Role of the Kinesin-2 Family Protein, KIF3, during Mitosis*

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During mitosis, kinesin and dynein motor proteins play critical roles in the equal segregation of chromosomes between two daughter cells. Kinesin-2 is composed of two microtubule-based motor subunits, KIF3A/3B, and a kinesin-associated protein known as KAP3, which links KIF3A/3B to cargo that is carried to cellular organelles along microtubules in interphase cells. We have shown here that the kinesin-2 complex is localized with components of the mitotic apparatus such as spindle microtubules and centrosomes. Furthermore, we found that expression of a mutant KIF3B, which is able to associate with KIF3A but not KAP3 in NIH3T3 cells, caused chromosomal aneuploidy and abnormal spindle formation. Our data suggested that the kinesin-2 complex plays an important role not only in interphase but also in mitosis.

Although kinesin-2 has mainly been reported to participate in intracellular transport in interphase cells, it has been shown to localize at the mitotic apparatus. In Chlamydomonas, the KIF3A homolog FLA10 protein is most abundant near the centrioles and the mitotic spindle during mitosis (17). The sea urchin kinesin-2 homolog, kinesin II, is present transiently in the mitotic apparatus of dividing embryos (18). However, detailed functional analyses have revealed that kinesin-2 is not critical for the progression of mitosis in both Chlamydomonas and sea urchin embryos (see "Discussion") (19, 20). In the present study, we have attempted to investigate the function and regulation of kinesin-2 during mitosis in mammalian cells and have found that kinesin-2 localizes to the mitotic apparatus and contributes to molecular events important for the progression of mitosis.

MATERIALS AND METHODS

Cell Culture—HeLa, HEK293T and Plat-E cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceuticals, Taito, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum. NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) calf serum.

Antibodies—Rabbit polyclonal antibody to KAP3 (N1 and C2) were prepared as described previously (14). Rabbit polyclonal antibodies to KIF3A and KIF3B were prepared by immunizing rabbits with peptides containing amino acids 563–671 of KIF3A and 657–747 of KIF3B, respectively. Antibodies were purified by affinity chromatography using columns to which the antigens used for immunization had been linked. Monoclonal antibody to KIF3A/3B (Kinesin II) was obtained from Covance (Princeton, NJ). Monoclonal antibodies to α-tubulin and cyclin B1 were from Oncogene Research (Boston, MA) and Santa Cruz Biotechnology (sc-245, Santa Cruz, CA), respectively. Monoclonal and polyclonal antibodies to GFP were from Quantum (Montreal, Quebec, Canada) and Clontech, respectively.

Immunoprecipitation and Immunoblotting—Cells were lysed in buffer A (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA at pH 7.5, 2 mM Na3VO4, 10 mM NaF) containing 1% Triton X-100. Lysates were incubated with antibodies for 1 h at 4°C. The immunocomplexes were adsorbed to protein G-Sepharose 4B (Amersham Biosciences) for 1 h at 4°C. Blocking of antibodies was performed by preincubating antibodies (1–2 µg) for 1 h at 4°C with a 100-fold molar excess of the antigens used for immunization. After washing extensively with buffer A containing 0.1% Triton X-100, samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane filter (Immobilon, Millipore, Bedford, MA). The blot was probed with antibodies (1:1,000) for KIF3A and KIF3B and transferred to a polyvinylidene difluoride membrane filter (Immobilon, Millipore, Bedford, MA). The blot was probed with antibodies (1:1,000) for KIF3A and KIF3B.

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§ The abbreviations used are: KIF, kinesin superfamily of proteins; APC, adenomatous polyposis coli; GFP, green fluorescent protein; PBS, phosphate-buffered saline.
Immunostaining—HeLa cells were fixed with 4% paraformaldehyde in PBS for 20 min at 37 °C and then fixed with methanol-acetone (1:1, v/v) for 10 min at −20 °C. Fixed cells were incubated with blocking solution (1% bovine serum albumin in PBS) and were double-stained with antibodies against KAP3 C2 (polyclonal) and α-tubulin (monoclonal), KIF3A or KIF3B (polyclonal) and α-tubulin (monoclonal), or GFP (polyclonal) and α-tubulin (monoclonal). Staining patterns obtained with these antibodies were visualized by incubation with secondary antibodies: fluorescein-5-isothiocyanate-labeled anti-rabbit IgG (for KAP3, KIF3A/3B, and GFP) and tetramethylrhodamine-5 (and -6)-isothiocyanate-labeled anti-mouse IgG (for α-tubulin). Chromosomes were stained with TOTO3 (Molecular Probes, Eugene, OR). Dilutions of primary antibodies were as follows: anti-KAP3 C2 polyclonal antibody, 1:100; anti-KIF3A or KIF3B polyclonal antibody, 1:50; anti-GFP polyclonal antibody, 1:100, anti-α-tubulin monoclonal antibody, 1:100. Cells were photographed with a Carl Zeiss LSM510 laser microscope (Göttingen, Germany).

Phosphatase Treatment—KAP3 immunoprecipitates were washed three times with bacterial alkaline phosphatase buffer (20 mM Tris–HCl, pH 8.0, 1 mM MgCl₂) and incubated at 50 °C for 30 min with 0.2 units of bacterial alkaline phosphatase (TAKARA, Kyoto, Japan) in the absence or presence of phosphatase inhibitors (2 mM Na₃VO₄ and NaF). Dilutions of primary antibodies were as follows: anti-KAP3 C2 polyclonal antibody, 1:100; anti-KIF3A or KIF3B polyclonal antibody, 1:50; anti-GFP polyclonal antibody, 1:100, anti-α-tubulin monoclonal antibody, 1:100. Cells were photographed with a Carl Zeiss LSM510 laser microscope (Göttingen, Germany).

Cell Cycle Synchronization—HeLa cells were synchronized by thymidine at the G₁/S boundary as described (21). In brief, cells were treated with 2.5 mM thymidine (Sigma) for 12 h, washed three times with PBS, and incubated for an additional 12 h under normal growth conditions. Finally, thymidine was added again for 12 h to block cells at the G₁/S boundary. Cells were then washed three times with PBS and placed under fresh growth medium. Cells were harvested at various time points as indicated. To obtain more enriched M phase cells, exponentially growing HeLa cells were treated with 100 ng/ml nocodazole (Sigma) for 24 h. Mitotic rounded-up cells were collected by gentle pipetting.

Retroviral Infection—DNA fragments encoding GFP-KIF3A or GFP-KIF3B were cloned into the retroviral vector pMX-puro (22, 23). The retroviral vectors were transiently transfected into Plat-E cells using Effectene (Qiagen, Hilden, Germany). After 24 h, cells were changed into fresh medium. After an additional 24 h, culture medium was collected and centrifuged at 2,000 g for 30 min. The supernatants, together with 10 µg/ml polybrene (Sigma), were added to NIH3T3 cells. The infected cells were selected using 2 µg/ml puromycin (Sigma) for 3 days.

RESULTS

KAP3 Is Phosphorylated during Mitosis—To investigate the role of kinesin-2 in mitosis, we first examined the expression and modification of KIF3A/3B and KAP3 using HeLa cells released from double thymidine block. Immunoblotting analysis revealed that the levels of KIF3A/3B and KAP3 expression increased slightly during G₂/M progression (Fig. 1A). Furthermore, a protein larger than the normal KAP3 was found to appear concomitant with the start of cyclin B destruction. Treatment of KAP3 immunoprecipitates with bacterial alkaline phosphatase resulted in the disappearance of this protein and a concomitant increase in the normal migrating form of KAP3, and this conversion was
not observed in the presence of phosphatase inhibitors (Fig. 1B). These results suggest that KAP3 is phosphorylated during mitosis.

**KIF3A/3B and KAP3 Form a Complex during Mitosis**—We next examined whether KIF3A/3B is associated with KAP3 in HeLa cells that were synchronized at mitosis by nocodazole treatment. Lysates from nocodazole-treated cells were subjected to immunoprecipitation with anti-KAP3 N1 antibodies, fractionated on a 7% SDS-PAGE gel, and immunoblotted with anti-KIF3A/3B and KAP3 antibodies. We found that both phosphorylated and unphosphorylated forms of KAP3 coprecipitated with KIF3A/3B and that coprecipitation was inhibited by preincubation of anti-KAP3 antibody with the antigen used for immunization (Fig. 2). Thus, KIF3A/3B are associated with KAP3 during mitosis.

**Regions Required for KIF3A/3B and KAP3 Interaction**—To determine the regions of KIF3A/3B responsible for their interaction with KAP3, we generated various deletion mutants of KIF3A/3B. When ectopically expressed in HEK293T cells, the two KIF3A/3B mutants lacking the C-terminal regions failed to coprecipitate with KAP3 (Fig. 3A). In contrast, KIF3A/3B mutants containing the C-terminal regions coprecipitated with KAP3. Thus, the C-terminal regions of KIF3A/3B are important for their interaction with KAP3. Similar experiments with various deletion mutants of KAP3 revealed that a fragment containing amino acids 528–694 is able to interact with KIF3A/3B (Fig. 3B). In addition, a fragment of KAP3 containing amino acids 1–614 was found to retain KIF3A/3B binding activity. Thus, amino acids 528–614 of KAP3 may be important for its interaction with KIF3A/3B.

**KIF3A/3B and KAP3 Localize to the Mitotic Apparatus**—We next examined the subcellular localization of KIF3A/3B and KAP3 in mitosis. Since commercially available anti-KIF3A/3B monoclonal antibodies were not suitable for immunostaining analysis, we generated polyclonal antibodies against the C-terminal regions of KIF3A and KIF3B, respectively. Immunoblotting analysis using HeLa cell lysates revealed that each antibody specifically recognizes KIF3A and KIF3B, respectively (Fig. 4A). This result is consistent with the fact that there is little amino acid sequence similarity among the C-terminal regions of the KIF family of proteins. Immunostaining analysis with these antibodies revealed intense KIF3A staining localized at the centrosomes in interphase and prophase cells but only weak staining at the centrosomes after prometaphase (Fig. 4B). When chromosomes began to condense and the mitotic spindle was formed in prometaphase, KIF3A was localized mainly at the spindle microtubules. From metaphase through telophase, KIF3A was concentrated at the midzone and was present mainly at the centrosomes during cytokinesis. KIF3A was also localized around the cellular cortex throughout mitosis. We also examined KAP3 localization during mitosis using a polyclonal antibody against the C-terminal region of KAP3 (C2) (14). When cells entered prometaphase, KAP3 was found to be localized at the centrosomes and spindle microtubules (Fig. 4C). In metaphase, KAP3 was also detected on the chromosomes, and this localization was especially prominent on the chromosomes. From

![FIGURE 2. Association of KIF3A/3B with KAP3 in mitotic HeLa cells. Lysates (Ly) prepared from HeLa cells arrested in mitosis by nocodazole treatment were subjected to immunoprecipitation (IP) with anti-KAP3 N1 antibodies, fractionated on a 7% SDS-PAGE gel, and immunoblotted with anti-KIF3A/3B and KAP3 antibodies. Pep. + indicates that antibodies were preincubated with antigen before immunoprecipitation. WB, Western blot.](image)

![FIGURE 3. Mapping of the regions in KIF3A/3B and KAP3 required for their interaction. A, mapping of the regions in KIF3A and KIF3B required for their binding to KAP3. Various deletion constructs of GFP-KIF3A and GFP-KIF3B were transfected into HEK293T cells. Cell lysates were subjected to immunoprecipitation (IP) with anti-GFP monoclonal antibodies followed by immunoblotting with the indicated antibodies. +, detectable activity; −, no detectable activity. WB, Western blot; WT, wild type; Mut, mutation; motor, motor subunit. B, mapping of the regions in KAP3 required for binding to KIF3A and KIF3B. Various deletion constructs of GFP-KAP3 were transfected to HEK293T cells, and cell lysates were subjected to immunoprecipitation with anti-GFP monoclonal antibodies followed by immunoblotting with the indicated antibodies. Arm, armadillo.](image)
metaphase to telophase, KAP3 was concentrated intensively at centro-
somes and midzone and remained at the centrosome during cytokine-
sis. Thus, the immunostaining patterns of KAP3 were partially similar to
those of KIF3A. These results suggested that a certain population of
KIF3A/3B and KAP3 colocalizes at the mitotic apparatus.

**Effects of KIF3 Mutants on Mitotic Progression**—To elucidate the
function of kinesin-2 in mitosis, we expressed a fragment of KIF3B fused
to GFP (Fig. 3A, GFP-KIF3B Mutant 3 (Mut 3)) in NIH3T3 cells by
retrovirus and examined its effects on the progression of mitosis. This
fragment lacks the C-terminal one-third of KIF3B and is unable to form
a complex with KAP3 (Fig. 3A) but can still associate with KIF3A (data
not shown). Although this mutant was localized at the spindle microtu-
bules, similar to wild-type KIF3B, cells expressing this mutant showed
an unusual number of centrosomes and abnormal spindle formation
(Fig. 5A). By contrast, cells expressing the full-length KIF3B fused to
GFP or control retrovirus vector did not show any abnormality in mito-
sis. Furthermore, flow cytometric analysis revealed that the frequency
of chromosomal aneuploidy in cells expressing this mutant was about
three times higher than that of cells expressing the full-length KIF3B
fused to GFP or control retrovirus vector (Fig. 5B). Similar aberrant
mitosis was found to occur when a fragment of KIF3A fused to GFP (Fig.
3A, GFP-KIF3A Mutant 3 (Mut 3)), which can form a complex with
KIF3B but cannot interact with KAP3, was expressed in NIH3T3 cells
(data not shown). These results suggest that the interaction of
KIF3A/3B with KAP3 may be important for the proper progression of
mitosis.

**DISCUSSION**

In the present study, we demonstrated that human KIF3A/3B and
KAP3 form a complex in mitosis and that they localize at the mitotic
apparatus, mainly at the spindle microtubules and centrosomes in HeLa
cells. Furthermore, we showed that expression of a dominant-negative
mutant of KIF3A/3B results in aberrant mitosis. Our findings suggested that the kinesin-2 complex plays a critical role not only in interphase but also in mitosis.

Our domain analysis revealed that amino acids 528–614 of KAP3 may interact with the C-terminal regions of KIF3A/3B. This result is consistent with previous data obtained by low angle rotary-shadowing electron microscopy showing that KAP3 is a globular protein and is associated with the tail domain of KIF3A/3B (16). We utilized deletion constructs of KIF3A/3B used for domain analysis (Fig. 3A, Mutant 3 (Mut 3)) to assess the significance of the kinesin-2 complex in mitosis since we could not efficiently knock down expression of KIF3A/3B and KAP3 using small interfering RNA. These mutants could form a heterodimer but could not interact with KAP3. Interestingly, when expressed in NIH3T3 cells, these mutants caused chromosomal aneuploidy and aberrant spindle formation. Thus, the interaction between KIF3A/3B and KAP3 may be required for spindle formation and chromosome segregation, although one cannot exclude the possibility that a protein(s) other than KAP3 also interacts and is critical for this function. We speculate that there may be an important mitosis-specific cargo protein(s) to which KIF3A/3B associate via an interaction with KAP3. Intriguingly, it has been reported that APC localizes to the ends of microtubules embedded in kinetochores and plays a critical role in chromosome segregation (24, 25). Since we have previously found that APC is associated with KIF3A/3B via an association with KAP3 (14), APC may be one of these critical cargo proteins involved in the progression of mitosis.

FIGURE 5. Effect of KIF3 mutant on progression of mitosis. A, expression of GFP-KIF3B Mutant 3 resulted in aberrant mitosis. Cells were stained with anti-GFP polyclonal and anti-α-tubulin monoclonal antibodies. TOTO3 was used for staining of chromosomes. Scale bar, 10 μm. Right panel, the number of mitotic cells positive for GFP and cells exhibiting the multipolar spindles were counted. WT, wild type; Mut, mutant. B, aneuploidy in NIH3T3 cells expressing GFP-KIF3B Mutant 3. Cells were stained with propidium iodide, sorted based on GFP fluorescence, and analyzed for ploidy with flow cytometer. The ploidy was determined from analysis of 30,000 cells expressing GFP-KIF3B wild-type or Mutant 3. In a parallel experiment, cell lysates were subjected to immunoblotting analysis with anti-GFP or anti-α-tubulin antibody (right panel). WB, Western blot.
ing that kinesin-2 is not critical for the progression of mitosis. These findings appear to conflict with our results. However, it may be possible that the importance of kinesin-2 function in mitosis may differ depending on the cell type and developmental stage. To further clarify the role of kinesin-2 in mitosis, it would be necessary to establish knock-out cell lines and to scrutinize the function of kinesin-2 in mitosis.

We showed that KAP3 is phosphorylated during mitosis. In this regard, it is interesting that some members of the KIF family have been reported to be phosphorylated by mitotic kinases. For example, CENP-E and Eg5 are phosphorylated by cdc2-cyclin B1 (29, 30). Xenopus Aurora kinase pEg2-mediated phosphorylation of Eg5 is required for spindle formation and stabilization (31). Also, cdc2-cyclin B1-mediated phosphorylation of Kid controls its distribution to spindle and chromosomes (32). Thus, it is possible that phosphorylation of KAP3 may play a role in the regulation of mitotic progression. Although phosphorylation of KAP3 did not affect its KIF3A/3B binding ability, it is interesting to speculate that phosphorylation of KAP3 may regulate its interaction with cargo proteins that are involved in spindle formation and chromosome segregation. Identification of mitosis-specific cargo proteins and kinases responsible for KAP3 phosphorylation may provide insights into the significance of KAP3 phosphorylation.

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