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Involvement of Nek2 in Mammalian Development as a Cell Cycle Regulator

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Nek2 is a mammalian protein kinase that is structurally homologous to NIMA, a mitotic regulator in *Aspergillus nidulans*. To understand cellular processes in which Nek2 participates during mammalian development, we investigated the expression and subcellular localization of Nek2 in vivo. The Nek2 protein was detected in spermatocytes and in a fraction of actively dividing ovarian follicle cells and of embryonic tissues. We also observed that Nek2 was localized in both the nucleus and centrosome in embryonic cells. Such localization pattern supports the proposal that Nek2 is a mitotic regulator that is involved in multiple cell cycle events during mammalian development.

During development of multicellular organisms, proliferation is an essential process along with differentiation. Proliferation should be controlled in close coordination with differentiation steps. Problems in controlling mechanisms of cell proliferation can result in embryonic lethality or birth defects. Therefore, the cell cycle machinery that controls cell proliferation should be linked tightly to the developmental program.

It is well known that cyclin-dependent kinases (Cdks) are important for overcoming checkpoints of cell cycle. However, a number of other protein kinases are also known to play critical roles in executing cell cycle events. NIMA is a serine/threonine kinase that functions as a mitotic regulator in *Aspergillus nidulans* (reviewed in Osmani and Ye, 1996). Loss-of-function mutations in the *nimA* gene caused cell cycle arrest at G2 phase, while overexpression of *nimA* induced premature mitosis exhibiting chromosome condensation and bipolar spindle formation (Osmani et al., 1988, 1991). Based on these observations, it was proposed that NIMA plays a critical role in progression of the M phase by controlling chromosome condensation/decondensation. Recently, it was reported that NIMA might help promote chromosome condensation through histone H3 phosphorylation (De Souza et al., 2000). Ectopic expression of *nimA* can induce premature mitotic events in yeasts, frog or human cells as in *Aspergillus*, suggesting that NIMA-like regulatory mechanisms may be present in other eukaryotes (O'Connell et al., 1994; Lu and Hunter, 1995).

So far seven mammalian genes have been known to share structural similarity with *Aspergillus nimA*, and have been named *Nek*s (Letwin et al., 1992; Schultz et al., 1994; Tanaka et al., 1999; Chen et al., 1999; Li et al., 1999; Kandli et al., 2000). Among them, Nek2 has been studied most extensively. Similarity between Nek2 and NIMA was not limited to their structures but extended to their cell cycle stage-specific expression and substrate specificity in kinase assays in vitro (Fry et al., 1995). We previously observed that *Nek2* was expressed in the mouse testis, most predominantly in spermatocytes (Rhee and Wolgemuth, 1997). These results suggested that, like *Aspergillus* NIMA, Nek2 might function as a cell cycle regulator in mammalian cells.

In the present study, we asked if Nek2 is involved in cell cycle events during development. To begin to address this question, we studied the expression and subcellular localization of Nek2 in actively dividing cells in vivo. Our observation revealed that the Nek2 protein was present in a fraction of actively dividing ovarian follicle cells and of embryonic cells. Nek2 was localized in multiple sites within the cell. These results suggest that Nek2 may be involved in multiple cell cycle events in vivo.

Materials and Methods

Immunoblot analysis

Immunoblot analysis was carried out as described previously (Rhee and Wolgemuth, 1997). 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 TBS with 0.1% Tween20) at room temperature for 1 h. After the blocking, the membrane was incubated with a primary antibody at room temperature for 3 h, washed with 0.1% TBST 3 times for 10 min each, incubated with a

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horseradish peroxidase-conjugated secondary antibody (1:5,000) for 1 h, and washed again with 0.1% TBST 3 times for 10 min each. Specific signals were detected with ECL reaction. The affinity-purified anti-Nek2 (Rhee and Wolgemuth, 1997) was diluted 1:100 and anti-Cdc2 antibody (Upstate Biotech) was diluted 1:1,000.

Immunohistochemical analysis

We used two immunostaining methods: the DAB and immunofluorescence staining methods. The testis and ovary from adult mouse were fixed, embedded in paraffin, sectioned and analyzed immunohistochemically as described previously (Rhee and Wolgemuth, 1997). After deparaffinization, slides were boiled in 0.01 M citrate buffer, pH 6.0, in a microwave (Shi et al., 1991) for 10 min and washed extensively with H_2O. The slides were treated with 0.03% H_2O_2 in methanol for 20 min, washed with PBST (PBS, 0.1% Triton X-100), and blocked for over 1 h with 2.5% goat serum in PBST. The slides were incubated with the primary antibodies in a humidified chamber overnight at 4℃, washed three times with PBST and stained with the Vectastain ABC kit (Vector Laboratories). Then DAB-stained slides were counterstained with hematoxylin. Alternatively, sections from mouse embryos were washed 3 times with PBS, incubated with FITC- or TRITC-conjugated secondary antibodies, mounted with a mounting solution that contained DAPI, and observed with a fluorescence microscope (Zeiss). The affinity-purified Nek2 antibody was diluted to 1:10, the monoclonal γ-tubulin antibody (Sigma) was diluted to 1:50, and the FITC- or TRITC-conjugated secondary antibodies were diluted 1:100.

Results

Expression of Cdc2 and Nek2 in the mouse testis

To confirm the expression of the Cdc2 and Nek2 proteins in the mouse testis, we carried out immunoblot analysis with total lysates from adult testes (Fig. 1). Single specific bands that corresponded to Cdc2 and Nek2 proteins with expected sizes, were detected (Th'ng et al., 1990; Rhee and Wolgemuth, 1997), confirming the presence of these proteins in the testicular cells. Moreover, these results strongly suggested that the Cdc2 and Nek2 antibodies that we used were suitable for immunohistochemical analyses.

We carried out immunohistochemical analysis to determine tissue- and developmental stage-specificity of *Cdc2* and *Nek2* expression at the protein level (Fig. 2). Cdc2 antibody stained spermatocytes specifically in all seminiferous tubules (Fig. 2A). No specific signal was detected in haploid germ cells that had undergone meiosis. This result is consistent with the fact that Cdc2 is a key regulator for meiosis (Dunphy et al., 1988; Gautier et al., 1988; Labbe et al., 1988). Nek2 antibody also stained spermatocytes specifically and no signal was detected in haploid cells (Fig. 2B). Such staining pattern disappeared when the Nek2 fusion protein was included in the incubation mix of the Nek2 antibody, supporting that the staining pattern was specific (Fig. 2C). These results suggest that, like Cdc2, Nek2 has a specific role in meiosis. The intracellular distribution of Nek2, however, appeared different from that of Cdc2. The Cdc2 antibody uniformly stained nuclei of spermatocytes, whereas the Nek2 antibody stained nuclei of spermatocytes with a uniquely punctate pattern (Fig. 2A,B). This suggests that biological functions of Nek2 might be distinct from those of Cdc2.

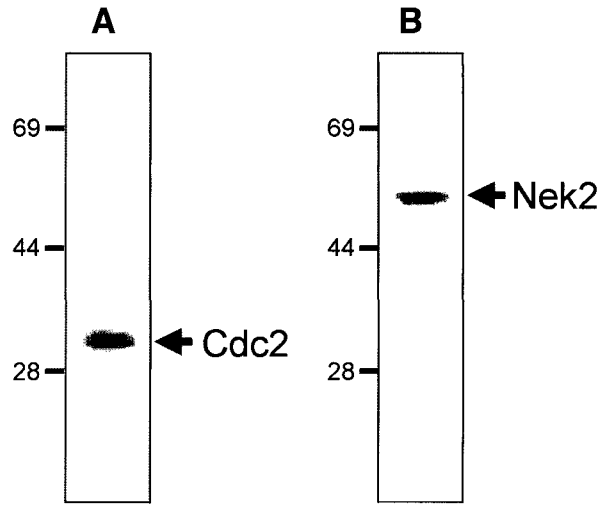

Fig. 1. Detection of Cdc2 and Nek2 in the mouse testis. Testicular lysates were prepared and subjected to immunoblot analysis with antibodies against Cdc2 (A) and Nek2 (B). Specific bands of Cdc2 (34 kDa) and Nek2 (48 kDa) were detected without an additional band.

Expression of Cdc2 and Nek2 in the mouse ovary and embryonic tissues

Specific expression of Nek2 in meiotic cells raised the possibility that Nek2 might be expressed in a cell cycle-dependent manner in mitotic cells as well. To test this possibility, we examined expression of *Cdc2* and *Nek2* in a tissue with a high proliferation activity. We initially chose the mouse ovarian follicle cells that were known to divide very actively in response to proper hormonal signals from the pituitary (Rao et al., 1978). Cdc2 antibody stained the nuclei of a fraction of ovarian follicle cells (Fig. 3A). This staining pattern suggests that Cdc2 is expressed in a cell cycle-specific manner. The Nek2 antibody also stained a fraction of ovarian follicle cells (Fig. 3B), suggesting that Nek2 might also be expressed in a cell cycle- specific manner. As observed in spermatocytes, the Nek2 antibody stained the nuclei distinctly in a punctate manner.

The Nek2 protein was also detected in the mouse embryo (Fig. 4). Most of embryonic tissues were immunostained with Nek2 antibody, without tissue-

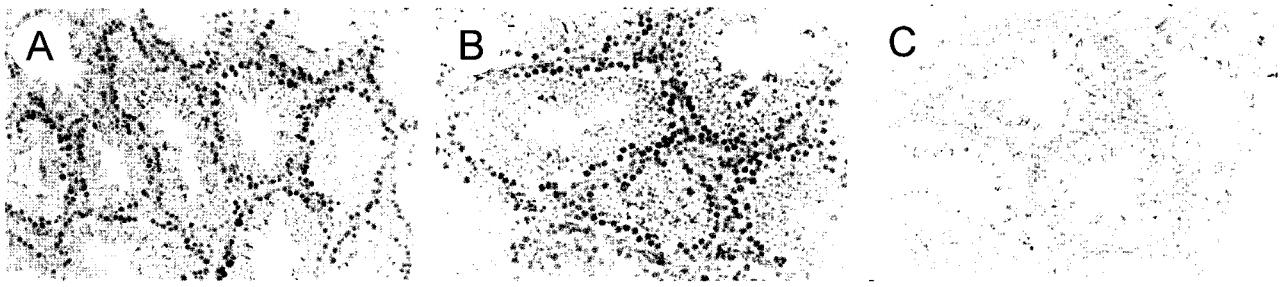

Fig. 2. Immunohistochemical analysis of Cdc2 and Nek2 in the mouse testis. Sections from mouse adult testes were immunostained with antibodies against Cdc2 (A) and Nek2 (B). As a control, Nek2 fusion protein was added into the incubation mix of the Nek2 antibody (C).

specificity. However, not all cells within the tissue were stained with the antibody. Rather, only a fraction of cells were positive of Nek2 protein. This expression pattern, again, supports the notion that Nek2 is expressed in a cell cycle-specific manner in vivo.

Subcellular localization of Nek2 in the centrosome

In the experiments described above, the Nek2 antibody stained the nucleus in a punctate manner. Such staining pattern suggests that Nek2 may be associated with the nuclear structure. In fact, we previously reported that Nek2 was in association with meiotic chromosomes (Rhee and Wolgemuth, 1997). However, association of Nek2 with centrosome was also reported in mitotic cells (Fry et al., 1998a). One possibility may be that subcellular localization of Nek2 is different in meiotic cells and in mitotic cells. Alternatively, differences in antibodies, methods, and sampling procedure could result in different staining patterns.

To resolve this somewhat contradictory results in subcellular localization of Nek2, we tried to detect its centrosomal localization in a mouse embryonic tissue. Since the centrosome forms a discrete under microscopic observation, it was difficult to detect centrosome by the DAB staining in which positive signals produce brown color. Instead, the centrosomal localization could be detected nicely with the immunofluorescent staining method. Therefore, we co-immunostained embryonic tissues with antibodies against Nek2 and γ-tubulin with the immunofluorescent staining method (Fig. 5). The results showed that the Nek2 antibody immunostained dot-like structures within the cells (Fig. 5A), which were co-stained with γ-tubulin antibody, a centrosomal marker (Fig. 5B). These results confirmed the centrosomal localization of Nek2 in embryonic cells. When we used a higher titer of Nek2 antibody, we were able to observe specific nuclear immunostaining in addition to centrosome (data not shown). These results revealed that Nek2 is localized in multiple sites within the cell, including the nucleus and centrosome.

Discussion

The results presented in this study revealed the cell cycle-specific expression of Nek2 in vivo. Nek2 protein was detected in a fraction of ovarian follicle cells and embryonic cells, suggesting that the Nek2-positive cells are likely at an actively proliferating stage. Moreover, a line of evidence suggests that Nek2 is present in a cell cycle stage-specific manner. First, in male meiotic germ cells, Nek2 was present in spermatocytes that corresponded to G2 phase of the meiotic cell cycle. Therefore, it is possible that Nek2 protein is also present specifically at G2/M phase of the mitotic cell cycle. Second, the expression pattern of Nek2 in the testis and ovary was similar to that of Cdc2 whose function is closely related to overcoming of checkpoints at G2/M transition. Third, in cultured cells, Nek2 was present more abundantly in cells at S/G2 phase than

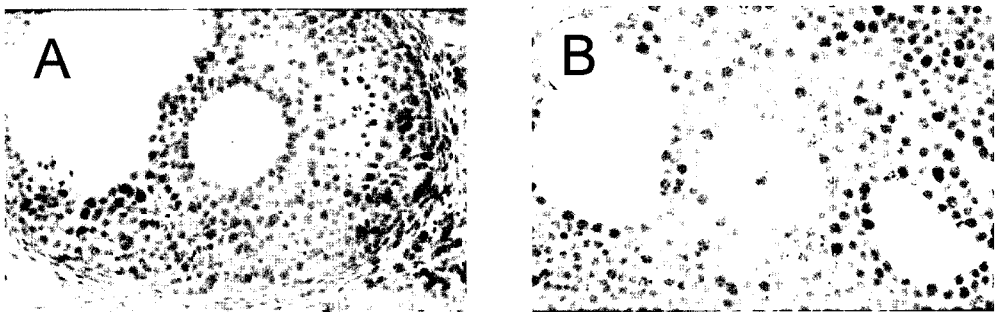

Fig. 3. Immunohistochemical analysis of Cdc2 and Nek2 in the mouse ovary. Sections from mouse adult ovaries were immunostained with antibodies against Cdc2 (A) and Nek2 (B).

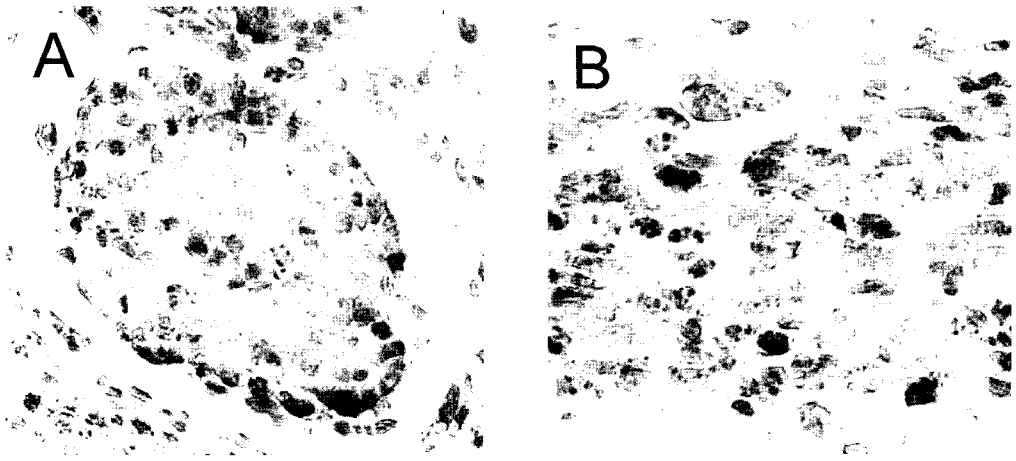

Fig. 4. Immunohistochemical analysis of Nek2 in the mouse embryonic tissues. Sections from the 11.5 dpc mouse embryo were immunostained with Nek2 antibody. (A) somite, (B) lateral mesoderm tissue next to the neural tube.

those at G1 phase (Schultz et al., 1994). Lastly, Nek2 protein was known to be a substrate of the anaphase-promoting complex which is activated during late M phase (Pfleger and Kirschner, 2000). Therefore, it is likely that Nek2 is involved in specific cell cycle events during G2/M phase.

Biological functions of Nek2 are not clear yet. *Aspergillus* NIMA was known to be important for chromosome condensation/dencondensation for mitosis (De Souza et al., 2000). However, somewhat unexpected function of Nek2 in the centrosome was reported. Fry et al. (1998a) showed that the human Nek2 protein was associated with centrosomes in cultured cells. Overexpression of *Nek2* induced splitting of centrosomes, whereas prolonged expression of *Nek2* led to dispersal of the centrosomal material and a loss of the focused microtubule-nucleating activity (Fry et al., 1998a). Centrosomal localization of Nek2 was also observed in *Xenopus* early embryos (Fry et al., 2000; Uto and Sagata, 2000). Specific inhibition of Nek2B function 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 (Fry et al., 2000; Uto and Sagata, 2000). Based on these results, it was proposed that Nek2 was specifically required for centrosome assembly and/or maintenance (Fry et al., 2000; Uto and Sagata, 2000). A centrosomal protein has also been identified as a Nek2-associated protein (C-Nap1) (Fry et al., 1998b). When the function of C-Nap1 was interfered with by antibody injection, centrosome splitting was induced (Mayer et al., 2000). 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 (Mayer et al., 2000).

In the present study, we observed that Nek2 was localized in both the nucleus and centrosome in in vivo cells. Such localization pattern supports the idea that Nek2 is involved in diverse mitotic events that occur in the nucleus and centrosome. In fact, it was recently reported that *Aspergillus* NIMA was in association not only with the chromatin but also with the spindle pole

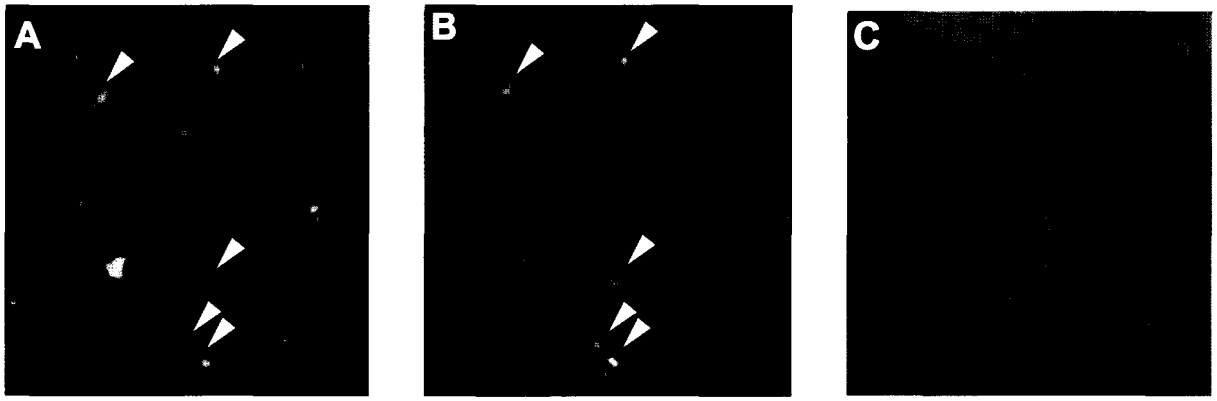

Fig. 5. Subcellular localization of Nek2 in the centrosome in the mouse embryo. Sections of mesenchymal tissues from the 11.5 dpc mouse embryo were immunostained with antibodies against Nek2 (A) and -tubulin (B). Nuclei were stained with DAPI (C).

body that corresponds to the centrosome in mammalian cells (De Souza et al., 2000), suggesting that NIMA also serves multiple functions as a cell cycle regulator.

Acknowledgments

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