Autophosphorylation of a Newly Identified Site of Aurora-B Is Indispensable for Cytokinesis*

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Mitotic kinases regulate cell division and its checkpoints, errors of which can lead to aneuploidy or genetic instability. One of these is Aurora-B, a key kinase that is required for chromosome alignment at the metaphase plate and for cytokinesis in mammalian cells. We report here that human Aurora-B is phosphorylated at Thr-232 through interaction with the inner centromere protein (INCENP) in vivo. The phosphorylation of Thr-232 occurs by means of an autophosphorylation mechanism, which is indispensable for the Aurora-B kinase activity. The activation of Aurora-B spatio-temporally correlated with the site-specific phosphorylation of its physiological substrates, histone H3 and vimentin. Overexpression of the TA mutant of Aurora-B, in which Thr-232 was changed into alanine, frequently induced multinuclearity in cells. These results indicate that the phosphorylation of Thr-232 is an essential regulatory mechanism for Aurora-B activation.

Drosophila Aurora- and Saccharomyces cerevisiae Ipl1p-like protein kinases form an evolutionarily conserved family of enzymes from budding yeast to mammals (1, 2). Although the yeast genome encodes only one kinase, mammals have at least three subfamilies of Aurora/Ipl1p-related kinases. Among these kinases, Aurora-B (also called Aurora 1 or AIM-1) functions in both early and late mitotic events, including chromosome congression to the spindle equator, chromosome segregation, and cytokinesis (1–2). Aurora-B has been reported to phosphorylate histone H3 (3–6), centromere protein A (CENP-A) (7), inner centromere protein (INCENP) (8), myosin II regulatory light chain (9), topoisomerase II α (10), McgRac1GAP (11), and type 3 intermediate filaments including vimentin, glial filibrillary acidic protein, and desmin (12, 13).

Aurora-B directly binds INCENP, the first chromosomal passenger protein to be described, and INCENP is required to correctly target Aurora-B to centromeres and the central spindle (14, 15). S. cerevisiae Ipl1p phosphorylates S. cerevisiae INCENP Sli15p, and the binding of Sli15p and Ipl1p produces an increase in Ipl1p kinase activity (16). It was also found that Caenorhabditis elegans Aurora-B AIR-2 specifically phosphorylates the C. elegans INCENP ICP-1 and phosphorylated ICP-1 up-regulates the AIR-2 activity in vivo (8). We recently observed the phosphorylation of histone H3 at Ser-10 and Ser-28 when Aurora-B is co-expressed with INCENP, indicating that Aurora-B is activated through its interaction with INCENP in vivo as well as in vitro (13). However, the molecular mechanism by which Aurora-B is activated after the association with INCENP remained unclear.

We report here evidence that human Aurora-B is phosphorylated at Thr-232 through interaction with the INCENP in vivo. This phosphorylation of Thr-232 occurs by means of an autophosphorylation mechanism and is essential for the Aurora-B kinase activity.

EXPERIMENTAL PROCEDURES

Mass Spectrometry—Silver-stained protein bands were in-gel digested by trypsin. For peptide sequencing by electrospray tandem mass spectrometry, samples were analyzed on a Q-TOF Ultima quadrupole time-of-flight instrument (Waters Ltd.).

Cell Culture and Transfection—COS-7, HeLa, and Epstein-Barr nuclear antigen-expressing T24 (17) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. These cells were transfected using LipofectAMINE Plus (Invitrogen). For Aurora-B and INCENP introduced into COS-7, the cells were transfected with expression plasmids for Aurora-B and INCENP at the rate of 1:10. Twenty-four or 48 h after transfection, the cells were fixed for immunocytochemical studies. In some experiments, 48 h after transfection, the mitotic cells were prepared as described (17) and incubated for 3 h to allow for cell cycle progression. Intermediate filament (IF) bridge formation and multinuclear cells were analyzed immunochemically. Metabolic labeling was carried out by the addition of 0.1 μCi/ml [35S]orthophosphate for 4 h in phosphate-free medium.

Immunofluorescence and Antibodies—The transfected cells or cells grown on glass slips were fixed with 3.7% formaldehyde in ice-cold phosphate-buffered saline for 10 min and then treated with methanol at −20 °C for 10 min or with 0.1% Triton-X100 at room temperature for 10 min. Cells were incubated with primary antibodies overnight at 4 °C and then with secondary antibodies for 1 h at 37 °C. The DNAs were stained with 0.5 mg/ml propidium iodide or 0.5 μg/ml 4′,6-diamidino-phenylindole-dihydrochloride. An anti-pT232 antibody (pAB2.1) was generated by immunizing mice with the synthetic peptide SLRRKTp-SLRRKT

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(anti-Myc, from Berkeley Antibody) mouse mAb diluted 1:100, Alexa Fluor 488-conjugated anti-mouse, anti-rat, or anti-rabbit IgG (Molecular Probe) diluted 1:400, Cy3-conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences) diluted 1:400, H9251 Aurora-B rabbit pAb diluted 1:100, H9251 Aurora-A mouse mAb diluted 1:500, pT288 (anti-phospho-Ser 288 on Aurora-A from Cell Signaling Technology) diluted 1:250, vi- mentin goat pAb (18) diluted 1:1000, fluorescein isothiocyanate-conju- 
gated anti-goat IgG (American Qualex) diluted 1:200.

Gene Construction—Wild type or KR mutant Myc-Aurora-B has been reported (6). Aurora-B TA (T232A) and TE (T232E) were mutated using the QuikChange site-directed mutagenesis kit (Stratagene). Wild type or mutant HA-INCENP was introduced in mammalian expression vec- tor pCMV. INCENP1 (residues 509–880), INCENP2 (residues 729–880), and INCENP3 (residues 1–834) were obtained by PCR from pCMV-INCENP-HA. For the INCENP SA (S861A and S862A) mutants, XbaI fragment was generated by PCR with oligonucleotide mutation primers, and then the fragment was replaced by the corresponding XbaI fragment in clone pCMV-INCENP-HA. pEGFP-Aurora-B TA and -INCENP SA were con- structed by inserting a fragment of Aurora-B TA clone into the EcoRI and XbaI sites of pEGFP-C2 (Clontech) and by inserting the HindIII-XhoI fragment of INCENP-SA clone into the HindIII-SalI sites of pEGFP-N1 (Clontech), respectively. Wild type or mutant vimentin, with mutations at Aurora-B phosphorylation sites, have been described elsewhere (12).

Aurora-B Kinase and Binding Assay—Mammalian cell extracts were prepared in buffer: 50 mM Hepes (pH 8.0), 150 mM NaCl, 2.5 mM ATP, 5 mM MgCl2, and 0.1 mM calyculin A) containing 50 μg of histone H3 as a substrate. The kinase activities were detected by Western blot analysis with PH10 or HTA28 antibody.

RESULTS AND DISCUSSION

To further examine the mechanism of Aurora-B activation, we first studied the phosphorylation state of Aurora-B during the cell cycle (Fig. 1A). Endogenous Aurora-B was immunoprecipitated with anti-Aurora-B from Transduction Laboratories and evaluated by autoradiography and by immunoblot with anti-Aurora-B antibody. B, anti-phospho-Thr-232 specific antibody (Ab) was generated. The site of phosphorylation, Thr-232, is conserved in human Aurora-B, A, and Ipl (top). Amino acid sequences of the synthetic peptides T232 and PT232 (left) and specificity of pT232 determination by enzyme-linked immunosorbent assay (right) are shown. Micrometer plates were coated overnight with 1 μg/ml of the peptides, blocked, and reacted with the antibody, C, specificity of anti-pT232 antibody using dephosphorylation assay or immunoabsorption assay. GST-Aurora-B (200 ng) was immunoblotted with anti-pT232 antibody after preincubation with or without A-protein phosphatase (PPase) (400 units) or absorption with PT232 or T232 synthetic peptide (50 μg/ml). CBB, Coomassie Brilliant Blue. D, the obtained antibody pT232 recognizes a single protein in crude extracts prepared from mitosis (M)-enriched cells or interphase (I) cells. Each sample was resolved and immunoblotted with anti-Aurora-B (top) or pT232 (bottom) antibody.
sites of human Aurora-B using mass spectrometry. This analysis identified phosphorylation sites in Aurora-B, including Thr-232 within the activation loop of the catalytic domain of the kinase (data not shown). Thr-232 of human Aurora-B is an equivalent residue of Thr-288 of human Aurora-A and Thr-260 of budding yeast Ipl1p, both of which are known to be phosphorylated in vivo (21–23) (Fig. 1B). We examined the significance of the phosphorylation of Thr-232 of Aurora-B in this study, and for this, we generated an anti-phosphoT232 (pT232) antibody, the specificity of which was confirmed in enzyme-linked immunosorbent assay assays (Fig. 1B). We have previously reported that the bacterially produced glutathione S-transferase (GST)-Aurora-B was able to efficiently phosphorylate histone H3 and type 3 intermediate filaments including vimentin, glial fibrillary acidic protein, and desmin in vitro, and therefore, GST-Aurora-B was thought to be in an active state. The anti-pT232 antibody reacted with GST-Aurora-B, suggesting that GST-Aurora-B is phosphorylated at Thr-232 (Fig. 1C). The reactivity against GST-Aurora-B was abolished after the treatment of GST-Aurora-B with the λ protein phosphatase (λ-PPase, a dual specificity phosphatase) or after the incubation of the antibody with the PT232 peptide antigen (Fig. 1C), which further indicated the specificity of the antibody. The anti-pT232 antibody reacted with mitotic Aurora-B, confirming that Thr-232 of Aurora-B is phosphorylated during mitosis in vivo (Fig. 1D). In mitosis, ~90% of Aurora-B is phosphorylated at Thr-232 (data not shown).

As described earlier, we recently demonstrated that the co-expression of Aurora-B and INCENP results in Aurora-B activation in vivo (13). Using this co-expression system, we examined the phosphorylation state of Thr-232 when Aurora-B is activated (Fig. 2A). When only wild type Aurora-B was overexpressed, phosphorylation of Thr-232 was not observed. In contrast, when wild type Aurora-B and wild type INCENP were co-expressed, marked phosphorylation of Thr-232 was detected, thereby indicating that the phosphorylation of Thr-232 is associated with the activation state of Aurora-B. Next, the kinase-inactive mutant of Aurora-B (KR), which has a mutation at the ATP binding site, and wild type INCENP were co-expressed. In this case, we found no phosphorylation of Thr-232, suggesting that Thr-232 by itself is phosphorylated by Aurora-B (i.e. autophosphorylation). The frequency of detection of pT232-positive cells after these transfections is shown in Fig. 2B. The reactivity of the anti-pT232 antibody in the cell stain-
expression of Aurora-B and INCENP were also detected by immunoblots with pT232, anti-Myc, or anti-HA antibody.

Procedures.

in COS-7 cells. After 24 h, Aurora-B was immunoprecipitated, and the kinase reaction was carried out as described under BHTA28 (C. elegans) CENP, the phosphorylation of Thr-232 occurs through autophosphorylation. In vitro phosphorylation assay showed that recombinant GST-Aurora-B was phosphorylated at Thr-232 and was unable to phosphorylate histone H3 at Ser-10 and Ser-28 in vitro, whereas immunoprecipitated Myc-Aurora-B KR was not phosphorylated at Thr-232 and was unable to phosphorylate histone H3. In vitro phosphorylation assay showed that recombinant GST-Aurora-B was phosphorylated at Thr-232 and was able to phosphorylate histone H3, whereas recombinant GST-Aurora-B KR was not phosphorylated at Thr-232 and was unable to phosphorylate histone H3. In vitro phosphorylation assay showed that recombinant GST-Aurora-B was phosphorylated at Thr-232 and was able to phosphorylate histone H3, whereas recombinant GST-Aurora-B KR was not phosphorylated at Thr-232 and was unable to phosphorylate histone H3 (Fig. 2D). In addition, GST-Aurora-B was able to directly phosphorylate MBP-Aurora-B KR at Thr-232 in vitro (Fig. 2E). These results indicate that when Aurora-B is activated through association with INCENP, the phosphorylation of Thr-232 occurs through autophosphorylation.

In the next set of experiments, we determined whether the phosphorylation of Thr-232 of Aurora-B is important for Aurora-B kinase activity (Fig. 3). When wild type INCENP and wild type Aurora-B were co-expressed, the phosphorylation of histone H3 at Ser-10 and Ser-28 occurred, thereby indicating the activation of Aurora-B (Fig. 3, A and B). When wild type INCENP and TA mutant of Aurora-B, in which Thr-232 was changed to alanine, were co-expressed, the phosphorylation of histone H3 at Ser-10 and Ser-28 was not detected (Fig. 3, A and B). This indicates that the TA mutant is kinase-dead in vivo. We next examined whether the replacement of Thr-232 with Glu (TE mutant) might elevate the kinase activity. Aurora-B TE mutant, however, was not active even when it was co-expressed with INCENP (Fig. 3C), suggesting that Aurora-B TE was not able to mimic the activated Aurora-B. To further clarify the effect of Thr-232 phosphorylation on the activation of Aurora-B, we examined the kinase activity of the TA mutant of Aurora-B. As shown in Fig. 3D, when wild type INCENP and wild type Aurora-B were co-expressed, immunoprecipitated Aurora-B was phosphorylated at Thr-232 and could phosphorylate histone H3 at Ser-10 and Ser-28 in vitro. In contrast, when wild type INCENP and TA mutant of Aurora-B were co-expressed, immunoprecipitated TA mutant of Aurora-B could not phosphorylate histone H3 in vitro. Taken together, these results suggest that Thr-232 phosphorylation of Aurora-B is indispensable for the activation of Aurora-B.

It was reported that C. elegans INCENP is phosphorylated at serines 598 and 599 (Ser-598/Ser-599) by Aurora-B and then increases Aurora-B kinase activity in vitro (8). We next examined the effects of the phosphorylation of mouse INCENP at Ser-861/Ser-862, which are equivalent residues of Ser-598/Ser-599 of C. elegans INCENP, on Aurora-B activity in vivo. To this end, we co-transfected wild type Aurora-B and various INCENP mutants (Fig. 4A) and then checked the Thr-232 phosphorylation of Aurora-B and Ser-28 phosphorylation of histone H3 (pS28) (Fig. 4, B and C). Among the co-transfection of INCENP Δ1, Δ2, or Δ3 mutants with Aurora-B, that of INCENP Δ3 resulted in a marked decrease in the frequency of pT232- and pS28-positive cells as compared with the co-transfection with wild type INCENP and Aurora-B. This indicates that the carboxyl-terminal IN-box domain of INCENP, contain-

![Figure 3](image-url)

**Fig. 3. Aurora-B kinase activity is required for phosphorylation of Thr-232.** A and B, COS-7 cells were transfected with expression plasmids for Myc-Aurora-B WT, TA, or TE and together, with or without INCENP-HA as indicated, and double-stained with αPH10 (A and C) or HTA28 (B), and anti-Myc antibodies. Scale bars, 20 μm. The percentage of αPH10- (A and C) or HTA28- (B) positive cells was scored (right in A, B, or C). Data are means ± S.E. of at least triplicate determination. D, Myc-tagged Aurora-B WT or TA and HA-tagged INCENP were expressed in COS-7 cells. After 24 h, Aurora-B was immunoprecipitated, and the kinase reaction was carried out as described under “Experimental Procedures.” Aurora-B kinase activities were detected in immunoblots with anti-PH10 and HTA28 antibodies. Aurora-B autophosphorylation and expression of Aurora-B and INCENP were also detected by immunoblots with pT232, anti-Myc, or anti-HA antibody.
ing the Aurora-B binding domain and Aurora-B phosphorylation sites, is indispensable for Aurora-B activation in vivo and that the NH2-terminal domain of INCENP has mild effects on the activation of Aurora-B. When the SA mutant of INCENP, in which serine residues of Aurora-B phosphorylation sites were changed to alanine (S861A/S862A), was co-expressed with Aurora-B, the phosphorylation of Thr-232 and Ser-28 markedly decreased. In contrast, the co-transfection of INCENP/H90041o or INCENP/H90042 SA and Aurora-B resulted in only mild reduction of pT232- and pH28-positive cells as compared with the co-transfection with INCENP Δ1 or Δ2 and Aurora-B, respectively. These results indicate that the phosphorylation of the IN-box domain is important for the “full-length” INCENP to activate Aurora-B, although the phosphorylation of the IN-box domain is not so necessary for the INCENP lacking its amino-terminal domain to activate Aurora-B. We next asked whether the binding between Aurora-B and INCENP was regulated by their phosphorylations (Fig. 4D). After wild type or the TA mutant Aurora-B was co-transfected with wild type or SA mutant INCENP, co-immunoprecipitation of INCENP with Aurora-B was detected. When wild type Aurora-B and SA mutant INCENP were co-transfected, Thr-232 phosphorylation of Aurora-B moderately decreased as compared with that of the co-transfection of wild type Aurora-B and wild type INCENP. We detected no differences in affinities between wild type or TA mutant Aurora-B and wild type or SA mutant INCENP. Thus, the phosphorylation of Aurora-B and INCENP may not affect their interaction.

We examined the subcellular localization of activated Aurora-B during the cell cycle, using an anti-pT232 antibody (Fig. 5). We first compared the subcellular distribution of Aurora-B and that of Thr-232-phosphorylated Aurora-B (Fig. 5A). Aurora-B expression is induced at the late G2 phase as described, and pT232 is also detected at the late G2 phase. Activated Aurora-B has a general chromosomal localization in G2 and prophase, moves to chromosomal centromeres during prometaphase and metaphase, and subsequently relocates to the spindle midzone during anaphase and telophase. We next observed the subcellular distribution of Aurora-A, another essential Aurora family kinase (Fig. 5B). As described earlier, activated Aurora-A is phosphorylated at Thr-288 (22) and can be detected by anti-pT288 Aurora-A antibody. In contrast to Aurora-B, activated Aurora-A was first detected in prophase and was found to localize to centrosomes and bipolar mitotic spindle poles. We further checked whether the activation of Aurora-B spatio-temporally correlated with the phosphorylation of its physiological substrates, histone H3 and vimentin. For this, we compared the subcellular distribution of pT232 and that of pS10 of histone H3 (Fig. 5C). pS10 was observed in the pT232-positive area, where Aurora-B was activated, from late G2 to metaphase. Recently, we demonstrated that Aurora-B phosphorylates vimentin at Ser-72, which may play an important role in the segregation of vimentin filaments during cytokinesis (12). We observed the subcellular distribution of pT232 and Ser-72-phosphorylated vimentin (pS72) (Fig. 5D). In this case, the localization of pT232 substantially overlapped with that of pS72 from anaphase to cytokinesis. Taken together, these results demonstrate that activated Aurora-B spatio-temporarily
phosphorylates histone H3 from the late G2 to metaphase and vimentin from anaphase to cytokinesis.

Since the overexpression of kinase-inactive Aurora-B (KR) or wild type Aurora-B is reported to disrupt cleavage furrow formation without affecting nuclear division, leading to multinucleate cells (20, 24), we checked whether the overexpression of the TA mutant of Aurora-B induces multinuclearity (Fig. 5E). Overexpression of the TA mutant frequently induced multinuclearity in cells, suggesting that the phosphorylation of Thr-232 and activation of Aurora-B is necessary to exert its function during cytokinesis. On the other hand, the overexpression of INCENP SA mutant did not produce multinucleate cells (Fig. 5E). Because the INCENP SA mutant can partially activate Aurora-B as described in Fig. 3, the overexpression of INCENP SA mutant might not completely perturb the Aurora-B function. To further investigate the mechanism related to the formation of multinucleate cells due to the impairment of Aurora-B activity, we examined the phenotype of the overexpression of vimentin mutants, in which phosphorylation sites by Aurora-B were changed to alanine or glycine (Fig. 5).

Our evidence shows that Aurora-B is phosphorylated at Thr-232 through association with INCENP and then is activated. It was reported that human Aurora-B forms complexes with the protein serine/threonine phosphatase type 1 (PP1) or PP2, and these phosphatases may negatively regulate Aurora-B kinase activity (25). Thus, the regulation of the Thr-232 phosphorylation state of Aurora-B is crucial for kinase activity and function. These observations pave the way for ongoing research on upstream regulators and downstream targets of Aurora-B as well as for a better understanding of the role of Aurora-B in carcinogenesis.

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