Degradation of human Aurora-A protein kinase is mediated by hCdh1

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Abstract Human Aurora-A is related to a protein kinase originally identified by its close homology to Ipl1p from *Saccharomyces cerevisiae* and aurora from *Drosophila melanogaster*, which are key regulators of the structure and function of the mitotic spindle. We previously showed that human Aurora-A is turned over through the anaphase promoting complex/cyclosome (APC/C)–ubiquitin–proteasome pathway. The association of two distinct WD40 repeat proteins known as Cdc20 and Cdh1, respectively, sequentially activates the APC/C. The present study shows that Aurora-A degradation is dependent on hCdh1 in vivo, not on hCdc20, and that Aurora-A is targeted for proteolysis through distinct structural features of the destruction box, the KEN box motifs and its kinase activity.

Key words: Aurora-A; Cdh1; Cdc20; KEN box; Anaphase promoting complex/cyclosome

1. Introduction

The most studied kinase of M-phase progression is cyclin-dependent kinase 1 (Cdk1). In addition to Cdk1, the polo-like kinases, as well as the NimA-related (NEK), Bub1, LATS and Aurora families are implicated in a variety of mitotic processes, such as centrosome separation, chromosome segregation and cytokinesis [1]. The first members of the Aurora family to be identified were the serine/threonine kinases, budding yeast Ipl1p and *Drosophila* aurora, which are required for chromosome segregation, centrosome maturation and function of the mitotic spindle [2,3]. Three genes in human and mouse, two each in the fly, frog and worm, and one in budding yeast encode the Aurora family of protein kinases. Human Aurora2/Aik/STK15/BTAK/ARK1/AIRK and mouse IAK1/Ayk1 appear to constitute a subfamily, because these protein kinases have closely related N-terminal as well as C-terminal kinase domains. Herein we refer to these proteins as Aurora-A according to the recommended nomenclature [1,4]. Aurora-A localizes to mitotic structures such as centrosomes and spindle poles, and both the message and protein levels are cell-cycle regulated [5–9]. The *Xenopus* kinesin-related protein Eg5 [10] and the cytoplasmic polyadenylation element-binding factor [11] are putative substrates of *Xenopus* Aurora-A/Eg2, but in mammals the physiological substrates and upstream regulators of Aurora-A remain unknown. Human Aurora-A is amplified in several human cancers [5,12–14] and Aurora-A overexpression causes malignant transformation in cultured cells [5,12]. These findings suggest that Aurora-A protein is important for the proliferation and genomic integrity of human cells.

The ubiquitin-proteasome pathway plays an important role in various cellular processes, including cell-cycle regulation, signal transduction, differentiation, antigen processing and degradation of tumor suppressors [15]. The ubiquitin protein ligase (E3) catalyzes the covalent attachment of polyubiquitin chains priming substrates for degradation by proteasomes. Two E3s play a role in the cell cycle: the anaphase promoting complex/cyclosome (APC/C) and the SCF (Skp1, cullin, F-box) complex [16]. The APC was the first multisubunit E3 to be described and is required for the degradation of cyclin B and sister chromatid separation [17–19]. The APC/C-dependent degradation requires the conserved activators, Cdc20/p55^{CDC}/Fizzy and Cdh1/Hct1/Fizzy-related. These two classes of proteins containing WD40 repeats bind to the APC/C and act as substrate recognition subunits, although it is not clear whether these APC/C activators regulate the activity and substrate specificity of the APC/C [18–22]. Two important destruction signals have been identified in substrates targeted for destruction by the APC/C, the destruction box and the KEN box. Cdc20-APC/C requires the destruction box. Mutations in the destruction box stabilize cyclins and significantly reduce or abolish their ubiquitination [23]. The KEN box is the transportable degradation signal of human Cdc20 for Cdh1-APC/C that has been identified using in vitro ubiquitination and proteolysis assays [24]. The KEN box is necessary, but not sufficient by itself to target human Cdc6, securin/pituitary tumor transforming gene (PTTG) and other proteins required for destruction in vivo [25–30].

2. Materials and methods

2.1. Materials

N-Acetyl-Leu-Leu-norleucinal (LLnL) was purchased from Sigma and used at a final concentration of 25 μM. Polyclonal anti-MAPK antibody was obtained from Cell Signaling Technology. Monoclonal

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Abbreviations: APC/C, anaphase promoting complex/cyclosome; PTTG, pituitary tumor transforming gene; LLnL, *N*-acetyl-Leu-Leu-norleucinal; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline

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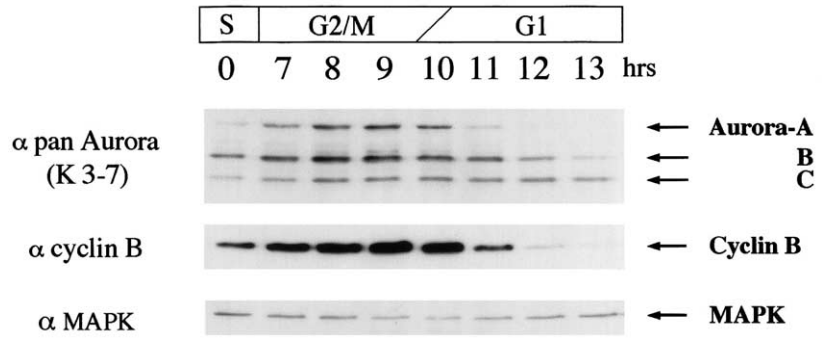

Fig. 1. Cell-cycle dependent protein expression of Aurora family. HeLa cells were synchronized at G1/S boundary by double thymidine block, then cells were collected at indicated times after release from arrest. Protein levels of Aurora family, cyclin B and MAPK were determined by immunoblotting with anti-pan Aurora (upper panel), anti-cyclin B (middle panel), or with anti-MAPK antibodies (lower panel), respectively. Cell-cycle synchronization was confirmed by flow cytometry (data not shown).

anti-cyclin B1 and anti-glutathione *S*-transferase (GST) antibodies were obtained from Santa Cruz and Amersham Biosciences, respectively. Anti-glu and anti-myc monoclonal antibodies were gifts from Dr. Larry A. Feig (Tufts University, Boston, MA, USA). Anti-Aurora-A monoclonal antibody (M11-17) has been described [9]. The monoclonal antibody K3-7 was raised against a recombinant GST-tagged Aurora-A [9] that recognizes the kinase domain of the Aurora family. Antibody specificity was tested by immunoblotting.

2.2. Plasmid Constructs

Complementary DNAs corresponding to the human Cdc20 [accession no. AF099644.1], Cdh1 [accession no. AF083810.1] and PTTG [accession no. AJ223953.1], were obtained by the reverse transcription-polymerase chain reaction (PCR) using total RNA from DLD-1 colon cancer cells [9]. We created a mammalian expression vector by inserting cDNA into an altered version of pMT3 that contained a modified glu (MEFMPME) 5′ of the cloning site [31] or into myc/pcDNA3 (Invitrogen). The plasmids, glu-Aurora-A/pMT3, Aurora-A/pGEX4T-2 and mammalian GST expression pME4T-2 have been described [9,32]. Point mutations within Aurora-A were engineered by standard double PCR mutagenesis. All PCR-amplified cDNA products were confirmed by sequencing.

2.3. Cell-cycle synchronization

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Sigma). Tissue culture dishes (10 cm) were seeded at a density of 3×10^6 with exponentially growing HeLa cells. The cells were then synchronized at S-phase using a double exposure to thymidine as follows [9]. On the day after seeding, thymidine (Sigma) was added to the medium at a final concentration of 2.5 mM and the plates were incubated for 16 h at 37°C. The plates were then washed three times with phosphate-buffered saline (PBS), then the cells were incubated in normal growth medium for 8 h at 37°C. Thereafter, the cells were incubated with thymidine again for 16 h. The cells were washed three times with PBS to remove the thymidine and normal growth medium was added. We monitored cell-cycle distribution by staining cellular DNA with propidium iodide then analyzing the cells with a FACScan (Becton Dickinson).

2.4. Transient transfection

COS-7 cells were maintained in DMEM supplemented with 10% FCS. COS-7 cells (3×10^5 cells in a 60 mm culture dish) were transfected using DEAE-Dextran [31].

2.5. In vivo degradation assay

Total cell lysates were prepared from COS cells transfected with 1 μg of glu-Aurora-A alone or together with the indicated amounts of myc-hCdc20 or myc-hCdh1. Empty vector myc/pcDNA3 was added to 3 μg of DNA. COS cells were incubated in the presence (+) or absence (–) of proteasome inhibitor LLnL for 10 h, then Aurora-A expression was measured by immunoblotting with anti-glu mAb. Levels of proteins containing WD repeats were measured by immunoblotting with anti-myc.

3. Results and discussion

3.1. Expression of human Aurora proteins is cell-cycle dependent

Mammals possess at least Aurora-A, -B and -C [4], which are cell-cycle regulated [5–7,9,33,34]. To further examine endogenous Aurora-A expression with respect to other Aurora proteins during different phases of the cell cycle, we raised the monoclonal antibody K3-7 against recombinant GST-tagged full-length human Aurora-A. This antibody cross-reacted with Aurora-B and Aurora-C recognizing the kinase domain of the Aurora family but not that of other kinases such as MAPK (data not shown). HeLa cells were synchronized by double thymidine block then released from G1/S boundary. Human Aurora proteins were immunoblotted on the same membrane

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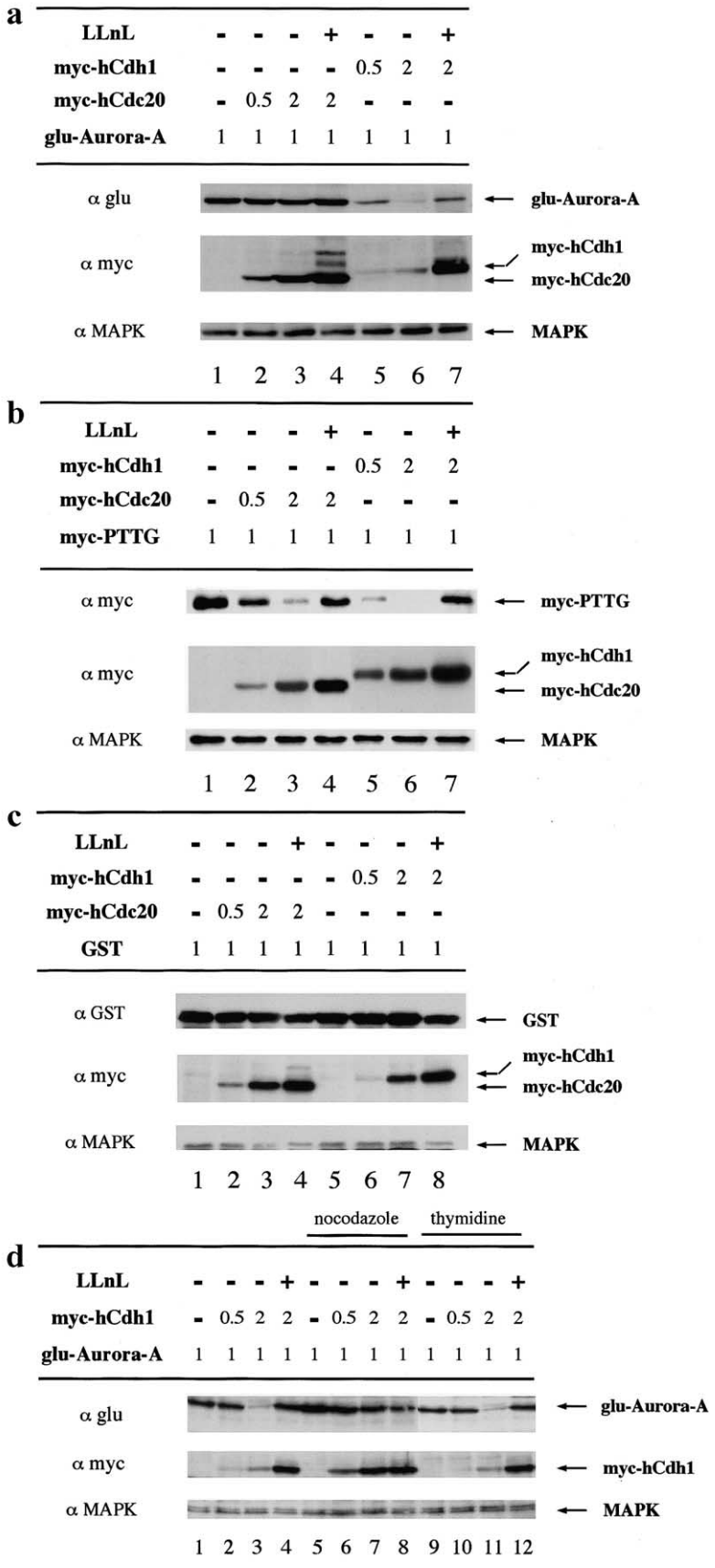
Fig. 2. Expression of hCdh1 decreases steady-state level of Aurora-A in vivo. a: Increasing amounts of hCdh1 correlate with decreased Aurora-A expression. Total cell lysates were prepared from COS cells transfected with 1 μg of glu-Aurora-A alone (lane 1) or together with 0.5 and 2 μg of myc-hCdc20 (lanes 2 and 3) or myc-hCdh1 (0.5 and 2 μg, lanes 5 and 6). Empty vector (myc/pcDNA3) was added to 3 μg of DNA. COS cells were incubated in the presence (+) or absence (–) of proteasome inhibitor LLnL for 10 h, then Aurora-A expression was measured by immunoblotting with anti-glu mAb (upper panel). Levels of proteins containing WD repeats were measured by immunoblotting with anti-myc (middle panel). b: Both hCdc20 and hCdh1 decrease steady-state level of hPTTG. Proteins levels of hPTTG were determined in COS cells transfected with myc-tagged hPTTG and increasing concentrations of myc-hCdc20 or myc-hCdh1. Amounts of DNA are shown in micrograms. Empty vector myc/pcDNA3 was added to 3 μg of DNA. c: Neither hCdc20 nor hCdh1 affects steady-state levels of GST. COS cells were transfected with GST expression plasmid pME4T-2 (1 μg) together with increasing amounts of myc-hCdc20 (lanes 2–4) or myc-hCdh1 (lanes 6–8). Transfected cells were incubated for 10 h in either the presence (+) or absence (–) of LLnL prior to lysis. Immunoblots of total cellular lysates were probed with anti-GST (upper panel), anti-myc (middle panel), or anti-MAPK (lower panel) antibodies. d: The in vivo degradation assay using S- and M-phase synchronized cells. COS cells were transfected with 1 μg of glu-Aurora-A alone or together with 0.5 and 2 μg of myc-hCdh1. The cells were then synchronized at S-phase using a single exposure to thymidine for 14 h or at M-phase by nocodazole (400 ng/ml) for 12 h. COS cells were incubated in the presence (+) or absence (–) of proteasome inhibitor LLnL for 10 h, then Aurora-A expression was measured by immunoblotting with anti-glu mAb (upper panel).


a
LLnL - - - + - - +
myc-hCdh1 - - - - 0.5 2 2
myc-hCdc20 - 0.5 2 2 - - -
glu-Aurora-A 1 1 1 1 1 1 1
α glu
glu-Aurora-A
α myc
myc-hCdh1
myc-hCdc20
α MAPK
MAPK
1 2 3 4 5 6 7
b
LLnL - - - + - - +
myc-hCdh1 - - - - 0.5 2 2
myc-hCdc20 - 0.5 2 2 - - -
myc-PTTG 1 1 1 1 1 1 1
α myc
myc-PTTG
α myc
myc-hCdh1
myc-hCdc20
α MAPK
MAPK
1 2 3 4 5 6 7
c
LLnL - - - + - - - +
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GST
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myc-hCdc20
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nocodazole
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myc-hCdh1 - 0.5 2 2 - 0.5 2 2 - 0.5 2 2
glu-Aurora-A 1 1 1 1 1 1 1 1 1 1 1 1
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1 2 3 4 5 6 7 8 9 10 11 12

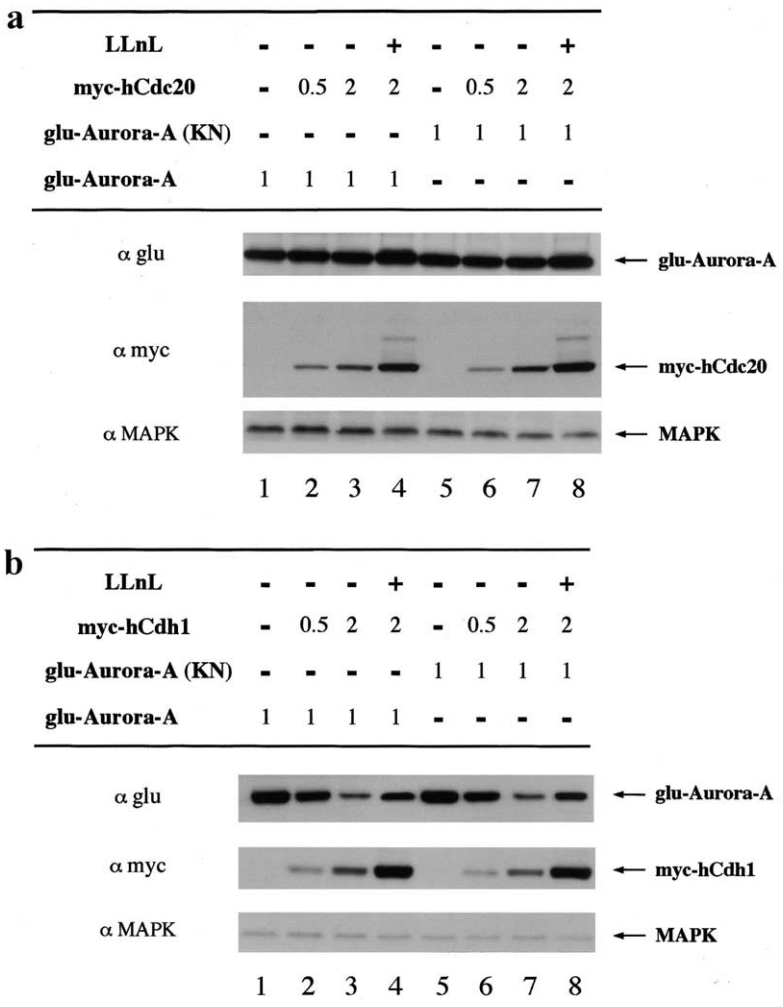

Fig. 3. Kinase activity of Aurora-A does not affect its degradation. COS cells were transfected with expression plasmids (1 μg) for glu-Aurora-A (lanes 1–4) or glu-Aurora-A (KN) (lanes 5–8) together with increasing amounts of myc-hCdc20 (a) or myc-hCdh1 (b). Transfected cells were incubated for 10 h in either the presence (+) or absence (–) of LLnL prior to lysis. Immunoblots of total cellular lysates were probed with anti-glu (upper panel), anti-myc (middle panel), or anti-MAPK (lower panel) antibodies.

using HeLa cell lysates. Consistent with the earlier findings, Aurora-A protein was absent through G1, began to accumulate in S-phase, and peaked late in mitosis (Fig. 1, upper panel). Endogenous Aurora-A was expressed in a manner similar to that of cyclin B (Fig. 1, middle panel). Although the expression level of Aurora-B and Aurora-C proteins oscillated, Aurora-B and Aurora-C were expressed for a longer period than Aurora-A (Fig. 1, upper panel). In particular, the levels of Aurora-B and of Aurora-C remained obvious during S- and early G1-phase, respectively. The control MAPK protein level did not significantly change during cell-cycle progression (Fig. 1, lower panel).

3.2. Degradation of Aurora-A by hCdh1 in vivo, but not by hCdc20

We have shown that Aurora-A is turned over through the APC/C–ubiquitin–proteasome pathway [9]. Activation of the APC/C requires association with the WD-repeat protein, Cdc20 or Cdh1 [18,19]. In this regard, Cdc20 interacts with Aurora-A in both *Xenopus* egg extracts and human cells [35]. We therefore investigated which WD-repeat protein activates APC/C for Aurora-A degradation in vivo. Aurora-A co-expressed with hCdh1 in COS cells produced a significant, dose-dependent decrease in the steady-state levels of Aurora-A protein (Fig. 2a, compare lane 1 to lanes 5 and 6), while the

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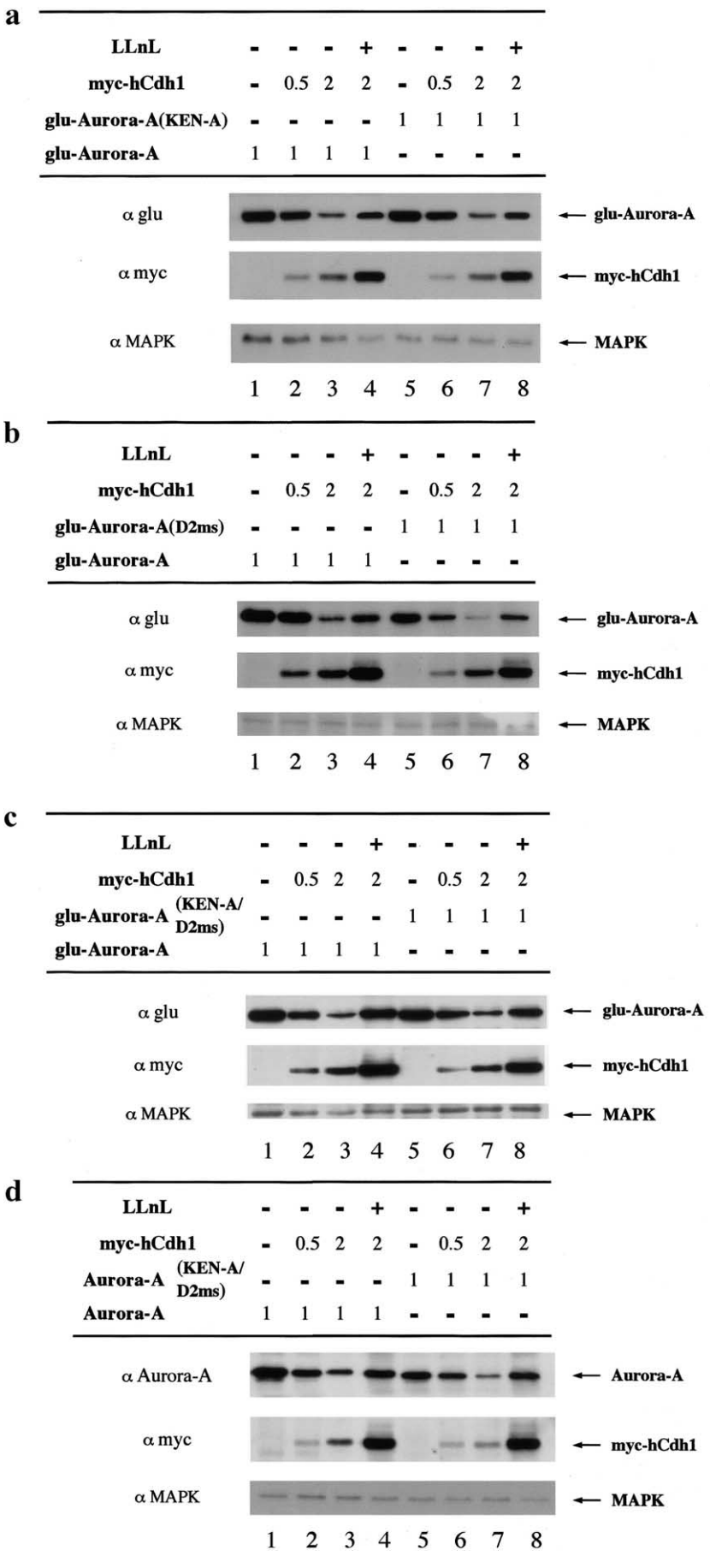
Fig. 4. KEN box and destruction box-like motifs in Aurora-A are not signals for degradation. a: Wild-type or KEN box mutant (KEN-A) forms of Aurora-A (glu-tagged) with indicated amounts of myc-hCdh1 were transiently expressed in COS cells. b: COS cells were transfected with cDNA encoding glu-Aurora-A or glu-Aurora-A (D2ms) together with indicated amounts of myc-hCdh1. c: Wild-type or KEN box/destruction box double mutant (KEN-A/D2ms) forms of Aurora-A (glu-tagged) with indicated amounts of myc-hCdh1 were transiently expressed in COS cells. Transfected cells were incubated for 10 h in either the presence (+) or absence (–) of 25 μM LLnL. Immunoblots of total cellular lysates were probed with anti-glu (upper panel), anti-myc (middle panel), or anti-MAPK (lower panel) antibodies. d: Wild-type or KEN box/destruction box double mutant (KEN-A/D2ms) forms of Aurora-A with indicated amounts of myc-hCdh1 were transiently expressed in COS cells. Levels of Aurora-A, myc-hCdh1 and MAPK were determined by immunoblotting with anti-Aurora-A (upper panel), anti-myc (middle panel), or with anti-MAPK antibodies (lower panel), respectively.


a
LLnL - - - + - - - +
myc-hCdh1 - 0.5 2 2 - 0.5 2 2
glu-Aurora-A(KEN-A) - - - - 1 1 1 1
glu-Aurora-A 1 1 1 1 - - - -
α glu
α myc
α MAPK
glu-Aurora-A
myc-hCdh1
MAPK
1 2 3 4 5 6 7 8
b
LLnL - - - + - - - +
myc-hCdh1 - 0.5 2 2 - 0.5 2 2
glu-Aurora-A(D2ms) - - - - 1 1 1 1
glu-Aurora-A 1 1 1 1 - - - -
α glu
α myc
α MAPK
glu-Aurora-A
myc-hCdh1
MAPK
1 2 3 4 5 6 7 8
c
LLnL - - - + - - - +
myc-hCdh1 - 0.5 2 2 - 0.5 2 2
glu-Aurora-A (KEN-A/D2ms) - - - - 1 1 1 1
glu-Aurora-A 1 1 1 1 - - - -
α glu
α myc
α MAPK
glu-Aurora-A
myc-hCdh1
MAPK
1 2 3 4 5 6 7 8
d
LLnL - - - + - - - +
myc-hCdh1 - 0.5 2 2 - 0.5 2 2
Aurora-A (KEN-A/D2ms) - - - - 1 1 1 1
Aurora-A 1 1 1 1 - - - -
α Aurora-A
α myc
α MAPK
Aurora-A
myc-hCdh1
MAPK
1 2 3 4 5 6 7 8

MAPK protein level did not significantly change, thus confirming that protein loading was comparable. This consequence was specific to hCdh1 because hCdc20 overexpression did not affect Aurora-A protein (Fig. 2a, lanes 1 to 3). The proteasome inhibitor LLnL restored hCdh1-dependent decreases in Aurora-A (Fig. 2a, compare lanes 6 and 7). Thus, the hCdh1-mediated down-regulation of Aurora-A was not caused by a decrease in its synthesis rate, but by accelerated turnover through proteasomes.

To rule out the possibility that hCdc20 cannot function in this system, we tested whether hCdc20 regulates steady-state levels of hPTTG, which is degraded by proteolysis mediated by the APC/C in a manner that is dependent on destruction and KEN boxes [26]. We transfected COS cells with a fixed amount of hPTTG and varying amounts of proteins containing WD repeats. Levels of hCdh1 or hCdc20 in the extracts increased in concert with a significant, dose-dependent decrease in steady-state levels of hPTTG protein (Fig. 2b). The proteasome inhibitor LLnL inhibited the decreases in hPTTG expression by both hCdc20 and hCdh1 (Fig. 2b, lanes 4 and 7). Therefore, both hCdc20 and hCdh1 mediate degradation of hPTTG in this system. In contrast, neither hCdc20 nor hCdh1 influenced steady-state levels of GST (Fig. 2c). These findings demonstrated that the specificities of the hCdc20- and hCdh1-activated forms of APC/C differed in the in vivo degradation system and that Aurora-A degradation is mediated by hCdh1, but not by hCdc20.

The level of hCdc20 is cell-cycle regulated with a peak in G2/M-phase, but its ability to activate the APC/C appears to be limited by the prior phosphorylation of APC/C core subunits by both Cdk and polo-like kinases. In contrast to hCdc20, hCdh1 is expressed throughout the cell cycle but Cdk-dependent phosphorylation of hCdh1 prevents it from binding to the APC/C core particle until late anaphase. In addition to phosphorylation, both hCdc20- and hCdh1-dependent APC/C activations are regulated by Mad2 (and its related protein Mad2b) in response to the mitotic checkpoint signals [16,18,19]. It is not clear whether the increment of hCdh1 is enough for Aurora-A degradation. To address this, we performed the in vivo degradation experiments using S- and M-phase synchronized cells. hCdh1-dependent decreases of Aurora-A in non-treated COS cells and in thymidine treated COS cells were comparable (Fig. 2d, lanes 1–4 and 9–12). In contrast to S-phase synchronized cells, hCdh1 did not significantly influence steady-state levels of Aurora-A protein in nocodazole-treated COS cells (Fig. 2d, lanes 5–8). Cdk1 kinase activity is a peak at the prometaphase, thereby inhibiting hCdh1 binding to the APC/C core particle despite the increased hCdh1 levels. These data suggest that Aurora-A is degraded in late anaphase. This assay is useful because it can test the effect of specific factors such as drugs, DNA damage, or co-transfection with other genes.

To re-examine the association of Aurora-A with hCdc20 or hCdh1, we transfected myc-tagged hCdc20 or hCdh1 expression vector with glu-Aurora-A into COS cells with or without LLnL. Glu-Aurora-A immunoprecipitates contained neither hCdc20 nor hCdh1 (data not shown). Aurora-A binding to hCdc20 or hCdh1 was undetectable, possibly because the affinity was too low to measure.

3.3. Aurora-A kinase activity does not affect its degradation

Phosphorylation and dephosphorylation exquisitely regulate APC/C activity [1]. Furthermore, Aurora family kinases form complexes with the protein serine/threonine phosphatases that regulate the kinase activation of Aurora families [32,36]. Therefore we examined whether Aurora-A kinase activity affects its degradation. We prepared a catalytically inactive mutant of human Aurora-A, in which an invariant lysine residue (K162) was replaced with arginine in the ATP-binding motif of the catalytic domain [37]. Fig. 3 shows that the kinase-deficient mutant was also degraded upon co-expression with hCdh1, but not with hCdc20. These experiments showed that Aurora-A kinase activity is not required for its own destruction and that other sequences are involved in its degradation.

3.4. The KEN box in Aurora-A is not a signal for degradation

Aurora-A lacks a functional destruction box [9] so the degradation signal is recognized by hCdh1-APC through other structural features. The KEN box (composed of the amino acids K-E-N) is an hCdh1-APC recognition signal distinct from the destruction box [24]. Aurora-A has KEN residues at the extreme amino terminus, position 5–7. The KEN box-like motif is not evolutionarily conserved in the Aurora family, even in human Aurora-C [38]. To investigate whether the KEN box in Aurora-A serves as a functional signal for degradation, we constructed a derivative of Aurora-A (Aurora-A^{KEN-A}) in which the invariant KEN residues were changed to alanines within the full-length protein. The KEN box mutant was also degraded by co-expression with hCdh1 (Fig. 4a). We have previously demonstrated that one stretch (^{205}RVYLILEYA) similar to the destruction box in Aurora-A might serve as a signal for polyubiquitination, but that this stretch alone was not sufficient to act as the degradation signal [9]. We then tested the stability of two mutants, one in the destruction box like stretch (D2ms) and a second with both a D2ms and KEN-A mutation. Fig. 4b,c shows that hCdh1 overexpression also leads to degradation of these Aurora-A proteins. To rule out any effects of the epitope tag, we performed a similar experiment using untagged Aurora-A. The hCdh1 degradative phenotype of cells transfected with wild or mutant Aurora-A (untagged) was the same as that caused by the glu-tagged versions (Fig. 4d). These results suggested that the KEN box and the destruction box-like motifs in Aurora-A are not signals for degradation in vivo. In summary, the rapid loss of human Aurora-A at the end of mitosis is due to protein degradation targeted by the hCdh1-APC/C–ubiquitin–proteasome pathway through other structural features functioning as the degradation signal.

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