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The enhancement of histone H4 and H2A serine 1 phosphorylation during mitosis and S-phase is evolutionarily conserved

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Abstract Histone phosphorylation has long been associated with condensed mitotic chromatin; however, the functional roles of these modifications are not yet understood. Histones H1 and H3 are highly phosphorylated from late G2 through telophase in many organisms, and have been implicated in chromatin condensation and sister chromatid segregation. However, mutational analyses in yeast and biochemical experiments with *Xenopus* extracts have demonstrated that phosphorylation of H1 and H3 is not essential for such processes. In this study, we investigated additional histone phosphorylation events that may have redundant functions to H1 and H3 phosphorylation during mitosis. We developed an antibody to H4 and H2A that are phosphorylated at their

respective serine 1 (S1) residues and found that H4S1/H2AS1 are highly phosphorylated in the mitotic chromatin of worm, fly, and mammals. Mitotic H4/H2A phosphorylation has similar timing and localization as H3 phosphorylation, and closely correlates with the chromatin condensation events during mitosis. We also detected a lower level of H4/H2A phosphorylation in 5-bromo-2-deoxyuridine-positive S-phase cells, which corroborates earlier studies that identified H4S1 phosphorylation on newly synthesized histones during S-phase. The evolutionarily conserved phosphorylation of H4/H2A during the cell cycle suggests that they may have a dual purpose in chromatin condensation during mitosis and histone deposition during S-phase.

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Introduction

In eukaryotic cells, the relaxed interphase chromatin undergoes pronounced changes resulting in the formation of highly condensed mitotic chromosomes. Phosphorylation of histone proteins is believed to be involved in the transition from interphase to mitotic chromatin; however, definitive roles of these modifications in this process have not yet been elucidated. For example, mitotic chromosome condensation is accompanied by the hyperphosphorylation of histones H1 and H3 (Guo et al. 1995; Hendzel et al. 1997; Van Hooser et al. 1998). The mitotic kinase cdc2-mediated phosphorylation of histone H1 is enhanced in early prophase and reaches peak levels during metaphase (Gurley et al. 1973; Bradbury et al. 1974; Langan et al. 1989). Although the timing of this event suggests a role for this modification in chromosome condensation, experiments with *Xenopus* egg extracts demonstrate that in the absence of linker histones chromosome condensation still occurs normally (Ohsumi et al. 1993; Dasso et al. 1994). In addition, *Tetrahymena* mutant strains lacking H1 also do not display any mitotic defects (Shen et al. 1995) and therefore, both these studies suggest that H1 is not essential to the mitotic chromatin condensation process.

Similar to H1 phosphorylation, an increase in histone H3 serine 10 phosphorylation (phospho-H3S10) is also a well-known hallmark for mitosis and meiosis in ciliate, yeast, worm, and vertebrate organisms (Gurley et al. 1973; Paulson and Taylor 1982; Hendzel et al. 1997; Cobb et al. 1999; Hsu et al. 2000). The kinase responsible for this modification is the Ipl1/aurora kinase family, whereas dephosphorylation is mediated by protein phosphatase 1 (Hsu et al. 2000; Zeitlin et al. 2001b). Consistent with a role for H3S10 phosphorylation in chromosome function, mutation of the endogenous H3S10 residue to a non-phosphorylatable residue in *Tetrahymena thermophila* results in chromosome segregation defects (Wei et al. 1999). However, similar mutational analyses in *Saccharomyces cerevisiae* showed that the H3S10 to alanine mutant does not have altered generation times or cell cycle progression defects compared with the wild-type strain (Hsu et al. 2000). Also, in vitro chromatin condensation assays using *Xenopus* extracts indicated that neither H3S10 phosphorylation nor the H3 N-terminal tail is required for mitotic chromatin condensation (de la Barre et al. 2001). Therefore, although H3S10 phosphorylation may play a role in chromosome condensation or segregation in *Tetrahymena*, the requirement for this histone modification in these processes is not conserved in budding yeast and *Xenopus*.

Because faithful cell division is essential to life, it is possible that additional phosphorylation events on other histones or histone sites may provide a redundant role to H1 and H3S10 phosphorylation during mitosis. Indeed, more recent reports have shown that H3 is also phosphorylated at serine 28 (Goto et al. 1999, 2002), threonine 11 (Preuss et al. 2003), and threonine 3 (Polioudakia et al. 2004) during mitosis. In this study, we report that histones H4 and H2A are highly phosphorylated at their respective serine 1 residues (hereafter phospho-H4S1/H2AS1) during mitosis in worm, fly, and mammalian cells. The first five amino acids of H4 and H2A in the human sequence are identical to each other and are highly conserved throughout evolution. Therefore, these additional sites of mitotic phosphorylation on histones H4 and H2A may share redundant functions with H3 phosphorylation in the process of chromosome condensation during mitosis.

In addition to our discovery of novel sites of mitotic phosphorylation, we also detected a lower level of H4/H2A phosphorylation during S-phase. This finding is consistent with previous studies showing H4S1 phosphorylation on newly synthesized histones (Ruiz-Carrillo et al. 1975). Therefore, H4S1/H2AS1 phosphorylation is an evolutionarily conserved modification that may have important roles during mitosis and S-phase-associated events.

Materials and methods

Generation of site-specific (S1) antibody against phosphorylated H4/H2A

A short peptide from the amino-terminus of H4/H2A (amino acids 1–9, SGRGKGGK) was synthesized containing an amino-acetylated and phosphorylated S1. The peptide was constructed with an artificial cysteine at the C-terminus for conjugation of the peptide to keyhole limpet hemocyanin using standard protocols. Rabbits were immunized with injections of 1 mg of conjugated peptide. This antibody is commercially available from Upstate Biotechnology, Lake Placid, N.Y.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assays were performed as described by Muller et al. (1987); the bound enzyme conjugate was assayed using p-nitrophenyl phosphate as substrate (Sigma), and detected by absorbance at 492 nm. Seven H4/H2A peptides were used in the ELISA: S1 phosphorylated H4 peptide was synthesized corresponding to amino acids 1–22 of the amino-terminus of H4 (S[P]GRGKGGKGLGKGGAKRHRKVL); R3 methylated H4 peptide 1–22; S1 phosphorylated and R3 methylated peptide 1–22; S1 phosphorylated and K5 acetylated peptide 1–22; S1 phosphorylated, R3 methylated, and K5 acetylated peptide 1–22; R3 methylated and K5 acetylated peptide 1–22; and the unmodified H4 peptide 1–22. Each of the seven peptides contained an amino-terminal acetyl group that is present on H4 in vivo.

Cell culture and preparation of protein samples

HeLa cells were cultured in DMEM (GibcoBRL) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were maintained in a humidified 37°C incubator with 5% CO₂ atmosphere. Adherent cells were collected by trypsinization, washed in PBS, pH 7.5 and then pelleted. Mitotic cells were isolated by treating cultures for 18 h with nocodazole (30 ng/ml) followed by agitation to release the mitotic cells that were loosely adhered to the tissue culture plates. Cell pellets from asynchronous or mitotic cultures were resuspended and lysed in nuclear isolation buffer (NIB250) and the released nuclei were gently pelleted by centrifugation. Pelleted nuclei were extracted using 0.4 N sulfuric acid and the acid soluble histones were precipitated with trichloroacetic acid and resuspended in distilled H₂O.

Cell culture and synchronization

To arrest cell cultures at the G1/S-phase border, HeLa cells were treated with 2 mM thymidine (Sigma) for 16 h, released into fresh medium for 8 h, and blocked at the G1/S-phase border again by subsequent treatment with 0.4 mM mimosine (Sigma) for 16 h (as described in Rice et al. 2002). Cells were released into fresh medium and samples were taken at 1 h to 2.5 h intervals as indicated.

Reverse phase HPLC (RP-HPLC) purification of histones

Separation of HeLa core histones by RP-HPLC was done as previously described (Sobel et al. 1994). Under these conditions H4 and H2A co-elute in one peak. The H4/H2A fraction was dried under vacuum and stored at –80°C.

Immunoblot analyses

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). The specificity of the antibody used was analyzed by immunoblotting analyses and by ELISA. Balanced protein loads were ensured by staining parallel gels of equivalently loaded samples with Coomassie Brilliant Blue and by staining of the immunoblots with Ponceau Red. All blots were blocked with 5% nonfat dry milk and incubated with the immune sera diluted in TBS with 0.1% Tween 20 and 2% milk. The membrane-bound primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (Amersham). For peptide competition assays, 1 μ l of antibody was preincubated with 1 μ g modified phospho-H4S1 peptide, modified phospho-H2AS1 peptide, or unmodified H4/H2A peptide prior to application.

Immunofluorescence

HeLa and mouse fibroblast cells were fixed in 1% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 15 min. Cells were blocked with 10% goat serum for 30 min prior to

incubation with a 1:1000 dilution of α -phospho-H4S1/H2AS1, and a 1:250 dilution of α -phospho-H3S10 or 1:500 dilution of α -phospho-H3S28. Cells were then incubated with a Cy3-conjugated donkey anti-rabbit IgG antibody (Jackson Laboratories) or a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse antibody (Jackson Laboratories) followed by 4',6-diamidino-2-phenylindole (DAPI) staining. Peptide competitions were done as described above for immunoblot analysis.

Antibody labeling of *Drosophila* embryos was carried out as previously described (Wang et al. 2001). Briefly, embryos (0–3 h) were dechlorinated in a 50% chlorine bleach solution, washed with 0.7 M NaCl with 0.2% Triton X-100, and fixed in a 1:1 heptane: Bouin's fluid (0.66% picric acid, 9.5% formalin, 4.7% acetic acid) mixture for 20 min at room temperature with vigorous shaking. Vitelline membranes were then removed by shaking embryos in heptane:methanol (1:1) at room temperature for 30 s. Embryos were blocked with blocking solution (PBS with 0.4% Triton X-100, 1% normal goat serum, and 2% BSA) for 30 min before incubation with α -phospho-H4S1/H2AS1 (1: 2000 diluted in blocking solution) at 4°C for 16 h. The α -phospho-H4S1/H2AS1 staining was visualized with Cy3-conjugated goat anti-rabbit secondary antibody. DNA was visualized by DAPI staining (1 μ g/ml in PBS). Stained embryos

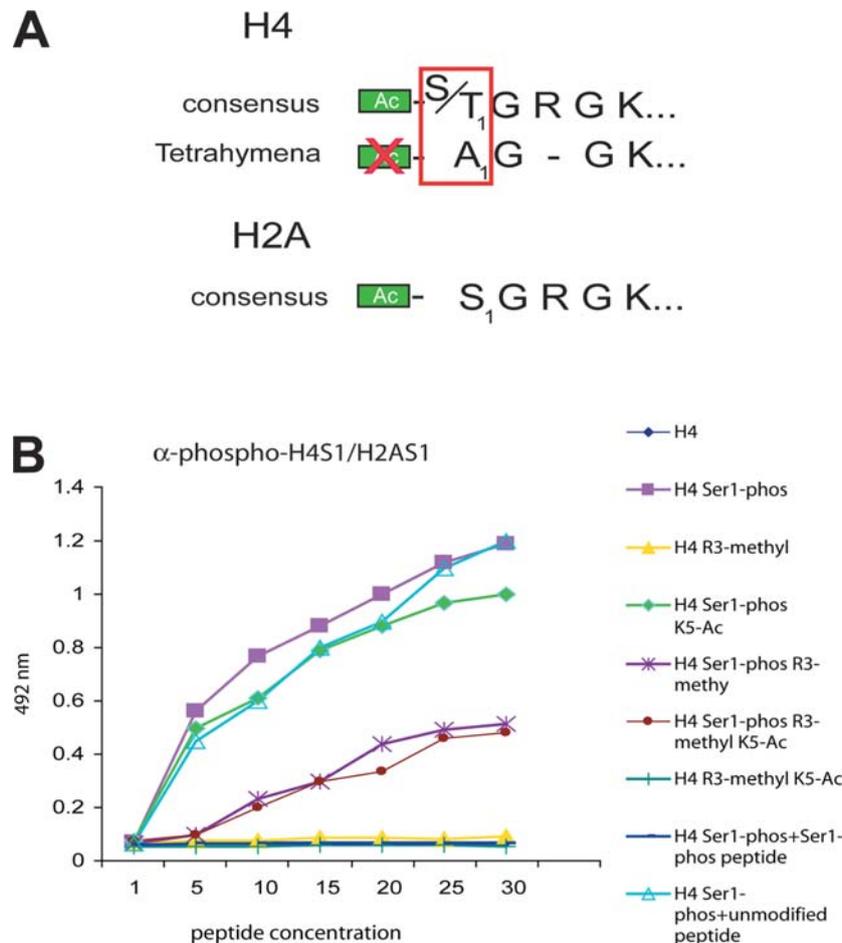


Fig. 1A, B Characterization of a novel site-specific antibody to phosphorylated H4 and H2A. **A** The amino-terminal sequences of histones H4 and H2A are highly conserved throughout evolution, although the *Tetrahymena thermophila* H4 alanine 1 (A1) is a non-conserved substitution. The initial serine or threonine residue in H4 and H2A is a phospho-acceptor site; this residue is also amino-acetylated in every organism tested to date except *T. thermophila* H4. **B** Enzyme-linked immunosorbent assay (ELISA) analysis of the antibody raised to the H4 peptide (9 amino acids) containing

acetylated and phosphorylated S1. ELISA experiments were performed using unmodified H4, phospho-H4S1, methyl-H4R3, phospho-H4S1/methyl-H4R3, phospho-H4S1/acetyl-H4K5, methyl-H4R3/acetyl-H4K5, and phospho-H4S1/methyl-H4R3/acetyl-H4K5 peptides immobilized on microtiter dishes. For peptide competition, α -phospho-H4S1/H2AS1 was incubated with 1 mg/ml of the phosphorylated or unmodified H4 peptide prior to ELISA. Peptide concentrations ranged from 1–30 ng (*x*-axis) and quantitation of absorbance was measured at 492 nm (*y*-axis)

were visualized using a 63× objective on a Zeiss 200 M microscope. Pictures were analyzed using Spot Camera Advanced software.

Caenorhabditis elegans embryo staining was performed as previously described (Howe et al. 2001). Embryos were fixed with 2% paraformaldehyde and permeabilized by freeze cracking on dry ice, followed by exposure to -20°C in 95% ethanol and then warmed to room temperature. The embryos were rehydrated and hybridized to primary antibody as follows: α -phospho-H4S1/H2AS1 was used at a 1:1000 dilution and α -phospho-H3S10 was used at a 1:500 dilution. Embryos were then incubated with donkey Cy3-anti-mouse (Jackson Laboratories) or FITC-anti-rabbit (Jackson Laboratories) followed by DAPI (blue) or Hoechst (red) staining. Fluorescently labeled samples were visualized using a confocal microscope.

Mass Spectrometry Analysis

Histones from nocodazole-arrested or asynchronously growing HeLa cells were isolated and digested with Glu-C (Roche) in 100 mM ammonium acetate (pH 4) for 4 h at 37°C. Peptides were fractionated by HPLC (Applied Biosystems model 130A) on a C18, HAILSIL-300 column (Higgins, Winter Park, Fla.). Fractions were collected manually. Those containing histones, residues ranging from 1 to 80, were concentrated using a Speed Vac Concentrator (Savant), brought to pH 8.5 with 100 mM ammonium bicarbonate, treated with propionylation reagent comprised of methanol and propionic anhydride in a ratio of 1:3 for 15 min at 37°C and lyophilized to dryness. Peptides were further digested with chymotrypsin in 100 mM ammonium bicarbonate (pH 8.5) for 6 h at 20°C. Newly generated amine termini were converted to amides by treatment with a second aliquot of the propionylation reagent, lyophilized to dryness, and the mixture was then resuspended in 0.1% acetic acid (pH 3). An aliquot of ~10 pmol of the resulting mixture was loaded onto a nano-HPLC column (360 x 50 μ m fused silica packed with 7 cm of C18 beads (YMC ODS-AQ; Waters), eluted with a linear gradient of 0-60% B in 60 min and 60-100% B in 5 min (A=0.1% acetic acid in nanopure water, B=70% acetonitrile in 0.1% acetic acid) and analyzed on a Thermo Electron LTQ-FT mass spectrometer equipped with a nano-HPLC microelectrospray ionization source. Synthetic peptides of histones were synthesized on an AMS 1400 multiple peptide synthesizer (Gilson Medical Electronics) using solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry and Wang resins. The peptides were cleaved from the resin using TFA and subsequently HPLC purified on a C-8 column (Vydac) using a gradient of 0-100% B in 12 min (A=0.1% TFA in nanopure water; B=100% acetonitrile in 0.085% TFA). Identities of synthetic peptides were confirmed by infusion of a 1 pmol/ μ l peptide solution in 40% acetonitrile and 0.1% acetic acid in nanopure water on a Thermo Electron LTQ-FT mass spectrometer.

Results

Generation and characterization of the phospho-H4S1/H2AS1 antibody

Histones H4 and H2A share an identical five amino acid sequence at their N-terminal ends in most eukaryotic organisms examined to date (Fig. 1A). Using mass spectrometry, we show that the serine 1 residues of both H4 and H2A are phosphorylated in vivo (Electronic Supplementary Material, Fig. 1). To facilitate investigation of the phosphorylation of these two histones, we raised an antibody (α -phospho-H4S1/H2AS1) against a peptide corresponding to the first nine amino acids of the human H4 sequence with the S1 residue amino-acetylated and phosphorylated (Fig. 1B). In an ELISA, this antibody

recognized the phospho-H4S1 peptide, but not the unphosphorylated peptide (Fig. 1B). In addition, pre-incubation of the antibody with excess phosphorylated peptide, but not with the unmodified peptide, competed away the binding of this antibody to the ELISA plate bound peptides, further confirming the phospho-specific recognition of H4 by this antibody.

The extreme amino-terminus of H4 is a region where multiple post-translational modifications may exist within a short stretch of amino acid residues (Fig. 1A). This region has been proposed as a possible 'modification cassette' in which specific combinations of histone modifications may elicit distinct biological functions (Fischle et al. 2003b). For example, in addition to serine 1, the arginine 3 and lysine 5 residues of H4 are known to be methylated and acetylated, respectively, in association with different nuclear processes. Methylation of H4R3 is mediated by PRMT1, and is associated with hormone-induced transcriptional activation (Strahl et al. 2001). In unstimulated conditions, the levels of methyl-H4R3 remain constant over the cell cycle (Rice et al. 2002). In addition, H4K5 is acetylated on newly deposited histones (Ruiz-Carrillo et al. 1975; Sobel et al. 1995) and is also enriched in regions of active transcription (Brownwell et al. 1996; reviewed in Jenuwein and Allis 2001). At present, it is not known which combinations of these modifications occur together on H4 in vivo, nor is it clear what percentage of total H4 molecules have multiple modifications at their N-terminal tails. Nevertheless, the potential for H4 to be multiply modified on residues that are in close proximity could disrupt the recognition of the phospho-S1 epitope by this antibody. Therefore, we used ELISA to test the ability of the phospho-H4S1/H2AS1 antibody to detect synthetic peptides containing phospho-H4S1 in combination with methyl-H4R3 and/or acetyl-H4K5. As shown in Fig. 1B, the antibody effectively detected the phospho-H4S1/methyl-H4R3 peptide, albeit at a lower efficiency. Similar to this result, the antibody also detected a tri-modified peptide (phospho-S1/methyl-R3/acetyl-K5). Finally, this antibody was equally effective in detecting a di-modified peptide phosphorylated at S1 and acetylated at K5 as compared with the singly-modified S1 phosphorylated peptide (Fig. 1B). Together, these results show that neither methylation of R3 nor acetylation of K5 on H4 precluded recognition of the phospho-S1 epitope by the phospho-H4S1/H2AS1 antibody.

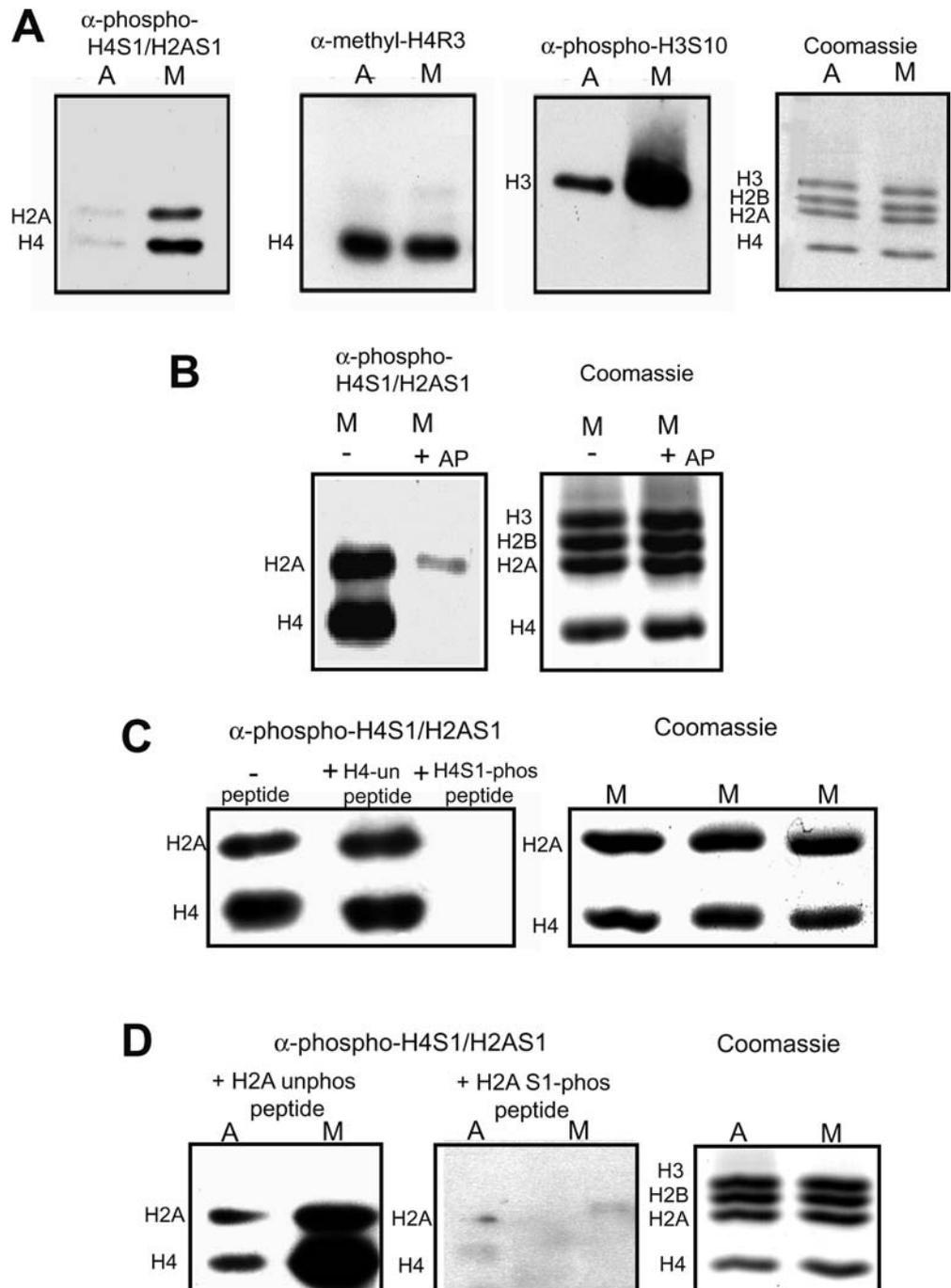
Phosphorylation of H4/H2AS1 is enhanced during mitosis

In immunoblot analysis, α -phospho-H4S1/H2AS1 detected two proteins from acid-extracted nuclear lysates that correspond to the molecular weights of H2A and H4 (Fig. 2). Using this antibody, we examined the levels of phosphorylated H2A and H4 in asynchronous (A) and nocodazole-arrested mitotic (M) HeLa cell cultures. Immunoblotting of acid-extracted histones from asynchronous and mitotic HeLa cells indicated a dramatic induction

of phospho-H4S1/H2AS1 in the nocodazole-arrested mitotic cells (Fig. 2A), which is consistent with the known increase in H3 phosphorylation during mitosis (Hendzel et al. 1997). Immunoblotting of the histone samples pre-treated with alkaline phosphatase (AP) abolished or greatly reduced detection of H4/H2A by the antibody, indicating that the recognition of histones by this antibody is indeed phospho-dependent (Fig. 2B). We also demonstrated that phospho-H4S1 and phospho-H2AS1 peptides abolish the recognition of both antigens by the antibody (Fig. 2C, D), further supporting the specificity of this antibody.

The ELISA data showed that the presence of a methyl group at H4R3 does not preclude recognition of the phospho-S1 epitope by α -phospho-H4S1/H2AS1 (Fig. 1B); we also confirmed this by immunoblot. Our analysis showed that the levels of methyl-H4R3 are identical in asynchronous and nocodazole-arrested samples (Fig. 2A); the same results were seen when the samples were treated with alkaline phosphatase to remove any phosphate groups on S1 that might interfere with detection by α -methyl-H4R3 (Electronic Supplementary Material, Figs. 2, 3). Taken together with earlier studies (Rice et al. 2002), these data confirm that the increase in

Fig. 2A–D Enhancement of phospho-H4S1/H2AS1 in mitosis. **A** Nuclei from asynchronous (*A*) or nocodazole-blocked mitotic (*M*) HeLa cells were acid-extracted and resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were examined by immunoblotting using antibodies as follows: α -phospho-H4S1/H2AS1, α -methyl-H4R3, and α -phospho-H3S10. Equal protein loading is estimated by Coomassie stain. **B** Whole-cell acid-extracted histones from HeLa mitotic samples treated (+) or mock-treated (-) with alkaline phosphatase (*AP*) for 30 min were resolved by SDS-PAGE and immunoblotted with α -phospho-H4S1/H2AS1. **C** Immunoblot analysis of HPLC-purified H4/H2A from mitotic (*M*) HeLa cells with α -phospho-H4S1/H2AS1. The antibody was pre-absorbed in the presence (+) of 1 mg/ml phospho-H4S1 peptide, unmodified H4 peptide, or mock treated (-) for 1 h prior to immunoblotting. **D** Immunoblot analysis of histones from asynchronous (*A*) or nocodazole-blocked mitotic (*M*) HeLa cells with α -phospho-H4S1/H2AS1. The antibody was preabsorbed in the presence (+) of 1 mg/ml phospho-H2AS1 peptide or phospho-H2AS1 peptide treated with alkaline phosphatase (unphosphorylated peptide) for 1 h prior to immunoblotting



the phospho-S1 levels detected by α -phospho-H4S1/H2AS1 is a result of the mitosis-specific increase in S1 phosphorylation.

Mitotic phosphorylation of H4S1/H2AS1 is evolutionarily conserved

Because the amino-terminal sequences of H4 and H2A are largely conserved throughout evolution (Fig. 1A), we tested whether the H4/H2A phosphorylation during mitosis is conserved among different organisms. We first examined *C. elegans* embryos with the α -phospho-H4S1/H2AS1 antibody by immunofluorescence microscopy (Fig. 3A, B). During early embryogenesis, worm embryos have rapid cell divisions, and therefore a high percentage of those cells are undergoing mitosis. As shown in Fig. 3A, these mitotic cells can be easily distinguished from those in interphase by their distinct chromosome morphology (DAPI stain, blue), and by their intense staining with the α -phospho-H3S10 antibody (red). Moreover, we find that in a *C. elegans* multicellular embryo, chromosomes in prophase (p) and metaphase (m) cells, but not in interphase (i) cells, are also stained by α -phospho-H4S1/H2AS1 (green). Fig. 3B shows a one-cell embryo (large arrowhead) in which the condensed chromosomes are aligned on the metaphase plate and are stained by both α -phospho-H4S1/H2AS1 (green) and α -phospho-H3S10 (red) antibodies. Similar co-staining was seen only in the mitotic cells (small arrowhead; prophase cell) in the surrounding cells of this field.

Even though the first amino acid on the *Drosophila* H4 sequence is a threonine instead of a serine, we found that our α -phospho-H4S1/H2AS1 also recognizes the phosphorylated form of *Drosophila* H4 (Electronic Supplementary Material, Fig. 4). The specificity of α -phospho-H4S1/H2AS1 was tested by immunoblots with acid-extracted proteins from *Drosophila* S2 cells (Electronic Supplementary Material, Fig. 4) and immunofluorescence microscopy in S2 cells (data not shown). In both experiments, phospho-H4S1 peptide successfully competed away the recognition of α -phospho-H4S1/H2AS1 from *Drosophila* H4 and H2A, suggesting that the antibody is indeed recognizing phospho-H4/H2A. Therefore, we were able to use this antibody to explore further the evolutionary conservation of S1 phosphorylation during mitosis in *Drosophila*. Early *Drosophila* embryos undergo an extended burst phase of growth characterized by rapidly successive mitosis and S-phases, with negligible gap phases. Upon staining the *Drosophila* embryos with α -phospho-H4S1/H2AS1 (Fig. 3C, red) and DAPI DNA dye (Fig. 3C, blue), we found that the individual cells of a 3 h embryo are highly synchronized at mitosis with the chromosomes of each cell lined up along the metaphase plate (Fig. 3C, left panels). Our results show that α -phospho-H4S1/H2AS1 localizes specifically to these metaphase chromosomes, which is consistent with the results from the nocodazole-treated human cells, as well as those seen with *C. elegans* embryos (Fig. 3A, B).

Interestingly, the antibody also stained *Drosophila* embryos that consist mostly of cells that are in S-phase (Fig. 3C, right panels), indicating that H4S1/H2AS1 are also phosphorylated during S-phase. Given that earlier studies have reported that H4S1 phosphorylation is associated with newly synthesized H4 during S-phase (Ruiz-Carrillo et al. 1975), our results may reflect this process in the *Drosophila* embryo.

In addition to *C. elegans* and *Drosophila*, other organisms were investigated for an increase in phospho-H4S1/H2AS1 throughout the cell cycle. Similar results were obtained with the slime mold *Physarum polycephalum* in immunoblots with α -phospho-H4S1/H2AS1 (*C. Thiriet and J. Hayes, personal communication*). Interestingly, the only organism investigated that contains the conserved H4S1/H2AS1, but does not exhibit a cell cycle stage-specific S1 phosphorylation is *S. cerevisiae*. In immunoblots with α -factor-arrested yeast, the levels of phospho-H4S1/H2AS1 did not change as the synchronized cells progressed through the cell cycle (*W.L. Cheung, personal communication*).

Phospho-H4S1/H2AS1 is localized to mitotic chromatin in situ and to a lesser extent in select interphase cells

Our immunoblotting data showed that the bulk levels of H4/H2A phosphorylation are highly increased in mitotically arrested cultures. We also used immunofluorescence microscopy to examine this phenomenon at the single cell level. Staining of a population of asynchronously growing mouse fibroblast cells with DAPI (blue) and α -phospho-H4S1/H2AS1 (red) showed that approximately 5% of the total cell population were in various stages of mitosis (prophase through telophase) as measured by the characteristic DAPI-dense morphology of mitotic chromosomes. Consistent with our immunoblot analysis, all of these cells stained intensely with α -phospho-H4S1/H2AS1 (Fig. 4A, arrowhead). Interestingly, some (approx. 10% of the total population, arrows), but not all interphase cells also stained with α -phospho-H4S1/H2AS1, albeit to a lesser degree than those in mitosis. We predicted that these phospho-H4S1/H2AS1-positive interphase cells were S-phase cells, which would be consistent with earlier reports (Ruiz-Carrillo et al. 1975) and our observation of S-phase staining in the *Drosophila* embryo (Fig. 3C). A greater magnification (Fig. 4B) of a mitotic (arrowhead) and a selected interphase cell illustrated the two levels of α -phospho-H4S1/H2AS1 staining that are above the baseline levels seen in the remaining interphase cells (Fig. 4A). This second population of α -phospho-H4S1/H2AS1-staining cells was investigated further below (Fig. 5).

The onset and localization of phospho-H3S10 and phospho-H3S28 during mitosis have been well characterized in mammalian cells (Hendzel et al. 1997; Goto et al. 1999; Zeitlin et al. 2001a). Phospho-H3S10 initiates during late G2, and is detected as punctate regions of staining in the pericentric heterochromatin. As cells

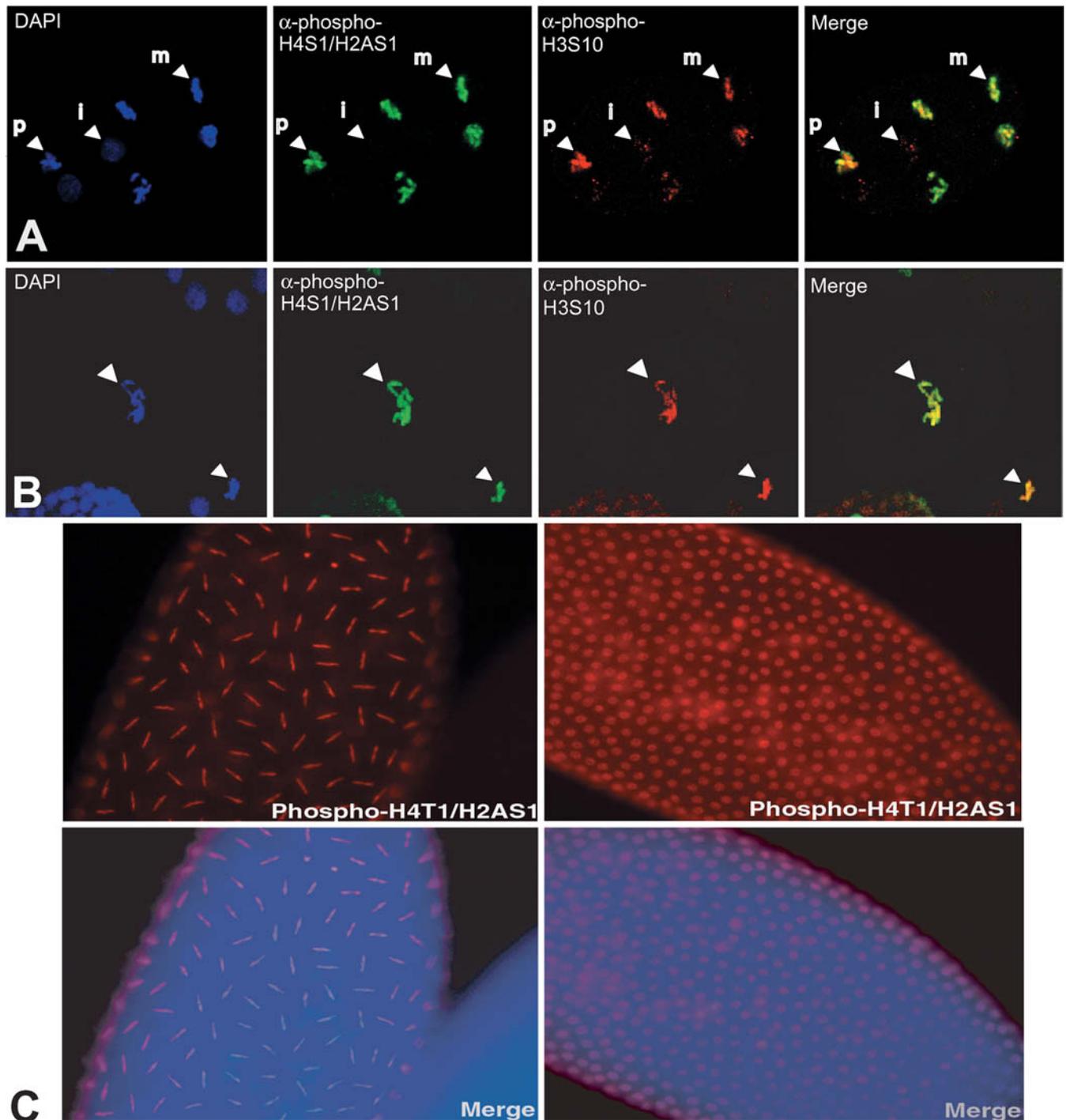


Fig. 3A–C Conservation of H4/H2A phosphorylation in *Caenorhabditis elegans* embryos and *Drosophila* early embryos. **A, B** Confocal images of *C. elegans* embryos costained with 4',6-diamidino-2-phenylindole (DAPI) (blue), α -phospho-H4S1/H2AS1 (green), and α -phospho-H3S10 (red). **A** Within an early *C. elegans* embryo, individual nuclei in prometaphase (arrowhead, p) and metaphase (arrowhead, m) show strong chromosomal colocalization of α -phospho-H4S1/H2AS1 (green) and α -phospho-H3S10 (red), while interphase (arrowhead, i) cells do not. **B** Both α -phospho-H4S1/H2AS1 (green) and α -phospho-H3S10 (red)

localize along prometaphase chromosomes (large arrowhead) in a one-cell embryo. In surrounding multicellular embryos, colocalization of α -phospho-H4S1/H2AS1 (green) and α -phospho-H3S10 (red) is observed in cells with mitotic chromosomal morphology (small arrowhead; prometaphase cell). **C** Early *Drosophila* embryos in which cells are undergoing mitosis (left panels) and S-phase (right panels) are stained with α -phospho-H4S1/H2AS1 (red) and DAPI (blue). Upon merging the images, the co-localization of signals is purple (bottom panels)

advance through mitosis, larger domains of chromatin become phosphorylated on H3S10. This signal peaks during metaphase, declines to baseline levels upon the completion of telophase, and is largely undetectable following mitosis. Occurring at a narrower time frame compared with the phospho-H3S10 profile, phospho-H3S28 is largely undetectable in pericentric heterochromatin during G2 and early prophase, then increases rapidly through metaphase and anaphase, and decreases in telophase just prior to the decline of the phospho-H3S10 signal.

To compare directly the timing of H3 and H4/H2A phosphorylation during the different stages of mitosis, we used dual-labeling immunofluorescence techniques on mouse fibroblasts (Fig. 4C) with α -phospho-H4S1/H2AS1 (red) in conjunction with α -phospho-H3S10 (green, top set) or α -phospho-H3S28 (green, lower set). Phospho-H4S1/H2AS1 staining initiated during early prophase and remained at high levels through anaphase, then similar to phospho-H3S10, declined upon the completion of telophase (Fig. 4C, top set). We also directly compared phospho-H4S1/H2AS1 and phospho-H3S28 using dual labeling, and found that the overall timing of these modifications was similar, yet the phospho-H4S1/H2AS1 signal persisted slightly longer in telophase than phospho-H3S28 (Fig. 4C, bottom set). The specificity of the antibody in immunofluorescence experiments was confirmed by peptide competition (Fig. 4D), whereby excess phospho-H4S1 peptide effectively competed away the recognition by the antibody of phospho-H4S1/H2AS1 in mitotic cells that are positive for phospho-H3S10 staining (arrowheads). Mouse fibroblasts were used for this series of experiments because the characteristically distinct pericentric heterochromatin structures during prophase are easily detectable by staining the chromosomal DNA with DAPI (blue). Essentially the same results were found using mouse fibroblasts (Fig. 4) and HeLa cells (data not shown).

Synchronized human cells exhibit a biphasic pattern of phospho-H4/H2AS1 phosphorylation with peaks of phosphorylation at early S-phase and mitosis

As shown in our immunofluorescence microscopy analyses of *Drosophila* embryos and mouse cells, a population of non-mitotic cells was positive for α -phospho-H4S1/H2AS1 staining. Given that earlier studies showed that H4S1/H2AS1 phosphorylation is associated with new histones, the α -phospho-H4S1/H2AS1-stained interphase cells could represent cells that are in S-phase, a time when histones are synthesized. To test this possibility, HeLa cells were treated sequentially with thymidine and mimosine (ribonucleotide reductase inhibitors that block cells at the G1/S border) and then released into drug-free medium to allow the cells to resume their cell cycle progression in synchrony (as previously done in Rice et al. 2002). Cells were harvested at various time points post release (0 h and successive 1 to 2.5 h intervals) and the cell

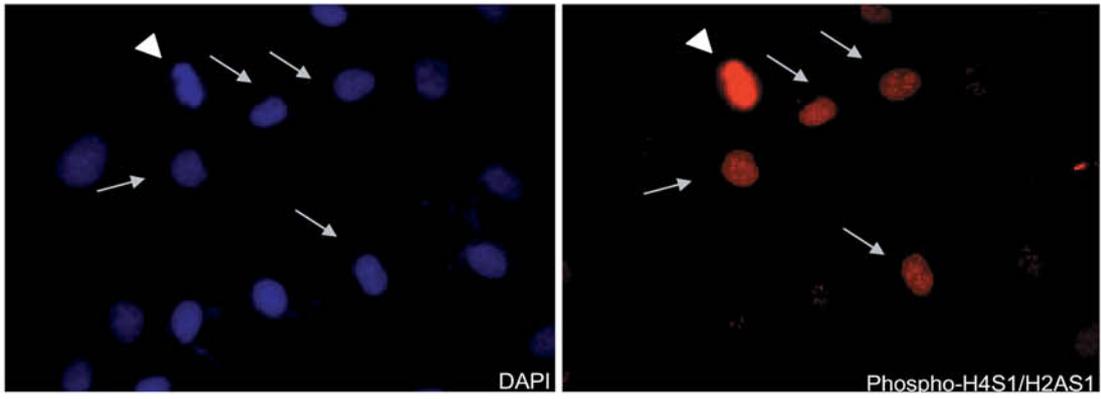
cycle stages were determined by fluorescence-activated cell sorting analysis (Fig. 5A). In parallel samples, histones were harvested by acid-extraction and analyzed by immunoblot with α -phospho-H4S1/H2AS1, α -phospho-H3S10, α -phospho-H3S28, and α -H4 (Fig. 5B). By this analysis, we found that the levels of phospho-H4S1/H2AS1 exhibited a biphasic pattern, in which levels were high at the G1/S border and during early S-phase (0 to 2.5 h), then declined to baseline levels during mid to late S-phase/early G2 (5 to 8.5 h), and reached peak levels during mitosis (10 to 15 h). The extent of H4S1/H2AS1 phosphorylation appeared higher in mitosis (10 to 15 h) as compared with early S-phase (0 to 2.5 h) time points, consistent with our immunofluorescence data (Fig. 3). The enhanced H4S1/H2AS1 phosphorylation lasted until telophase/early G1 (15 h), and was at baseline levels by 18 h (data not shown). In contrast, phospho-H3S10 and phospho-H3S28 specifically increased at the mitotic time points (10–15 h) and were largely undetectable in G1 or S-phase cells. Equal protein loading was estimated by immunoblotting a parallel blot with a general antibody against histone H4 (α -H4). Therefore, in synchronized human cells, phospho-H4S1/H2AS1 levels exhibit a biphasic pattern, with enhanced phosphorylation found at both early S-phase and mitosis.

Using indirect immunofluorescence to investigate α -phospho-H4S1/H2AS1 staining in non-mitotic cells (Fig. 4A, B), we were not able to detect discrete α -phospho-H4S1/H2AS1 staining in the cytoplasm, where new histones are synthesized. However, our detection of this modification in the nucleus of non-mitotic cells may be reflective of newly synthesized and deposited H4. To investigate whether the α -phospho-H4S1/H2AS1-positive cells detected by indirect immunofluorescence were indeed in S-phase, asynchronous HeLa cells were pulse-labeled with 5-bromo-2-deoxyuridine (BrdU), fixed, and dually stained with α -phospho-H4S1/H2AS1 (red) and α -BrdU (green). As shown in Fig. 5C, the number of BrdU-positive cells that were also labeled with α -phospho-H4S1/H2AS1 was approximately 56% (see also Electronic Supplementary Material, Fig. 5). We expect that the BrdU-positive cells that lack α -phospho-H4S1/H2AS1 staining are in the late stages of S-phase when phospho-H4S1/H2AS1 levels decline to baseline levels. Our detection of phospho-H4/H2AS1 in the nucleus of S-phase cells is consistent with our immunoblot results using synchronous HeLa cells (Fig. 5B), suggesting that, in addition to mitosis, H4S1/H2AS1 phosphorylation may also be an S-phase-specific modification of newly deposited histones.

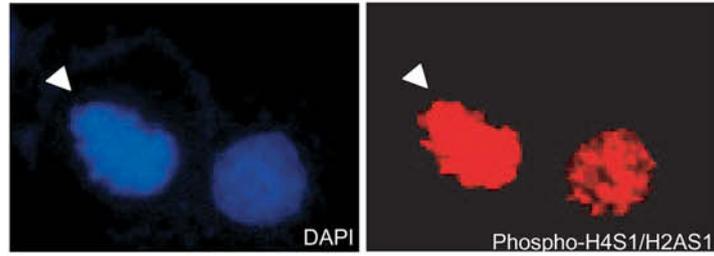
Discussion

Through the development of a phospho-specific antibody to H4S1/H2AS1, we found that these histones are phosphorylated at their serine 1 residues during mitosis in a wide range of eukaryotic cells. The onset, duration, and subcellular localization of H4/H2A phosphorylation are similar to histone H3 phosphorylation on S10 and S28,

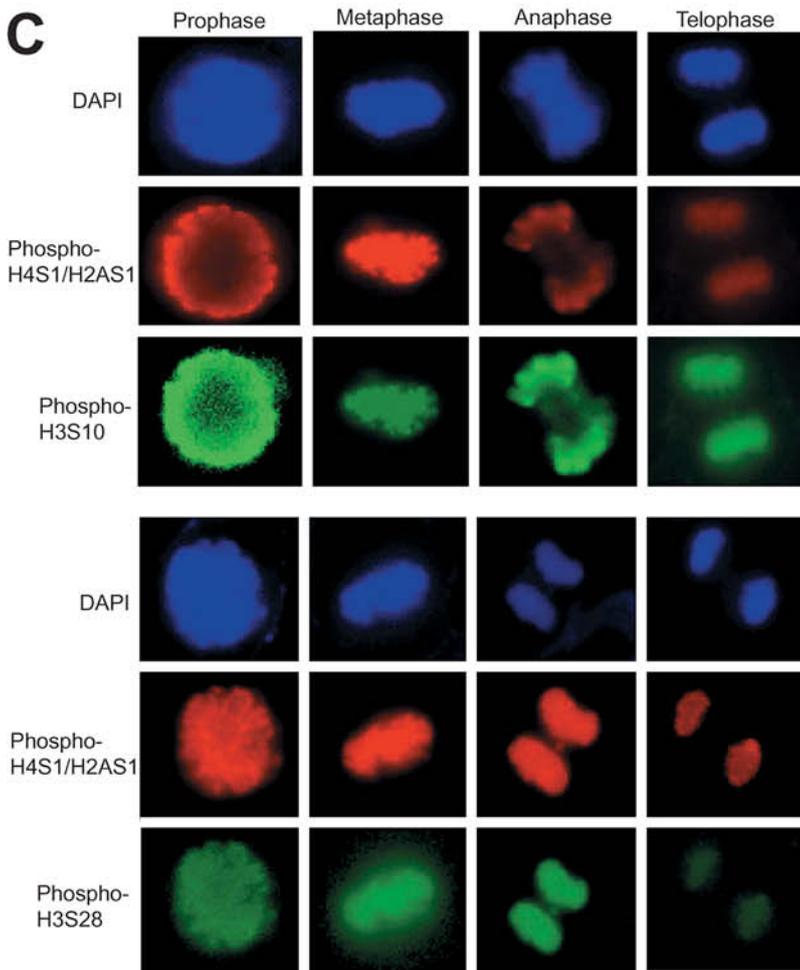
A



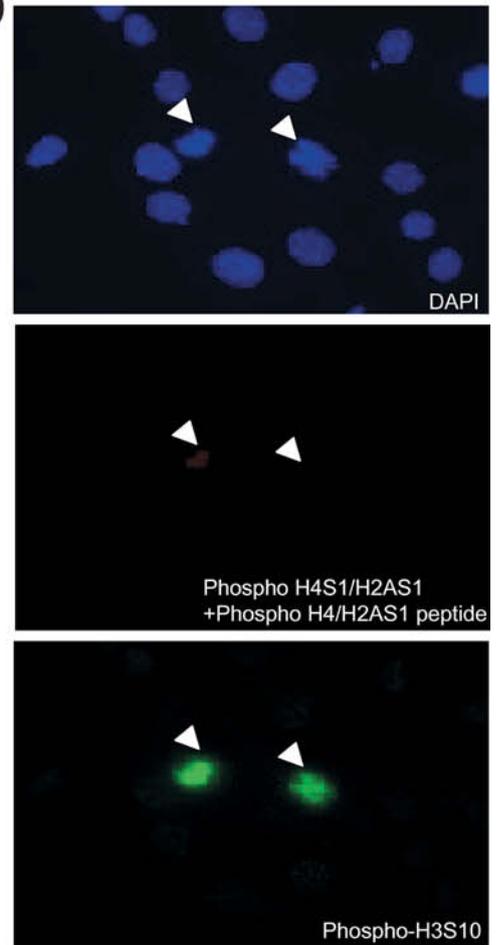
B



C



D



◀ **Fig. 4A–D** Detection of phospho-H4S1/H2AS1 by immunofluorescence microscopy. **A** Immunofluorescence staining of α -phospho-H4S1/H2AS1 (red) in a field of asynchronous mouse fibroblasts. A single cell with characteristic mitotic chromosome morphology (as defined by DAPI staining, blue) is marked with an arrowhead, and interphase cells containing above-background levels of α -phospho-H4S1/H2AS1 staining are marked with arrows. **B** Greater magnification of a mitotic and a selected interphase cell exhibiting the two levels of α -phospho-H4S1/H2AS1 staining. The mitotic cell (arrowhead) stains intensely with α -phospho-H4S1/H2AS1 (red); 10% of the interphase cells exhibit a lower level of α -phospho-H4S1/H2AS1 staining that is above the baseline staining seen in the remaining interphase cells. **C** Asynchronous mouse fibroblasts were doubly stained with α -phospho-H4S1/H2AS1 (red) and α -phospho-H3S10 (green) in one experiment (top set) and with α -phospho-H4S1/H2AS1 (red) and α -phospho-H3S28 (green) in a second experiment (bottom set). The mitotic stage of each cell was determined by the chromatin morphology with the DAPI stain (blue). **D** Immunofluorescence staining of α -phospho-H4S1/H2AS1 (red) pre-absorbed with 1 mg/ml phospho-H4S1 peptide in mouse fibroblast cells. The cell cycle stage was determined by DAPI staining (blue) and the presence of mitotic cells (arrowheads) was confirmed by staining with α -phospho-H3S10 (green)

suggesting that these histone phosphorylation events may together participate in the condensation or segregation of mitotic chromosomes. We also detected a lower level of nuclear H4S1/H2AS1 phosphorylation in early S-phase cells, possibly on newly deposited histones on replicating DNA as previously shown (Ruiz-Carrillo et al. 1975). Therefore, the H4/H2A S1 phosphorylation is an evolutionarily conserved modification that may have separate roles during mitosis and S-phase.

Our finding that H4S1/H2AS1 are highly phosphorylated in association with mitotic chromosome condensation adds to the original finding that H1 and H3 are hyperphosphorylated during this process. In *Tetrahymena*, the aberrant chromosome segregation phenotype seen in the H3 S10A mutation offers compelling evidence that phosphorylation of H3 at this site has an important functional role in this process; however, similar effects were not seen in the H3 S10A mutant in budding yeast. It is interesting to note that the first residue of histone H4 in *Tetrahymena* is an alanine (A1) (Fig. 1A). Given our finding that S1 residues of H4 and H2A are phosphorylated during mitosis, it is an intriguing possibility that H3S10 and H4S1/H2AS1 phosphorylation may have compensatory or perhaps redundant functions. This may explain why the H3 S10A mutation in *Tetrahymena*, in which H4S1 phosphorylation is not possible, resulted in mitotic defects. This hypothesis of redundancy of phospho-H4S1 and phospho-H3S10 was tested in budding yeast by generating yeast strains that have both H3S10 and H4S1 mutated to alanines; however, such mutants did not exhibit a mitotic phenotype (J.Y. Hsu, unpublished observation). On the other hand, the chromosomes of *S. cerevisiae* do not condense to a great extent during mitosis, and the levels of phospho-H4S1/H2AS1 do not increase during mitosis in yeast (W.L. Cheung, personal communication). Therefore, lower eukaryotes such as *S. cerevisiae* may have different histone modification patterns in mitosis. Aside from *Tetrahymena*, we have not yet been able to link a mitotic modification to a

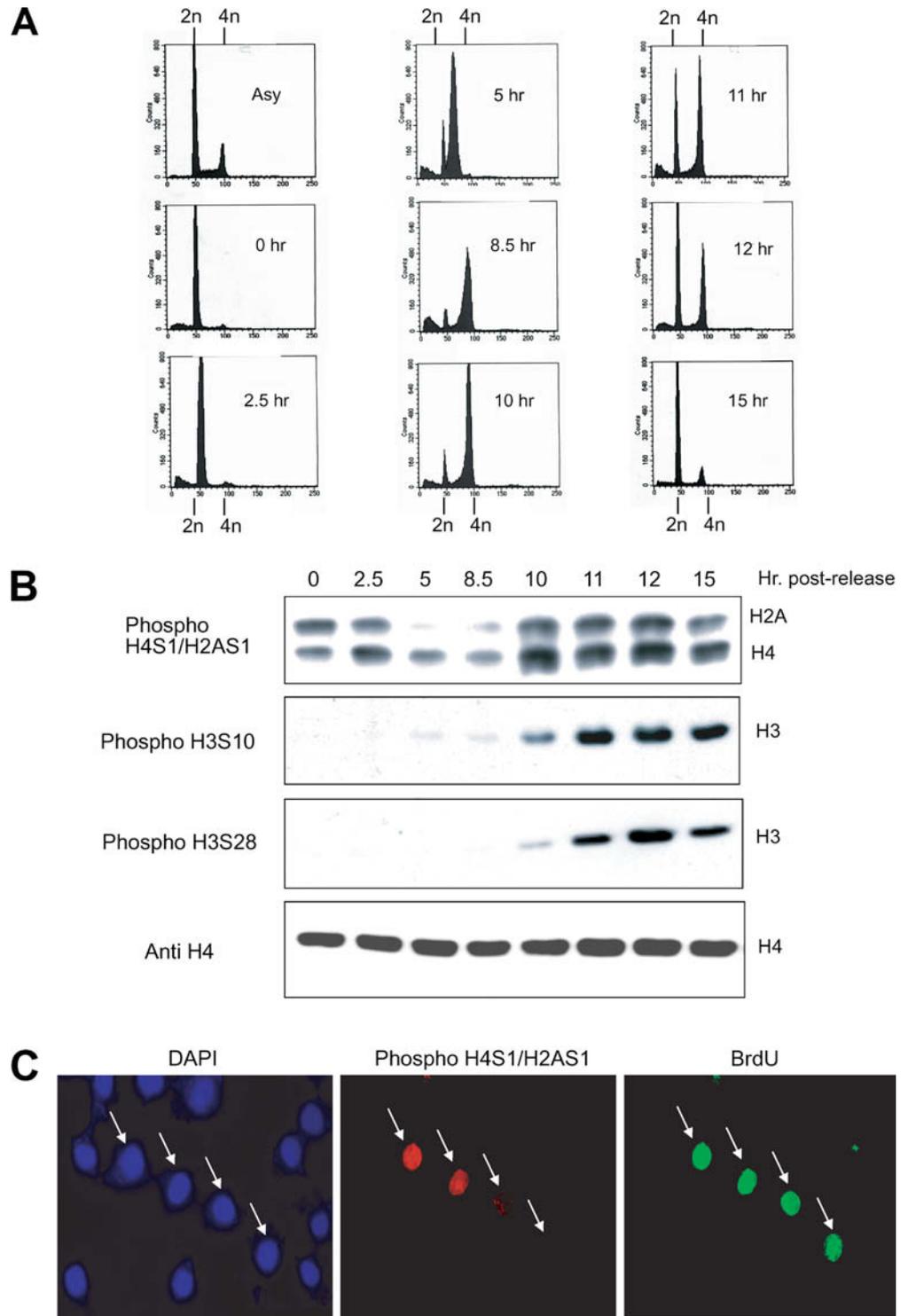
resulting phenotype. However, the discovery of additional highly conserved phosphorylation events, such as H4S1/H2AS1, makes it clear that we have yet to determine all the histone modifications that may play a role in this process.

Although several possible functions have been proposed for mitosis-specific phosphorylation of histones, it is still unclear what these modification events do. Among the proposed functions, it is thought that the addition or removal of a phosphate group alters the overall charge of the nucleosome, which in turn affects intra- or inter-nucleosomal structure and stability. Such alterations could potentially facilitate chromatin condensation and/or sister chromatid separation (Prigent and Dimitrov 2003). This theory is less plausible if only one or two phosphate groups per nucleosome are added during mitosis; however, as described above, multiple phosphorylation events on several histone tails may be associated with this process, and collectively they may produce a more substantial alteration in charge. A second possibility is that mitotic histone phosphorylation may contribute to the resolution of sister chromatids by increasing the electrostatic repulsion between the two chromatids (Swedlow and Hirano 2003). Thus, similar to the previous model, the net change in charge brought about by histone phosphorylation may contribute to chromatin condensation and sister chromatid separation.

Finally, the phosphorylated histones may serve as binding or docking sites for effector proteins that may mediate chromatid condensation and separation. Phosphate-dependent protein-protein interactions have been well documented for many proteins, especially those involved in the propagation of signal transduction cascades (Hunter 2000). Indeed, other histone modifications, namely acetylation and methylation, have been shown to mediate the binding of proteins to chromatin. Examples of such interactions include the binding of bromodomain-containing proteins to acetyl-lysine residues, such as TAF(II)250 to acetylated H4 lysines (K5, K8, K12, and K16) in transcriptionally active regions (Jacobson et al. 2000; Kanno et al. 2004). Furthermore, chromodomain-containing proteins bind methyl-lysines, such as HP1 to methyl-H3K9 (Jacobs et al. 2001) and *Drosophila* Polycomb protein to methyl-H3K27 (Fischle et al. 2003a). Therefore, it is possible that the window of histone phosphorylation from late G2 through telophase may serve as an efficient way to recruit effector proteins to mitotic chromatin.

While contemplating the potential of phospho-H4/H2A S1 as a binding site for effector proteins, it is intriguing to consider the effect of neighboring modifications that may occur on the H4 and H2A tails. S1 is proximal to arginine 3 (R3) and lysine 5 (K5) (Fig. 1A), which are known methylation and acetylation sites, respectively (Jacobson et al. 2000; Rice et al. 2002). Recently, a ‘binary switch’ hypothesis proposed that the binding of an effector protein to a specific histone modification may be affected by modifications on neighboring residues (Fischle et al. 2003b). Indeed, different combinations of modifications

Fig. 5A–C Enhancement of H4/H2A serine 1 phosphorylation during mitosis and early S-phase. HeLa cells were synchronized at the G1/S border by sequential, overnight treatments with thymidine and mimosine as described in Materials and methods. Cells were released into fresh medium in the absence of drug and harvested at the indicated times post-release. **A** At each time point (1 to 3.5 h increments), an aliquot of cells were fixed with ethanol, stained with propidium iodide, and analyzed by fluorescence-activated cell sorting (FACS). The chromatographs of the synchronized HeLa cells are aligned with a chromatograph of an asynchronous population of HeLa cells. The time of harvest is indicated in the *upper right corner* of each panel. *Vertical bars* above and below the FACS chromatographs indicate the peaks containing the cells with 2n and 4n DNA content. **B** The synchronized HeLa cells were acid-extracted, and the histones were resolved by electrophoresis on a 15% SDS-polyacrylamide gel and immunoblotted with α -phospho-H4S1/H2AS1, α -phospho-H3S10, α -phospho-H3S28, and α -H4. **C** Asynchronous HeLa cells were seeded on coverslips, pulse labeled with 5-bromo-2-deoxyuridine (BrdU), fixed with paraformaldehyde, and dually stained with α -phospho-H4S1/H2AS1 (*red*) and α -BrdU (*green*). Approximately 56% of the BrdU-positive S-phase cells are also positive for α -phospho-H4S1/H2AS1 staining (*arrows*) (see also Electronic Supplementary Material, Fig. 4); the remaining BrdU-positive cells are likely in later S-phase when phospho-H4S1/H2AS1 levels decline



may represent binding motifs for different effector proteins. The identical five amino acids of the H4 and H2A amino-termini were described as potential ‘modification cassettes’ that may participate in diverse biological functions by having distinct patterns of modifications within these cassettes. In this study, we take the first important steps towards testing this hypothesis by introducing a new immunological tool that detects a biphasic pattern of H4S1/H2AS1 phosphorylation in

mitosis and S-phase. Thus, different combinations of modifications within the proposed H4 and H2A cassettes may relate distinct roles of S1 phosphorylation in different phases of the cell cycle. How these multiple modifications are regulated and functionally affect one another will be interesting questions to address.

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