Histone H1 Phosphorylation Occurs Site-specifically during Interphase and Mitosis

IDENTIFICATION OF A NOVEL PHOSPHORYLATION SITE ON HISTONE H1

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Bettina Sarg, Wilfried Helliger, Heribert Talasz, Barbara Förg, and Herbert H. Lindner

From the Division of Clinical Biochemistry, Biocenter, Innsbruck Medical University, Innsbruck A-6020, Austria

H1 histones, isolated from logarithmically growing and mitotically enriched human lymphoblastic T-cells (CCRF-CEM), were fractionated by reversed phase and hydrophilic interaction liquid chromatography, subjected to enzymatic digestion, and analyzed by amino acid sequencing and mass spectrometry. During interphase the four H1 subtypes present in these cells differ in their maximum phosphorylation levels: histone H1.5 is tri-, H1.4 di-, and H1.3 and H1.2, only monophosphorylated. The phosphorylation is site-specific and occurs exclusively on serine residues of SP(K/A)K motifs. The phosphorylation sites of histone H1.5 from mitotically enriched cells were also examined. In contrast to the situation in interphase, at mitosis there were additional phosphorylations, exclusively at threonine residues. Whereas the tetraphosphorylated H1.5 arises from the triphosphorylated form by phosphorylation of one of two TPKK motifs in the C-terminal domain, namely Thr137 and Thr154, the pentaphosphorylated H1.5 was the result of phosphorylation of one of the tetraphosphorylated forms at a novel nonconsensus motif at Thr10 in the N-terminal tail. Despite the fact that histone H1.5 has five (S/T)P(K/A)K motifs, all of these motifs were never found to be phosphorylated simultaneously. Our data suggest that phosphorylation of human H1 variants occurs randomly during both interphase and mitosis and that distinct serine- or threonine-specific kinases are involved in different cell cycle phases. The order of increased phosphorylation and the position of modification might be necessary for regulated chromatin decondensation, thus facilitating processes of replication and transcription as well as of mitotic chromosome condensation.

The nucleosome core, which consists of 146 bp of DNA wrapped 1.75 times around an octamer of core histones, represents the fundamental subunit of chromatin (for review, see Ref. 1). The H1 or linker histones are associated with the core histone-DNA complex and with the linker DNA between adjacent nucleosomes. Histone H1 is phosphorylated in a cell cycle-dependent manner: levels of H1 phosphorylation are usually lowest in the G1 phase and rise continuously during S and G2. The M phase, where chromatin is highly condensed, shows the maximum number of phosphorylated sites. The individual H1 subtypes, however, differ in their degree of phosphorylation during the cell cycle (2, 3). A number of studies indicate that H1 phosphorylation is more likely involved in chromatin decondensation than in condensation (4). H1 phosphorylation seems to destabilize chromatin structure, thus weakening its binding to DNA. This decondensation of chromatin may give the DNA access to factors involved in transcription and replication in G1 and S as well as to condensing factors active during mitosis (5). Recent studies demonstrate that H1 phosphorylation regulates specific gene expression in vivo and that it acts by mimicking the partial removal of H1 (6).

The H1 histones consist of a globular central region flanked by short N- and long C-terminal domains (7). These two domains contain the serine and threonine residues that are cell cycle-dependently phosphorylated in both interphase and mitosis. The state of H1 phosphorylation is thought to depend on a balance of protein phosphatase 1 and CDC2/CDC2 kinase activities in the cell (8). These kinases, when complexed with various cyclins, require the consensus sequence (S/T)PAXZ, where X is any amino acid and Z is a basic amino acid (for review, see Ref. 9). The p34cdc2/cyclin B kinase, having maximum activity at mitosis (10), was expected to be the enzyme responsible for phosphorylation of the mitotic-specific serine and threonine sites of histone H1. Surprisingly, in Chinese hamster ovary cells mitotic-specific phosphorylation of both serine and threonine residues was found to be located at the end of the N-terminal region of H1, which has no (S/T)PAXZ consensus sequences (11). Only few data exist about site-specific phosphorylation during interphase. Gurley et al. (11) assumed that there is no absolute cell cycle-specific site of phosphorylation and that during interphase, the number of phosphates per H1 molecule is more important than which sites are phosphorylated. On the other hand, Mizzen et al. (12) demonstrated that phosphorylation of macronuclear H1 in the ciliated protozoan Tetrahymena occurs hierarchically during interphase.

So far it is not clear how phosphorylation of histone H1 brings about the DNA-H1 interactions that result in different chromatin states at interphase and mitosis. This is also true for the moderate phosphorylation of H1 occurring during interphase, which is associated with the process of DNA replication (13, 14). To gain more information on how phosphorylation influences the interaction of H1 with DNA and, in turn, modulates chromatin structure during interphase and mitosis, it is necessary to investigate in more detail the phosphorylation of H1 during the different cell cycle phases. The present paper demonstrates that human lymphoblastic T-cells have an unambiguous site specificity for the phosphorylation of histone H1. During interphase, phosphorylation occurs exclusively on serine residues in the consensus sequences SP(K/A)K. The up-phosphorylation occurring during mitosis, however, is the result of an additional specific phosphorylation of threonine residues. We found two tetraphosphorylated H1.5 forms (with phosphorylation at either Thr137 or Thr154) and two pentaphosphorylated forms consisting of the two tetraphosphorylated forms each being additionally phosphorylated at Thr10, which does not occur in a TPK(A)K consensus sequence. Interestingly, a hyperphosphorylated H1.5 protein with a phosphate group at both Thr137 and Thr154 was not detected.
H1 Phosphorylation

EXPERIMENTAL PROCEDURES

Materials—Sodium perchlorate (NaClO₄), trifluoroacetic acid, acetonitrile, and TEA were purchased from Fluka (Buchs, Switzerland). Hydroxypropylmethyl cellulose (4000cP) was obtained from Sigma. All other chemicals were purchased from Merck if not otherwise indicated.

Cell Culture and Synchronization of Cells—Human lymphoblastic T-cells (line CCRF-CEM) and HeLa cells were cultured in RPMI 1640 medium (Biochrom; Berlin, Germany) supplemented with 10% (v/v) fetal calf serum (Sigma), 60 μg/ml penicillin, and 100 μg/ml streptomycin in the presence of 5% CO₂. Exponentially growing CCRF-CEM cells were labeled for 18 h in sodium phosphate-deficient Dulbecco’s modified Eagle’s medium with carrier-free ³²P (10 mCi/1.5 × 10⁸ cells, 8 μCi/ml medium; Amersham Biosciences). To obtain mitotic-enriched CCRF-CEM cells, exponentially growing cells were incubated with 0.06 μg of colcemid/ml of medium for 5 h. The cells were characterized using flow cytometry and light microscopy.

For cell synchronization, HeLa cells were grown as a monolayer and cultured at an initial cell density of 1.5 × 10⁵/dish (10 cm). After 24 h of growing in normal medium, cells were incubated with 2 mM thymidine for 24 h, recovered for 8 h in normal medium, and incubated further with 2 μM thymidine for an additional 14 h. Finally, cells were incubated again in normal medium for a further 5 h (90% of cells in S phase) or 10 h (40% of cells in late S phase and 60% in G2/mitosis).

Immunofluorescence—An amount of 1 × 10⁶ HeLa cells was cyto- spun onto slides. Cells were permeabilized using Triton X-100 (1% for 2 min and 0.1% for 10 min) in KCM buffer (120 mM KCl, 20 mM NaCl, 0.5 mM EDTA, 10 mM Tris/HCl, pH 7.5), blocked for 1 h in KCM buffer containing 2.5% bovine serum albumin, and sequentially incubated with rabbit polyclonal to histone H1.4 phosphothreonine 146 (Abcam, ab3596) as the primary antibody (1:200 diluted in KCM containing 1% bovine serum albumin, 0.1% Triton X-100). As secondary antibody we used goat anti-rabbit IgG fluorescein isothiocyanate conjugate (Sigma (1:160 diluted in KCM containing 1% bovine serum albumin, 0.1% Triton X-100). Cells were fixed for 10 min with 2% paraformaldehyde in KCM, stained with DAPI for 1 min, and mounted in ProLong antifade ware (Universal Imaging Corp.) and analyzed using Adobe Photoshop.

Preparation of H1 Histones—Human cells (1.5 × 10⁹) were collected for 10 min) and H1 histones extracted with 0.02% hydroxypropylmethyl cellulose (4000cP) at a constant voltage (12 kV) and operated at 23 °C.

Reversed Phase (RP)-HPLC—The separation of whole linker histones was performed on a Nucleosil 300-5 C₁₈ column (250 mm × 4 mm inner diameter; 5-μm particle size; end-capped; Macherey-Nagel). The his- tone fraction H1.5 (150 μg) isolated from exponentially growing CCRF-CEM cells by RP-HPLC was fractionated on a PolyCAT A column (250 mm × 4.6 mm inner diameter; 5-μm particle size; 100-nm pore size; ICT) at 23 °C and at a constant flow of 1.0 ml/min using a two-step gradient starting at solvent A:solvent B (100:0) (solvent A: 70% acetonitrile, 15 mM TEA/H₃PO₄, pH 3.0; solvent B: 70% acetonitrile, 15 mM TEA/H₃PO₄, pH 3.0, and 0.68 x 10⁻₅ NaClO₄). The concentration of solvent B was increased from 0 to 60% B in 5 min, from 60% to 100% in 35 min, and then maintained at 100% for 30 min.

H1.5 from colcemid-treated cells was analyzed using a two-step gradient starting at solvent A:solvent B (100:0) (solvent A: 70% acetonitrile, 10 mM TEA/methanephosphonate (TEA/MPA, pH 3.0); solvent B: 70% acetonitrile, 10 mM TEA/MPA, pH 3.0, and 1 mM NaClO₄). The concentration of solvent B was increased from 0 to 30% B in 5 min and from 30 to 100% B in 60 min. The isolated protein fractions were desalted using RP-HPLC.

Capillary Electrophoresis—High performance capillary electrophoresis (HPCE) was performed on a Beckman system P/ACE 2100. An untreated capillary (fused silica, 57 cm total length × 75 μm inner diameter) was used, protein samples were injected by pressure, and detection was performed by measuring UV absorption at 200 nm. Runs were carried out in 0.1 M sodium phosphate buffer, pH 2.0, containing 0.02% hydroxypropylmethyl cellulose at a constant voltage (12 kV) and at a capillary temperature of 25 °C.

Enzymatic Cleavage—Histone H1 fractions obtained by HILIC (20–100 μg) were digested with α-chymotrypsin (EC 3.4.21.1; Sigma type I-S, 1/150 w/w) in 100 μl of 100 mM sodium acetate buffer, pH 5.0, for 20 min at room temperature. The digest was subjected to RP-HPLC. The C-terminal fragments of the individual H1 histones obtained by digestion with chymotrypsin (~ 5 μg) were digested with trypsin (Roche

2 The abbreviations used are: TEA, triethylamine; CE, capillary electrophoresis; ESI-MS, electrospray ionization mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; HPCE, high performance capillary electrophoresis; HPLC, high performance liquid chromatography; MPA, methanephosphonate; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RP, reversed phase.
Applied Science sequencing grade, 1/50 w/w) in 100 μl of 5 mM NaHCO3 buffer, pH 8.3, for 24 h at 37 °C. The N-terminal fragments of the individual H1 histones obtained by digestion with chymotrypsin (~5 μg) were digested further with endoproteinase Glu-C (Roche Applied Science, 1/30 w/w) in 100 μl of 50 mM NH4HCO3 buffer, pH 7.8, for 18 h at 37 °C. The digests were subjected to RP-HPLC.

Amino Acid Sequence Analysis—Peptide sequencing was performed on an Applied Biosystems, Inc. (ABI) model 492 Procise protein sequenator.

Mass Spectrometric Analysis—Tryptic digests of the N- and C-terminal fragments of individual human H1 histones were analyzed using a LCQ ion trap instrument (ThermoFinnigan, San Jose, CA) equipped with a nanospray interface. The nanospray voltage was set at 1.6 kV, and the heated capillary was held at 170 °C. MS/MS spectra were searched against a histone data base using SEQUEST (LCQ BioWorks, ThermoFinnigan). Determination of the molecular masses of the histone H1.5 subfractions obtained by HILIC and of peptides obtained by enzymatic cleavages were carried out by electrospray ionization mass spectrometry (ESI-MS). Samples (5–10 μg) were dissolved in 50% aqueous methanol containing 0.1% formic acid and injected into an ion source.

RESULTS

HILIC Separation of Phosphorylated Histone H1.5—To examine the interphase phosphorylation sites, 32P-labeled H1 histones were isolated from exponentially growing human lymphoblastic T-cells (CCRF-CEM) and fractionated using RP-HPLC. As illustrated in Fig. 1A, the main component histone H1.5 clearly separated from the residual histones H1.2, H1.3, and H1.4. Of all H1 histones present in the cell line investigated (subtype H1.1 is absent from CEM cells) histone H1.5, which corresponds to the murine histone H1b in the nomenclature of Lennox et al. (2), exhibits the highest rate of phosphorylation during both interphase and mitosis (3, 16). For this reason and because pure histone H1.5 can be isolated in a simple HPLC procedure, we investigated this histone subtype first. To determine the extent of phosphorylation, the histone H1.5 subfraction obtained by RP-HPLC (Fig. 1A) was subjected to HPCE. As shown in Fig. 1B, H1.5 was separated into four peaks belonging to the nonphosphorylated (designated H1.5p0), mono-, di-, and triphosphorylated H1.5 forms of histone H1.5. To prove that the peaks shown in Fig. 1B actually are the result of differently phosphorylated forms of H1.5 and not caused by any contaminations or modifications other than phosphorylation, the H1.5 subfraction was digested with alkaline phosphatase. Thereafter, the sample was chromatographed and subjected to HPCE (data not shown).

In contrast to Fig. 1B, only two peaks were visible: one main peak consisting of nonphosphorylated histone H1.5, and one minor peak of monophosphorylated H1.5. The complete loss of the higher phosphorylated forms of H1.5 and not caused by any contaminations or modifications other than phosphorylation, the H1.5 subfraction was digested with alkaline phosphatase. Thereafter, the sample was chromatographed and subjected to HPCE (data not shown).

Previously, we demonstrated the efficacy of HILIC by applying this method to the analytical and semipreparative scale isolation of core histone variants, acetylated and methylated core histone variants, and phosphorylated histone variants (15, 17–19). When histone H1.5 isolated by RP-HPLC (Fig. 1A) was subjected to HILIC, surprisingly, not four peaks as in CE but five peaks were obtained (Fig. 2). By comparing the number and height of peaks obtained with HILIC (Fig. 2) and CE (Fig. 1B), respectively, we found that the single CE peak p1 was further resolved into two fractions (designated p1g and p1m, corresponding to the greater and minor monophosphorylated H1.5 fraction) by HILIC. We therefore assumed that HILIC has the potential to separate two monophosphorylated H1.5 subfractions differing in the position of the phosphate group.

Characterization of the Five H1.5 Subfractions Obtained by HILIC—Confirmatory assignment of the five fractions obtained by HILIC (Fig.
H1 Phosphorylation

TABLE 1
ESI-MS data of histone H1.5 peaks p0–p3

The individual HILIC fractions p0, p1g, p1m, p2, and p3 (Fig. 2) were analyzed using ESI-MS. The mass for the unphosphorylated intact molecule was calculated to be 22,491 Da. Mass differences found correlate with a molecular mass of additional phosphate groups (+80 Da per phosphate group).

| Histone fraction | ESI-MS analysis | Difference to calculated mass of 22,491 | No. of phosphate groups |
|------------------|-----------------|----------------------------------------|------------------------|
| p0               | 22,489          | 2                                      | +0                     |
| p1g              | 22,572          | 81                                     | +1                     |
| p1m              | 22,570          | 79                                     | +1                     |
| p2               | 22,651          | 161                                    | +2                     |
| p3               | 22,733          | 242                                    | +3                     |

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The peptide fraction designated 108–225 (Fig. 3), whereas the fraction with the lesser content of 32P was more radioactive. The more radioactive fraction was obtained by HILIC (Fig. 2) was subjected to chymotryptic digestion of the N-terminal fragments of differently phosphorylated H1.5 forms from exponentially growing CEM cells (interphase) and cells treated with colcemid (mitosis). p0, p1, p2, p3, p4, and p5 = non-, mono-, di-, tri-, tetra-, and pentaphosphorylated H1.5 histones, respectively; p1g = greater monophosphorylated H1.5 histone fraction; p1m = minor monophosphorylated H1.5 histone fraction.

2) was performed by mass spectrometry. Table 1 shows the ESI-MS analysis of the intact protein fractions: histones H1.5p0–H1.5p3. In fact, the two histones H1.5p1g and H1.5p1m contain one phosphate each, whereas H1.5p2 and H1.5p3 are di- and triphosphorylated, respectively.

To identify the phosphorylation sites, the individual H1.5 proteins obtained by HILIC (Fig. 2) were first subjected to chymotryptic digestion (schematically shown in Fig. 3). Under the conditions used, two main peptide fragments were obtained. Using ESI-MS and sequence analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected. The analysis by Edman degradation (data not shown) we found a cleavage at position 107 on the C-terminal side of phenylalanine, as expected.

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To identify the exact positions of phosphorylation, we subsequently digested the peptide fragments I obtained from H1.5, namely p1g, p1m, p2, and p3 by V8 endoproteinase Glu-C and the corresponding fragments II by trypsin. Resulting peptides were separated by RP-HPLC and identified using ESI-MS and amino acid sequencing. Human histone H1.5 sequence data were taken from the Swiss-Prot data base (accession no. P16401). Serine and threonine residues in bold letters were found to be phosphorylated; underlined residues are typical (S/T)P(K/A)K motifs.

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Distribution of phosphate groups in the N- and C-terminal fragments of differently phosphorylated H1.5 forms from exponentially growing CEM cells (interphase) and cells treated with colcemid (mitosis). p0, p1, p2, p3, p4, and p5 = non-, mono-, di-, tri-, tetra-, and pentaphosphorylated H1.5 histones, respectively; p1g = greater monophosphorylated H1.5 histone fraction; p1m = minor monophosphorylated H1.5 histone fraction.

TABLE 2
Phosphorylation status of histone H1.5 during interphase and mitosis

| Cell cycle phase | Histone fraction | No. of phosphate groups | N terminus | C terminus | N+C terminus |
|------------------|-----------------|-------------------------|-----------|-----------|-------------|
| Interphase       | p0              | 0                       | 0         | 0         |             |
|                  | p1g             | 1                       | 0         | 1         |             |
|                  | p1m             | 0                       | 1         | 1         |             |
|                  | p2              | 1                       | 1         | 2         |             |
|                  | p3              | 1                       | 2         | 3         |             |
|                  | p4              | 1                       | 3         | 4         |             |
|                  | p5              | 2                       | 3         | 5         |             |
| Mitotic-embryon  |                 |                         |           |           |             |
|                   | p0              | 0                       | 0         | 0         |             |
|                   | p1g             | 1                       | 0         | 1         |             |
|                   | p1m             | 0                       | 1         | 1         |             |
|                   | p2              | 1                       | 1         | 2         |             |
|                   | p3              | 1                       | 2         | 3         |             |
|                   | p4              | 1                       | 3         | 4         |             |
|                   | p5              | 2                       | 3         | 5         |             |
consisted of a peptide with 42 amino acids (residues 3–44; III 3–44 in Fig. 3). Thus, both of the radioactive fractions contain the same SPAK motif (positions 17–20). The precise site of phosphorylation was determined by amino acid sequencing and indeed found to be Ser\textsuperscript{17} of this SPAK sequence. H1.5p1m was analyzed in the same manner. As expected, we found only minute amounts of radioactivity in the fragments III 8–44 and III 3–44 caused by inevitable cross-contamination of H1.5p1m with H1.5p1g.

Tryptic digestion of fragments II of H1.5p1m and -p2, both containing one phosphate group in the C-terminal region, yielded two radioactive fractions after HPLC separation (data not shown). The individual peptides were identified by amino acid sequencing and proved to be different length peptides out of the C-terminal domain containing a part of a SPKK motif with a phosphorylated serine in position 172 (designated VI 168–174, 169–174 in Fig. 3). Cleavage of the C-terminal fragment II of H1.5p3 yielded three radioactive fractions, two of them identical to those obtained by enzymatic digestion of histones H1.5p1m and -p2. The third one was characterized by MS and designated VII 184–193 (Fig. 3). This peptide was analyzed by Edman degradation, which showed that the third phosphorylation site during interphase is Ser\textsuperscript{188}.

Proportion of Histone H1.5p1g to p1m in Various Cell Lines—The finding that monophosphorylation of H1.5 occurs at only two specific positions, namely at Ser\textsuperscript{17} in the N-terminal region (H1.5p1g) and at Ser\textsuperscript{172} (H1.5p1m) in the C-terminal domain, was unexpected and indicates a nonrandom distribution of phosphate groups. To clarify the question of whether this result is valid only for CEM cells or is generally true for other cell lines, we investigated the relative amounts of p1g and p1m in several human cell lines (Raji, U937) and in mouse erythroleukemia cells (line F4N) using the HILIC technique (Fig. 5). We found p1g and p1m forms in all of these cell lines, and, surprisingly, the patterns were very similar to those found for CEM cells. Fig. 5 shows that monophosphorylated H1.5 obtained from interphase consists of roughly 70% p1g and about 30% p1m.

Characterization of Hyperphosphorylated Histone H1.5 from Mitotic-enriched CEM Cells—To extend our investigations to the identification of mitotic phosphorylation sites, H1.5 was isolated from colcemid-treated CEM cells by RP-HPLC (Fig. 1A). The extent of its phosphorylation was examined by HPCE (Fig. 1B), and in addition to H1.5p0, p1, p2, and p3 forms, two further peaks belonging to the tetra- (p4) and pentaphosphorylated (p5) forms were detected (data not shown). When using the HILIC system as shown in Fig. 2, no elution of H1.5p4 and p5 was possible because of their strong binding to the column (data not shown). Elution was finally achieved by the addition of TEA/MPA instead of triethylammonium phosphate, thus permitting the use of higher sodium perchlorate concentrations (20). Resolution of p1g/p1m into two peaks, however, was not possible under the conditions applied. Fig. 6A shows the elution profile of histone H1.5 from mitotically enriched cells under optimized conditions. To identify the individual peaks the corresponding fractions were isolated, desalted, and digested with chymotrypsin as described (Figs. 3 and 4). MS analysis of fragments I and II on the one hand confirmed the distribution of phosphate groups of p1g, p1m, p2, and p3 already obtained for interphase cells and, on the other hand, allowed the localization of mitotic-specific phosphate groups of H1.5 (p4 and p5) in the N- and C-terminal domain (summarized in Table 2). As a result, both mitotic forms, p4 and p5, reveal three phosphate groups in the C-terminal tail. In the N-terminal domain, however, we proved the existence of a single phosphate group for p4 and, surprisingly, two phosphate groups for p5.

To characterize the precise location of the mitotic-specific phosphorylation in the N-terminal domain of histone H1.5p5, further enzymatic digestion using trypsin was performed. Trypsin cleavage yielded peptides in the preferred mass range for effective fragmentation by MS/MS. The protein digest was analyzed using capillary HPLC connected online to a LQO ion trap instrument equipped with a nanospray interface (data not shown). The most intense ions were selected for MS/MS analysis and searched against a human histone data base using SEQUEST.
with phosphorylation (+80 Da) on serine and threonine residues and α-N-acetylation (+42 Da) on serine as variable modifications. A peptide fragment with the molecular mass of 2185 Da was found and identified as residues 1–20 of H1.5 being N-terminally acetylated at Ser1 and phosphorylated at Ser172 and Thr10. Phosphorylation of Thr10 was unexpected and to our knowledge has not been reported previously.

The exact positions of the mitotic-specific phosphorylation in the C-terminal domain of histone H1p4 and H5 were determined by enzymatic digestion of chymotryptic fragments II (residues 108–225 in Fig. 3) using trypsin. Using MS/MS, two peptides spanning residues 150–218 and 150–157 (fragments V; Fig. 3) were identified containing a TPKK motif with a phosphorylated threonine in position 154. Moreover, a decapeptide containing residues 130–139 of histone H1.5 (fragment IV; Fig. 3) which contained a TPKK motif with a phosphorylated threonine at position 137 was found. Fragments IV and V of H1.5p4 and p5 were present in both their phosphorylated and their unphosphorylated form, indicating that either Thr137 or Thr157 becomes phosphorylated during mitosis. The results of histone H1.5 phosphorylation are summarized in Fig. 6B.

Like Histone H1.5, the Variants H1.2, H1.3, and H1.4 are Site-specifically Phosphorylated at Serine Residues during Interphase.—To clarify whether the other histone variants H1.2, H1.3, and H1.4 are also site-specifically phosphorylated at serine residues, the second prominent RP fraction eluting at about 35 min (shown in Fig. 1A) was digested by chymotrypsin as described for histone H1.5 (Fig. 3). The resulting three N- and C-terminal fragments corresponding to the variants H1.2, H1.3, and H1.4 were then further separated by RP-HPLC (data not shown) and subjected to MS analysis. The results are summarized in Table 3. In contrast to histone H1.5, none of the variants H1.2, H1.3, or H1.4 was notably phosphorylated in the N-terminal region. In the C-terminal domain, however, histone H1.4 was di- and H1.2 and H1.3 monophosphorylated. In the C-terminal end, the number of phosphate groups detected exactly correlates with the number of SP(K/A)K motifs present (Table 4); in the N-terminal end there are no such motifs. To identify the precise position of phosphate groups, the C-terminal fragments of the three variants were digested with trypsin (Fig. 3) and the peptides obtained characterized by LC-MS/MS analysis. Ser172, 186 in H1.2 and the serine residues 171 and 186 in H1.4 were found to be phosphorylated. Because of the minute amount of H1.3 present in the sample, which in addition was phosphorylated at a very low rate, immobilized metal affinity chromatography enrichment of phosphopeptides was performed. MS analysis of this fraction indicated a phosphorylation of Ser186 in H1.3. These results demonstrate that during interphase exclusively the serine, and none of the threonine residues, is phosphorylated. In the case of histone H1.4 this finding was further confirmed by indirect immunofluorescence. Using a histone H1.4 antibody detecting phosphorylated Thr145, we observed a very weak signal during interphase but a strong signal in mitotic cells (Fig. 7).

**DISCUSSION**

When investigating Chinese hamster ovary cells Gurley et al. (11) found that in interphase histone H1 is phosphorylated exclusively in the C-terminal region and that during mitosis only it is also phosphorylated in the N-terminal end. With regard to H1 interphase phosphorylation the authors supposed that there is no qualitative cell cycle specificity as to which site is phosphorylated first and that the number of phosphates per H1 molecule is more important than which sites are phosphorylated. Our results, however, demonstrate unambiguously that, depending on the H1 variant, phosphorylation also occurs in the N-terminal domain during interphase. In contrast to the variants H1.2, H1.3, and H1.4, which are indeed phosphorylated in the C-terminal tail only, histone H1.5 is phosphorylated in both the C- and N-terminal regions.

In this context it should be noted that in vitro four distinct CDKs, the interphase-specific p34CDC2/cyclin A (kinase B), the late interphase p34CDC2/cyclin A (kinase A), the mitotic-specific p34CDC2/cyclin B (kinase C), and the p34CDC2/unknown cyclin (kinase M), phosphorylate all of the H1 sites of Chinese hamster ovary cells (21), indicating random phosphorylation. Our in vivo findings using human CEM cells, however, clearly indicate site-specific phosphorylation during both interphase and mitosis.

In interphase, for example, phosphorylation of histone H1.5 starts mainly at Ser17, to a minor extent at Ser172, and ends with phosphorylation of Ser186. Similarly, we exclusively found phosphorylation of serine residues in the other H1 subtypes present in human CEM cells.

This finding also easily explains why the variants H1.2, H1.3, and H1.4 are not N-terminally phosphorylated because no SPXZ motifs are present in the N-terminal region of any of these three H1 variants. It should be pointed out, therefore, that no relevant threonine phosphorylation occurs during interphase in any of the four H1 subtypes investigated.

There are some evidences that histone H1 must be partially displaced...
from the chromatin fiber for it to be phosphorylated by an H1 kinase during mitosis (22). This possibly implies a different behavior of the various H1 subtypes (H1.5 versus H1.2, H1.3, and H1.4) in terms of weakening the binding of the N- and C-terminal domains in the chromatin during interphase.

In mitotically enriched CEM cells we found tetra- and pentaphosphorylated H1.5 histones. In contrast to interphase phosphorylation exclusively occurring on serine sites of SPK(A)K motifs, during mitosis threonine residues are phosphorylated, namely the Thr137 and Thr154 of the TPKK motifs in the C-terminal and the Thr10 in the N-terminal tail of the H1.5 molecule. Because only either Thr137 or Thr154 (but not both) is phosphorylated, the tetraphosphorylated H1.5 consists of two forms (with phosphate groups at serine in position 17, 172, 188, and at either Thr10 or Thr154). Whether this different threonine phosphorylation is caused by distinct kinases and/or occurs during different phases of mitosis remains an important question. The pentaphosphorylated H1.5, finally, is generated by additional phosphorylation of Thr10 in the N-terminal tail, but not at a CDK consensus sequence. The presence of distinctly phosphorylated H1.5 proteins (p4 and p5) also reflects the temporal progression of up-phosphorylation. As we found phosphorylated Thr10 in H1.5p5 only, and not in the tetraphosphorylated form, we conclude that Thr10 phosphorylation happens as the last step in the mitotic-specific cascade of phosphorylation and is most probably triggered by events other than those responsible for TPXZ motif phosphorylation.

Because the maximal number of phosphorylation sites frequently corresponds to the number of (S/T)PXZ motifs, we had previously assumed the existence of only a single pentaphosphorylated form of H1.5. This paper, however, clearly demonstrates that during mitosis there exist two pentaphosphorylated H1.5 histones. Therefore, despite the fact that histone H1.5 contains five (S/T)PXZ motifs and a phosphorylatable threonine in a nonconsensus sequence, we never detected a hexaphosphorylated H1.5 protein with a phosphate group at both Thr137 and Thr154. As the C-terminal domain of histone H1 is responsible for high affinity binding of histone H1 to chromatin in vivo and that binding is described as being directly modulated by phosphorylation at specific positions, it would be interesting to know whether the differences in the position of phosphorylation of Thr137 and Thr154 play a particular role in H1 binding to chromatin during mitosis (23, 24). The site-specific phosphorylation of the residual subtypes H1.2, H1.3, and H1.4 was not investigated in detail during mitosis. It is evident from our interphase data revealing phosphorylation of all SPXZ motifs present in these subtypes that additional phosphorylation during mitosis can occur only on Thr. This was confirmed by immunofluorescence using a histone H1.4 (phosphothreonine 145) antibody (Fig. 7). Only during mitosis was a significant binding of the antibody observed.

Interestingly, we found a threonine phosphorylation of histone H1.5 during mitosis occurring at the identical position 10. It was hypothesized that this Ser10 phosphorylation might modulate the interaction between the basic N-terminal end of histone H3 and DNA, thus facilitating access to various factors involved in condensation (38). Interestingly, we found a threonine phosphorylation of histone H1.5 during mitosis occurring at the identical position 10. It remains to be investigated whether this histone H1 phosphorylation plays a role similar to that of the corresponding histone H3 phosphorylation. Cells expressing various oncogenes or missing tumor suppressor...
genes exhibit elevated levels of phosphorylated H1 and H3 histones, and these modifications are thought to be responsible for the less condensed chromatin structure and aberrant gene expression found in the oncogene-transformed cells (5, 39–41). Overexpression of Aurora B kinase, for example, which has been observed in many cancer cell lines (42), causes increased phosphorylation of H3 at Ser10, and this occurrence is associated with the chromosome instability often seen in malignant cells (43). The question arises as to whether increased phosphorylation of Thr10 of histone H1.5 is also associated with tumorigenesis.

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