Current Biology, Vol. 12, 894–899, June 4, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S0960-9822(02)00848-5

The Kinase Activity of Aurora B Is Required for Kinetochore-Microtubule Interactions during Mitosis

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Summary

As a component of the "chromosomal passenger protein complex," the aurora B kinase is associated with centromeres during prometaphase and with midzone microtubules during anaphase and is required for both mitosis and cytokinesis [1–6]. Ablation of aurora B causes defects in both prometaphase chromosomal congression and the spindle checkpoint [4–6]; however, the mechanisms underlying these defects are unclear. To address this question, we have examined chromosomal movement, spindle organization, and microtubule motor distribution in NRK cells transfected with a kinase-inactive, dominant-negative mutant of aurora B, aurora B(K-R) [6–8]. In cells overexpressing aurora B(K-R) fused with GFP, centromeres moved in a synchronized and predominantly unidirectional manner, as opposed to the independent, bidirectional movement in control cells expressing a similar level of wild-type aurora B-GFP. In addition, most kinetochores became physically separated from spindle microtubules, which appeared as a striking bundle between the spindle poles. These defects were associated with a microtubule-dependent depletion of motor proteins dynein and CENP-E from kinetochores. Our observations suggest that aurora B regulates the association of motor proteins with kinetochores during prometaphase. Interactions of kinetochore motors with microtubules may in turn regulate the organization of microtubules, the movement of prometaphase chromosomes, and the release of the spindle checkpoint.

Results and Discussion

A kinase-inactive mutant of aurora B and its GFP-fused form, referred to as aurora B(K-R) and aurora B(K-R)-GFP, respectively, were previously found to function as dominant-negative inhibitors of endogenous aurora B [6–8]. At a low level of expression, as judged by the intensity of GFP, some of the chromosomes failed to congress or congressed very slowly into the metaphase plate (data not shown). Over 60% (5/8) of these cells showed subsequent defects in anaphase chromosomal segregation [6]. At high levels of expression, aurora B(K-R)-GFP induced a strong inhibitory effect on prometaphase chromosomal congression (Figure 1A). However, these cells were able to exit mitosis by forming a nuclear envelope around miscongressed chromosomes [6].

To understand the mechanism of the disruption of chromosomal congression, we monitored the movement of individual centromeres in cells expressing a high level of aurora B(K-R)-GFP or control cells expressing a similar level of wild-type aurora B-GFP (Figure 1B). Both aurora B-GFP and aurora B(K-R)-GFP were localized at centromeres and spindle poles [6] and to a less extent along chromosomal arms (see Figures 3A and 3D).

In control cells, the centromeres of neighboring chromosomes moved independently of each other at an average rate of 1.8 $\pm$ 1.2 μm/min (mean $\pm$ SD, n = 22), with frequent changes in direction (Figure 1B, left). They eventually accumulated at the metaphase plate $\sim$20 min after nuclear envelope breakdown (19/19, Figures 1Aa–1Ad; Supplementary Movie 1 available with this article online). In contrast, in cells expressing aurora B(K-R)-GFP, centromeres of neighboring chromosomes moved synchronously at an average rate of 1.3 $\pm$ 0.9 μm/min (mean $\pm$ SD, n = 22) (Figure 1B, right). The movement also showed a higher degree of persistence in direction than did the movement of control centromeres. Furthermore, instead of congressing into a metaphase plate perpendicular to the spindle axis, chromosomes became segregated into two elongated clusters that lie along the sides of the spindle axis (10/10, Figures 1Ae–1Ah; Supplementary Movie 2).

From the defective chromosomal movement and peculiar chromosomal distribution, we surmised that aurora B(K-R) might affect the organization of spindle microtubules and/or the interactions of kinetochores with microtubules. Microtubule organization was visualized by microinjecting transfected cells with rhodamine-labeled tubulin (Figure 2A; Supplementary Movies 3 and 4) and by immunofluorescence and 3D image reconstruction (Figure 2B). In control prometaphase cells expressing wild-type aurora B-GFP (Figure 2A), microtubules formed multiple, discrete kinetochore bundles that terminated near centromeres (5/5) (Figures 2Aa–2Ac; Figure 2B, left; Supplemental Movie 3). Strikingly, cells expressing aurora B(K-R)-GFP formed a single microtubule bundle between the two spindle poles, with a cluster of chromosomes lying on each side (8/9) (Figures 2Ad–2Af; Figure 2B, right; Supplemental Movie 4). Most centromeres remained separated from microtubules. These results suggest that the kinase activity of aurora B is required for establishing or maintaining microtubule-kinetochore associations.

To address the possibility that aurora B might affect the kinetochore localization of motor proteins, which have been suggested to play a role in proper spindle organization [9–12], prometaphase chromosomal congression [11, 13–16], and the spindle checkpoint [11, 12, 17–20], we examined the distribution of two kinetochore-associated motors: cytoplasmic dynein and CENP-E. In control cells, dynein was found at kinetochores during prometaphase and along astral microtubules during metaphase [10, 21–23] (Figures 3A and 3A′). In contrast, aurora-B(K-R)-GFP-transfected cells showed a diminished level of dynein at prometaphase kinetochores (Figures 3B and 3B′) and prominent local-

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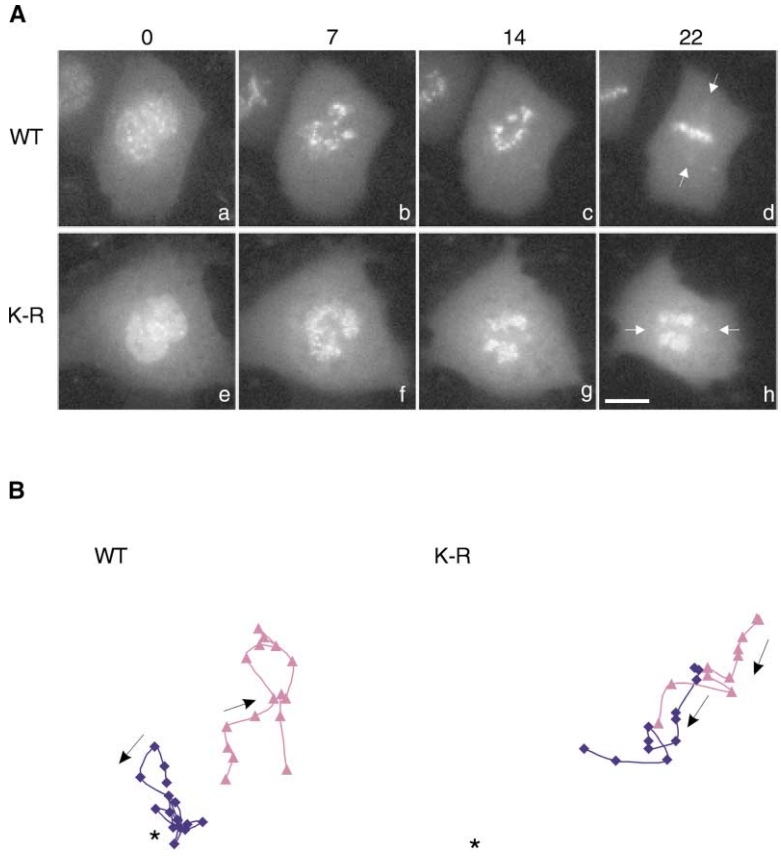


Figure 1. Effects of Kinase-Inactive Aurora B on Prometaphase Chromosomal Movements

(A) Aurora B-GFP (Aa–Ad) or aurora B(K-R)-GFP (Ae–Ah), transiently expressed in NRK cells, are localized at centromeres and serve also as markers for tracking the movement of chromosomes. In cells expressing aurora B-GFP (Aa–Ad), prometaphase chromosomes congress normally into the metaphase plate between the two spindle poles (arrows). In cells expressing aurora B(K-R)-GFP (Ae–Ah), chromosome movements are defective, and chromosomes fail to congress into a metaphase plate. They instead form two elongated clusters along the spindle axis as defined by the two spindle poles (arrows). Time in minutes is indicated above the images. Scale bar, 10 μm.

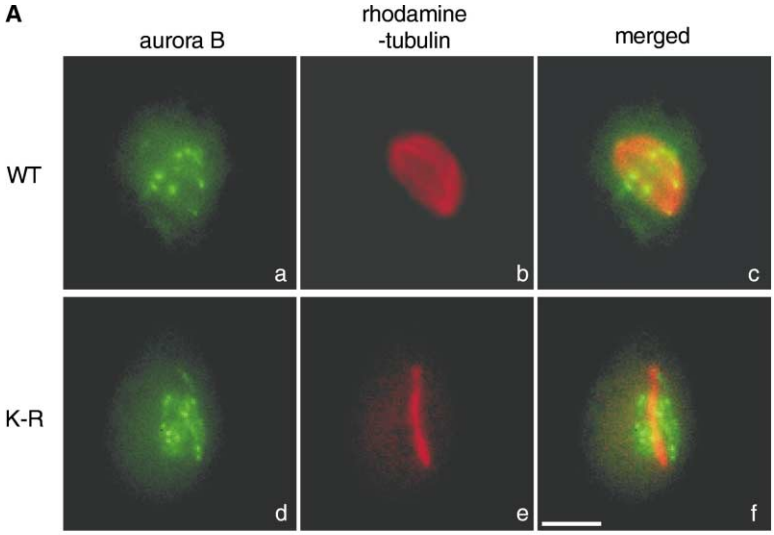
(B) Traces of the movement of centromeres of two representative neighboring chromosomes during late prometaphase, in cells expressing either aurora B-GFP (left) or aurora B(K-R)-GFP (right). Arrows indicate the direction of the movement. Asterisks indicate the position of the center of the spindle. The spindle axis is oriented along the north-south direction. Centromeres in control cells move independently of each other, with a highly variable speed and direction (left), whereas centromeres containing aurora B(K-R)-GFP move in a more coordinated fashion (right).

ization of dynein along the microtubule bundle that lies between the spindle poles (Figures 3B and 3B′). Staining of CENP-E, a kinesin-like motor, showed localization at kinetochores and spindle poles in control prometaphase cells as reported previously [24–27] (Figures 3D and 3D′). In aurora B(K-R)-GFP-transfected cells (Figures 3E and 3E′), CENP-E showed reduced localization at kinetochores, although the localization at spindle poles appeared unaffected.

To determine if the association/dissociation of dynein and CENP-E with kinetochores was dependent on microtubules, we treated aurora B(K-R)-GFP-transfected cells with 20 μM nocodazole for 4 hr before staining for motor proteins. Both dynein and CENP-E became prominently associated with kinetochores following nocodazole treatment (Figures 3C, 3C′, 3F, and 3F′), suggesting that microtubules were required not for the association of these motors with kinetochores but for their dissociation in cells overexpressing aurora B(K-R)-GFP. A similar microtubule-dependent mechanism was suggested for the dissociation of dynein from kinetochores following normal chromosomal congression [28]. In addition, our observations suggest that aurora B(K-R) did not inhibit the association of motor molecules with kinetochores but promoted their dissociation upon contact with microtubules.

We propose that a substrate of the aurora B kinase, which may be an adaptor protein or motor proteins themselves, is required for the stable association of dynein and CENP-E at kinetochores. In the absence of the kinase activity, these motors were released prematurely from kinetochores as soon as they were in contact with microtubules (Figure 4), whereas in control cells they were released only following chromosomal congression. As dynein contributes to the poleward movement of the chromosomes while CENP-E may be involved in the attachment of chromosomes to microtubules [29, 30], loss of these motor proteins may account for the defects in chromosomal movements and kinetochore-microtubule interactions. The residual, synchronized chromosomal movements, without kinetochore fibers, were likely dragged by the "polar ejection forces" on chromosome arms [31–33], which sweep the chromosomes into elongated clusters along the spindle axis. In addition, without the attachment to kinetochores, the microtubule bundling activity responsible for the formation of kinetochore fibers would induce the formation of a single microtubule bundle between the spindle poles.

We have recently found that a spindle checkpoint protein, Mad2, was released prematurely from the kinetochores in cells overexpressing aurora B(K-R) [6] but remained on the kinetochores after nocodazole treatment (our unpublished data). Furthermore, in budding yeast, aurora kinase homolog Ipl1p appeared to function in monitoring forces at kinetochores for the spindle checkpoint control [34]. As dynein is involved in the localization of Mad2 [19], defects in dynein localization could easily explain the defects in spindle checkpoint in cells overexpressing aurora B(K-R). The present results, together with our recent observations that the kinase activity of aurora B is required for maintaining the association of chromosomal passenger proteins at the spin-



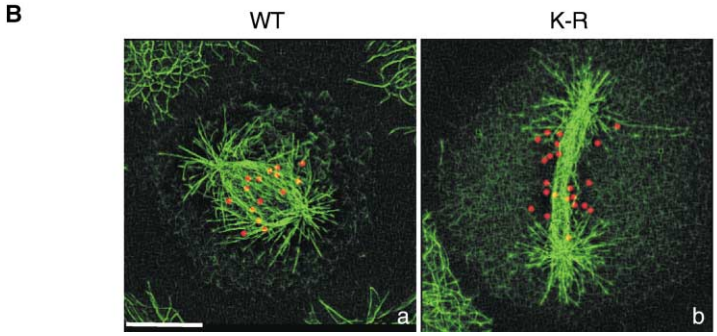


Figure 2. Separation of Kinetochores from Microtubule Bundles in Cells Expressing Kinase-Inactive Aurora B

(A) NRK cells transiently expressing aurora B-GFP (Aa–Ac) or aurora B(K-R)-GFP (Ad–Af) were microinjected with rhodamine-labeled tubulin at prometaphase. Cells expressing aurora B-GFP show well-organized kinetochore fibers (Ab), while those expressing aurora B(K-R)-GFP show a single microtubule bundle that extends between the spindle poles with chromosomes lying along the sides (Ae). Scale bar, 10 μm.

(B) NRK cells transiently expressing aurora B-GFP (Ba) or aurora B(K-R)-GFP (Bb) were fixed and stained with anti-β-tubulin antibodies. 3D reconstruction of microtubules and centromeres indicate that most centromeres (red dots) are separated from microtubules in aurora B(K-R)-GFP cells. Scale bar, 10 μm.

dle midzone during telophase [6], suggest that the primary functions of aurora B may be to regulate the anchorage of microtubule motors at specific target sites during various stages of mitosis and cytokinesis.

Experimental Procedures

Cell Culture, Microscopy, and Image Processing

Normal Rat Kidney epithelial cells (NRK-52E; American Type Culture Collection, Rockville, MD) were cultured in Kaighn's modified F12 (F12K) medium supplemented with 10% FBS (JRH Bioscience, Lenexa, KS), 1 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained at 37°C in a stage incubator built on top of an Axiovert S100TV inverted microscope (Carl Zeiss, Thornwood, NY) and viewed with a 100×, NA 1.30 Fluar objective lens. All images were acquired with a cooled charge-coupled device camera (ST133 controller and CCD57 chip; Roper Scientific, Treton, NJ) and processed with custom software for background subtraction. To track centromere movement, the images of aurora B-GFP or aurora B(K-R)-GFP were acquired every 15 s during prometaphase, and coordinates of the spindle poles and several centromeres were determined frame by frame using a custom interactive program. The center of the spindle was calculated as the midpoint between the two spindle poles. Before plotting, normalized positions of the centromeres were calculated by treating the center of the spindle as the stationary origin and rotating the images such that the spindle axis lies along the north-south direction. For 3D reconstruction, stacks of wide-field images were collected at a vertical interval of 0.25 μm. Microtubule images were processed with custom pattern-recognition software that enhances linear structures. Aurora B images were processed manually for the determination of each centromere in the 3D space. Normal views of the stack were then constructed with a maximum-intensity projection algorithm.

Transfection and Microinjection

Both aurora B-GFP and aurora B(K-R)-GFP were constructed as described previously [6]. NRK cells were plated at a density of 5×10^4 cells/ml on a coverslip chamber dish and incubated for 18–24 hr. Immediately before transfection, the cells were rinsed once in serum-free F12K or Opti-MEM I medium (Life Technologies, Rockville, MD). The cells were transfected with the DNA construct (2 μg) using LipofectAMINE according to manufacturer's instructions (Life Technologies, Rockville, MD). After 4 hr incubation, the medium containing DNA-LipofectAMINE was replaced with the F12K medium containing 10% FBS, and the cells were cultured for an additional 14–18 hr.

Tubulin was prepared and labeled with 5- (and 6-) carboxytetramethylrhodamine (TAMRA; Molecular Probes, Eugene, OR) as described previously [35, 36]. Microinjection was performed using a low-pressure, continuous-flow method with custom drawn glass needles and a custom-designed pressure control system as described previously [35–37].

Drug Treatment and Immunofluorescence

Nocodazole (Sigma, St. Louis, MO) was stored at −20 °C as 10^3 x stocks in DMSO and diluted into pre-warmed medium before application to cells. Immunofluorescence staining of microtubules was performed essentially as described previously [35]. For dynein and CENP-E staining, cells were rinsed with PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, and 4 mM $MgSO_4$ [pH 6.9]), then lysed for 5 min in 0.5% Triton X-100 in PHEM buffer and fixed with 4% formaldehyde in PHEM buffer for 20 min. Fixed cells were rinsed with PBST (PBS with 0.05% Tween 20), blocked for 45 min

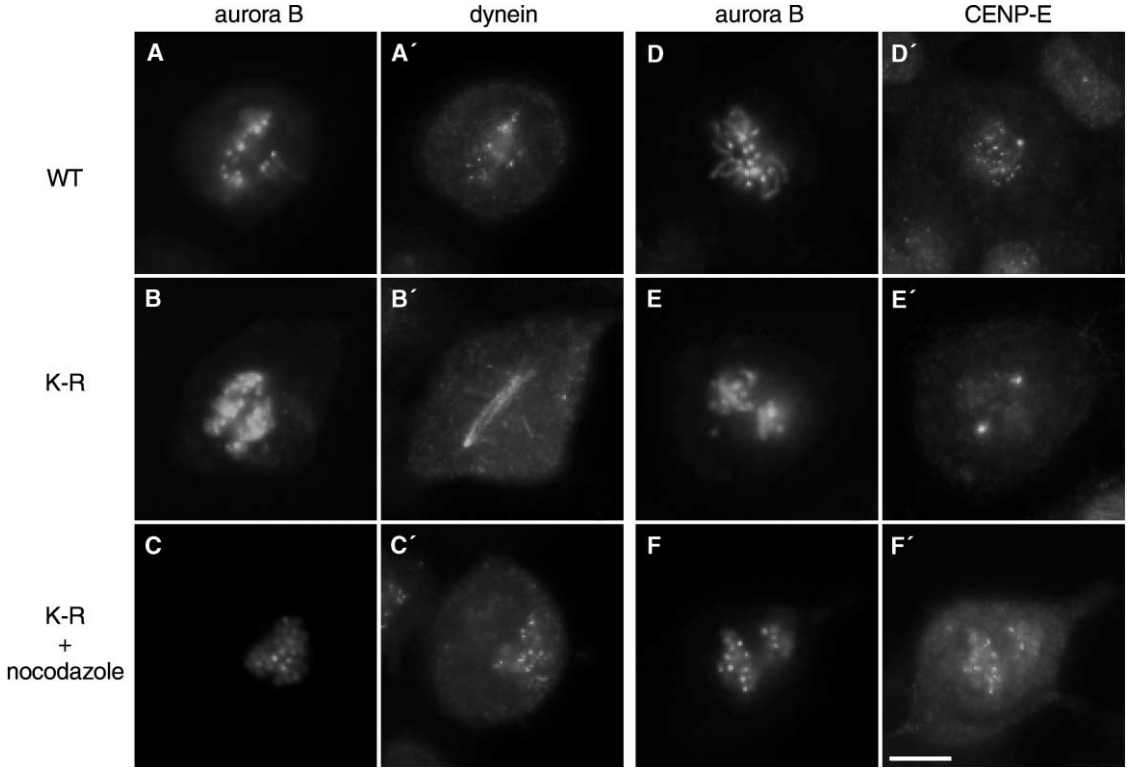


Figure 3. Premature Dissociation of Cytoplasmic Dynein and CENP-E from Kinetochores in Cells Expressing Kinase-Inactive Aurora B

Immunofluorescence shows little cytoplasmic dynein (B′) and CENP-E (E′) staining at kinetochores in prometaphase cells expressing aurora B(K-R)-GFP (B, B′, E, and E′), while control cells expressing aurora B-GFP show dynein (A′) and CENP-E (D′) staining at a number of kinetochores (A, A′, D, and D′). Treatment with nocodazole causes the association of both dynein (C′) and CENP-E (F′) at kinetochores in cells expressing aurora B(K-R)-GFP (C, C′, F, and F′). Scale bar, 10 μm.

with 2.5% BSA in PBS, and incubated with rabbit anti-dynein polyclonal antibodies (gift of Dr. R. Vallee, Columbia University) at a dilution of 1:750 in BSA/PBS, or rabbit anti-CENP-E polyclonal antibodies (gift of Dr. T. Yen, Fox Chase Cancer Center) at a dilution of 1:500 for 45 min at room temperature or overnight at 4°C. After washing with PBST, cells were incubated with Alexa 546-conjugated

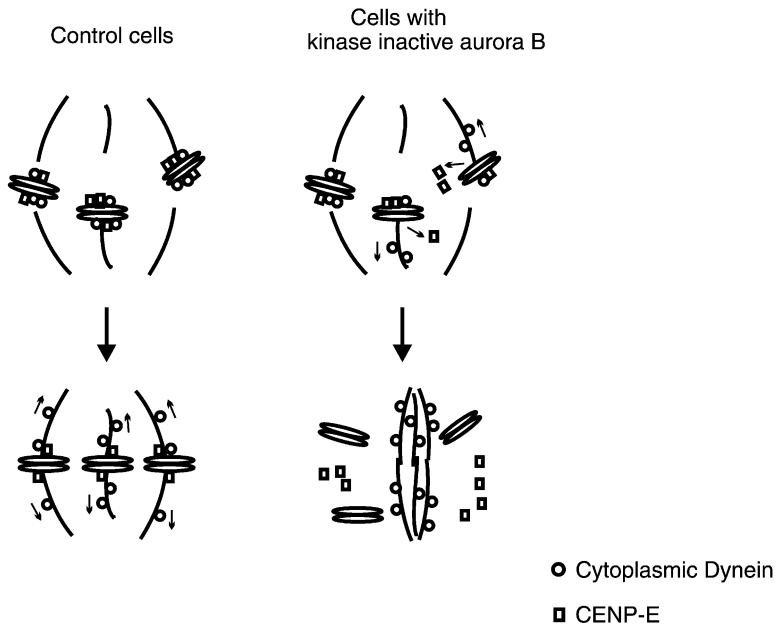


Figure 4. A Simplified Model of the Function of Aurora B during Prometaphase

In control cells, kinetochore-associated dynein and CENP-E contribute to the movement of prometaphase chromosomes, the attachment of kinetochores to microtubules, and the spindle checkpoint. They are released from kinetochores only following chromosomal congression. Deactivation of aurora B causes premature dissociation of these motors from kinetochores and leads to separation of chromosomes from microtubules and release of the spindle checkpoint. The lack of kinetochore association also causes microtubules to form a single bundle instead of multiple kinetochore fibers.

goat anti-rabbit antibodies (Molecular Probes) for 45 min at room temperature. Before observation, cells were rinsed with PBST and mounted in an anti-bleaching medium [35].

Supplementary Material

Time-lapse movies of the images in Figures 1A and 2A can be found at http://images.cellpress.com/supmat/supmatin.htm. These supplementary movies show the movements of centromeres (Movies 1 and 2) and the dynamics of kinetochore microtubules (Movies 3 and 4) in mitotic cells expressing aurora B-GFP or aurora B(K-R)-GFP.

Acknowledgments

We thank Dr. Richard Vallee (Columbia University) for providing anti-dynein antibodies; Dr. Tim Yen (Fox Chase Cancer Center) for anti-CENP-E antibodies; and Anne K. Warner for reading the manuscript. This project was supported by a National Institutes of Health grant GM-32476 to Y.-L.W.

Received: February 13, 2002
Revised: March 25, 2002
Accepted: March 27, 2002
Published: June 4, 2002

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