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Aurora-A Kinase Phosphorylation of Aurora-A Kinase Interacting Protein (AIP) and Stabilization of the Enzyme-Substrate Complex

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Abstract Aurora-A is an oncogenic kinase that plays essential roles in mitosis as well as cell survival. Aurora-A interacting protein (AIP) was identified as a negative regulator of Aurora-A with its ectopic over expression inducing destabilization of Aurora-A protein. Here we present evidence that in human cells, contrary to the earlier report, AIP functions in stabilizing rather than destabilizing Aurora-A. Furthermore, AIP is phosphorylated on Serine 70 by Aurora-A but not Aurora-B and expression of phosphorylation mimic mutant of AIP results in prolonged protein stability compared to unphosphorylatable mutant. We observed that when co-expressed with AIP, protein levels of both Aurora-A and Aurora-B are markedly elevated regardless of their kinase activities and phosphorylation state of AIP. Interaction of Aurora kinases with AIP is necessary for this elevated stability. This phenomenon is commonly detected in several human cancer cell lines used in this study. Depletion of AIP by RNA interference decreased Aurora-A but not Aurora-B in two of the three cell lines analyzed, indicating that under physiological condition, AIP functions in stabilization of Aurora-A but not Aurora-B, though this regulation may be dependent on additional factors as well. Further, AIP siRNA induced cell cycle arrest at G2/M, which is consistent with anticipated loss of function of Aurora-A in these cells. Thus, our study provides the first evidence of a role for AIP in G2/M cell cycle progression by cooperatively regulating protein stabilization of its upstream regulator, Aurora-A kinase through protein–protein interaction as well as protein phosphorylation. J. Cell. Biochem. 102: 1318–1331, 2007.

Key words: Aurora-A kinase; AIP; phosphorylation; protein stability; cancer

Aurora kinase family members represented by Aurora-A, -B, and -C kinases play multiple essential roles in faithful chromosome segregation during mitosis in accordance with their specific localizations on centrosome, mitotic spindle and centromere [Carmena and Earnshaw, 2003]. Extensive studies have identified many interacting proteins as well as substrates for Aurora kinases, which are involved in not only mitotic regulation but also in cell survival [Katayama et al., 2003; Meraldi et al., 2004]. Aberrant regulation of cell survival by Aurora kinases due to their over expression leads to the generation of chromosome instability as detected in a number of human cancers [Katayama et al., 2003; Meraldi et al., 2004]. Ectopic over expression of Aurora-A in cultured cells results in centrosome amplification, aneuploidy and transformation [Zhou et al., 1998]. Several Aurora kinase interacting proteins, identified so far, are their *bona fide* substrates, though biological significance of these phosphorylations have been determined for only some of these substrate proteins. For instance, phosphorylation of type I protein phosphatase

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by Aurora-A results in its inactivation [Katayama et al., 2001]. Phosphorylation of CENP-A by Aurora-A is important for recruitment of Aurora-B to inner centromere [Kunitoku et al., 2003]. Aurora-B phosphorylation of INCENP has the positive feedback effect of up-regulating Aurora-B kinase activity [Honda et al., 2003]. Phosphorylation of HURP and p53 by Aurora-A positively and negatively regulate their protein stabilities respectively [Katayama et al., 2004; Yu et al., 2005]. Therefore, biological consequence of phosphorylation by Aurora kinases are quite varied and investigations on mapping of phosphorylation sites of substrates, and their effects on Aurora kinases and substrates are essential to understand the signal transduction pathways mediated by Aurora kinases.

AIP is one of the Aurora-A binding proteins but whether the protein is a substrate of Aurora kinases is not known yet. AIP was originally isolated as a gene which alleviates Aurora-A mediated cytotoxicity in yeast by using a dosage suppressor screen [Kiat et al., 2002]. Analysis of AIP function in mammalian cells found that carboxyl terminus of AIP, the same region isolated in dosage suppressor screen, interacts with Aurora-A, and ectopic over expression of AIP leads to degradation of Aurora-A through ubiquitin independent yet proteasome dependent pathway [Kiat et al., 2002; Lim and Gopalan, 2007]. Intriguingly however, northern blotting of AIP in this report showed elevated expression in several cancer cell lines in which over expression of Aurora kinases has also been detected [Kiat et al., 2002]. Besides these paradoxical findings it was also surprising that cells over expressing AIP, a negative regulator of a critical cell survival and cell cycle regulatory protein Aurora-A kinase, could still survive and proliferate. Therefore, identification of the molecular mechanisms maintaining equilibrium between Aurora-A and AIP in normal and cancer cells is of central importance.

Here we report that AIP phosphorylation on serine 70 by Aurora-A prolongs half-life of the protein. Furthermore significant increase in intracellular content of Aurora kinases is detected with over expression of AIP that contradicts the observations made in the previous study described above. Depletion of AIP by RNA interference leads to decline of Aurora-A protein but not Aurora-B. These findings suggest that under physiological conditions, AIP functions in stabilization of Aurora-A and not destabilization. In addition, AIP depletion caused cell cycle arrest at G2/M consistent with anticipated loss of function of Aurora-A in these cells. Our findings provide the first evidence of a role for AIP in cell cycle progression through cooperative stabilization of its upstream kinase Aurora-A through protein–protein interaction as well as protein phosphorylation.

MATERIALS AND METHODS

Plasmids and siRNA

A full length AIP cDNA was cloned by polymerase chain reaction (PCR) using primers (forward primer: 5′-gaattcggatgctcctggggcgcctgac-3′ and reverse primer: 5′-ctcgaggcggcgccagactcatttgccc-3′) from Hela cell cDNA library, and subcloned into pGEM-T Easy Vector (Promega). The sequence of AIP cDNA was confirmed, and subcloned into pGEX6p-2 vector (GE HealthCare), pCMV-myc vector (Invitrogen) and pEGFP-C1 vector (Clontech). S70A and S70E mutant AIPs were created by QuikChange site direct mutagenesis kit (Stratagene) and each mutation was confirmed by sequencing. Carboxyl half deletion mutant AIP (amino acids 88–199) was subcloned by PCR with primers (5′-gaattccggatggataccgggaccgcagggactg-3′) and reverse primer mentioned above into pCMV-myc vector. Aurora-A amino half (amino acids 1–129) and carboxyl half (amino acids 130–401, kinase domain) deletion mutants, and Aurora-B amino half (amino acids 1–73) and carboxyl half (amino acids 74–343, kinase domain) deletion mutants were subcloned into pCR2.1 vector (Invitrogen) by PCR using following primers. For Aurora-A amino half, forward primer: 5′-ggatccatggaccgatctaaagaaaactgc-3′ and reverse primer: 5′-tctagactcgagctaagccactgcctctttttgattc-3′, for Aurora-A carboxyl half, forward primer: 5′-ggatccatgttggaagactttgaaattggtcgc-3′ and reverse primer: 5′-tctagactcgagctaagactgtttgctagctgattc-3′, for Aurora-B amino half, forward primer: 5′-ggatccatggcccagaaggagaactc-3′ and reverse primer: 5′-gaattctcaaattgtgaagtgccgcgttaaga-3′, for Aurora-B carboxyl half, forward primer: 5′-ggatccatggatgactttgagattgggcgt-3′ and reverse primer: 5′-gaattctcaggcgacagattgaaggg-3′. Each cDNA fragment was subcloned into pGEX4T-1 vector (GE HealthCare). GL2 siRNA as control, Aurora-A siRNA [Kufer et al., 2002] and smart

pool for AIP siRNA were purchased from Dharmacon. Each siRNA was used at a final concentration of 40 nM.

Semi-Quantitative Reverse Transcriptase Coupled PCR (RT-PCR)

Semi-quantitative RT-PCR was performed using a poly-dT primer and SuperScript II transcriptase (Invitrogen), followed by PCR with AIP cloning primers described above, Aurora-A specific primers (5′-cggatccctctg-gagccttggagtt-3′ and 5′-cctcgagctaagactgtttgc-tagc-3′), Aurora-B and β-actin specific primers described earlier [Raff et al., 1997; Sasai et al., 2004].

Glutathione *S*-Transferase (GST) Fusion Protein, In Vitro Kinase Assay, and In Vitro Binding Assay

GST-Aurora kinase proteins and GST-AIP proteins were produced in BL21 pLys bacteria according to the manufacturer's protocol (Amersham Pharmacia Biotech). An in vitro kinase assay and in vitro binding assay were carried out as described earlier [Katayama et al., 2001; Sasai et al., 2004].

Transfection and Cell Synchronization

Transfection of mammalian expression vectors and siRNA were carried out using Lipofectamine and Oligofectamine (Invitrogen), respectively. For protein stability study in Figure 5A–C, flag-Auroras and myc-AIPs were used at molar ratio of 1:9 respectively. For in vivo phosphorylation study in Figure 2 and in vivo interaction study in Figure 5D, flag-Auroras and myc-AIPs were used at molar ratio of 1:1 respectively. Hela cells were synchronized at G1/S boundary with double thymidine block and release as described [Katayama et al., 2001], and then total RNA and protein were extracted. For FACS analysis, cells were stained with propidium iodide and analyzed by Becton Dickinson FACSCAN flow cytometer. To assay stability of AIP, human embryonic kidney (HEK) 293 cells transfected with myc-AIPs were treated with 80 μg/ml of cycloheximide for indicated times. Alternatively, cells were also metabolically labeled with [^{35}S] methionine (EXPRE^{35}S^{35}S Protein labeling mix; NEN). After 1 h, the cells were washed with phosphate-buffered saline and chased with complete culture medium for indicated times. For AIP stability in cell released from mitotic arrest, both HEK293 and Hela cells transfected with myc-AIP were first synchronized by thymidine for 16 h and then released into fresh medium for 5 h. Then cells were cultured in nocodazole containing medium (40 ng/ml for Hela and 300 ng/ml for HEK293) for 4 h followed by washing with PBS three times and then released into cycloheximide containing fresh medium without nocodazole for indicated times.

Immunoprecipitation and Immunoblotting

Cells extracts were prepared, as described [Sasai et al., 2004]. For immunoprecipitation, cell extracts were incubated for 3 h at 4°C with monoclonal myc antibody (Clontech) followed by incubation with protein-G agarose (Sigma) for 1 h. The immunocomplex was washed four times with lysis buffer and then resolved by SDS–PAGE and analyzed by immunoblotting with antibodies to Aurora-A, Aurora-B (BD Biosciences), myc and phospho-(ser/thr) PKA substrate, phospho-Thr288 Aurora-A (Cell signaling), Flag-M2, phosphoserine, β-actin (Sigma), and CDC37 (Affinity BioReagents).

Immunofluorescence Microscopy

Cells grown on poly-L-lysine-coated coverslips were transfected for 24 h and then fixed in 20 mM PIPES, pH 6.8, 0.2% Triton X-100, 1 mM $MgCl_2$, 10 mM EGTA, 4% formaldehyde for 10 min at room temperature followed by washing three times with PBS and incubated in 3% bovine serum albumin/PBS for 1 h. Anti-myc (1:1,000), phospho-Thr288 Aurora-A (1:300) and anti-hospho-histone H3 (Cell signaling) (1:2,000), antibodies diluted in blocking buffer were incubated at 4°C overnight. Primary antibodies were visualized using FITC, Texas-Red (PIERCE), and DNA was counterstained with DAPI. Cells were examined with a fluorescence microscope (Optiphoto-2: Nikon). Images were captured using MetaMorph software (Molecular Devices).

RESULTS

AIP mRNA Expression is Invariable During Cell Cycle

First, we investigated the expression profile of AIP mRNA during cell cycle by using synchronized Hela cells. While Aurora-A expression peaked at G2/M phase as reported earlier, AIP expression appeared very similar

through cell cycle (Fig. 1A). This expression pattern of AIP mRNA indicates that AIP functions in not only Aurora-A regulation but possibly also in additional regulatory pathways through the cell division cycle. We found that expression level of AIP is elevated in several human cancer cell lines derived from breast, pancreatic, and bladder cancers (Fig. 1B).

Aurora-A Phosphorylates AIP at Serine 70 In Vitro and In Vivo

While it has been reported that AIP destabilizes Aurora-A, whether Aurora-A reversibly controls AIP function has not yet been investigated. To examine this possibility, we first performed in vitro kinase assay to find out if AIP is a substrate of Aurora-A. Bacterially expressed GST and a GST-AIP fusion protein were incubated with either GST-wild-type-Aurora-A or GST-kinase-dead Aurora-A (K162R) in presence of γ^{32}P-ATP. Wild-type Aurora-A clearly phosphorylated AIP but not kinase-dead Aurora-A (Fig. 2A). By contrast, Aurora-B did not phosphorylate AIP, indicating that AIP is an Aurora-A specific substrate (Fig. 2A).

Consensus motif for phosphorylation site by Aurora kinases has been predicted as R/K-$(X)_{1-3}$-S/T [Meraldi et al., 2004]. There were six sites matching the consensus motifs in AIP (Fig. 2B). An in vitro kinase assay using GST-AIP with amino acid substitution of either serine or threonine in each site revealed that Aurora-A phosphorylates only serine 70 (Fig. 2C). To confirm in vivo phosphorylation of AIP at serine 70 by Aurora-A, anti-phospho-PKA substrate antibody was used because this antibody recognizes phosphorylated motif sequence, R/K-X-X-S/T, same as the motif for Aurora kinases. First, we tested the specificity of this antibody with

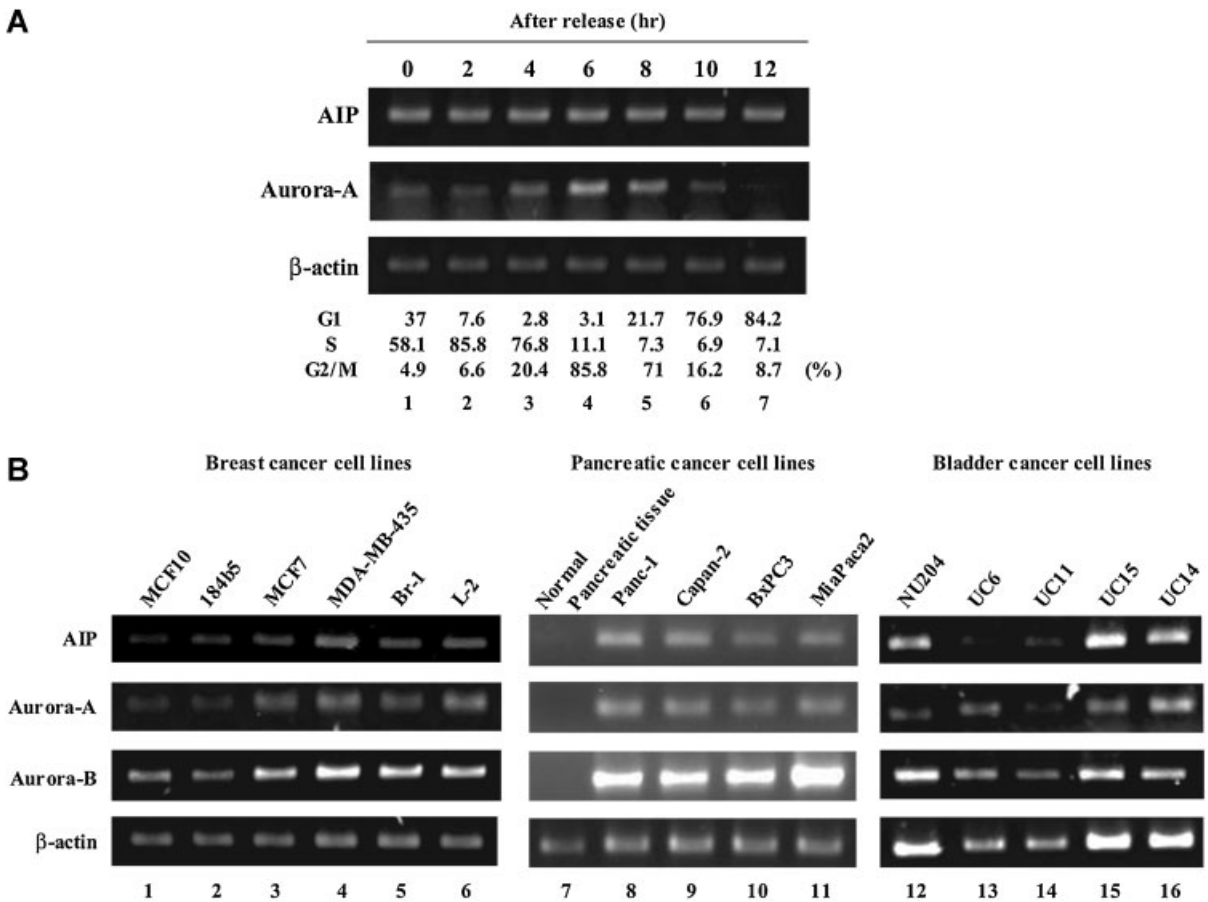

Fig. 1. Invariant expression of AIP mRNA through cell cycle. **A**: Hela cells were synchronized by double thymidine block and released into cell cycle for over 12 h, and total RNA was extracted at indicated time points. Expression level at each time point was analyzed by RT-PCR. Cell cycle distribution at each time point was determined by fluorescence-activated cell sorter analyses. **B**: Expression levels of indicated genes by RT-PCR in breast (**lanes 1–6**), pancreatic (**lanes 7–11**) and bladder (**lane 12–16**) cancer cell lines.

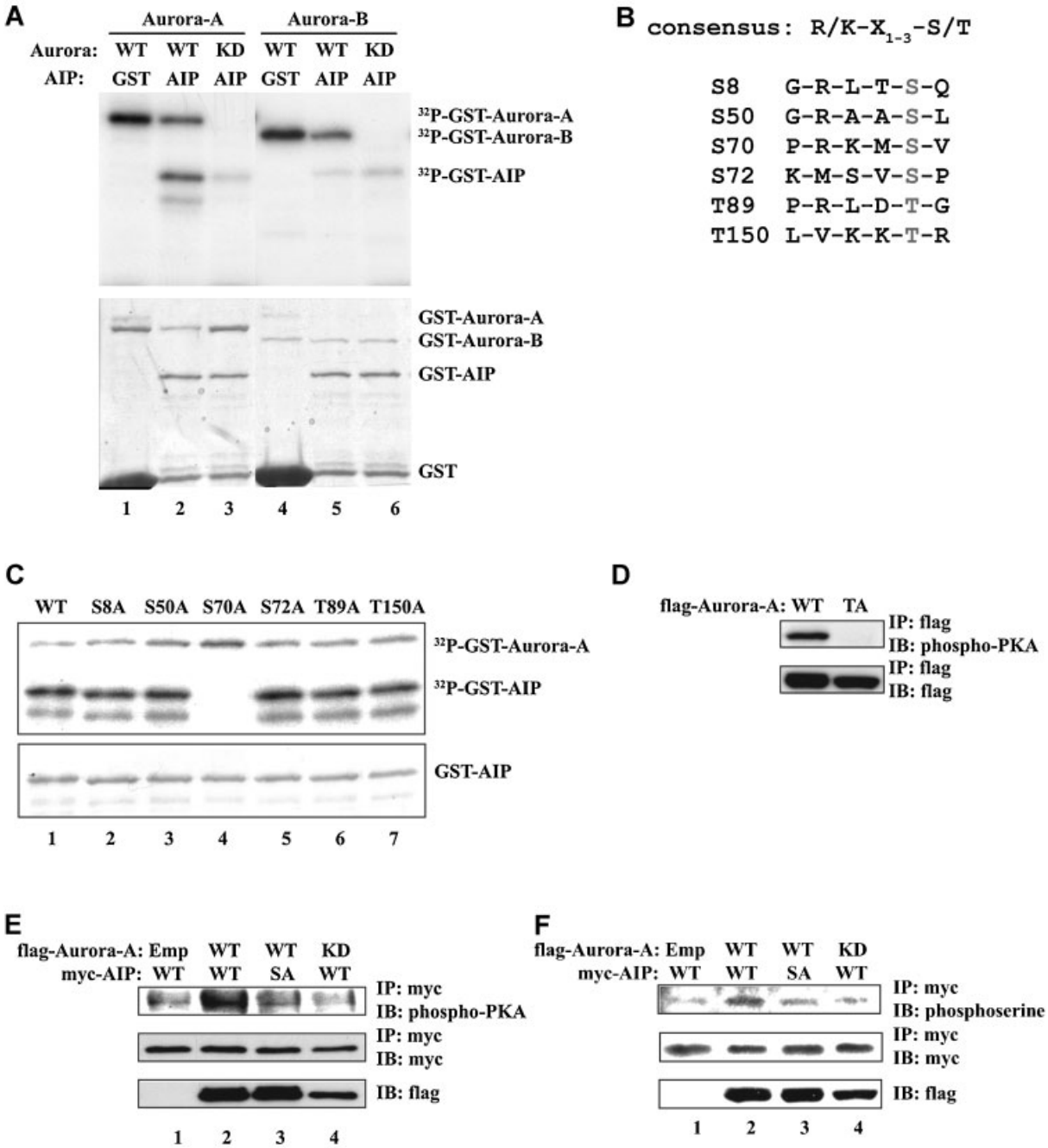

Fig. 2. Aurora-A but not Aurora-B phosphorylates AIP at serine 70 in vitro and in vivo. **A**: GST (**lanes 1** and **4**) or GST-AIP (**lanes 2**, **3**, **5**, and **6**) were incubated with GST-wild-type Aurora-A (WT) (lanes 1 and 2), GST-kinase-dead Aurora-A (KD) (lane 3), GST-Aurora-B WT (lanes 4 and 5) or GST-Aurora-B KD (lane 6) in the presence of [γ^{32}P]ATP. GST proteins were resolved by SDS-PAGE and visualized by autoradiography (for kinase assay, **top**) or Coomassie blue staining (**bottom**). **B**: Consensus Aurora kinases phosphorylation motifs (**top**) and similar amino acid sequences found in human AIP (**bottom**) with the putative phosphorylated residues shown in gray. **C**: GST-AIP WT (**lane 1**) or GST-AIP mutants (**lanes 2**–**7**) in which serine or threonine residue highlighted in gray in B replaced to alanine were incubated with GST-Aurora-A WT and [γ^{32}P]ATP as in A. Proteins were resolved and visualized as in (A). **D**: Lysates from HEK293 cells transfected with either flag-Aurora-A WT (**lane 1**) or T288A mutant (**lane 2**) were immunoprecipitated with anti-flag antibody followed by immunoblotting with anti-phospho PKA antibody (**top**) or anti-flag antibody (**bottom**). **E**: Myc-AIP WT (**lanes 1**, **2**, and **4**) or S70A mutant (**lane 3**) were co-transfected with empty vector (lane 1), flag-Aurora-A WT (lanes 2 and 3) or flag-Aurora-A KD (lane 4) into HEK293 cells at a 1:1 ratio. Twenty four hours after transfection, cells were treated with nocodazole for 16 h. Cell extracts were immunoprecipitated with anti-myc antibody followed by immunoblotting with anti-phospho-PKA antibody (**top**) or anti-myc antibody (**middle**). Expression of flag-Aurora-A was analyzed by direct immunoblotting with anti-flag antibody (**bottom**). **F**: Cell extracts as in (**E**) were immunoprecipitated with anti-myc antibody followed by immunoblotting with anti-phosphoserine antibody (**top**) or anti-myc antibody (**middle**). Expression of flag-Aurora-A was analyzed as in (E; **bottom**).

T288A mutant Aurora-A in which R-R-T-T_{288} is substituted to R-R-T-A_{288}. It has been shown that PKA phosphorylates this threonine 288, which in turn activates Aurora-A [Walter et al., 2000]. Flag tagged wild-type and T288A mutant Aurora-A were transfected into HEK293 cells and treated with nocodazole to accumulate cells in mitosis when T288 phosphorylation peaks. As expected, this antibody recognized wild-type but not T288A mutant Aurora-A, indicating specific recognition by this antibody of Aurora phosphorylated motif (Fig. 2D). Myc tagged wild-type and S70A mutant AIP were expressed with either flag tagged wild-type Aurora-A or kinase-dead Aurora-A in nocodazole treated mitotic HEK293 cells. A stronger signal in cells expressing wild-type Aurora-A was detected compared to cells expressing S70A mutant AIP or kinase-dead Aurora-A, confirming that Aurora-A phosphorylates AIP at serine 70 in vivo (Fig. 2E). Further, we also confirmed Aurora-A phosphorylation of AIP using phosphoserine antibody under the same conditions described above (Fig. 2F).

Phosphorylation of AIP Prolongs its Protein Stability

To examine the effect of AIP on Aurora-A kinase activity, an in vitro kinase assay of Aurora-A was performed in the absence or presence of either wild-type, unphosphorylatable S70A mutant or phospho-mimic S70E mutant of AIP with histone H3 as the substrate. Phosphorylation state of AIP had no significant effect on Aurora kinase activity compared to control reactions, though the latter showed slightly stronger histone H3 phosphorylation signal (Fig. 3A). This was probably due to less contaminant co-purified with GST proteins than GST-AIPs interfering with the kinase reaction, as suggested from the Coomassie blue stained proteins on the gel (Fig. 3A, bottom panel). Next, Aurora-A was co-transfected with either wild-type or phospho mutants of AIP into Hela cells to see in vivo effect of AIP on the presence of active Aurora-A kinase by using anti-phospho-threonine 288 Aurora-A antibody. As expected from the result of in vitro

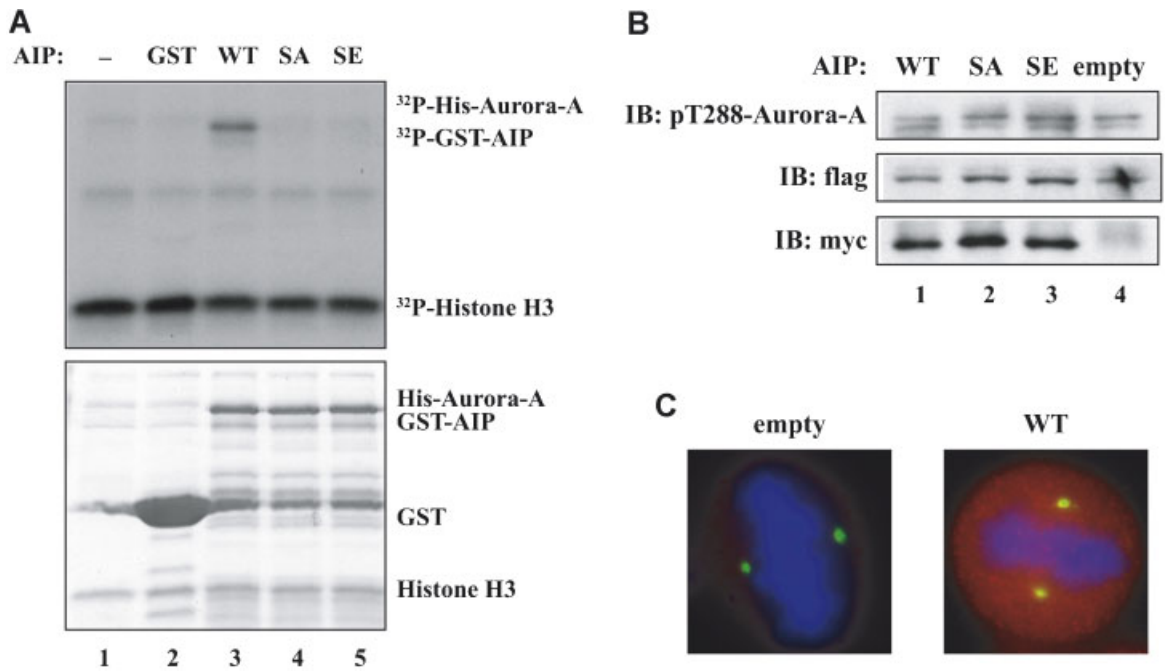

Fig. 3. AIP does not affect Aurora-A kinase activity. **A**: His-Aurora-A was incubated with histone H3 and [γ^{32}P] ATP in absence (–) (**lane 1**) or presence of GST (**lane 2**), GST-AIP WT (**lane 3**) or GST-AIP mutants (**lanes 4** and **5**). Proteins were resolved by SDS–PAGE and visualized by autoradiography (for kinase assay, **top**) or Coomassie blue staining (**bottom**). **B**: Flag-Aurora-A was co-transfected with myc-AIP WT (**lane 1**) or myc-AIP mutants (**lanes 2** and **3**) at a 1:1 ratio or with empty vector (**lane 4**) at a 3:1 ratio into Hela cells. Twenty four hours after transfection, cells were treated with nocodazole for 12 h. Cell extracts were immunoprecipitated with anti-flag antibody followed by immunoblotting with anti-phospho (p)T288 Aurora-A antibody (**top**) or anti-flag antibody (**middle**). Expression of myc-AIP was analyzed by direct immunoblotting with anti-myc antibody (**bottom**). **C**: Hela cells transfected with empty vector (left) or myc-AIP WT (right) for 24 h were fixed and stained with anti-phospho (p)T288 Aurora-A antibody (*green*) and anti-myc antibody (*red*). DNA was counterstained with DAPI (*blue*). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

kinase assay, phosphorylation state of AIP had no detectable effect on the intracellular level of active Aurora-A kinase (Fig. 3B). Indirect immunofluorescent staining of Hela cells transfected with either empty vector or wild-type AIP also revealed comparable level of pT288 phosphorylation of Aurora-A on centrosomes (Fig. 3C) further indicating that AIP does not regulate the intracellular content of active Aurora-A kinase.

We have previously reported that p53 phosphorylation by Aurora-A leads to destabilization of p53 through mdm2 mediated ubiquitination pathway [Katayama et al., 2004]. By contrast, recent finding on HURP phosphorylation by Aurora-A revealed phosphorylation dependent stabilization of HURP [Yu et al., 2005]. Thus, we assayed if Aurora-A phosphorylation of AIP affects its protein stability. HEK293 cells transfected with either S70A or S70E mutant of AIP were exposed to cycloheximide, a translational inhibitor in time course experiments. As shown in Figure 4A,B, phospho-mimic S70E mutant appeared more stable, though at a modest level, than unphosphorylatable S70A mutant. Similar result was also observed in pulse-chase experiments with 35S-methionine (Supplemental Fig. 1). Since Aurora-A is active in mitosis, we investigated if Aurora-A phosphorylation affects AIP stability in mitotic cells. For the purpose, Hela cells transfected with wild-type, S70A or S70E mutant of AIP were first synchronized by thymidine and then grown in nocodazole containing medium to arrest the cells at prometaphase followed by release in cycloheximide containing medium for indicated times. Figure 4C,D clearly demonstrate shorter steady state level of unphosphorylatable S70A mutant compared with wild-type and phospho mimic S70E mutant. Relative to S70E, protein stability of wild-type AIP dropped faster at 90 and 120 min, which coincided with expected decrease in the amount of Aurora-A during mitotic exit due to anaphase promoting complex/cyclosome mediated protein degradation as evident from the observed decline in the level of cyclin B1 in these cells. Similar observations were also made in HEK293 cells (data not shown). Taken together, these data demonstrate that decline in the level of AIP coincides with progression towards mitotic exit and Aurora-A phosphorylation of AIP in mitosis contributes to stabilization of AIP.

Aurora-A is Stabilized After Ectopic Over Expression of AIP

In view of earlier reports of AIP being a negative regulator of Aurora-A it was expected that stabilization of phosphorylated AIP might facilitate Aurora-A degradation. To investigate this possibility, we co-expressed either wild-type Aurora-A or kinase-dead Aurora-A with wild-type AIP or mutant AIPs at 1:9 molar ratio in HEK293 cells under conditions identical to those described in the earlier publication reporting degradation of Aurora-A after ectopic co-over expression with AIP [Kiat et al., 2002]. Surprisingly, marked increase of both wild-type and kinase-dead Aurora-A proteins in cells expressing both wild-type and mutant AIPs was observed compared with empty vector transfected cells (Fig. 5A). Intriguingly, both wild type and kinase-dead Aurora-B proteins also increased in amount in presence of AIP (Fig. 5A). This phenomenon was similarly detected by using GFP tagged AIP (data not shown). Both endogenous Aurora-A and Aurora-B proteins were also elevated after ectopic expression of AIPs (data not shown). Increase in Aurora kinase proteins occurred in dose dependent manner following co-transfection with increasing amount of AIP (data not shown). To confirm that this result is not an artifact of HEK293 cell environment, we repeated the same experiment in Cos-7 and Hela cells that were used in the original report. Again we observed similar results as seen in HEK293 cells in all the cell lines (Fig. 5B). We further investigated this finding, which contradicted the previously published results, by using carboxyl half deletion mutant AIP that was reported to be more effective in destabilization activity than full length AIP [Kiat et al., 2002]. We observed almost similar levels of Aurora-A in cells with the empty vector and those expressing the carboxyl half deletion mutant AIP whereas increased level of Aurora-A in cells expressing the full length AIP (Fig. 5C). Next, we analyzed in vivo interaction between Aurora kinases and AIP. For this purpose, we co-transfected Aurora-A or -B together with AIP at equal molar ratios in independent experiments with the amount of Aurora being comparable among the transfectants. Although protein levels of both Auroras were slightly increased in presence of the full length AIP even under this condition, we detected strong interaction

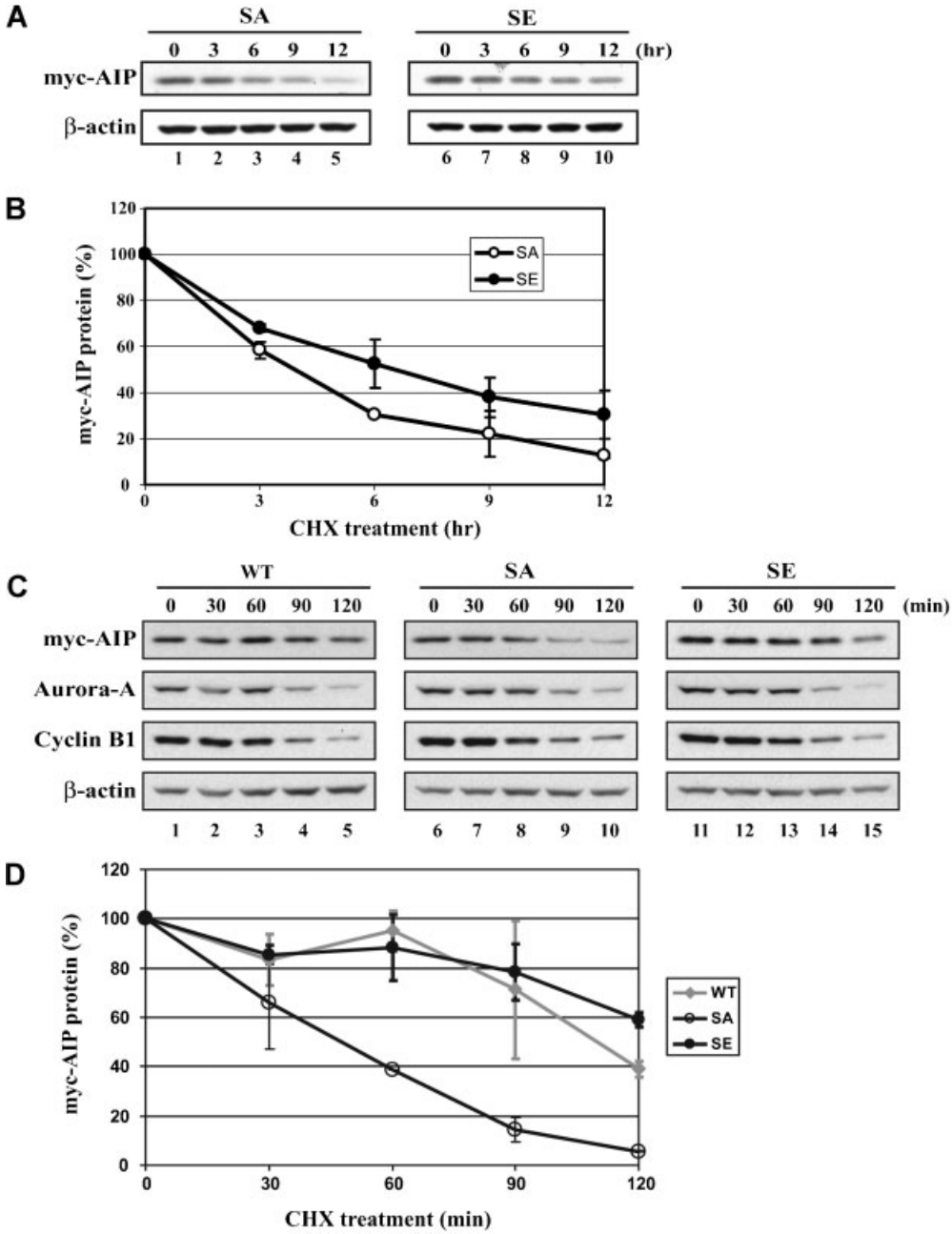

Fig. 4. Aurora-A phosphorylation stabilizes AIP. **A**: Lysates from HEK293 cells transfected with either myc-AIP S70A mutant (**lanes 1–5**) or S70E mutant (**lanes 6–10**) for 48 h were collected at the indicated time points after addition of cycloheximide (CHX) and subjected to immunoblotting with the indicated antibodies. **B**: The amount of myc-AIP in (A) was quantified by densitometry and is shown relative to the amount of myc-AIP expressed in absence of CHX (n = 3). **C**: Hela cells transfected with myc-AIP WT (**lane 1–5**), S70A mutant (**lane 6–10**) or S70E mutant (**lane 11–15**) were synchronized with thymidine and nocodazole then released into fresh medium containing CHX for indicated time points described in Materials and Methods. Cell lysates were subjected to immunoblotting with indicated antibodies. **D**: The amount of myc-AIP in (C) was quantified by densitometry and is shown relative to the amount of myc-AIP expressed in absence of CHX (n = 2).

between both Auroras and full length AIP but not the carboxyl-half deletion mutant AIP (Fig. 5D). We further examined in vitro binding between Aurora kinases and AIP by GST-Pull down assay with in vitro transcribed/translated AIP and glutathion sepharose bound Aurora kinase recombinant proteins. As shown in Figure 5E, AIP binds directly Aurora-A and -B

kinases through their kinase domains which are highly conserved among the members of the Aurora kinase family [Carmena and Earnshaw, 2003; Katayama et al., 2003]. These results indicate that protein–protein interaction is required for AIP mediated stabilization of Aurora kinases.

Effects of AIP Depletion on Stability of Aurora Kinases, Cell Morphology, and Cell Cycle

To confirm that AIP mediates stabilization of Aurora kinases under physiological condition, we examined the levels of Aurora kinases following small RNA interference mediated

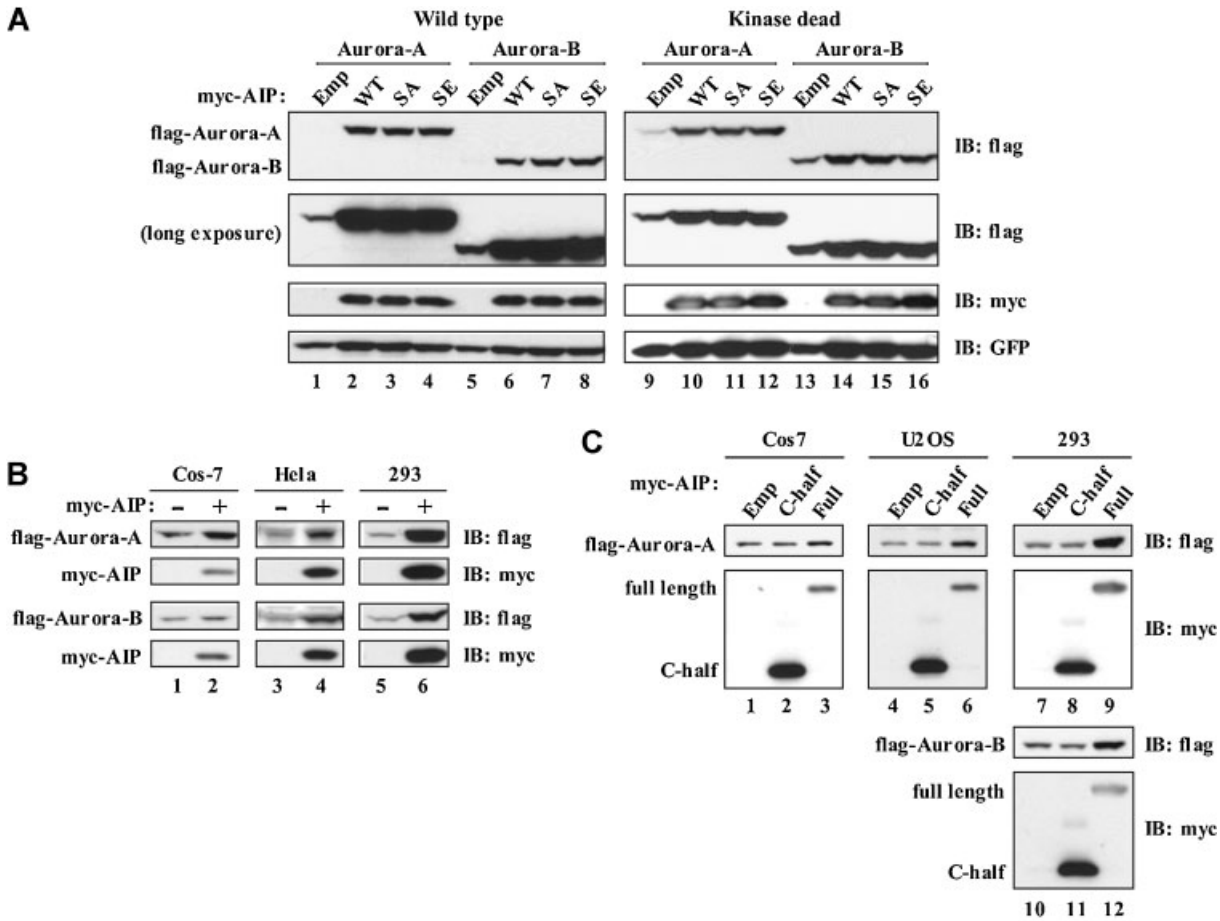

Fig. 5. AIP interaction stabilizes Aurora-A in vivo. **A**: Flag-wild-type Aurora-A (WT) (**lanes 1–4**), flag-Aurora-B WT (**lanes 5–8**), flag-kinase-dead Aurora-A (KD) (**lanes 9–12**) or flag-Aurora-B KD (**lanes 13–16**) were co-transfected with empty vector and GFP (lanes 1, 5, 9, and 13), with myc-AIP WT and GFP (lane 2, 6, 10, and 14), with myc-AIP S70A mutant and GFP (lanes 3, 7, 11, and 15), or with myc-AIP S70E mutant and GFP (lanes 4, 8, 12, and 16) into HEK293 cells. Molar ratio of DNA used was 1:9:1 of flag/myc/GFP 48 h after transfection, aliquots of the same total cell lysates were immunoblotted with the indicated antibodies. Similar GFP expression indicates comparable transfection efficiency (**bottom**). **B**: Flag-Aurora-A WT (**lanes 1–6**, **top panel**) or flag-Aurora-B WT (**lanes 1–6**, **third panel**) were co-transfected with empty vector (lanes 1, 3, and 5) or myc-AIP WT (lanes 2, 4, and 6) into Cos-7 (lanes 1 and 2), Hela (lanes 3 and 4) and HEK293 cells (lanes 5 and 6) respectively at 1:9 molar ratio, and analyzed as in (A). **C**: Flag-Aurora-A WT (**lanes 1–9**, **top panel**) or flag-Aurora-B (**lanes 10–12**, **third panel**) were co-transfected with empty vector (lanes 1, 4, 7, and 10), with carboxyl half of myc-AIP (C-half) (lanes 2, 5, 8, and 11) or with full length of myc-AIP WT (full length) (lanes 3, 6, 9, and 12) into Cos-7 (lanes 1–3), U2OS (lanes 4–6) and HEK293 cells (lanes 7–12), respectively at a 1:9 molar ratio, and analyzed as in (A). **D**: Flag-Aurora-A WT (**lanes 1–3**) or Flag-Aurora-B WT (**lanes 4–6**) were co-transfected with empty vector (lanes 1 and 4), with C-half of myc-AIP (lanes 2 and 5), or with full length of myc-AIP (lanes 3 and 6) into HEK293 cells at 1:1 molar ratio. Forty eight hours after transfection, cell lysates were subjected to direct immunoblotting (lanes 1–6) or immunoprecipitation with normal IgG (–) (lanes 7, 9, 11, 13, 15, and 17) or anti-myc antibody (+) (lanes 8, 10, 12, 14, 16, and 18) followed by immunoblotting with indicated antibodies. **E**: ^{35}S-labeled, in vitro-translated AIP was incubated with the beads bound either with GST (**lane 1**) or with a series of GST-Aurora-A (**lanes 2–4**) or a series of GST-Aurora-B (**lanes 5–7**) full length and deletion mutant proteins. After binding, the beads were resolved by SDS–PAGE and visualized by autoradiography (for binding, **top**) or Coomassie blue staining (**bottom**). Input (**lane 8**) indicates direct loading of ^{35}S-labeled AIP.

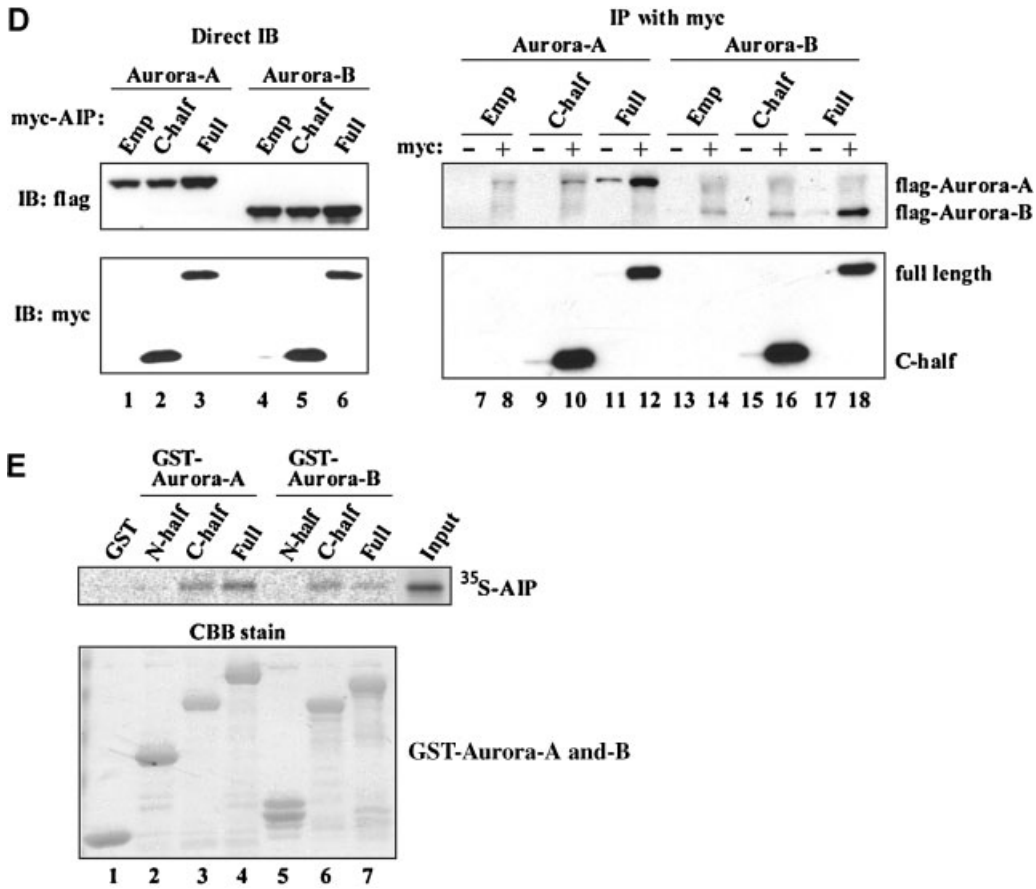

Fig. 5. (*Continued*)

depletion of AIP. AIP mRNA was effectively depleted in Hela, U2OS and HEK293 cells transfected with AIP siRNA compared to control siRNA (Fig. 6A). Immunoblotting of the cells revealed decreased amount of Aurora-A in Hela and U2OS cells whereas in HEK293 cells, no change was detected while Aurora-B amount was not altered in any of the three cell lines examined (Fig. 6B). Thus, it appeared that although AIP is capable of stabilizing Aurora-A and Aurora-B under ectopic over expression condition, its physiological function of stabilizing Aurora-A may not be exclusive in all cell types and additional factors may be involved in the stabilization of Aurora-A protein in some cells. On the other hand protein stability of Aurora-B is not physiologically regulated by AIP. Interestingly, AIP depletion of HEK293 cells resulted in a distinct change in the cell surface morphology with disappearance of filopodia-like structure and flattening of the cell surface compared to control and Aurora-A siRNA transfected cells (Fig. 6C), implying that AIP plays a role in controlling the surface morphology of HEK293 cells. We did not observe similar differences in cell shape of AIP depleted U2OS cells (Fig. 6C).

Since it is known that depletion of Aurora-A protein in cells by Aurora-A siRNA induces cell cycle arrest at G2/M phase [Hirota et al., 2003; Katayama et al., 2004], we examined whether AIP depletion in U2OS cells also has similar effects on cell cycle progression. FACS analysis revealed higher frequency of G2/M cell population in AIP depleted cells than in control siRNA transfected cells though the effect was relatively more modest than what was seen in Aurora-A depleted cells (Fig. 6D). Consistent with the observation that AIP silencing does not affect Aurora-A protein content in HEK293 cells, there was no change in cell cycle distribution observed in AIP depleted HEK293 cells compared with those transfected with control siRNA (data not shown).

To further investigate whether increase in the percentage of cells with 4C DNA content

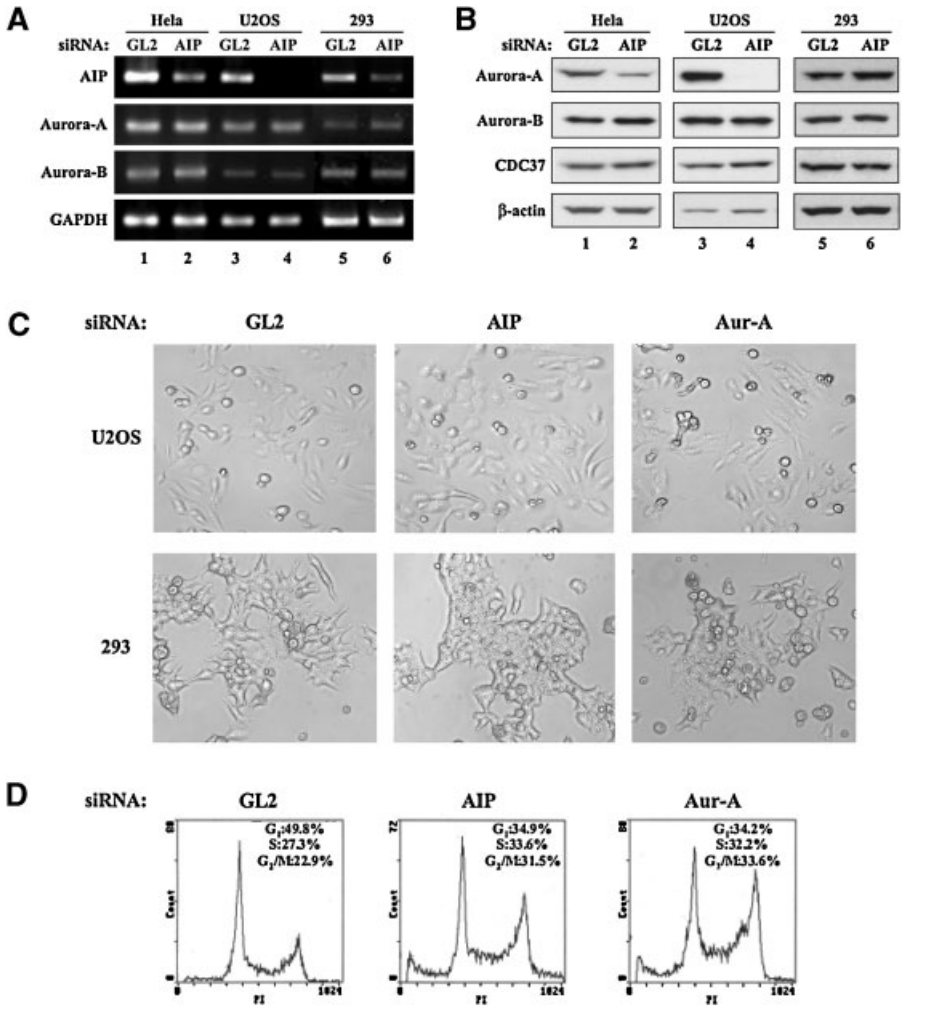

Fig. 6. Effects of AIP on cell morphology and cell cycle. **A**: Hela (**lanes 1 and 2**), U2OS (**lanes 3 and 4**) and HEK293 cells (**lanes 5 and 6**) were transfected with control siRNA duplex (GL2; lanes 1, 3, and 5) or siRNA duplex targeting AIP (lanes 2, 4, and 6) for 48 h. Total RNA and proteins were extracted. RNA from the cells was subjected to RT-PCR using primers targeting indicated transcripts. **B**: Cell extracts prepared from (A) were immunoblotted with indicated antibodies. **C**: Phase contrast picture of U2OS (**top**) and HEK293 cells (**bottom**) transfected with indicated siRNA duplex for 48 h. **D**: FACS analysis of indicated siRNA transfected U2OS cell from (A).

after AIP depletion, detected by FACS analyses, represented cells arrested in G2/M phase or an increase in G1 tetraploid cells, siRNA transfected U2OS cells were stained with anti-phospho-histone H3, a mitotic cell marker to count mitotic cells. We did not observe any G1 tetraploid cells and found that AIP depleted cells had lower frequency of cells in mitosis (0.59%, SD = 0.002) than those transfected with control (2.81%, SD = 0.34%) or Aurora-A (1.65%, SD = 1.19%) siRNA. This result indicates that AIP depletion in U2OS cells causes G2 arrest and not formation of G1 tetrapolid cells. Therefore, these results document direct involvement of AIP in the progression of cells through G2/M phase possibly through the regulation of protein stability of Aurora-A kinase.

DISCUSSION

Aurora kinases are regulated through phosphorylation, binding with specific partners and ubiquitination mediated proteolysis. Of these regulatory interactions, specific down regulation of Aurora-A by AIP was the first report of a protein–protein interaction directly facilitating degradation of Aurora kinase. While investigating Aurora-A controlled AIP function, we found that under ectopic over expression condition,

AIP stabilizes not only Aurora-A but also Aurora-B in contrast to earlier findings even though we utilized the same experimental conditions and cell lines used in the previous report. According to the previous study, AIP interacted with Aurora-A through its carboxyl half region and this interaction was necessary for ubiquitination independent protein destabilization of Aurora-A [Kiat et al., 2002; Lim and Gopalan, 2007]. Though we detected interaction of Aurora kinases with full length AIP in vitro and in vivo, the carboxyl half region was not found to be the interacting domain and only the presence of full length interactive AIP correlated with increased protein amount of Aurora kinases. This increase in amount was independent of Aurora kinase activity and the phosphorylation state of AIP catalyzed by Aurora-A. On the other hand, we observed that under physiological condition Aurora-A and not Aurora-B is stabilized in presence of AIP as evident from RNA interference experiments directed against AIP mRNA in U2OS and Hela cells. Intriguingly, AIP depletion in HEK293 cells did not affect Aurora-A stability but induced change in the cell surface morphology, indicating that AIP has a physiological function of controlling cell shape in HEK293 cells. These results demonstrate that AIP interaction positively controls protein stability of Aurora-A under AIP over expression condition, however its opposing effect on Aurora-A stability by AIP depletion is restricted to only some cell types. In cells revealing physiological function of AIP on Aurora-A stabilization, such as in U2OS cells, we observed that AIP depletion induced cell cycle arrest at G2 phase, possibly by affecting Aurora-A function in these cells. Taken together, our study suggests that AIP plays a role in G2/M phase progression by stabilizing Aurora-A protein and facilitating maintenance of its function during progression of mitosis in some cell types.

The current findings contradict the previous report where all experiments were done by ectopic co-over expression of both AIP and Aurora-A [Kiat et al., 2002; Lim and Gopalan, 2007]. Although the reason for this contradiction is currently unknown there are possibilities, which might explain the discrepant results obtained in the two studies. A polymorphism in Aurora-A gene, Phe31Ile has been identified and Aurora-A Phe31 but not Ile31 has been shown to interact with UBE2N, E2 ubiquitin ligase [Ewart-Toland et al., 2003]. It has been demonstrated that Aurora-A Phe 31 not Ile31, in a complex with UBE2N transiently over expressed, has an intrinsic E3 ubiquitin ligase activity and catalyses polyubiquitination of both Aurora-A itself for protein stabilization and IκBα for protein degradation [Briassouli et al., 2006]. It appears that the previous study utilized Aurora-A Phe31 isoform, while our study utilized the Ile31 isoform expression construct. Recent finding by the other group [Lim and Gopalan, 2007] that ectopic over expression of AIP leads to suppression of Aurora-A ubiquitination leading to degradation raises the possibility that AIP induces Aurora-A protein destabilization by antagonizing the complex formation of Aurora-A Phe31 isoform with UBE2N. However, since Aurora-A Ile31isoform, which is unable to interact with UBE2N, is protected from degradation in presence of AIP, we favor the notion that presence of UBE2N may be one of the components of a multi-protein complex required for Aurora-A stabilization. Absence of Aurora-A destabilization in AIP depleted HEK293 cells, unlike in Hela and U2OS cells, also lends credence to this idea. It is plausible that the components of the Aurora-A stabilizing complex may vary in different cell types depending upon the amino acid contents and the structural conformations of the proteins involved. Depleting the same component in different cells may thus have differential effects on the stability of Aurora-A based on the remaining proteins in each complex. Nonetheless, since our findings in this report are corroborated by siRNA mediated AIP silencing experiments, unlike the exclusively ectopic over expression systems involving AIP and Aurora-A at 9:1 molar ratios utilized in the previous study, we feel confident to conclude that under physiological conditions AIP functions to stabilize Aurora-A protein in human cells rather than destabilizing it as reported earlier. However the reason for Aurora-A stability in absence of AIP, as seen in the case of HEK293 cells, and the molecular mechanisms regulating stability of Aurora-A in vivo remain to be properly elucidated which, will help explain the apparent contradictory findings made in this study and the previously published report on the role of AIP in regulating Aurora-A protein stability.

The reason for increase in Aurora-B protein in AIP over expressing cells despite no apparent

change in protein amount after AIP depletion is also currently unknown. This may simply reflect a stabilizing effect of AIP binding on Aurora-B protein mediated through the sequence motifs in the kinase domain conserved among the Aurora family members. This is plausible since several other common binding partners for Aurora-A and -B kinases have been identified with conflicting findings on their physiological roles in regulating activation and stability of these proteins. For instances, RasGAP and Survivin form ternary complexes with Aurora-A and -B kinases through kinase domains in vitro and in vivo, and these associations peak at mitosis when activities of Aurora kinases are maximum, but RasGAP function inhibits both Aurora kinases under ectopic expression condition in vivo [Gigoux et al., 2002]. On the other hand, Survivin has been shown to up-regulate kinase activity of Aurora-B in vivo and in vitro whereas down-regulation of Aurora-A activity in vitro [Bolton et al., 2002; Chen et al., 2002; Eyers and Maller, 2004]. Furthermore, depletion of Survivin induces destabilization of Aurora-B not Aurora-A while its over expression does not affect the amount of either of the two kinases in vivo [Honda et al., 2003; our unpublished data]. These conflicting data revealing opposing functions of RasGAP and Survivin on Aurora kinases indicate the complexity of their intracellular regulation. In this context, it is possible that under physiological conditions, AIP interaction with Aurora-B serves to regulate functions other than stabilization of the protein. Another possibility is that multiple factors including AIP binding stabilize Aurora-B and depletion of AIP alone does not cause detectable change in Aurora-B stability. Further investigation into these possibilities will help improve our understanding of various Aurora kinase functional interactions during cell cycle progression.

It was of interest to investigate whether or not AIP is phosphorylated by Aurora-A. We identified serine 70 of AIP as Aurora-A phosphorylation site and this phosphorylation leads to prolonged protein half-life of AIP in asynchronous and synchronized cells exiting mitosis. In asynchronous cells, however, phospho mimic or unphosphorylatable AIP mutants showed comparable stabilizing effect on Aurora kinases. This observation could be explained by the fact that under ectopic over expression condition differential stability of AIP mutants in asynchronous cells is minimized since the steady state levels of both AIP mutants show relatively minimal difference at various times of cycloheximide treatment compared with those observed in cells exiting mitosis. It is, therefore, expected that in cells progressing through mitosis Aurora-A phosphorylation will have a more pronounced effect on the stability of AIP and in turn on its own stability through AIP interaction compared with cells at other stages of the cell cycle. p53 and HURP proteins are phosphorylated by Aurora-A, and these phosphorylations have opposing effects of negatively and positively regulating the stability of these proteins respectively [Katayama et al., 2004; Yu et al., 2005]. Thus, it appears that Aurora-A phosphorylations often serve to regulate the steady state level of the target proteins. It is, therefore, important to examine whether phosphorylations of other substrate proteins by Aurora-A also affect their stability.

We found that expression level of AIP mRNA is elevated in several cancer cell lines. Interestingly, elevated expression pattern of Aurora kinases overlap with those of AIP in many of these cell lines [Zhou et al., 1998; Li et al., 2003; Fraizer et al., 2004]. This observation is consistent with AIP being involved in the stabilization of Aurora-A, as reported in this study, rather than in the degradation, as reported earlier. It will now be important to investigate whether or not AIP mediated protein stabilization of Aurora kinases also contributes to increased protein amount of Aurora kinases in tumors which may or may not be expressing elevated level of Aurora kinase mRNAs due to aberrant transcriptional regulation and/or gene amplification. We detected almost constant expression level of AIP mRNA during cell cycle progression while Aurora-A peaks at G2-M phase. This observation suggests the existence of other AIP interacting proteins through different stages of the cell cycle and also additional functions of AIP unrelated to regulation of the cell cycle. Supporting the latter idea, we observed that AIP depletion in HEK293 cells induced flattening of the cell surface with disappearance of filopodia-like structures indicating a role for AIP in the regulation of surface morphology of some cell types. It will be of interest to investigate the detailed molecular interactions of AIP and elucidate their functional significance.

In conclusion, our findings provide the first account of Aurora-A kinase and its substrate protein AIP cooperatively regulating their stabilities through protein–protein interaction as well as protein phosphorylation.

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