Aurora-B/AIM-1 Regulates the Dynamic Behavior of HP1 α at the G₂–M Transition^{[D] [V]}

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Heterochromatin protein 1 (HP1) plays an important role in heterochromatin formation and undergoes large-scale, progressive dissociation from heterochromatin in prophase cells. However, the mechanisms regulating the dynamic behavior of HP1 are poorly understood. In this study, the role of Aurora-B was investigated with respect to the dynamic behavior of HP1 α . Mammalian Aurora-B, AIM-1, colocalizes with HP1 α to the heterochromatin in G₂. Depletion of Aurora-B/AIM-1 inhibited dissociation of HP1 α from the chromosome arms at the G₂–M transition. In addition, depletion of INCENP led to aberrant cellular localization of Aurora-B/AIM-1, but it did not affect heterochromatin targeting of HP1 α . It was proposed in the binary switch hypothesis that phosphorylation of histone H3 at Ser-10 negatively regulates the binding of HP1 α to the adjacent methylated Lys-9. However, Aurora-B/AIM-1-mediated phosphorylation of H3 induced dissociation of the HP1 α chromodomain but not of the intact protein in vitro, indicating that the center and/or C-terminal domain of HP1 α interferes with the effect of H3 phosphorylation on HP1 α dissociation. Interestingly, Lys-9 methyltransferase SUV39H1 is abnormally localized together along the metaphase chromosome arms in Aurora-B/AIM-1-depleted cells. In conclusion, these results showed that Aurora-B/AIM-1 is necessary for regulated histone modifications involved in binding of HP1 α by the N terminus of histone H3 during mitosis.

INTRODUCTION

Chromosomal passenger proteins are characterized by the unique property of translocation from the chromosome to the central spindle at the onset of anaphase (reviewed in Adams *et al.*, 2001a; Terada 2001). In G₂ and prophase, chromosomal passengers are localized on the chromosome, whereas during prometaphase and metaphase they translocate to the inner centromeres. During anaphase the chromosomal passengers transfer to the spindle midzone where they eventually become part of the midbody (Earnshaw and Cooke, 1991). At the onset of anaphase, separase cleaves the cohesin protein into separate sister chromatids and activates at the same time the phosphatase Cdc14, which dephosphorylates Inner Centromere Protein (INCENP) and triggers its relocalization to the spindle midzone (Pereira and Schiebel, 2003). Recent results show that Aurora-B, together with INCENP and Survivin/BIR, is part of a chromosomal passenger complex. Aurora-B binds to Survivin and the C-terminal domain of INCENP (reviewed in Adams *et al.*, 2001b; Terada 2001). It is of particular interest that all metazoan INCENPs as well as the INCENP homologue of *Saccharomyces cerevisiae* (Slp5p) share a highly conserved motif near the C terminus, the so-called IN-box (INCENP conserved box) (Adams *et al.*, 2000), which is required for interaction with Aurora-B. Disruption of Aurora-B, INCENP, or Survivin/BIR function leads in several species to severe

defects in chromosome segregation and cytokinesis (Tatsuka *et al.*, 1998, Terada *et al.*, 1998, Giet and Glover, 2001). In *Drosophila* cells and *D. elegans* embryos that lack INCENP or Survivin, Aurora-B cannot properly localize to the kinetochores and midbody (Speliotes *et al.*, 2000; Adams *et al.*, 2001b). Similarly, in *Drosophila* cells, depletion of Aurora-B by double-stranded RNA-mediated interference (RNAi) leads to aberrant localization of INCENP (Adams *et al.*, 2001b). These data indicate that INCENP is required for the proper localization of Aurora-B. Conversely, Aurora-B regulates the proper localization of INCENP by an unknown mechanism. Aurora-B is also responsible for the phosphorylation of histone H3 at Serine 10 (Hsu *et al.*, 2000, Murnion *et al.*, 2001). In addition, in some eukaryotes, the N-terminal tail of histone H3 is also phosphorylated during mitosis and meiosis. Phosphorylation at Serine 10 has been reported to be involved in proper chromosome condensation and segregation (Wei *et al.*, 1999). In *Tetrahymena*, mutation of Serine 10 has been reported to lead to defects in condensation, thus indicating a correlation between phosphorylation at Serine 10 and chromosome condensation and segregation (Hsu *et al.*, 2000). However, recent reports suggest a poor correlation between H3 phosphorylation and condensation levels in *Drosophila* S2 cells, *Xenopus*, and budding yeast (Adams *et al.*, 2001b; Lavoie *et al.*, 2002, MacCallum *et al.*, 2002). Thus, the exact role of H3 phosphorylation in mitotic progression remains elusive (Hans and Dimitrov, 2001).

Heterochromatin protein 1 (HP1), a chromosomal protein originally identified because of its localization in the heterochromatin in the polytene nuclei of *Drosophila*, is composed of two related functional domains, an amino-terminal chromodomain (CD) (chromatin organization modifier) and a carboxy-terminal chromo shadow domain (CSD) (Aasland and Stewart, 1995, Bannister *et al.*, 2001). HP1 functions as a chromatin organizer. Some of the molecular events leading

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to HP1-induced heterochromatin formation have recently been resolved due to the discovery of histone methyltransferase activity associated with the *Su(var)3-9* in *Drosophila*, and in homologues from fission yeast (Clr4) to humans (SUV39H1). Notably, the H3 Lys-9 methyl epitope induced an affinity for heterochromatin in HP1 proteins. This epigenetic signal was recognized through the CD of the protein (Jenuwein and Allis, 2001). In addition, Fischle and Allis have proposed a binary switch hypothesis, which suggests that phosphorylation of Serine 10 negatively regulates the binding of HP1 α to the adjacent methylated Lys-9 of histone H3 (Fischle *et al.*, 2003). Indeed, very recently it was shown that inhibition of H3 phosphorylation at Serine 10 inhibited the dissociation of HP1 (Hirota *et al.*, 2005; Fischle *et al.*, 2005). These observations suggest that H3 S10 phosphorylation causes displacement of HP1 from the heterochromatin at the onset of mitosis. However, it was also reported that H3 phosphorylation alone is insufficient for HP1 dissociation and that acetylation of Lysine 14 is required to prevent binding of HP1 to H3 (Fass *et al.*, 2002; Mateescu *et al.*, 2004). These data suggest that in addition to H3 phosphorylation another factor may regulate HP1 dissociation *in vivo*.

In metaphase cells, HP1 α is localized at the centromeric heterochromatin that contains histone H3 protein highly methylated at Lysine 9. Loss-of-function mutations in the HP1 gene will lead to a number of structural defects in mitotic chromosomes, including lack of condensation, and segregation and telomere fusions (reviewed in Eissenberg and Elgin, 2000). This suggests that HP1 is also essential for mitotic progression. In the G₂ phase, HP1 is associated with heterochromatin, but it progressively dissociates from it at the G₂-M boundary, concomitant with histone H3 phosphorylation (Hendzel *et al.*, 1997; Murzina *et al.*, 1999; Sugimoto *et al.*, 2001; Fass *et al.*, 2002). Currently, the mechanisms contributing to the dynamic behavior of heterochromatin during mitosis are poorly understood. Many of the proteins interacting with HP1, the CAF-1 subunit p150 (Murzina *et al.*, 1999), Ku70 (Song *et al.*, 2001), and the lamin B receptor (Ye and Worman, 1996), bind to CSD of HP1, but interaction of lysine 9-methylated histone H3 with the CD region (Lachner *et al.*, 2001) and INCENP with the hinge region (Ainsztein *et al.*, 1998) have also been documented.

The objective of the present study was to elucidate the mechanism of HP1 α protein dissociation from the chromosome arms at the G₂-M transition. Transfection experiments using Aurora-B/AIM-1 small interfering RNA (siRNA) or the kinase-negative form of Aurora-B/AIM-1 revealed that the kinase activity of Aurora-B/AIM-1 is required for the dissociation of HP1 α from the chromosome arms in mitotic cells. This indicates that histone H3 phosphorylation is required for HP1 α dissociation from the chromosome arms. In addition, it was shown that INCENP contributes to the proper localization of Aurora-B/AIM-1 in heterochromatin. Last, it was shown that Aurora-B/AIM-1 regulates the localization of SUV39H1. The implications of these findings for heterochromatin organization are discussed.

MATERIALS AND METHODS

Plasmid Construction

FLAG-tagged expression vectors (pEFhyg I carrying the EF1 α promoter; Terada *et al.*, 1995) for wild-type rat Aurora-B/AIM-1 (WT) and for its dominant negative form (K106R) have been described previously (Terada *et al.*, 1998). The entire coding sequence of human histone H3 cDNA was isolated by reverse transcription-PCR using HeLa mRNA as template. The (S10A) mutation in histone H3 was introduced by site-directed mutagenesis of pCMV C-terminal hemagglutinin (HA)-H3 wild type, using QuikChange (Stratagene, La Jolla, CA). Histone H3 S10A was generated using the follow-

ing primers: sense, 5'-GCAGACTGCCCGCAAAGCGACCGGTGGTAAAG-3' and antisense, 5'-CTTTACCACCGTCGCTTGCGGGGAGTCTGC. Human T7-HP1 α (pCDNA3) and full-length His₆-HP1 α was generously provided by I. Lee (Department of Biochemistry, University of Ulsan College of Medicine, Ulsan, Korea). The chromodomain of HP1 α (residue 15-72) was also expressed using the pRSET-A-His₆ vector. Green fluorescent protein (GFP)-HP1 α was generated from full-length human HP1 α cDNA, which was cloned into the BamHI and XbaI sites of the pEGFP-C1 vector (Clontech, Mountain View, CA) for expression of HP1 α as a GFP fusion protein in mammalian cells. FLAG-SUV39H1 (kindly provided by M. L. Cleary, Department of Pathology, Stanford University School of Medicine, Stanford, CA) was cloned into the pGEX vector (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) to obtain the glutathione-Sepharose fusion protein encoding glutathione S-transferase (GST)-SUV39H1 (3-412) and GST-SUV39H1 delta N (82-412).

Generation and Purification of GST and His₆ Fusion Proteins

Recombinant proteins were expressed in 1-l cultures of *Escherichia coli* strain BL21 and solubilized in 10 ml of radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% NP-40, and 0.5% sodium deoxycholate) containing a full set of protease inhibitors (Roche Diagnostics, Indianapolis, IN) and lysozyme (Sigma-Aldrich, St. Louis, MO) by freeze-thawing in liquid N₂, followed by sonication. Soluble proteins were cleared by centrifugation, purified with 400 μ l of glutathione-Sepharose beads (GE Healthcare) or Ni/NTA beads (QIAGEN, Valencia, CA), and washed in RIPA buffer. Coomassie staining of SDS-PAGE gels was used to determine protein concentration.

Antibodies

The following antibodies were used: polyclonal and monoclonal anti-AIM-1 (used at a dilution of 1:200; N12 at a dilution of 1:500; BD Biosciences Transduction Laboratories, Lexington, KY); monoclonal Aurora-A (used at a dilution of 1:200; Cell Signaling Technology, Beverly, MA); polyclonal anti-INCENP (used at 1:100; gift from W. C. Earnshaw, Wellcome Trust Centre for Cell Biology, Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, Scotland); polyclonal anti-H3, dimethyl K9, and pSer-10 histone H3 antibodies (Upstate Biotechnology, Lake Placid, NY); HP1 α (Mab3446, Chemicon International, Temecula, CA); polyclonal and monoclonal mouse anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA, and BabCO, Richmond, CA), polyclonal and monoclonal mouse anti-FLAG (Sigma-Aldrich); and anti-SUV39H1 (MG44) monoclonal antibody (gift from M. L. Cleary).

Antibody Staining and Microscopy

HeLa or Chinese hamster ovary (CHO) cells expressing enhanced green fluorescent protein (EGFP)-HP1 α were washed with phosphate-buffered saline (PBS), followed by swelling in a hypotonic solution of 37.5 mM KCl containing proteinase inhibitors, and fixed in 4% (wt/vol) paraformaldehyde in PHEM buffer (Cimini *et al.*, 2003). Cells were subsequently permeabilized for 10 min with 0.5% Triton X-100 in PHEM buffer and blocked for 1 h with 5% goat serum. Methanol-fixed cells were rehydrated with 0.05% Tween 20-containing PBS and treated with a mixture of primary antibodies. GFP fluorescence was directly observed by epifluorescence microscopy. Coverslips were incubated with 2-5 μ g/ml 4,6-diamidino-2-phenylindole (DAPI). Cells were analyzed using a Nikon Eclipse microscope. To determine HP1 α or SUV39H1 fluorescence at the centromeres or chromosome arms, the same camera settings were used for all digital images. The best in-focus image of a centromere was determined visually, and, based on the CREST signal, a region corresponding to the kinetochore was generated using Adobe Photoshop software (Adobe Systems, Mountain View, CA). The mean fluorescence intensity of the region was then recorded together with the mean fluorescence intensity of a randomly selected area around the chromosome arm outside the kinetochore and was quantified using MetaMorph software version 6.1 (Molecular Devices, Sunnyvale, CA). To localize either HP1 α or SUV39H1 at the centromere or the chromosomal arm, the average intensity values (at least 5 areas) of HP1 α or SUV39H1 around the centromeres or chromosome arms were calculated for each experimental condition. Cells with centromeric HP1 α or SUV39H1 were identified as those that displayed 5 times higher fluorescence intensity at the kinetochores than at the chromosome arms. Results were compared using a statistical *t* test.

Cell Line Establishment and Live Cell Microscopy

HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). CHO cells were cultured in Ham's F-10 supplemented with 10% FBS. PEGFP-C1-HP1 α and/or FLAG-tagged SUV39H1 and puromycin-resistant plasmid (10:1) was transfected into CHO1 or HeLa with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The cells were selected with G418 or puromycin added to the growth medium 48 h after transfection. Resistant cells were selected. Selection was over a period of 2 wk with regular medium changes. Several G418- or puromycin-resistant colonies were picked and

expanded for immunostaining. For live cell microscopy, after transfection with FLAG- Aurora-B/AIM-1 KR or control vector into cells expressing GFP-HP1 α , the cells were grown directly on glass coverslips. Cells were maintained during imaging in DMEM plus 10 mM HEPES buffer in a Rose chamber on a heated stage at 37°C (Bionomic Controller BC-100; 20/20 Technologies, Wilmington, NC). Cells were imaged at 5-min intervals on a Nikon TE300.

Cell Cycle Synchronization and Transfection

For cell cycle synchronization, G₁/S phase HeLa cells were obtained from a double-thymidine block as described previously. Under these conditions, HeLa cells will arrest in early S phase (Heintz *et al.*, 1983; Pines and Hunter, 1989); S-phase HeLa cells were obtained by releasing cells synchronized by a double-thymidine block into fresh medium for a period of 2 h; M-phase HeLa cells were obtained from a thymidine/nocodazole block.

RNAi in HeLa Cells

HeLa cells were synchronized in S phase using the double-thymidine block protocol (2 cycles of 15-h culture with thymidine and 9-h culture free of thymidine); enrichment of mitotic cells was achieved by nocodazole treatment after release from the thymidine block. When HeLa cells were transfected during the second thymidine block, the cells were washed 9 h after the initiation of the second thymidine block, transfected for 2 h in Opti-MEM using Lipofectamine 2000 (Invitrogen), and cultured again for 4 h in the original culture medium in the continued presence of thymidine before thymidine was removed. siRNA for human Aurora-B/AIM-1 was purchased from Cell Signaling Technology, and annealed siRNAs for human Aurora-B/AIM-1 (5'-CAG AAG AGC TGC ACA TTT G-3', 5'-GCA GAG AGA TCG AAA TCC A-3', 5'-CCA AAC TGC TCA GGC ATA A-3', and 5'-TGG GAC ACC CGA CAT CTT A-3') and for human Aurora-A (5'-AAC ACC CAA AAG AGC AAG CAG) were kindly provided by Z. Chen (Eisai Research Institute). siRNA for the scrambled sequence was used as a control. For INCENP RNAi, cDNA fragments corresponding to the N-terminal 600-base pair region of human INCENP were amplified by PCR and inserted into the pTOPO vector (Invitrogen). INCENP cDNA flanked by T7-promoter binding sites was amplified by PCR, using the M13F and T7-TOPO primers (5'-GCGTAATACGACTACTATAGGTAACGGCCGCGCAGTGTGCTG) and the pTOPO-INCENP plasmids as templates. RNA strands were synthesized using the MEGAscript kit (Ambion, Austin, TX) from PCR-derived linear template carrying a T7 promoter at both ends; double-stranded RNA (dsRNA) was formed by annealing as described previously (Yang D *et al.*, 2002). To prepare siRNAs for INCENP and *E. coli* endoribonuclease (RNase III) as a control RNAi, 100 μ g of dsRNAs was digested with 0.5 μ g of recombinant RNase III for 2 h in a 100- μ l reaction buffer at 25°C. The siRNAs were purified using Q-Sepharose column (GE Healthcare).

Chromatin Isolation

Chromatin fractions were prepared using a modified method described by Remboutsika *et al.* (1999). Briefly, HeLa cells were harvested, washed twice with ice-cold PBS, and resuspended in 15 mM Tris-HCl buffer, pH 7.5, containing 5 mM MgCl₂, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), 2 mM sodium vanadate, 250 mM sucrose, protease inhibitor cocktail (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride (buffer N). An equal volume of buffer N containing 0.6% NP-40 was added to the cells, and the suspension was gently mixed and incubated on ice for 5 min. Nuclei were pelleted at 2000 $\times$ g for 5 min at 4°C in a microfuge. The cytoplasmic fraction (C) was recovered and the nuclei washed three times with buffer N before lysis in 10 mM PIPES buffer, pH 6.5. After centrifugation at 6000 $\times$ g for 20 min at 4°C in a microfuge, the chromatin pellet was washed once in the same buffer and recovered in SDS-PAGE loading buffer (Ch). After centrifugation at 6000 $\times$ g for 10 min at 4°C in a microfuge, equivalent samples (in terms of initial nuclei) from the C and Ch fractions were subjected to 10% SDS-PAGE and immunoblot analysis.

In Vitro Histone Methyltransferase Assay

In vitro histone methyltransferase reactions were performed with GST-SUV39H1 (82–412) as described previously (Rea *et al.*, 2000). Briefly, reactions were carried out in a final volume of 50 μ l in methylase buffer (50 mM Tris-HCl, pH 8.5, 20 mM KCl, 10 mM MgCl₂, 10 mM β -mercaptoethanol, and 250 mM sucrose) containing as substrates histone H3 (Roche Diagnostics) and S-adenosyl-L-methionine (Sigma-Aldrich) as the methyl donor. After incubation at 37°C for 60 min, SDS loading buffer was added reaction, and the reactions boiled for 5 min.

In Vitro Kinase Assay

Aurora-B/AIM-1 kinase protein extracted from adenovirus-infected cells was incubated for 30 min at 30°C in a total volume of 20 μ l containing 0.5 mg/ml histone H3, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 5 mM NaF, 1 mM DTT, 0.05 mM sodium vanadate, and 0.1 mM ATP.

HP1 Binding Assays

His₆-HP1 α Ni/NTA beads were incubated for 1 h with methylated histone H3 peptide in binding buffer in a total volume of 100 μ l. The bound and unbound (supernatant) proteins were separated by 14% SDS-PAGE and visualized after immunoblotting with anti-H3 antibodies. For kinase reactions, the beads were equilibrated with a kinase buffer and incubated for 30 min at 30°C in a total volume of 20 μ l supplemented with 0.6 μ M purified Aurora-B/AIM-1. Beads were then washed extensively with washing buffer.

Purification of Kinase-Active Aurora-B/AIM-1

The adenovirus encoding Aurora-B/AIM-1 was generated by ligating a rat N-terminal FLAG-tagged Aurora-B/AIM-1 cDNA (Terada *et al.*, 1998) into pACCMV.pLpA and amplified as described previously (Albrecht and Hansen, 1999). E1-transformed human 293 cells were infected with the adenovirus, and mitotic cells were enriched after treatment with nocodazole for 12 h. The cells were collected 60 h after infection and were lysed for 20 min on ice in TBSN buffer consisting of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM Na₃VO₄, and a protease inhibitor cocktail (Roche Diagnostics). FLAG-tagged Aurora-B/AIM-1 protein was purified using a FLAG purification kit (Sigma-Aldrich).

RESULTS

In G₂ Cells, HP1 α Colocalizes with Aurora-B/AIM-1 to Heterochromatin and Dissociates from It at the G₂-M Transition Concurrent with Histone H3 Phosphorylation

To clarify the molecular behavior of HP1 α during G₂ phase and mitosis, stable CHO and HeLa cells expressing EGFP-HP1 α were established. The localization of HP1 α in these cells was analyzed by conventional immunofluorescence microscopy. In G₁/S-phase cells that usually contain unphosphorylated H3, HP1 α was distributed in discrete, dispersed nuclear foci and at the nucleolar periphery (Supplemental Figure 1A, G₁/S). In these cells, a similar nuclear distribution of large foci representing condensed chromatin masses as revealed by DAPI staining was observed for HP1 α . Global histone H3 phosphorylation begins in the pericentromeric heterochromatin in late G₂ and subsequently progresses to cover the entire heterochromatin before the onset of mitosis (Hendzel *et al.*, 1997). During interphase, Aurora-B/AIM-1, a mitotic H3 kinase, is not localized at G₁/S heterochromatin sites (Supplemental Figure 1B, G₁/S), but in G₂-phase cells, HP1 α is clearly localized in condensed DNA regions as shown by DAPI staining (Supplemental Figure 1B, G₂). During prophase, most of the HP1 α protein has dissociated from the heterochromatin, the starting point for H3 phosphorylation (Supplemental Figure 1A, prophase). In prophase cells, HP1 α underwent large-scale but progressive dissociation from heterochromatin into the cytoplasm. This dissociation correlated with H3 phosphorylation and Aurora-B/AIM-1 localization (Supplemental Figure 1A, prophase).

Although in prometaphase cells most of the HP1 α protein occurred as a diffuse signal in the cytoplasm, when cells were extracted with the nonionic detergent Triton X-100, which removed cytoplasmic HP1 α , before fixation, the centromeric localization of HP1 α and Aurora-B/AIM-1 became obvious (Supplemental Figure 1B, prometaphase). During anaphase, HP1 α began to target again the chromosome, and upon dissociation of Aurora-B/AIM-1 from the centromeres to the midspindle, H3 was dephosphorylated (Supplemental Figure 1, A and B, anaphase). During telophase, HP1 α was almost exclusively directed to the nuclei, concurrent with the translocation of Aurora-B/AIM-1 to the midbody and the nearly complete dephosphorylation of histone H3. Virtually identical results were obtained when HeLa cells were used (see below).

Figure 1. Aberrant localization of HP1 α by depletion of Aurora-B/AIM-1. Immunostaining of HeLa cells expressing EGFP-HP1 α with anti-H3 phos (H3P) after expression of Aurora-B/AIM-1 RNAi for 48 h. Scrambled siRNA was used as a control. After expression of Aurora-B/AIM-1 RNAi or kinase-negative Aurora-B/AIM-1 KR for 48 h, cells were swollen in a hypotonic solution, fixed in paraformaldehyde, and then permeabilized with 0.5 Triton X-100. During M phase, HP1 α still remained associated with the chromosome arms in mitotic cells lacking H3 phosphorylation due to Aurora-B/AIM-1 RNAi or overexpression of kinase-negative Aurora-B/AIM-1 K106R (KR), whereas HP1 α was associated with the centromeres in the control (control). Scale bar, 10 μ m. (B) Immunoblotting of cell extracts of mitotic HeLa cell 48 h after expression of RNAi with different antibodies: anti-AIM-1 (Aur-B/AIM-1), Aurora-A (Aur-A), and α -Tubulin antibodies as a loading control. (C) Quantification of HP1 α associated with the chromosome arms 48 h after expression of control, Aurora-A, or Aurora-B/AIM-1 RNAi. HeLa cells expressing EGFP-HP1 α were synchronized, and the mitotic cells were counted and classified as indicated. Results represent the average of three independent experiments (>200 cells each); bars indicate standard deviations as described in *Materials and Methods*.

Aurora-B/AIM-1 Is Required for Dissociation of HP1 α from Heterochromatin during the G₂-M Transition

Because Aurora-B/AIM-1 colocalizes with HP1 α to the heterochromatin during G₂, and dissociation of HP1 α protein occurs in parallel with histone H3 phosphorylation, it is likely that Aurora-B/AIM-1 regulates the dissociation of HP1 α from heterochromatin during G₂-M transition. To elucidate the functional relationship between Aurora-B/AIM-1 and HP1 α , the effect of Aurora-B/AIM-1 siRNA on mitotic progression was examined. HeLa cells treated for 48 h with either Aurora-B/AIM-1 or Aurora-A siRNA were substantially, albeit incompletely, depleted of the protein (Figure 1B). Depletion of Aurora-B/AIM-1 led to a significant decrease of histone H3 phosphorylation (H3P), and in mitotic cells HP1 α remained associated with the chromosome arms (Figure 1, A and C). In contrast, both HP1 α dissociation and phosphorylation status of H3 in cells expressing either Aurora-A (related to centrosome function; Glover *et al.*, 1995; Terada *et al.*, 2003) or control siRNA were not affected (Figure 1C). In addition, in HeLa cells expression of kinase-negative Aurora-B/AIM-1 K106R (KR) showed similar results, as did Aurora-B/AIM-1 RNAi (Figure 1A), thus indicating that the kinase activity of Aurora-B/AIM-1 is required for HP1 α dissociation. Moreover,

videomicroscopy of CHO cells expressing Aurora-B/AIM-1 KR demonstrated clearly that HP1 α failed to dissociate from the chromosome arms and that the chromosomes failed to align on the metaphase plate (Supplemental Movies 1 and 2). Eventually, both chromosome segregation and cytokinesis failed and could not be completed, leading to multinucleated cells as was shown previously (Terada *et al.*, 1998). To conclude, dissociation of HP1 α from the chromosomal arm depends on the kinase activity of Aurora-B/AIM-1 but not of Aurora-A.

Endogenous HP1 α Dissociates from Chromatin during Mitosis and Is Inhibited by Depletion of Aurora-B/AIM-1

To investigate whether endogenous HP1 α dissociates from mitotic chromatin, HeLa cells were synchronized by release from a double-thymidine block (Pines and Hunter, 1989). Most of the cells (96%) had entered S phase within 3 h of release from the thymidine block and had entered G₂-M phase 9 h after release from the block based upon the DNA content of the cells (Figure 2A). Twelve hours after release some cells started to enter the next S phase, and after 15 h most cells were in G₁. Differential nuclear extraction of synchronized cells showed that during G₁-S HP1 α was mostly associated with the Ch fraction. Only a low level of HP1 α

Figure 2. Aurora-B/AIM-1 inhibits its release of HP1 α from chromatin in M-phase HeLa cells. (A) Samples of HeLa cells were synchronized, released at different time points, collected, and the DNA content analyzed by flow cytometry. (B) Synchronized cells were lysed by addition of 0.3% NP-40 in buffer N, containing 5 mM MgCl₂. The Ch fraction was pelleted at 2000 $\times$ g for 5 min at 4°C in a microfuge. The C fraction was recovered. Equal amounts of proteins prepared from cytoplasmic and chromatin fractions were run on a 12% gel and blotted with anti-HP1 α . (C) HeLa cells were synchronized in S phase using the double-thymidine block protocol. Mitotic cells were enriched by nocodazole treatment after release from the thymidine block. Cells were transfected with Aurora-B/AIM-1 (+) or control (–) RNAi during the second thymidine block, washed 9 h after initiation of the second thymidine block, and cultured again in the original culture medium for 4 h in the continued presence of thymidine. After removal of the drug, cells were cultured in fresh medium for 5 h and

treated with nocodazole for 4–5 h. Both the Ch and C fractions were prepared from the exponentially growing cells (L) or mitotic cells (M). Immunoblots with anti-HP1 α antibody was performed as shown in C.

was detectable in the C fraction. This result was supported by the immunostaining data presented above. The level of HP1 α in the cytoplasmic fraction increased proportionally with the number of mitotic cells (Figure 2B, 9 and 12 h). To investigate whether depletion of Aurora-B/AIM-1 has an effect on the ability to extract endogenous HP1 α , Aurora-B/AIM-1 RNAi was expressed in HeLa cells. Depletion of Aurora-B/AIM-1 significantly inhibited in mitotic cells chromatin dissociation of HP1 α ; the protein remained unchanged during early S phase (Figure 2C; our unpublished data). These observations strongly suggest that the dynamic behavior of HP1 α , which was observed using EGFP-HP1 α , accurately reflects the behavior of endogenous HP1 α .

INCENP Is Required for Aurora-B/AIM-1 Recruitment to Heterochromatin but Not for HP1 α Recruitment

INCENP is required for the correct localization of Aurora-B/AIM-1 to the centromere and midbody (Adams *et al.*, 2001b). However, it is unknown whether INCENP plays a similar role in the recruitment of Aurora-B/AIM-1 to the heterochromatin during G₂ phase. To investigate the functional significance of INCENP in directing Aurora-B/AIM-1 to heterochromatin regions, the INCENP gene was silenced by siRNA in HeLa cells expressing EGFP-HP1 α . In control cells, HP1 α and Aurora-B/AIM-1 associated with heterochromatin in the G₂ phase (Figure 3, A and C, left). Immunoblots showed that the levels of INCENP were significantly decreased in cells treated for 48 h with INCENP siRNA (Figure 3B). Immunostaining showed that elimination of INCENP prevented endogenous Aurora-B/AIM-1 from localizing at the heterochromatin during the G₂ phase. Heterochromatin staining with Aurora-B/AIM-1 and Survivin antibodies was either low or nonexistent in these cells. However, in control cells both proteins colocalized with HP1 α to the heterochromatin (Figure 3A; our unpublished data). It is of particular interest in this context that depletion of the bulk of INCENP did not detectably interfere with the heterochromatin localization of HP1 α (Figure 3, A and C, right). This indicates that INCENP is dispensable for anchoring of HP1 α to the chromatin. In addition, inhibition of both, mitotic H3 phosphorylation and dissociation of HP1 α from the chromosome arms, was observed. Thus, INCENP is necessary for the phosphorylation of mitotic histone H3, recruitment of

Aurora-B/AIM-1 to the heterochromatin, and dissociation of HP1 α from the chromosome arms.

Phosphorylation of H3 Induces the Dissociation of the HP1 α Chromodomain but Not of the Intact Protein from H3 Methylated at Lysine 9

Because HP1 α failed to dissociate from the chromosome arms in cells lacking functional Aurora-B/AIM-1, it is likely that the phosphorylation of histone H3 regulates the dissociation of HP1 α from the same. To investigate whether the dissociation of HP1 α from mitotic chromosomes was related to the phosphorylation of histone H3 at Serine 10, a C-terminal HA-tagged nonphosphorylatable histone H3 mutant (S10A) and WT were transfected into HeLa cells. Cultures were harvested 30 min after release from nocodazole arrest. As shown in Figure 4, A and B, a detailed quantitative analysis of the transfected cells revealed that in 36% of the cells expressing H3 S10A, HP1 α was still associated with the chromosome arms, whereas the same happened in only 8% of cells expressing WT H3. In addition, chromosome segregation defects, such as lagging chromosomes and chromatin bridges in anaphase/telophase cells, but no cytokinesis defects, were observed in cells expressing mutant H3 S10A (Figure 4C). This indicates that H3 phosphorylation is essential for proper chromosome segregation but not cytokinesis progression. H3 phosphorylation is also necessary for the dissociation of HP1 α from the chromosome arms *in vivo*. As proposed by Fischle *et al.* (2003) in the binary switch hypothesis, phosphorylation of histone H3 at Serine 10 negatively regulates the binding of HP1 α to the adjacent methylated Lysine 9 of histone H3.

Next, the effect of Aurora-B/AIM-1-mediated H3 phosphorylation on HP1 α binding to H3 methylated at Lysine 9 was investigated, and an *in vitro* binding assay was performed. Histone H3 peptide was used in an *in vitro* histone methyltransferase assay in the presence of glutathione-Sepharose containing GST alone and GST-SUV39H1 lacking the N-terminal domain. As shown in Figure 4D, methylation of histone H3 occurred only with GST-SUV39H1 but not with GST alone as determined by anti-dimethylated (Lysine 9) histone H3 antibody (Figure 4D, lanes 1 and 2). Consistent with previous results, H3 methylated at Lysine 9 was pulled down by both the His₆-HP1 α protein and the CD (Figure 4D, lanes 4 and 7). However, H3 protein phosphorylated by

Figure 3. Depletion of INCENP by RNAi inhibits association of Aurora-B/AIM-1 but not HP1 α , with heterochromatin in the G₂ phase. HeLa cells expressing EGFP-HP1 α were transfected with control or INCENP siRNA. G₂ phase cells were obtained by releasing cells synchronized by a double-thymidine block into fresh medium for a period of 10–12 h. M-phase cells were obtained from a thymidine/nocodazole block as shown in Figure 2C. (A) Cells treated with INCENP (INCENP RNAi) or control (RNase III siRNA) siRNAs were immunostained with anti-AIM-1 or anti-phos H3 (H3P) antibodies. DNA was stained with 50 ng/ml Hoechst 33258 to monitor the location of heterochromatin. Aurora-B/AIM-1 colocalizes with HP1 α in heterochromatin. In INCENP-depleted cells, Aurora-B/AIM-1 does not associate with heterochromatin in G₂ phase. During M phase, HP1 α still remains associated with the chromosome arms. Bar, 10 μ m. (B) For an immunoblot analysis, equal amounts of proteins prepared from HeLa cells transfected with INCENP (+) or control siRNA (–) were run on a 10% gel, blotted with anti-INCENP, and with anti- α -tubulin antibodies as a loading control. (C) Quantification of HP1 α associated with interphase

Figure 4. H3 phosphorylation is required but not sufficient to trigger dissociation of HP1α from H3 methylated at Lysine 9. (A) HA-tagged wild-type histone H3 (WT) or HA-tagged nonphosphorylatable H3 (S10A) were transfected into an EGFP-HP1α-expressing HeLa cell line. Cells were synchronized and fixed as shown in Figure 2C and visualized by GFP signal for HP1α, DAPI for DNA, and immunostaining with anti-HA (H3). Overexpression of H3 S10A, but not H3 WT, inhibited HP1α dissociation from the chromosome arms. (B) Quantification of HP1α associated with the chromosome arms 48 h after transfection of HA-H3 WT or S10A. HeLa cells expressing EGFP-HP1α were synchronized as shown in Figure 2C, stained with anti-HA antibody, and classified as indicated. The mitotic cells expressing HA-H3 were counted. Results are the average from three independent experiments (>200 cells each), and bars indicate standard deviations as described in *Materials and Methods*. (C) Quantitative analysis of the induction of chromosome segregation (lagging chromosomes and chromatin bridges) and cytokinesis defects (multinucleated cells) after HA-histone H3 WT or S10A mutant expression. The graph represents HA-positive cells expressing histone H3 72 h after transfection. Results are the average from three independent experiments (>200 cells each), and bars indicate standard deviations. (D) Effect of Aurora-B/AIM-1-mediated histone H3 phosphorylation on in vitro HP1α binding to histone H3 methylated at Lysine 9. Histone H3 peptide was subjected to an in vitro histone methyltransferase assay as a methyl donor. In vitro histone methyltransferase reactions were performed with human histone methyltransferase (HMTase) SUV39H1 (lanes 2, 4, 5, 7, and 8), as detected with anti-dmLys-9 H3 antibody (top, lanes 1 and 2) and H3 antibody (bottom). The methylated H3 peptides were collected and used in an His₆-HP1α whole protein (lanes 3–5) or CD (lanes 6–8) pull-down assay after kinase assay in the presence or absence of FLAG-Aurora-B/AIM-1 protein. H3 phosphorylation was detected with anti-phos H3 (H3P) antibody (middle and bottom, lanes 3–8). Bound proteins were resolved by 14% SDS-PAGE and immunoblotted with anti-H3 (top).

Aurora-B/AIM-1 protein extracted from adenovirus-induced HeLa cells was also pulled down by intact HP1α, when an anti-H3 phos10 antibody was used for detection. This finding was opposite to what is proposed in the binary switch hypothesis (lanes 5 and 8) but it is consistent with two recent publications reporting that histone H3 phosphorylation is insufficient for HP1 dissociation from methylated H3 (Fass *et al.*, 2002; Mateescu *et al.*, 2004). To conclude, these data indicate that phosphorylation of hi-

stone H3 is necessary but not sufficient to trigger the dissociation of the HP1α protein from H3 protein methylated at Lysine 9.

Abnormal Localization of Histone Methyltransferase SUV39H1 in Cells Lacking Aurora-B/AIM-1

It was recently reported that histone methyltransferase SUV39H1 is dynamically distributed in mitotic chromatin. It displays dispersed staining at prophase, gives highly specific centromeric signals at prometaphase and metaphase, and again shows significantly reduced staining at the onset of anaphase (Aagaard *et al.*, 2000). SUV39H1 has been shown to form a complex with HP1, and its enzymatic activity is required for proper targeting of HP1 to the heterochromatin (Aagaard *et al.*, 2000). To investigate whether the abnormal localization of HP1α by Aurora-B/AIM-1 RNAi could be correlated with impaired targeting of SUV39H1 during mitosis, HeLa cells that stably express FLAG-tagged SUV39H1 were immunostained. These experiments showed that in

Figure 3 (cont). heterochromatin or the chromosome arms 48 h after transfection of control, or INCENP siRNAs. HeLa cells expressing EGFP-HP1α were synchronized, and the mitotic cells were counted and classified. Results are the average of three independent experiments (>200 cells each), and bars indicate standard deviations as described in *Materials and Methods*. Quantification of HP1α associated with the chromosome arms after expression of control, Aurora-A, or Aurora-B/AIM-1 RNAi for 48 h.

Figure 5. Aberrant localization of SUV39H1 in mitotic chromosomes lacking Aurora-B/AIM-1. Immunofluorescence of mitotic chromosomes in control and Aurora-B/AIM-1-depleted HeLa cells stably expressing EGFP-HP1 α and FLAG-tagged SUV39H1 with anti-FLAG antibody. (A) After expression of Aurora-B/AIM-1 RNAi for 48 h, cells were treated for 5 h with nocodazole, fixed, and stained with anti-FLAG antibody (SUV39H1). In cells depleted of Aurora-B/AIM-1, HP1 α and SUV39H1 were localized at the metaphase chromosome arms. (B) Quantification of SUV39H1 associated with the chromosome arms 48 h after transfection of control or Aurora-B/AIM-1 siRNAs. HeLa cells were synchronized. The mitotic cells were counted and classified as indicated. Results are the average of three independent experiments (>200 cells each), and bars indicate standard deviations as described in *Materials and Methods*. (C) HeLa cells were synchronized in S phase using the double-thymidine block protocol as shown in Figure 2C. Mitotic cells were enriched using nocodazole after release from the thymidine block. After transfection with Aurora-B/AIM-1 (+) or control (–) RNAi during the second thymidine block, HeLa cells were washed 9 h after the initiation of the second thymidine block and cultured again in the original culture medium for 4 h in the continued presence of thymidine. After removal of the drug, cells were further cultured for 5 h in fresh medium and treated with nocodazole for 4–5 h. Ch and C fractions were prepared from the exponentially growing cells (L) or mitotic cells (M). Immunoblot was performed with anti-SUV39H1 antibody.

cells depleted of Aurora-B/AIM-1 where the dissociation of HP1 α was affected, SUV39H1 was localized together with HP1 α along the entire metaphase chromosome arms, whereas in control cells SUV39H1 protein was concentrated at the centromere regions (Figure 5, A and B). These data were further verified by immunoblots. Depletion of Aurora-B/AIM-1 significantly inhibited the dissociation of SUV39H1 from the chromatin-bound fraction in mitotic cells, whereas it decreased in control cells (Figure 5C). These observations suggest that Aurora-B/AIM-1 is involved in the dissociation of both HP1 α and SUV39H1 from chromosome arms.

DISCUSSION

The data presented here provide new insight into the functions that passenger proteins play in the dynamic distribution of HP1 α at the G₂–M boundary. In late G₂ phase, H3 phosphorylation is initiated within the pericentric heterochromatin. It progresses along both chromosome arms during mitosis (Hendzel *et al.*, 1997). In parallel to H3 phosphorylation, proteins of the HP1 family undergo large-scale, but progressive, dissociation from the heterochromatin in G₂ cells (Murzina *et al.*, 1999). In plant cells, HP1 γ shows a similar localization pattern during mitosis (Fass *et al.*, 2002), indicating that the dynamic behavior of HP1 proteins has been conserved during evolution. HP1 is known to physically interact with certain components of the replication complex such as the ORCs (Pak *et al.*, 1997) and the MCMs (Christensen and Tye 2003), the lamin B receptor (Ye and Worman, 1996), chromatin assembly factor 1 (Shibahara and Stillman, 1999), and INCENP (Ainsztein *et al.*, 1998). The N-terminal domain of INCENP binds to the hinge region of

HP1, whereas its C-terminal domain interacts with Aurora-B/AIM-1. Supplemental Figure 1 and Figure 3A show that in CHO and HeLa cells, HP1 α and Aurora-B/AIM-1 colocalize at the G₂ heterochromatin and that HP1 α dissociation occurs concomitantly at the heterochromatin, the starting point for H3 phosphorylation. These data suggest that passenger proteins colocalize with HP1 α at the G₂ heterochromatin, possibly through direct interaction of HP1 with INCENP. In fact, depletion of INCENP prevented the positioning of endogenous Aurora-B/AIM-1 at the heterochromatin (Figure 3A). Thus, INCENP is necessary for the proper targeting of Aurora-B/AIM-1 to the heterochromatin, centromere, and midspindle but not for recruitment of HP1 α and heterochromatin maintenance.

Trimethylation of histone H3 at Lysine 9 is important for targeting HP1 to heterochromatin region. As proposed in the binary switch hypothesis, H3 phosphorylation negatively regulates the binding of HP1 to the adjacent methylated Lysine 9 (Fischle *et al.*, 2003). Furthermore, inhibition or depletion of Aurora-B/AIM-1 triggered the retention of HP1 in chromatin during mitosis (Figures 1 and 2). In addition, an *in vitro*-binding assay revealed that Aurora-B/AIM-1-mediated phosphorylation of H3 induced the dissociation of the HP1 chromodomain but not of the intact protein (Figure 4D). This observation concurs with that of previous studies showing that in GST pull-down assays, phosphorylation of H3 at Serine 10 does not prevent binding of plant HP1 γ (Fass *et al.*, 2002) and that phosphomethylated H3 peptide stably interacts with the intact HP1 α protein (Mateescu *et al.*, 2004). While this manuscript was in review, similar findings on the regulation of HP1-chromatin binding were reported by others (Fischle *et al.*, 2005; Hirota *et al.*, 2005). However, these

observations suggest that H3 phosphorylation at Serine 10 is both necessary and sufficient for the dissociation of HP1 from the mitotic chromosomes. One reason for the different results could be that Aurora-B/AIM-1 protein purified from HeLa cells in this study may exhibit little enzymatic activity to induce dissociation of the intact HP1 α . Nonetheless, results presented here suggest that the center and/or C-terminal domain of HP1 α interferes with or reduces the effect of H3 phosphorylation on the dissociation of HP1 α . It was recently reported that the combined effects of H3 phosphorylation at Serine 10 and Lysine 14 acetylation are responsible for the dissociation of HP1 from chromatin during mitosis and that an H3 peptide carrying a triple MetLys-9-pSer-10-AcLys-14 modification no longer binds to HP1 (Mateescu *et al.*, 2004). In addition to H3 phosphorylation, the decrease of Lysine 9 methylation and/or Lysine 14 acetylation may be required for the dissociation of HP1 α from chromatin during mitosis in mammalian cells.

The histone methyltransferase SUV39H1 has been implicated to play, through selective methylation of H3 at Lysine 9, an essential role in the initial steps of heterochromatin formation, and to generate a binding site for HP1 (Rea *et al.*, 2000; Bannister *et al.*, 2001; Lachner *et al.*, 2001; Nakayama *et al.*, 2001). Furthermore, loss of methylated H3 tails was shown to be accompanied by delocalization of HP1 from heterochromatin (Maison *et al.*, 2002). These findings underscore the essential role SUV39H1 plays in targeting of HP1 and in establishing heterochromatin. Interestingly, methylation of Lys-9 interferes with Aurora-B/AIM-1-dependent phosphorylation of Ser-10. It is also inhibited by the preexisting phosphorylation of Serine 10 (Jenuwein and Allis, 2001). Thus, Aurora-B/AIM-1-mediated phosphorylation of H3 around the heterochromatin may interfere with additional methylation by SUV39H1 and thereby trigger the sequential dissociation of the HP1 and SUV39H1 complex, which eventually leads to the disruption of the heterochromatin structure. Consistent with this model, HP1 α and SUV39H1 are localized along the metaphase chromosome arms in mitotic cells lacking Aurora-B/AIM-1 (Figure 5, A and B). These observations suggest that Aurora-B/AIM-1 may decrease the methylation status of the mitotic chromosome arm by regulating the localization of SUV39H1 and thereby inducing the dissociation of HP1 from heterochromatin, although bulk trimethylation levels at Lysine 9 remain unchanged during the cell cycle (Fischle *et al.*, 2005). However, the possibility that in Aurora-B/AIM-1-depleted cells the retention of SUV39H1 at the chromatin is due to the direct binding of HP1 α to SUV39H1 cannot be excluded because SUV39H1 can bind directly to HP1. To conclude the present studies established a correlation between H3 phosphorylation, heterochromatin dynamics, and chromosomal passenger proteins. Further studies are needed to establish the exact role the proteins play during dissociation from heterochromatin during mitosis.

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