specific stage of the ovarian follicle cell cycle. To determine the cell cycle stage-specific expression of *Nek2*, ovarian sections were prepared from mice in which BrdU had been injected for indicated time periods and co-immunostained with antibodies against Nek2 and BrdU. The results showed that the Nek2 antibody immunostained about 10% of the ovarian follicle cells (Fig. 6C). Interestingly, most of the Nek2-positive cells were also immunostained with the BrdU antibody in mice treated with BrdU for 2 h. These results indicate that the levels of the Nek2 protein in the ovarian follicle cells fluctuated during the cell cycle, and were most abundant at around the S phase.

## DISCUSSION

The results presented in this study revealed a dynamic expression and subcellular distribution of Nek2 during the cell cycle. Nek2 proteins were localized in both the nucleus and cytoplasm throughout the cell cycle; however, the nuclear distribution of Nek2 was particularly abundant in cells at S phase. An association of Nek2 with chromosomes was observed when cells entered into mitosis and its association was maintained until the end of metaphase. Once the cell entered anaphase, Nek2 was dissociated from the condensed chromosome and re-distributed into the whole mitotic matrix. Distinct localization of Nek2 to the midbody appeared in cells at telophase. Nek2 was also associated with the centrosome throughout the cell cycle as reported previously (15). This diverse and dynamic behavior of Nek2 suggests that Nek2 may be a mitotic regulator involved in diverse cell cycle events.

Dynamic subcellular localization was also observed in *Aspergillus* NIMA (4). At mitosis, NIMA becomes enriched on chromatin and subsequently localizes to the mitotic spindle and spindle pole bodies, suggesting its functional roles at these sites, in addition to chromosomes (4). Therefore, it is likely that homology between Nek2 and NIMA extends from structure to expression, biochemical properties, subcellular localization, and even to cellular functions.

This diverse distribution of Nek2 within cells is not surprising. In fact, Fry *et al.* (15) estimated that only about one tenth of the endogenous Nek2 was associated with the centrosome. Therefore, the rest of the Nek2 protein was expected to be located elsewhere within a cell. However, the previous study limited the localization of the Nek2 protein to the centrosome (15, 17). We could consider three possible differences between our work and the previous reports. First, the different antibodies used in the studies might recognize different sets of epitopes within the Nek2 protein and resulted in slightly different staining patterns. Second, difference in the experimental methods might produce somewhat different staining patterns. In support of this notion, we observed that fixation with cold methanol produced clear nuclear staining, including the chromosomes, whereas fixation with paraformaldehyde yielded cytoplasmic staining, including the centrosomes. There may be other differences in the experimental procedures that might affect immunostaining patterns. Finally, we cannot rule out the possibility of species- or cell type-specific distribution of Nek2.

It has been known that other mitotic kinases showed dynamic distributions during M phase progression (22, 23). For example, polo-like kinases (Plks) are localized at the centrosome from prophase to anaphase. Plks are also present in the centromeres in cells from prophase to anaphase. At the beginning of anaphase, Plks are no longer detected in the centromere. Instead, Plks accumulates at the central spindle and in the midbody of the telophase cells after the centrosomal staining is lost (22). These multiple localizations are in keeping with the observation that Plks influence multiple events during the cell cycle, including modulation of Cdc2 activity, centrosome and spindle maturation and function, chromosome segregation, regulation of anaphase-promoting complex activity and execution of cytokinesis (22, 24).

Another example of multiple roles can be found in a group of proteins named “chromosome passengers” (23). To date, four chromosomal passenger proteins have been described in detail: inner centromere protein (INCENP) (25), TD-60 (26), aurora B (27), and survivin (28, 29). These proteins associate with chromosomes along their length during prophase, becoming concentrated at the inner centromere by metaphase. Upon the transition to anaphase, the passengers abruptly transfer to the central region of the mitotic spindle and to

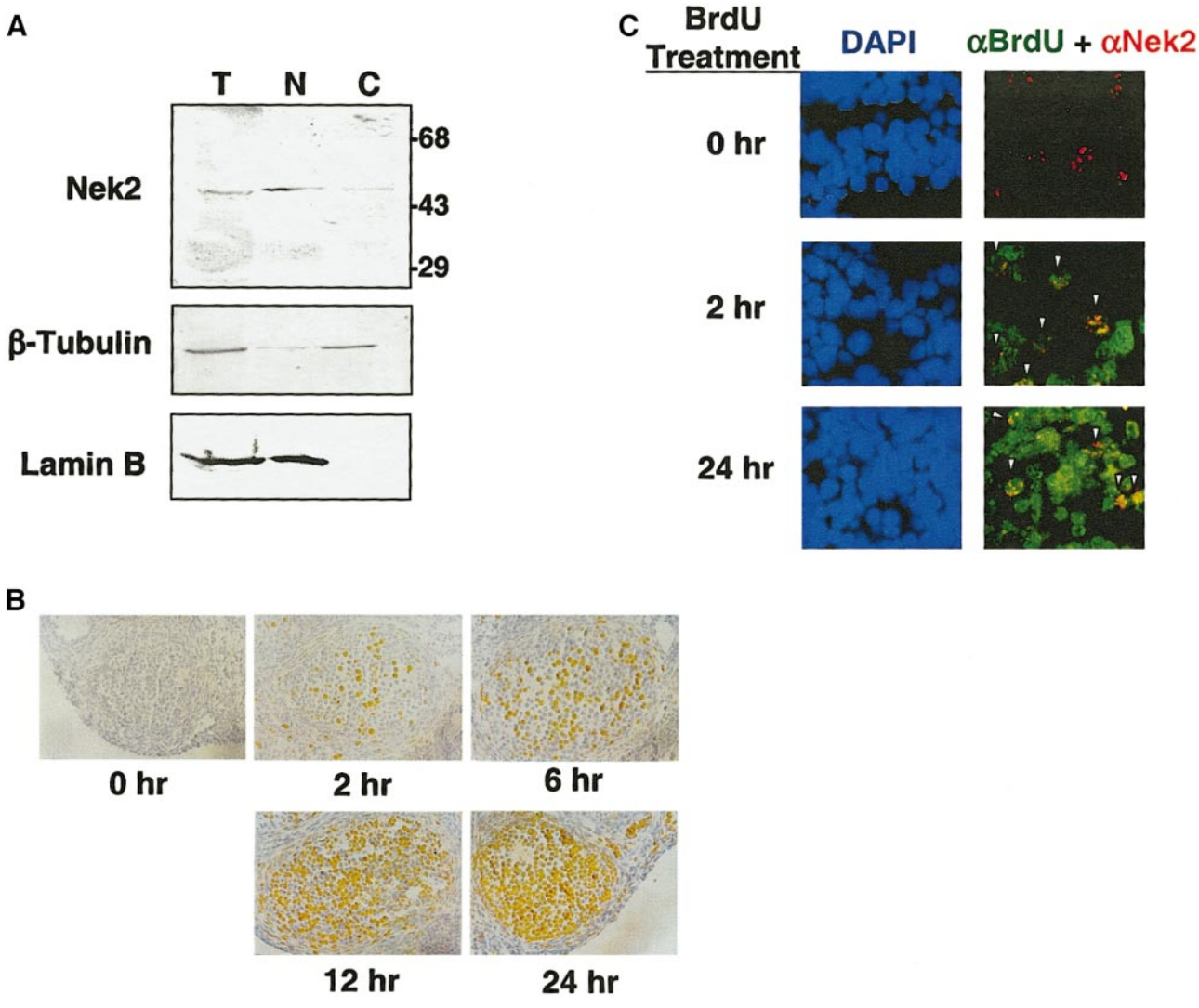


**FIG. 6.** Localization of endogenous Nek2 in the nuclear and cytosolic fractions and cell cycle stage-specific expression of Nek2 protein in mouse ovarian follicle cells of the ovarian follicle cells. (A) Ovarian follicular cells were fractionated into nuclear and cytosolic parts. Localization of Nek2 was determined by immunoblot analysis. Antibodies against lamin B and $\beta$-tubulin were used to confirm the nuclear and cytosolic fractions, respectively. (B) The proliferative activity of mouse ovarian follicle cells was determined. Ovary samples were isolated from mice that had been injected with BrdU for 0, 2, 6, 12, or 24 h. Cells that were in the S phase during the injection periods were detected with BrdU antibody. Most nuclei of the follicle cells that had been treated with BrdU for 24 h appeared labeled. (C) Coimmunostaining of mouse ovarian follicle cells with antibodies against Nek2 and BrdU. Ovarian sections were prepared from mice into which BrdU had been injected for 0, 2, or 24 h, and immunostained with antibodies against Nek2 (red) and BrdU (green). Nuclei were stained with DAPI (blue). Most of the Nek2-positive cells were colabeled with the BrdU antibody after 2-h treatment (arrowheads).

the cell cortex in the region where the contractile ring will form. Based on this behavior, it was suggested that these proteins might have chromosomal functions during early mitosis, but then perform essential cytoskeletal functions during anaphase and telophase (23).

The dynamic distribution of the Nek2 protein in mitotic cells both *in vitro* and *in vivo,* observed in the present study, allowed us to propose that Nek2 may be involved in diverse cellular processes in the mitotic cells. To identify these processes, determining the Nek2 partner proteins will be helpful. Attractive candidates that remain to be tested include the aurora kinase or INCENP. There may be novel Nek2 interacting proteins in other subcellular compartments as well. Recently, we identified a novel Nek2 interacting protein, NIP1, which is localized in the ER/Golgi, suggesting that Nek2 may be involved in the ER/Golgi inheritance during the mitosis (J.C.Y., S.K.J., D.J.W., K.K., K.R., submitted).

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