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C-Terminal Phosphorylation of Murine Testis-Specific Histone H1t in Elongating Spermatids

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Abstract

Previous studies gave differing results as to whether the testis-specific histone H1t was phosphorylated during rodent spermatogenesis. We show here that histones extracted from germ cell populations enriched with spermatids at different stages of development in rat testes reveal an electrophoretic shift in the position of H1t to slower mobilities in elongating spermatids as compared to that from preceding stages. Alkaline phosphatase treatment and radioactive labeling with ³²P demonstrated that the electrophoretic shift is due to phosphorylation. Mass spectrometric analysis of histone H1t purified from sexually mature mice and rat testes confirmed the occurrence of singly, doubly, and triply phosphorylated species, with phosphorylation sites predominantly found at the C-terminal end of the molecule. Furthermore, using collision-activated dissociation (CAD) and electron transfer dissociation (ETD), we have been able to identify the major phosphorylation sites. These include a new, previously unidentified putative H1t-specific cdc2 phosphorylation site in linker histones. The presence of phosphorylation at the C-terminal end of H1t and the timing of its appearance suggest that this post-translational modification is involved in the reduction of H1t binding strength to DNA. It is proposed that this could participate in the opening of the chromatin fiber in preparation for histone displacement by transition proteins in the next phase of spermiogenesis.

Keywords

histone H1t; collision-activated dissociation; electron transfer dissociation

Introduction

Mammalian linker histones bind to the linker DNA regions that connect adjacent nucleosomes along the chromatin fiber and, in doing so, participate in the modulation of the folding dynamics of the fiber.^{1,2} They encompass a highly heterogeneous family of histones, also known as the

histone H1 family, which in mammals consists of at least 12 functionally identified components. Common to all the protein members of this family is a tertiary structure organization consisting of a central winged fold domain,³ that is flanked by N- and C-terminal tails that are highly unstructured in solution. It has been shown that C-terminal regions can, under certain circumstances and upon interaction with DNA, adopt a helical organization.⁴⁻⁷ The C-terminal domain is also critical for the folding of the chromatin fiber.^{8,9} Phosphorylation of histone H1 at this domain has been shown to significantly weaken the interaction with the chromatin fiber and reduce its binding affinity.¹⁰

Histone H1 and its related chromosomal proteins play an important role in the organization of sperm chromatin in highly diverse organisms.¹¹ In mammals, the somatic-like histones, which are present at the onset of spermatogenesis, are partially replaced by testis-specific histones, including a highly specific histone H1t.¹² The histones are almost completely replaced by the transition nuclear proteins and then the protamines as the mature sperm are formed.

During mammalian spermatogenesis,¹³ the transcription of numerous haploid specific genes is initiated during the round spermatid phase (steps 1–8 in the rat) and is followed by a cessation of all transcription in the elongating spermatids (steps 9–12). Profound chromatin reorganization occurs as the histones are initially replaced by transition proteins¹⁴ in the condensing spermatids (steps 12–15) followed by their eventual replacement by protamines in condensed spermatids (steps 16–19). The mechanisms by which histones are displaced and transition proteins and protamines are deposited are not known. Acetylation of core histones appears to be involved in this process. In particular, histone H4 has been shown to be highly acetylated in spermatids, and *in vitro* studies showed that H4 acetylation resulted in an enhanced ability of protamines to displace histones from chromatin.¹⁵

We characterize here the modification of histone H1t during the steps of spermiogenesis just prior to histone displacement. H1t is first synthesized during the primary spermatocyte stage; in the round spermatids H1t is the predominant form of linker histones,¹⁶ where it accounts for approximately 55% of the histone H1 complement. H1a represents about 26% and the other somatic types (H1b, c, d, and e) are more minor contributors.

There has been some disagreement in the literature as to whether or not H1t is phosphorylated during spermatogenesis. In studies of adult mouse testes, it was first reported using two-dimensional AU-SDS gels that H1t exhibited several more slowly migrating spots that disappeared upon treatment with alkaline phosphatase.¹⁷ An additional band in a corresponding position was also shown for adult rat testes and a decrease in H1t mobility was detected in elongating spermatids of vitamin A synchronized rats.¹⁸ In contrast, in a study of testes from 40-day old rats, it was reported that H1t was not phosphorylated based on absence of detectable ³²P incorporation into the band corresponding to H1t.¹⁹

In the present study, we have revisited this problem and show that in both mice and rats H1t is phosphorylated, and that the phosphorylation occurs in elongating spermatids and mainly affects the C-terminal region of the molecule. A powerful mass spectrometric approach utilizing both CAD and ETD mass spectrometry has been used to identify the main phosphorylation sites involved.

Experimental Section

Histone H1t Purification

The testis tissues from either rat or mouse were homogenized using a Kinematica Polytron in 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA buffer containing a protease inhibitor mixture ("Complete" from Roche Diagnostics, Laval, QC) at the ratio of 1 tablet per 100 mL

buffer. After homogenization, the samples were centrifuged at 2 000g for 10 min at 4 °C. The pellet was then resuspended in 0.6 N HCl (at approximately 6 mL per gram of starting tissue), homogenized and centrifuged as above. The HCl supernatant extracts were precipitated with 6 volumes of acetone at −20 °C overnight and then centrifuged at 2000 g for 10 min at 4 °C. The acetone pellets were dried using a speedvac concentrator. The dried pellets were resuspended in approximately 1 mL of water. An equal volume (1 mL) of 10% perchloric acid (PCA) was added to reach a final concentration of 5% PCA. The 5% PCA solution was incubated on ice for 5 min and then centrifuged at 12 000 g for 10 min. HCl was added to the supernatant to a final concentration of 0.25 N HCl. The HCl supernatant extracts were precipitated with 6 volumes of acetone at −20 °C overnight. The acetone pellet was dried as above. The dried pellet after PCA extraction was resuspended in water, filtered through a 0.45 μ m Nanosep centrifugal device (Pall, Ann Arbor, MI) and injected onto a 4.6 $\times$ 250 mm Vydac C₁₈ reversed-phase high performance liquid chromatography (RP-HPLC) column (Grace Vydac, St. Hesperia, CA). Histone H1s were eluted starting from a 0.1% trifluoroacetic acid (TFA) solution and an increasing acetonitrile (ACN) shallow gradient: 1–5% ACN for 1 min, 5–25% ACN for 10 min, 30–40% for 40 min.

Cell Separation and Protein Isolation

In general, cell suspensions prepared from testes of adult Sprague–Dawley rats (Harlan–Sprague–Dawley, Indianapolis, IN) were separated by centrifugal elutriation^{20,21} to obtain fractions enriched in round spermatids (steps 1–8) and elongating spermatids (steps 9–12). The contribution of each cell type to the histones in the fraction was calculated from the percentage of nucleated cells, except for condensing (steps 13–15) and condensed (steps 16–19) spermatids, because they primarily contain transition proteins or protamines in place of histones. The percentage of each cell type was then multiplied by the amount of DNA in the cell, assuming that the histone:DNA ratios would be similar and the percentage recalculated. In the round spermatid fraction, the round spermatids accounted for 73% of histones, with early spermatocytes and somatic cells each contributing 11%. It is difficult to purify elongating (step 9–12) spermatids by a single step of centrifugal elutriation.¹⁸ In the elongating spermatids fraction, these cells contributed 42% of the histones, with somatic cells, which resembled macrophages and white blood cells from the interstitial region, contributing 50%.

In one experiment, highly purified fractions of round and elongating spermatids at specific steps of development were prepared from vitamin A synchronized rats by centrifugal elutriation followed by equilibrium density centrifugation.¹⁸

Nuclei were prepared by lysis in hypotonic buffer containing Triton X-100^{22,23} with PMSF, *p*-chloromercuriphenyl sulfonic acid, and, in some cases, iodoacetamide, as protease, phosphatase, and isopeptidase inhibitors, respectively. Proteins were extracted from nuclei with 0.25 N HCl and precipitated with trichloroacetic acid (TCA). TCA precipitations were done with either 25% TCA or in two steps: 4% TCA to remove the core histones and most nonhistone proteins followed by 25% TCA precipitation to collect the H1 histones. All of the bands shown here were observed both in the 25% precipitate and the 4–25% cut, which is consistent with them representing H1 histones.

Alkaline Phosphatase Treatment of Histones

The incubation mixture contained bacterial alkaline phosphatase (60 units per milligram, Sigma Chemical Co, St Louis, MO) at 100 μ g/mL, nuclear protein extracted from spermatids, and PMSF in a volume of 150 μ L of 100 mM Tris-HCl (pH 8.0). The mixture was incubated for 1 h at 37 °C. To control for the possibility that the observed result was due to some possible nonspecific reaction of the enzyme or a contaminant in the preparation, a different alkaline phosphatase from calf intestine (Worthington Biochemical Corp. Freehold, Lakewood, NJ)

was used in one experiment. The enzyme concentration was 400 $\mu\text{g/ml}$ (2100 U/mL) in 100 5005 > 1 of the same buffer but with the addition of para-aminobenzoic acid to 1 mM and leupeptin to 10 $\mu\text{g/mL}$. Reaction was incubated at for 1.5 h. Proteins were collected by TCA precipitation.

Radioactive Labeling with ^{32}P

Prior to and during the preparation of the cell suspension by the trypsin method,²¹ 10 mCi of ^{32}P -orthophosphate was added to 200 mL of PBS and distributed among 4 flasks. The free ^{32}P was removed during the centrifugation of the cell suspension prior to elutriation.

Gel Electrophoresis and Detection of Proteins

The nuclear proteins were usually analyzed by acid-urea poly acrylamide gel electrophoresis (AU)-PAGE (15% acrylamide, 2.5 M urea).^{24,25} In some cases, sodium dodecyl sulfate (SDS)-PAGE²⁶ or acetic acid-urea-Triton X-100 (AUT)-PAGE^{27,28} were used. Gels were stained with either Coomassie blue or amido black. H1t bands were identified by Western-blotting with a polyclonal antiserum to H1t²⁹ used at a dilution of 1:200. Procedures, including transfer to nitrocellulose membranes and detection using an ABC kit (Vector Laboratories, Burlingame, CA), have been described in detail previously.²⁰ Autoradiography was performed by drying the gel and exposure to Kodak AR film for 3 days.

Mass Spectrometric Analysis

Upon purification of murine H1t as described above, the H1t-containing HPLC fraction was lyophilized and then resuspended in 50 μL H_2O . An aliquot (5% of the resuspended fraction) was diluted 3-fold with 100 mM ammonium bicarbonate, pH 8. The aliquot of H1t was then digested with AspN protease (Roche Diagnostics) at a substrate-to-enzyme ratio of 20:1 for 7 h at 37 °C and the resulting peptide mixture was acidified to pH 3.5 with glacial acetic acid. An aliquot (5%) of AspN-digested H1t was pressure-loaded (200 PSI) onto a self-packed, polyimide-coated, fused-silica capillary precolumn (360 μm O.D. $\times$ 75 μm I.D., Polymicro Technologies, Phoenix, AZ) packed with 4 cm of C18 resin (5–20 μm diameter, 120 Å pore size, YMC Inc. Milford, MA). The precolumn was washed for 5 min with 0.1% acetic acid at 1 $\mu\text{L/min}$. The precolumn was then connected to an analytical column (360 μm O.D. $\times$ 50 μm I.D. fused silica) packed with 7 cm of C₁₈ resin (5 μm diameter, 120 Å pore size, YMC Inc., Milford, MA) as detailed previously.³⁰ The analytical column was equipped with an emitter tip having a 2 μm -diameter orifice that was pulled using a laser puller as described in Martin et al.³¹ H1t peptides were eluted using nanoflow HPLC with an 1100 series binary HPLC pump (Agilent Technologies, Santa Clara, CA) coupled to an electrospray ionization source, modified for nanospray, on a LTQ-Orbitrap mass spectrometer (Thermo Scientific, Waltham, MA). A flow rate of 60 nL/min was achieved at the reverse phase column by splitting the flow delivered from the HPLC as described in Martin et al.³¹ Briefly, a splitter enabled the excess solvent to flow to waste through a stainless steel union where the high-voltage was applied for electrospray ionization.³¹ The gradient consisted of 0–60% B in solvent A in 60 min and 60–100% B in 10 min, where solvent A and solvent B were 0.1 M acetic acid and 0.1 M acetic acid in 70% acetonitrile, respectively. Full scan (m/z 300–2000) mass spectra were acquired with the Orbitrap as the analyzer using a resolution of 30 000 (at m/z 400) and an AGC target of 5e5.

A second aliquot (5%) of purified H1t was again diluted 3-fold with 100 mM ammonium bicarbonate, pH 8. H1t was then treated with propionic anhydride to derivatize endogenously monomethylated or unmodified ϵ -amino groups of lysine residues. Chemical derivatization with propionic anhydride converts amino groups of lysines to their corresponding propionyl amides and has been detailed previously.³² Briefly, equal volumes of propionylation reagent and protein were reacted, and derivatization was repeated three times to ensure full conversion

of amino groups. H1t was then digested with trypsin (Promega, Madison, WI) at a substrate-to-enzyme ratio of 20:1 for 6 h at 37 °C. Trypsin cleaved C-terminal to arginine residues only since lysine residues were derivatized and blocked from cleavage. The resulting H1t peptide mixture was acidified with glacial acetic acid. Similar to as described above, an aliquot of the H1t peptide mixture was loaded onto a reverse phase capillary column and H1t peptides were analyzed via tandem mass spectrometry (MS/MS) using a hybrid quadrupole linear ion trap Fourier transform (LTQ-FT) mass spectrometer (Thermo Scientific, Waltham, MA). The LTQ-FT instrument was operated in data-dependent scan mode with dynamic exclusion enabled. The data-dependent method consisted of acquisition of a full scan mass spectrum using the FT as analyzer (resolution 100,000, full scan AGC target of 5e5) followed by ten MS/MS scans of the ten most abundant ions in the initial full scan. Precursor ions selected for MS/MS were fragmented via collision-activated dissociation (CAD) and MS/MS scans were acquired using the ion trap as the analyzer. The precursor ions were allowed to be selected for fragmentation two times (repeat count of two) within 20 s before they were placed on the exclusion list for 30 s. Precursor ions were isolated with a 3D isolation window and an AGC target of 1e4.

An aliquot of trypsin-digested H1t was also analyzed on a LTQ (Thermo Scientific, Waltham, MA) equipped with a Thermo upgrade for electron transfer dissociation (ETD).³³ ETD MS/MS spectra were acquired data-dependently with the six most abundant ions in the initial full scan mass spectrum being selected for fragmentation via ETD. Dynamic exclusion was enabled as described above, precursor ions were isolated with a 3 Da isolation window, and the AGC target for MS/MS scans was 1e4. The reagent anions used for ETD were radical anions of fluoranthene and the maximum ion injection time for reagent anions was 50 ms. The AGC target for fluoranthene anions was 1e5. Peptide cations were reacted with fluoranthene radical anions for 200 ms and the average scan duration for MS/MS spectra was 400 msec. In a separate ETD MS/MS experiment, trypsin-digested H1t peptides were again analyzed via LC-MS/MS, and specific *m/z* values corresponding to precursors of singly and doubly phosphorylated peptides of H1t were targeted for isolation and fragmentation via ETD. After ETD was performed, the charge-reduced species, $[M+4H]^{+3\cdot}$ and $[M+4H]^{+2\cdot}$, that result from electron transfer to the H1t peptide precursors were subjected to a supplemental, low-energy collisional activation (12% collision energy) to convert these nondissociative electron transfer products to c- and z--type fragment ions. All CAD and ETD MS/MS spectra were interpreted via manual examination for identification of phosphorylation sites on murine H1t.

The HPLC fraction containing rat H1t was resuspended in 100 μ L H₂O. An aliquot (5% of the fraction) was diluted 4-fold with 100 mM ammonium bicarbonate. Rat H1t was digested with AspN at a substrate-to-enzyme ratio of 20:1 for 7 h at 37 °C and the resulting peptide mixture was acidified with glacial acetic acid. Similar to that described above for murine H1t, AspN-digested rat H1t was analyzed via nanoflow LC-MS/MS on the LTQ Orbitrap.

Results

Histone H1t is Phosphorylated in Rat

Because of the past disagreement on whether or not rat H1t was phosphorylated and to determine the generality of the H1t phosphorylation process, we re-examined the phosphorylation of rat H1t.

Histones from specific stages of highly purified spermatids obtained by vitamin A synchronization, elutriation, and density gradient centrifugation were analyzed by AUT-PAGE. The results (Figure 1A) showed that highly purified spermatids contained almost exclusively H1t and H1a; hence the majority of the somatic forms [b, c, d, e following the nomenclature of Seyedin and Kistler 34] seen in moderately enriched round spermatid (RS) populations must have been largely derived from contaminating cells. Although H1a and H1t

are poorly separated on these gels, they can be distinguished by the metachromasia of staining with amido black; H1a stains black while H1t stains blue. Within the band marked H1a/t in steps 1–8, spermatids with both black (slowly migrating) and blue components were seen (Figure 1A). At steps 9–10, this band was black, representing H1a, and at steps 11–12, it was absent. Concurrently, an additional distinctively blue, more slowly migrating component (P) was observed weakly in steps 7–8 and as a more major component in steps 9–10 and 11–12 in spermatids. Thus, H1t was essentially the only form of H1 present in steps 11–12 in spermatids, and all the H1t had a modified mobility in these cells. Subsequent experiments done with moderately enriched cells subjected to elutriation only were interpreted using this information.

Histones from moderately enriched populations of spermatids at different stages of development were analyzed by SDS-PAGE. The results (Figure 1B) confirmed the presence of H1t as the major linker histone in the elongating spermatids. Most of the H1c, H1bde, and H1° was likely a result of the somatic cell contamination in this fraction. H1a levels were quite low in the elongating spermatids. Next, histones were separated by AU-PAGE using long gels (Figure 1C). In the fraction enriched in elongating spermatids, a series of four bands was observed extending from the position of H1a/t to slower mobilities; some of these clearly had slower migrations than the bands in the round spermatid fraction. The identity of the shifted bands as modified forms of H1t was confirmed by Western blotting with antibodies to H1t (Figure 1D). The slowly migrating bands were observed in the 4–25% TCA cut (not shown) as well as in the 25% TCA precipitate, ruling out the possibility that transition protein 4 (TP4), which also migrates slower than H1t, but is precipitated by 4% TCA,²⁰ contributes appreciably to these bands.

The shifted bands were demonstrated to be phosphorylated forms of H1t by their disappearance after treatment of the preparations with alkaline phosphatases (Figure 2). In the samples not treated with alkaline phosphatase, four bands were present in the H1a/t region, but after treatment with alkaline phosphatase, only the fastest two bands are observed (Figure 2B). The loss of the more slowly migrating forms is clearly shown in the scan of the gel (Figure 2A). Identical results were obtained by treatment with calf intestinal alkaline phosphatase (not shown). Immunostaining with antibodies to H1t shows that the slowest two bands represent phosphorylated H1t (Figure 2C).

To further investigate whether the modified form of H1t was due to phosphorylation, we examined the incorporation of ³²P into histones. There was a more than 2-fold increase in the specific activity of H1t detected as the spermatids matured (Table 1). This increase in labeling was observed in the H1t region in both SDS and AU gels (Figure 3), and it supports the interpretation that H1t is preferentially phosphorylated in elongating spermatids.

Purification of Mouse and Rat Histone H1t

The results provided in the preceding section show that, like mouse H1t, rat H1t is also phosphorylated. The core histones of unfractionated testes from both mouse and rat consist of a heterogeneous mixture of variants including H2A.X, H2A.Z, and the testis-specific H2Bt as visualized by AUT PAGE (Figure 4A). Gel electrophoresis by either AUT- or SDS-PAGE (Figure 4A–B) of the 5% PCA extracts show that the linker histones also exhibit compositional complexity with 4–5 clearly distinctive bands. These bands can be chromatographically resolved into at least 8 distinctive elution peaks by reversed-phase HPLC (Figure 4C). As a result, it is possible to obtain an electrophoretically pure H1t fraction that is amenable to mass spectrometry analysis for the identification of the sites of phosphorylation of H1t.

Murine Histone H1t is Mainly Phosphorylated at the C-Terminus

Because H1t is a lysine-rich protein, a conventional peptide analysis utilizing trypsin for protein digestion would provide minimal sequence coverage of H1t. Therefore, derivatization of H1t with propionic anhydride was performed to convert the ϵ -amino groups of lysine residues into their corresponding propionyl amides and limit tryptic cleavage to arginine residues³² Mouse H1t derivatization and subsequent treatment with trypsin resulted in the generation of peptides that were more readily retained on a reverse phase column for analysis via LC-coupled tandem mass spectrometry (MS/MS).

Both CAD and ETD were employed to fragment mouse H1t peptides. ETD was necessary to unambiguously assign phosphorylation sites on these peptides as peptide fragmentation via ETD is indifferent to the presence of labile modifications such as phosphorylation.^{33,35} This ETD-based method is a sensitive technique which can be combined with online nanoflow LC-MS/MS and facilitated the identification of multiple phosphorylation sites on mouse H1t from femtomole amounts of material. Four sites of phosphorylation at the C-terminal region of H1t were identified on residues S140, T155, S162, and S177 (see Figure 5). The relative abundances of the phosphorylation sites, S140, S177, S162, and T155, are in the ratio of 8:4:2:1, respectively. Furthermore, LC-MS/MS analysis employing ETD facilitated the interrogation of doubly phosphorylated H1t peptides, and the various forms of phosphorylated H1t peptides are shown in Figure 5b. Phosphorylation of S140 was found to coexist with T155 or S162 phosphorylation. Interestingly, T155 represents a putative cdc2-dependent phosphorylation site that is not conserved within any other linker histone. Cdc2 is a mitotic kinase that is a well-known participant in the phosphorylation of histone H1.³⁶ The other identified phosphorylation sites are putative substrates of Cdc2, PKA or PKC as these H1t residues are present within regions that resemble recognition motifs for these kinases. PKA and PKC have long been suspected to be involved in the phosphorylation of spermiogenic chromosomal proteins because of the presence of basic amino acids in their phosphorylation recognition motifs.^{37,38}

While singly and doubly phosphorylated H1t peptides were successfully interrogated following tryptic digestion, digestion with endoproteinase AspN was utilized to generate larger peptides compared to that from the tryptic digestion and also enabled the detection of H1t peptides concurrently modified with multiple phosphate groups. The C-terminal peptide of murine H1t generated with AspN is an 82-residue peptide (residues 117–198) and harbors all four residues that were found to be phosphorylated upon LC-MS/MS analysis of trypsin-digested H1t. Of all the forms of peptide 117–198 that were detected, approximately 70% of the H1t peptide was unmodified, while the various phosphorylated forms were present at a combined 30% relative abundance and include forms harboring one to four phosphate groups. The multienzyme approach, utilizing both trypsin and AspN, followed by mass spectrometric analysis of H1t peptides allowed for the interrogation of 90% of the murine H1t sequence and facilitated the interrogation of 94% of the Ser, Thr, and Tyr residues that are present within H1t.

In order to determine the location of phosphorylation occurring on rat H1t, Asp-N digestion and subsequent LC-MS analysis was performed on rat H1t. Similar to that found for murine H1t, the major phosphorylation sites of rat H1t were located within the C-terminal region and were detected on the 82-residue rat H1t peptide generated with the Asp-N protease. The notable similarity in the phosphorylation patterns of mouse and rat H1t is demonstrated in the MS spectra shown in Figure 6. The $[M+15H]^{+15}$ ions of the C-terminal peptide comprised of residues 117–198 of rat H1t were detected unphosphorylated and phosphorylated, harboring one to four phosphate groups. These phosphorylated species of rat H1t are analogous to the phosphorylated species identified in murine H1t.

In summary, this mass spectrometric approach enabled the identification of novel phosphorylation sites within H1t, with these sites being located in the C-terminal region of both mouse and rat H1t.

Discussion

Histone H1t is Ubiquitously Phosphorylated in Elongating Spermatids of Murine Species

The results of this study support and extend the observation of phosphorylation of H1t in spermatids of the mouse¹⁷ to the rat. The first appearance of phosphorylated forms at 4 weeks of age in the mouse and their increase at 16 weeks indicated that the phosphorylation was occurring in spermatids. Here, we confirmed these results in the rat and localized this event to elongating spermatids. Since the phosphorylation is localized to the elongating spermatids, the failure to observe such phosphorylation in 40-day old rat testes might be explained. Normal elongating spermatids first appear in the testes at day 36 in the rat, but initially their numbers constitute a smaller percentage in the testis than at maturation, which in the rat occurs later.³⁹

Post-Translational Modifications of Murine Histone H1t

The post-translational modifications of somatic linker histones have been studied extensively in recent years⁴⁰⁻⁴⁴ [see⁴⁵ for a recent review]. However, little attention has been paid to the developmental-specific histones such as the testis-specific H1t.

As is typical of other members of the histone H1 family,⁴⁰⁻⁴³⁻⁴⁶ boar histone H1t was also described to be N-terminally acetylated.⁴⁷ This also appears to be the case for both mouse and rat H1t. Upon ETD MS/MS analysis of trypsin-digested mouse H1t, the N-terminal peptide identified was SETAPAASSTLVAPVEKPSSKR (residues 1–23). This peptide was observed to have a 42 Dalton increase compared to the expected molecular weight, which is consistent with acetylation at the N-terminus of the H1t protein. In addition, an N-terminal peptide was observed with the sequence SETAPAASSTLVAPVEEKPSKR (residues 1–24), which indicates the presence of a second, lower-abundant isoform of mouse H1t. Note the addition of a glutamic acid residue, which is underlined in the peptide sequence above. The molecular weight and ETD MS/MS information acquired of the H1t peptide 1–24 is also consistent with N-terminal acetylation. Furthermore, the observed masses of the N-terminal mouse H1t peptides derived using AspN (H1t 1–72 or 1–73) contained a 42-Dalton shift, consistent with acetylation. This shift in mass was also observed upon LC–MS analysis of AspN-derived rat H1t 1–72. Following N-terminal acetylation, phosphorylation appears to be the next most prominent posttranslational modification (PTM) present on H1t.

Phosphorylation of a somatic histone H1 has been extensively characterized both in the past [see ref 48 for a review] and more recently through MS analysis.⁴⁰⁻⁴²⁻⁴⁴ It was initially shown that its distribution along the molecule (in Chinese hamster CHO cells) depended on the stage of the cell cycle with the C-terminus becoming increasingly phosphorylated as the cell cycle progressed from G1 to mitosis. In the mitotic chromosome, H1 became additionally phosphorylated at its N-terminal end.⁴⁸ This pattern is nonrandom, and in humans, distinct serine- and threonine-specific kinases participate in different cell cycle stages.⁴⁴

As a result of the global proteomic analysis of linker histones, histone H1t was detected to be present in low amounts in mouse spleen extracts⁴³ and in HeLa cells⁴⁹ where a KPRApTpTPK peptide, which corresponds to T155 and T157 of the mouse sequence shown in Figure 5, was identified. However, this peptide was identified from a mixture of thousands of phosphopeptides using a database searching algorithm and the MS/MS data were not manually examined as we have done here. The occurrence of minute amounts of H1t in somatic cells, which probably is the result of gene “leaking”,⁴³ is not surprising. It was shown that rat sarcoma

XC10 cells transfected with a glucocorticoid responsive MMTV-H5 gene resulted in viable cells expressing large amounts of the highly developmental-dependent avian erythrocyte histone H5.⁵⁰ Hence, the physiological relevance, if any, of the presence of H1t and its two putative phosphorylation sites in somatic cells remains to be determined.

Phosphorylation of H1t and the Replacement of Histones by Transition Proteins

The C-terminal localization of the main phosphorylation sites of H1t obtained from mature testes of murine species is interesting in different regards. First, the pattern of phosphorylation observed is similar to that which is found at the C-termini of other somatic histone H1s in what appears to be a consensus region.⁴⁵ This consensus region is approximately 20 amino acids away from the winged helix domain and 20–30 amino acids upstream from the C-terminus. This is the region that is mainly involved in the participation of histone H1 in the folding of the chromatin fiber.⁵¹ Furthermore, a serine to glutamic acid substitution, used as a phosphorylation mimic at S183 of human histone H1d, dramatically impaired its binding to chromatin.¹⁰ Based on primary sequence alignment, phosphorylation of S183 in human H1d is equivalent to S177 phosphorylation in murine H1t, the latter of which was identified in the current study. This is a site that is also present in murine somatic linker histones and which appears to have been quite conserved throughout mammalian evolution. Thus, it is highly likely that phosphorylation of H1t in spermatids is associated with the decondensation of chromatin.

This suggestion raises the question as to what is the possible function of chromatin decondensation in elongating murine spermatids. H1t phosphorylation cannot be related to transcriptional activity because transcription has already ceased earlier in round spermatids. Instead, we propose that the phosphorylation of H1t in conjunction with the hyperacetylation of histone H4 that is observed in elongating spermatids²³ are part of a process of loosening the histone-DNA interactions in preparation for the displacement of the histones by transition proteins.^{13,52} The fact that pS177 is a prominent phosphorylation site together with the relevance of this site in lowering the DNA-binding affinity at an equivalent position in a somatic linker histone counterpart¹⁰ would explain why this highly tissue-specific histone H1 is not indispensable for normal spermatogenesis in mice.⁵³ The compensatory effect of the somatic histone H1 complement that increases upon depletion of H1t⁵³ could be possibly explained by the phosphorylatable RKSPAK sequence around S177 of H1t (Figure 5). This sequence is present at a similar position in all somatic mouse and human H1 variants and for which evidence of phosphorylation has been well documented.^{40,43,44} Interestingly, this sequence can be a target for both the sperm ubiquitous PKA and cdc2. The presence of the other specific phosphorylation sites detected here as well as the more open conformation conferred by H1t to chromatin,⁵⁴ while not strictly required, may contribute to enhancing the efficiency of the spermiogenic process.

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Figure 1.

(A) Histone H1t is the main histone H1 component of spermatids and exhibits a decreased electrophoretic mobility in elongating spermatids. The H1 region of an amido-black stained AUT gel of nuclear protein extracted from the following cell types: RS, round spermatids enriched by elutriation; and highly purified s1, step1–2; s7, step7–8; s9, step 9–10; s11, step 11–12 spermatids. (B–D) Protein profiles of the H1 region of nuclear proteins extracted from moderately enriched populations of round spermatids (RS) or elongating spermatids (ES). (B) SDS gel (Coomassie blue stain). (C) AU gel (Coomassie blue stain). (D) Immunoblot of AU gel reacted with antibodies to H1t. The putative phosphorylated forms of H1t are marked with P.

Figure 2.

Effect of treatment with alkaline phosphatase on mobility of H1 proteins extracted from the elongating spermatid fraction in an AU gel. (A) Scan of the gel. Dashed line is protein treated with alkaline phosphatase; solid line is protein incubated without the enzyme. (B) Coomassie blue stained gel. U, untreated proteins; AP, protein treated with bacterial alkaline phosphatase; Inc., protein incubated as in previous lane but without the enzyme. (C) Immunoblot with antibodies to H1t. (Note: the Immunoblot was overstained spreading the bands, but differences between the AP-treated sample and the others can still be observed.)

Figure 3. Incorporation of ^{32}P into H1 proteins extracted from fractions enriched in round spermatids (RS) and elongating spermatids (ES). (A) Stained AU gel. (B) Autoradiogram of AU gel. (C) Stained SDS gel. (D) Autoradiogram of SDS gel. The bands in the autoradiogram corresponding to the phosphorylated forms of H1t are marked with P.

Figure 4. Purification of histone H1t. (A–B) Protein composition of rat and mouse testis histones after different purification steps: HCl, hydrochloric acid extracted histones; PCA, perchloric acid extracted histones; H1t, histone H1t purified by RP-HPLC fractionation. (A) AUT gel. (B) SDS gel. (C) RP-HPLC elution profile of rat (upper plot) and mouse (lower plot) PCA histone extracts.

Figure 5.

(A) Amino acid sequence alignment of mouse and rat H1t. The boxed amino acid sequences correspond to the predicted kinase phosphorylation consensus sequences. The serines highlighted in red represent the novel phosphorylation sites that were identified by mass spectrometry. (B) Singly and doubly phosphorylated peptides observed following LC-MS/MS analysis of trypsin-digested H1t.

Figure 6.

MS spectra of the $[M+15H]^{+15}$ ions of the C-terminal peptides (residues 117–198) derived from mouse (A) and rat (B) H1t. The monoisotopic ions of the unphosphorylated mouse and rat peptides are denoted with asterisks and consist of m/z 566.6645 and m/z 575.6696, respectively. The observed m/z values are within 3 ppm of the theoretical values calculated for the monoisotopic $[M+15H]^{+15}$ ions. Accurate mass measurements were also observed that correspond to phosphorylated forms of the H1t peptides and the addition of phosphate groups (1–4) are indicated above the corresponding peaks. Note the remarkable similarity in relative abundances of the phosphorylated murine and rat peptides. The species indicated with filled circles are oxidized forms (present at $\geq 10\%$ relative abundance) of the H1t peptides. Oxidation was more prominent in the mouse H1t sample.

Table 1

Relative Specific Activity of ^{32}P -Labelled Histones in Enriched Fractions of Testicular Cells Resolved Using SDS Gels

histone variant	round spermatid fraction	elongating spermatid fraction
H1t	9	22
H1abcde	10	12
H2A	100	54