Destruction Box–Dependent Degradation of Aurora B Is Mediated by the Anaphase-Promoting Complex/Cyclosome and Cdh1

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Abstract

Aurora B kinase, a subunit of the chromosomal passenger protein complex, plays essential roles in spindle assembly, chromosome bi-orientation, and cytokinesis. The kinase activity of Aurora B, which peaks in mitosis, is tightly controlled in the cell cycle. Modulation of Aurora B protein levels could partly account for the regulation of its kinase activity in the cell cycle. However, little is known on the molecular mechanism of regulation of Aurora B levels. Here, we examined Aurora B protein levels and confirmed that they fluctuate during the cell cycle, peaking in mitosis and dropping drastically in G_1. This profile for Aurora B in the cell cycle is reminiscent of those for substrates of the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase essential for mitotic progression. Indeed, Aurora B is a substrate of APC/C both *in vitro* and *in vivo*. Aurora B is efficiently ubiquitinated in an *in vitro* reconstituted system by APC/C that had been activated by Cdh1. The recognition of Aurora B by APC/C-Cdh1 is specific as it requires the presence of a conserved D-box at the COOH terminus of Aurora B. Furthermore, endogenous Aurora B and Cdh1 form a complex exclusively in mitotic cells. Degradation of Aurora B at the end of mitosis requires Cdh1 *in vivo* as a reduction of the Cdh1 level by RNA interference stabilizes the Aurora B protein. We conclude that, as a key mitotic regulator, Aurora B is regulated both by its activation during early mitosis and by its destruction by APC/C-Cdh1 in late mitosis and in G_1. (Cancer Res 2005; 65(19): 8730-5)

Introduction

The ubiquitin-proteasome system is a common mechanism used in eukaryotic cells to regulate biological transitions through protein destruction (1, 2). In this system, ubiquitin chains are conjugated to target proteins in an ATP-dependent manner, thus marking them for degradation by the 26S proteasome. Ubiquitination of a target protein requires three enzymes, a ubiquitin-activating enzyme (E1), a conjugating enzyme (E2), and a ubiquitin ligase (E3). In this cascade of enzymatic reactions, ubiquitin is first activated by E1 via a thioester linkage; the activated ubiquitin is then transferred to E2 and finally to a substrate through the action of a substrate-specific E3. The polyubiquitinated substrate is then degraded by the proteasome. This irreversible process of protein destruction has the defining characteristic of providing switchlike control of cellular transitions essential for the cell cycle regulation (2).

The anaphase-promoting complex/cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase essential for mitotic progression, was originally identified as a ubiquitin ligase for cyclin B (2–5). Subsequent studies showed that APC/C is not only active at anaphase to degrade cyclin B, but its activity persists until the end of G_1 (6–8), targeting a large group of cell cycle regulators for degradation. Activation of APC/C in the cell cycle is attributed to mitosis-specific phosphorylation of APC/C subunits (9, 10) and to the binding of *Fizzy* family proteins, Cdc20 and Cdh1 (7, 11). Genetic and biochemical studies indicate that Cdc20 and Cdh1 are essential regulators of APC/C activity; they directly bind to and activate APC/C. In the cell cycle, Cdc20 activates APC/C at the onset of anaphase to trigger chromosome separation, whereas Cdh1 replaces Cdc20 at late anaphase and Cdh1 remains associated with APC/C until late G_1.

APC/C controls mitosis and G_1 by targeting different substrates for destruction at different cell cycle stages. Examples of APC/C substrates include securin, Cdc20, Aurora A, Plk1, and geminin (2). APC/C recognizes either a KEN-box (K-E-N) or a destruction box (D-box; R-x-x-L, x = any amino acid) in substrates. D-box–containing substrates are recognized by either APC/C-Cdc20 or APC/C-Cdh1, whereas KEN box–containing substrates are only ubiquitinated by APC/C-Cdh1 (12–18). In addition, a unique sequence in Aurora A, termed the A-box (RxLxPSN), is also required for its efficient ubiquitination by APC/C-Cdh1 (19, 20). Although it is not clear whether the A-box sequence is directly recognized by APC/C-Cdh1, this sequence is required to activate the silent D-box in Aurora A (19, 20).

Aurora A and B are two essential mitotic protein kinases, initially identified as a result of oncogenic activities in tumor cells and mitotic phenotypes in *Drosophila* (21–24). These two kinases control many cell cycle events, ranging from centrosome maturation to mitotic entry, spindle formation, kinetochore-microtubule dynamics, and cytokinesis (21–24). Members of the mammalian Aurora kinases share a high degree of sequence identity, especially in the kinase domain (70% amino acid identity between human Aurora A and B). However, even with such a high degree of sequence similarity, members of Aurora family each exhibit a distinct pattern of cellular localization and control discrete cellular processes. For instance, Aurora A is associated with centrosomes from G_2 until mitotic exit. In contrast, Aurora B, which complexes with inner centromere protein and survivin to form a chromosomal passenger complex, is localized at inner centromeres at prometaphase and metaphase, then at the spindle midzone during anaphase, and finally at the midbody during cytokinesis (23, 24).

Aurora kinases function through their phosphorylation of mitotic regulators and of structural components of centrosomes, mitotic spindle, kinetochores, and central spindle (25). To carry out their function in mitosis, the kinase activities of both Aurora A and B are tightly regulated in the cell cycle; both peak in mitosis and drop to nondetectable levels in G_1 (26). Consistent with this,

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levels of both proteins also peak in mitosis and drop drastically in G_1 (19, 20, 24, 27), implying the existence of an active protein degradation mechanism during mitotic exit. It has been shown previously that Aurora A is degraded by the APC/C pathway at the M to G_1 transition (19, 20, 28–30). However, the mechanism of Aurora B turnover remains unclear.

In this report, we set out to investigate the mechanism that regulates the levels of the Aurora B protein in the cell cycle. We observed that the stability of Aurora B is also under the control of the APC/C pathway. Aurora B is efficiently ubiquitinated *in vitro* by APC/C-Cdh1, but only weakly by APC/C-Cdc20. Endogenous Aurora B and Cdh1 specifically associate *in vivo* during mitotic exit, at a time when Aurora B is being degraded. Furthermore, a reduction of the Cdh1 protein level by RNA interference leads to stabilization of Aurora B. Efficient ubiquitination requires the presence of a D-box in the COOH-terminal region of the protein; mutation in this D-box prevents ubiquitination by APC/C-Cdh1 *in vitro* and results in stabilization of the mutant protein *in vivo*. We conclude that the APC/C-Cdh1 pathway confines the Aurora B kinase to mitosis through proteolytic degradation of the kinase as cells exit from mitosis into G_1.

Materials and Methods

Plasmids and reagents. Human Aurora B cDNA was subcloned into pCS2 HA-FA to tag Aurora B with a hemagglutinin antigen (HA) epitope at its NH_2 terminus. Point mutants of Aurora B were generated by the QuickChange kit per instruction of the manufacturer (Stratagene, La Jolla, CA). Cdc20, Cdc27, and Cdh1 antibodies have been described previously (7). p38 mitogen-activated protein kinase (MAPK) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) mouse Cdh1 antibody from NeoMarkers (Fremont, CA) Aurora B (AIM-1) antibody from BD Transduction Labs, Aurora A was from Cell Signaling (Beverly, MA) and α-HA antibody from Covance (Princeton, NJ).

Cell culture, cell synchronization, and transfection. HeLa S3 and HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA) and antibiotics. Cells were synchronized at either the G_1-S boundary by a double-thymidine block or at prometaphase by a thymidine-nocodazole block (7). Arrested cells were released into fresh media to allow synchronous progression through the cell cycle.

Transfections were done with Effectene (Qiagen, Valencia, CA) per instruction of the manufacturer. Knockdown of Cdh1 (Fig. 3*B*) was done by transfecting cells with small interfering RNAs (Dharmacon, Lafayette, CO) directed against green fluorescent protein or Cdh1 using Oligofectamine as directed by the manufacturer (Invitrogen, Carlsbad, CA). RNA interference target sequences are (in the sense orientation) green fluorescent protein: 5′-GCAAGCTGACCCTGAAGTTC-3′ and Cdh1: 5′-GGATTAACGAGAATGA-GAAGT-3′, 5′-CAAGCTGCTGGTCTGGAAT-3′. Two small interfering RNA sequences targeted to Cdh1 gave comparable knockdown efficiency. Twenty-four hours post transfection, cells were incubated with 100 ng/mL nocodazole for 20 hours and nocodazole-arrested mitotic cells were released into fresh media containing 10 μg/mL cycloheximide (Calbiochem, San Diego, CA). At the indicated time points, cells were harvested by trypsinization and whole-cell extracts were Western blotted with the indicated antibodies.

To determine the stability of Aurora B and Aurora B DB1 *in vivo* (Fig. 4), HeLa cells were transiently transfected with the indicated HA-tagged Aurora B expression vector and, 24 hours posttransfection, cells were arrested at prometaphase by a thymidine-nocodazole block. Mitotic cells were shaken off and then released into fresh media containing 10 μg/mL cycloheximide. Cells were collected at the indicated time points and whole-cell extracts were subjected to Western blot analysis. Exit from mitosis and entry into G_1 was monitored by 4′,6-diamidino-2-phenylindole (DAPI) staining and observed under microscopy.

Recombinant protein expression and purification. Human Cdc20 and Cdh1 were expressed and purified from baculovirus-infected *Sf9* cells as described previously (7). Human UbcX and wheat E1 were expressed in *E. coli* and purified by nickel chromatography (Qiagen) and ubiquitin affinity chromatography, respectively. Ubiquitin aldehyde was purchased from Boston Biochemicals (Cambridge, MA) and ubiquitin was from Sigma (St. Louis, MO).

Ubiquitination assays. Interphase extracts from *Xenopus* eggs (31) were immunoprecipitated with α-Cdc27 antibody-protein A beads for 2 hours at 4°C to purify APC/C (7). The APC/C beads were collected by centrifugation and washed five times in buffer XB [10 mmol/L HEPES-KOH (pH 7.8), 100 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.1 mmol/L $CaCl_2$, 50 mmol/L sucrose] plus 500 mmol/L KCl and 0.5% NP40, and five times in buffer XB. Purified APC/C, on beads, was then incubated with recombinant Cdh1 or Cdc20 (18 pmol each) for 1 hour at 25°C followed by washing in buffer XB thrice. Ubiquitination reactions were initiated by mixing ^{35}S-labeled substrates with E1 (50 μg/mL), E2 (50 μg/mL), ubiquitin (1.25 mg/mL), ubiquitin aldehyde (1 μmol/L), and an energy regeneration mix (7). Reactions were preformed at 25°C and stopped at various times in SDS sample buffer. Samples from time points were analyzed by SDS-PAGE and by Phosphor-Imager (Molecular Dynamics, Piscataway, NJ).

Immunoprecipitation and Western blotting. Cells were lysed in buffer [20 mmol/L HEPES-KOH (pH 7.6), 150 mmol/L KCl, 0.1 mmol/L EDTA, 0.5% NP40, 10% glycerol, containing a protease inhibitor cocktail (Complete, Roche, Indianapolis, IN), 0.5 μmol/L microcystin, and 1 mmol/L dithiol-threitol] on ice for 30 minutes followed by centrifugation at maximum speed in a microfuge for 30 minutes at 4°C. Clarified extracts were immuno-precipitated with the indicated antibodies overnight at 4°C. Immunocom-plexes were collected by centrifugation, washed three times in the lysis buffer at 4°C, and then processed for SDS-PAGE and Western blotting. For Western blotting, whole-cell extracts or immunoprecipitates were separated by SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, blocked in 10% nonfat dry milk in buffer [10 mmol/L Tris-Cl (pH 7.8), 150 mmol/L NaCl, 0.1% Tween 20], and probed overnight with indicated antibodies in blocking buffer. Blots were developed using Enhanced Chemiluminescence Plus (Amersham, Piscataway, NJ) followed by scanning with a PhosphorImager (Molecular Dynamics).

Results

Levels of the Aurora B protein peak in mitosis and decline in G_1. We first examined levels of the Aurora B protein in synchronized tissue culture cells. HeLa S3 cells were arrested at the G_1-S boundary by a double-thymidine block and then released into fresh media to allow cells to progress through cell cycle synchronously from the G_1-S boundary to mitosis until the next G_1 phase. Whole-cell extracts were prepared and protein levels analyzed by Western blotting. Aurora B was not detectable at the G_1-S boundary but gradually accumulated as cells progressed into S and G_2 (Fig. 1*A*). Aurora B levels peaked at 9 hours post release, a time at which the majority of cells had entered mitosis and were positive for phospho-histone H3 staining and contained 4N DNA content (data not shown). As cells exit mitosis and enter G_1, Aurora B levels rapidly decreased to very low levels (Fig. 1*A*). Noticeably, Aurora B levels behaved similarly to those of Cdc20 (Fig. 1*A*), cyclin B, and Plk-1 (data not shown), three known mitotic substrates of APC/C (2, 7). To further address the kinetics of Aurora B down-regulation at mitotic exit, we next synchronized HeLa S3 cells at prometaphase using a thymidine-nocodazole block, followed by releasing into fresh media, allowing cells to synchronously exit mitosis and enter G_1. We observed that Aurora B levels decreased as cells completed mitosis and that Aurora B levels again mimicked those of Cdc20 (Fig. 1*B*). Thus, Aurora B protein levels fluctuate in the cell cycle in a manner similar to known substrates of APC/C.

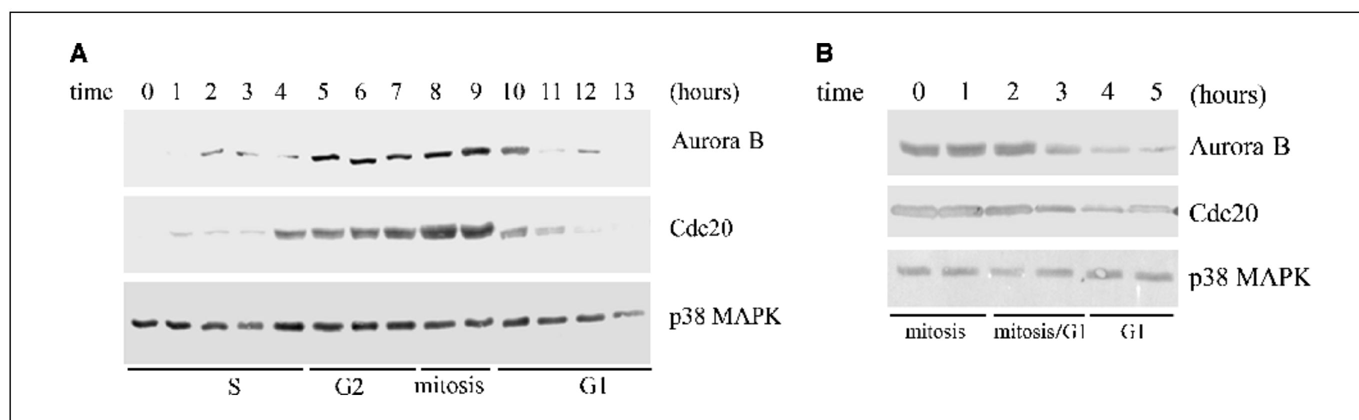

Figure 1. Levels of the Aurora B protein fluctuate in the cell cycle. *A,* HeLa S3 cells were arrested at the G_1-S boundary by a double-thymidine treatment, released into fresh media, and harvested every hour. Cell lysates were Western blotted with Aurora B, Cdc20, and p38 MAPK antibodies. p38 MAPK serves as a loading control. Cell cycle stages were determined by flow cytometry (data not shown) and indicated in the figure. *B,* HeLa S3 cells were arrested at prometaphase through a thymidine-nocodazole treatment, released into fresh media, and harvested at the indicated times. Cell lysates were Western blotted with Aurora B, Cdc20, and p38 MAPK antibodies. Cell cycle stages were determined by flow cytometry (data not shown) and are indicated in the figure.

Aurora B is ubiquitinated by anaphase-promoting complex/cyclosome-Cdh1. Because the pattern of expression of the Aurora B protein mimics those of other APC/C substrates, we directly tested whether Aurora B can be ubiquitinated by APC/C *in vitro*. Inactive APC/C was isolated from *Xenopus* egg extracts by immunoprecipitation with antibodies against Cdc27, an APC/C subunit (3). The immunopurified APC/C was then activated by incubation with equimolar amounts of recombinant Cdc20 or Cdh1. The resulting active APC/C-Cdc20 and APC/C-Cdh1 were incubated with E1, E2, ubiquitin, ATP, and ^{35}S-labeled substrates synthesized by *in vitro* translation. Aurora A, a known substrate of APC/C, was used in this assay as a positive control. We observed, as expected, that APC/C-Cdh1, and, to a slightly lesser extent, APC/C-Cdc20, efficiently ubiquitinated Aurora A. Surprisingly, Aurora B was also efficiently ubiquitinated by APC/C-Cdh1. Although Aurora B was ubiquitinated by APC/C-Cdc20, the extent of ubiquitination is much less and conjugates formed only contained mono- and di-ubiquitin. This is not due to low activity of the APC/C-Cdc20 complex because it strongly ubiquitinates Aurora A, securin, and Plk-1 (Fig. 2*A*, and data not shown).

We next determined the recognition of Aurora B by APC/C-Cdh1. APC/C-Cdh1 recognizes both KEN-box and D-box sequences in substrates. Sequence analysis indicates one KEN-box and two D-boxes in human Aurora B (Fig. 2*B*), all of which are conserved in Aurora B from human, mouse, and *Xenopus*. Interestingly, DB1 is also conserved in Aurora A in all vertebrates and is required for Aurora A ubiquitination and degradation (20, 32). Because recognition of Aurora A by APC/C-Cdh1 depends on a COOH-terminal D-box (20, 32), we mutated both DB1 and DB2 in Aurora B. Site-directed mutational analyses indicated that DB1 is essential for recognition by APC/C-Cdh1, as mutating RxxL to AxxA in this region abolished APC/C-Cdh1–dependent ubiquitination (Fig. 2*C*). In contrast, mutation of DB2 had no detectable effect on the ability of Aurora B to be ubiquitinated by APC/C in a Cdh1-dependent manner. Thus, our data indicate that the D-box in Aurora B at amino acids 315 to 318 is required for ubiquitination by APC/C-Cdh1. Recognition of Aurora A by APC/C-Cdh1 seems to also require this same conserved D-box (19, 20, 28, 32). It is also interesting to note that Aurora A is more robustly ubiquitinated by APC/C-Cdh1 than Aurora B under identical conditions (Fig. 2*A*).

Aurora B is a target of Cdh1 *in vivo*. To confirm that Aurora B is a target of APC/C-Cdh1 *in vivo*, we examined whether Aurora B and Cdh1 form a complex in mitotic cells. HeLa S3 cells were synchronized at prometaphase by a thymidine-nocodazole treatment and then released into fresh media. Cdh1 was immunoprecipitated at various time points after release and the immunocomplexes analyzed by Western blotting with Cdh1 and Aurora B antibodies. Aurora B specifically associated with Cdh1 during mitotic exit (Fig. 3*A*). The kinetics of association between Aurora B and Cdh1 is consistent with our above observations because levels of Aurora B decrease sharply just after the time at which we detect association between Cdh1 and Aurora B (Fig. 3*A*).

We next showed a requirement of Cdh1 in degradation of Aurora B *in vivo*. The level of endogenous Cdh1 was partially reduced through transfection of a small interfering RNA targeted to Cdh1 (Fig. 3*B*; ref. 33). Transfected cells were arrested at mitosis by incubating with nocodazole for 20 hours, and mitotic cells were then released into fresh media and examined at various time points in the presence of cycloheximide to determine the half-life of Aurora B *in vivo* (Fig. 3*B*). We observed that knockdown of Cdh1 resulted in a significant increase in the stability of Aurora B. Similarly, the stability of other well-characterized APC/C-Cdh1 substrates, such as Aurora A, was also increased in the Cdh1-knockdown cells (Fig. 3*B*). Under our partial knockdown conditions, a reduction of the Cdh1 level did not significantly alter the kinetics of mitotic exit as determined by examination of DAPI-stained cells under microscopy. Three hours post release, all of the transfected cells had completed mitosis and entered G_1 (data not shown). Therefore, an elevation of Aurora B levels in Cdh1 knockdown cells could not be attributed to a delay in mitotic exit. Thus, the stability of Aurora B is under the control of the APC/C-Cdh1 pathway *in vivo*.

Aurora B DB1 mutant is stable *in vivo*. We next examined the recognition of Aurora B by APC/C-Cdh1 *in vivo*. HeLa cells were transfected with vectors expressing HA-Aurora B or HA-Aurora B DB1. Cells were arrested at prometaphase by a thymidine-nocodazole block. Mitotic cells were shook off and released into fresh media containing cycloheximide and the levels of HA-Aurora B and HA-Aurora B DB1 mutant were determined by Western blot analysis. Similar to the endogenous Aurora B,

ectopically expressed HA-Aurora B was degraded on exit from mitosis (Fig. 4). In contrast, HA-Aurora B DB1 remained stable on exit from mitosis into G_1. We conclude that degradation of Aurora B *in vivo* requires D-box 1, which provides independent support for the conclusion that Aurora B is degraded through the APC/C pathway *in vivo*.

Discussion

In this study, we present evidence that Aurora B is degraded during mitotic exit and in G_1 in an APC/C-Cdh1–dependent manner. Although it has been previously shown that Aurora B protein levels fluctuate in the cell cycle, the precise molecular mechanism behind this phenomena is not clear (24). In fact, it has been assumed that Aurora B is not subject to regulation by APC/C-Cdh1 (28), although ubiquitination of Aurora B has been observed *in vitro* (34) and Aurora B has been shown to interact with the proteasome (35). The data presented here show that the APC/C-Cdh1 pathway is responsible for down-regulation of Aurora B during mitotic exit. First, the profile of Aurora B protein in the cell cycle closely mimics those of other APC/C-Cdh1 substrates, such as Cdc20 and cyclin B. Second, in an *in vitro* reconstituted assay, we found that APC/C-Cdh1 efficiently ubiquitinates Aurora B. Third, endogenous Aurora B interacts with Cdh1 at a time when its level is down-regulated. Fourth, knockdown of Cdh1 by RNA interference stabilizes the Aurora B protein. Fifth, mutation in a specific recognition element, D-box 1, abolishes ubiquitination of Aurora B by APC/C-Cdh1 *in vitro* and leads to stabilization of the mutant protein in mitotic and G_1 cells. Thus, we conclude that Aurora B is a physiologic substrate of the APC/C-Cdh1 ubiquitin ligase.

The observation that Aurora B is a substrate for APC/C-Cdh1 is in sharp contrast to the previous observation that Aurora B is unable to be degraded by the APC/C pathway in *Xenopus* extracts that contain exogenous Cdh1 (28). A likely explanation for this

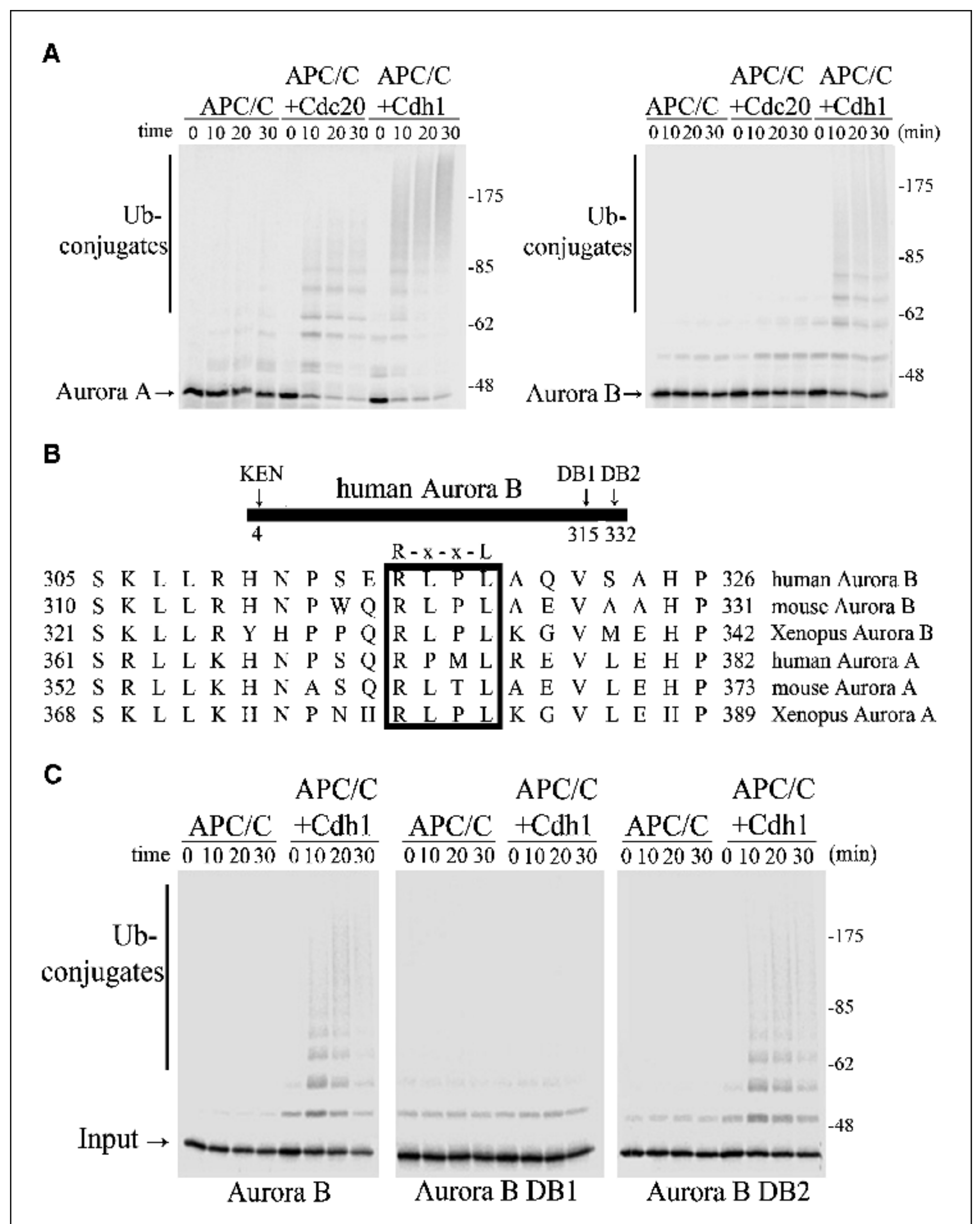

Figure 2. Aurora B is ubiquitinated by APC/C-Cdh1 *in vitro. A, in vitro* ubiquitination of Aurora A and B. APC/C was immunopurified from *Xenopus* interphase extracts and activated with equal molar amounts of recombinant Cdc20 or Cdh1. APC/C-dependent ubiquitination of ^{35}S-labeled Aurora A and B was analyzed in the presence of E1, E2, ubiquitin, and an energy mix. Note that APC/C-Cdh1 ubiquitinates both Aurora A and B whereas APC/C-Cdc20 efficiently ubiquitinates only Aurora A. *B, top,* a schematic representation of Aurora B with positions of the KEN-box and the two D-boxes (DB1 and 2) indicated. *Bottom,* conservation of DB1 in Aurora A and B from various species. The conserved amino acids in DB1 are outlined. *C,* recognition of Aurora B by APC/C-Cdh1. Wild-type and D-box mutants of Aurora B were analyzed in the APC/C-Cdh1 ubiquitination assay as described in *A*.

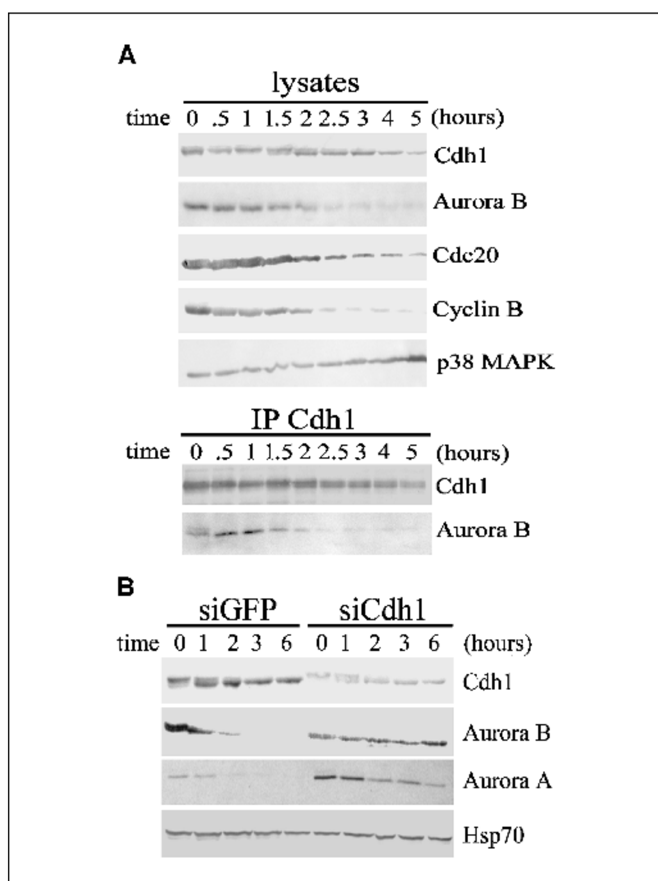

Figure 3. Aurora B is a substrate of APC/C-Cdh1 *in vivo. A,* HeLa S3 cells were synchronized at prometaphase by a thymidine-nocodazole block and released for the times indicated. Cdh1 was immunoprecipitated and the immunocomplexes were analyzed by Western blotting with antibodies indicated. Levels of Aurora B, Cdh1, Cdc20, cyclin B, and p38 MAPK in the whole-cell extracts were also shown. *B,* knockdown of Cdh1 stabilizes Aurora B in HeLa cells. Expression of Cdh1 was knocked down by a small interfering RNA directed against Cdh1. Cells were arrested at prometaphase by incubating with 100 ng/mL nocodazole for 20 hours and mitotic cells were then released into fresh media containing cycloheximide, an inhibitor of protein synthesis, for the indicated times. Whole-cell extracts were Western blotted with the indicated antibodies. The heat shock protein 70 (*Hsp70*) Western blot serves as a loading control. Knockdown of Cdh1 to this level did not significantly alter the kinetics of mitotic exit (data not shown).

discrepancy is that exogenously added recombinant Cdh1 may not fully activate APC/C in extracts. Indeed, of five physiologic substrates (Aurora A, securin, Kid, Cdc20, and Plk1) tested in our laboratory, only three (Aurora A, securin, and Kid) were degraded in *Xenopus* extracts in which APC/C had been activated by recombinant Cdh1.[1]

Aurora A and B are two key kinases controlling many different events in mitosis. Although both Aurora A and B are substrates of APC/C-Cdh1, the efficiency of their ubiquitination in our reconstituted system is different. Aurora A is ubiquitinated more efficiently than Aurora B. The biochemical basis for this differential ubiquitination may result from the way by which Aurora A and Aurora B are recognized by APC/C-Cdh1. Although APC/C-Cdh1 recognizes D-boxes in the COOH-terminal regions of both proteins, the recognition toward Aurora A is greatly enhanced by a NH_2-terminal A-box (RxLxPSN), also known as the D-box activation domain (28), which includes a known phosphorylation site in Aurora A (20). Mutation of this phosphorylation site within the A-box to aspartic acid stabilizes Aurora A (20). Interestingly, this phosphorylation site in Aurora A is conspicuously absent in Aurora B, although some elements of the A-box are partially conserved, including the RxL motif. Thus, it is possible that differential recognition of the A box in Aurora A and B may contribute to their differential ubiquitination by APC/C-Cdh1. At the functional level, this differential efficiency in ubiquitination between Aurora A and B may imply that, relative to that of Aurora A, a higher local concentration of Aurora B may be required for APC/C-Cdh1–mediated ubiquitination. This higher local concentration could be achieved through spatial localization of the Aurora B kinase (e.g., at the midbody microtubule during cytokinesis; ref. 24). Thus, the differential efficiency in ubiquitination of Aurora A versus Aurora B may offer a temporal as well as a spatial regulation on the destruction of these two protein kinases during mitotic exit. This is consistent with our observation that, kinetically, Aurora A is degraded earlier than Aurora B during mitotic exit (data not shown).

What is the physiologic importance for degradation of Aurora B during mitotic exit and in G_1? In mitosis, Aurora B regulates chromosome condensation, microtubule-kinetochore interaction, and cytokinesis. Kinetics of the degradation of Aurora B by the APC/C-Cdh1 pathway suggests that this degradation may be coupled to the initiation, progression, or completion of cytokinesis. Given that Aurora B is a key regulator of cytokinesis (22, 23), destruction of Aurora B may provide a temporal as well as a spatial control of cytokinesis. Consistent with this hypothesis, it has been shown that overexpression of Aurora B leads to multinuclearity and increased ploidy (22–24), underscoring the importance of regulating the Aurora B protein level. Aurora B is also involved in chromosome condensation through phosphorylation of histone H3 during mitosis. Thus, unscheduled activity of Aurora B kinase in interphase cells, if the protein fails to be degraded in G_1, may interfere with S-phase events, such as replication of DNA and cohesion of duplicated daughter DNA. We noted that expression of a nondegradable mutant of Aurora B (the DB1 mutant in Fig. 4) to moderate levels in HeLa cells did not lead to an obvious defect in mitotic exit or S-phase entry (data not shown), suggesting that degradation of Aurora B is one of the mechanisms, but not the

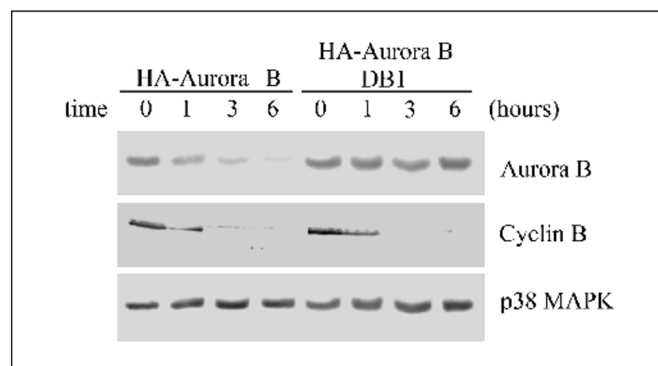

Figure 4. D-box 1 is required for degradation of Aurora B *in vivo*. HeLa cells were transiently transfected with HA-tagged Aurora B and Aurora B DB1 and synchronized at prometaphase by a thymidine-nocodazole block. Mitotic cells were isolated by shake-off and released in the presence of cycloheximide for times indicated. The levels of HA-Aurora B, HA-Aurora B DB1, and cyclin B were analyzed by Western blotting with HA and cyclin B antibodies. The p38-MAPK Western blot serves as a loading control.

[1] W. Zhao and G. Fang, unpublished observation.

sole mechanism, in the down-regulation of the kinase activity of the chromosomal passenger protein complex (Aurora B-inner centromere protein-survivin). We conclude that APC/C-mediated destruction of Aurora B provides a mechanism for precise control of the Aurora B kinase in the cell cycle. The fact that Aurora B is frequently overexpressed in various cancer cells may underscore the importance of this regulatory mechanism in the control of cell proliferation (22–24).

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