Peptide Mass Mapping of Acetylated Isoforms of Histone H4 from Mouse Lymphosarcoma Cells Treated with Histone Deacetylase (HDACs) Inhibitors

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The acetylated isoforms of histone H4 from mouse lymphosarcoma cells treated with HDAC inhibitors trichostatin A (TSA) and depsipeptide (DDP) were separated by acetic acid urea-polyacrylamide gel electrophoresis (AU-PAGE), in-gel digested, and analyzed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS). The acetylation pattern of histone H4 in mouse lymphosarcoma cells induced by TSA was established in which acetylation initially occurred at K16 followed by K12 and then K8 and/or K5. An identical order of acetylation was found for cells treated with DDP. (J Am Soc Mass Spectrom 2005, 16, 1641–1653) © 2005 American Society for Mass Spectrometry

The core histones form an octamer that consists of a tetramer of H4-H3 and two dimers of H2A-H2B which organize with double stranded DNA to form nucleosomes [1, 2]. Histones are highly positively-charged and highly conserved throughout eukaryotic evolution. Their posttranslational modifications (acetylation, methylation, phosphorylation, ADP ribosylation, and ubiquitination, etc.) are proposed to affect the interactions between DNA and other chromatin associated proteins, thus regulating the higher order structure of chromatin and in turn gene transcription [3]. Histone acetylation and deacetylation are controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HDAC inhibitors increase acetylation levels and induce open chromatin structure and increased gene transcription. HDAC inhibitors have been found to function as potential chemotherapeutic reagents as they arrest cell growth, differentiation, and/or apoptosis in many tumor cell types [4].

Although nucleosome formation is essential to compact the genome within the nuclei, it also impedes access of most transcription factors to their cognate sites on DNA. Most of the extrinsic and intrinsic signal transduction pathways manifest their biological response by modulating gene expression that requires

access of transcription factors to the DNA. Mobilization of nucleosomes is mediated through altering posttranslational modifications of NH₂-terminal tails of histones as well as by ATP-dependent chromatin remodeling machinery (for review, see references [5–7]). Depending upon the modification states of core histones, the associated genes can be activated or repressed as they interact with coactivators or corepressors. In eukaryotic cells, acetylation of core histones at the NH₂-terminal lysines and phosphorylation at serine-10 of H3 usually are markers of active genes, whereas methylated histones are associated with both active and repressed promoters depending upon the site of methylation. For example, K9 methylation (K9-Me) in H3 is exclusively associated with transcriptionally repressed genes whereas K4-Me in H3 is a marker of active promoters. In contrast, methylation of R17 in H3 and R3 in H4 is a signature of genes activated by steroid hormone receptors [8, 9]. Lysines can be mono-, di- or trimethylated whereas arginines can be mono- or di- (asymmetrical or symmetrical) methylated [3, 6, 8–10]. Gene activation also depends on the degree of histone methylation [9]. Gene expression can be turned on or off very rapidly by reversible acetylation/deacetylation or phosphorylation/dephosphorylation by cellular HATs/HDACs and kinases/phosphatases. In contrast, methylation was thought to be irreversible as no specific histone demethylase had been identified [11]. However, LSD1 (KIAA0601), a nuclear homolog of amine oxidases, has recently been reported to function as a histone demethylase and transcriptional corepressor, which is specific

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to demethylate lysine 4 of histone H3 [12]. Another study has shown that methylated arginines of H3 and H4 can be converted to citrulline by peptidylarginine deiminase 4, releasing methylamine [13, 14]. These findings suggest that methylation marks on histones can be also dynamically regulated by histone methylases and demethylases or by cellular enzymatic machinery.

Posttranslational modifications on core histones also act in a combinatorial, sequential or antagonistic manner to activate or repress gene expression. For example, K9- and K14-acetylation (Ac), and S10-phosphorylation (S10-P) are signature markers on early growth response genes stimulated with growth factors [15]. Similarly, K4-Me and K14-Ac on the same H3 and R3-Me and K5-Ac on H4 are symbols of active genes indicating that modifications at one site sequentially or synergistically modulate those at other sites [6]. In contrast, some modifications are mutually exclusive, e.g., S10-P prevents methylation at K9 of H3 and S18-P of H4 excludes methylation at K20 but facilitates K16 acetylation. Therefore, analysis of different posttranslational modifications occurring upon a single molecule is critical to explore their potential role in gene regulation. To identify these modification patterns, we explored systematic mass spectrometric analysis of histone H4 from mouse lymphosarcoma cells treated with HDAC inhibitors.

Traditional methods, such as micro-sequencing and immunoassay, are limited by the dependence on large amounts of purified samples and the availability of site specific antibodies [16, 17]. The successful development of soft ionization techniques, such as electrospray ionization (ESI) [18–20] and matrix assisted laser desorption/ionization (MALDI) [21–23], have spurred the adoption of mass spectrometry as a powerful technique for protein characterization. Mass spectrometry has been demonstrated to be a very powerful technique to identify the posttranslational modifications of core histones [24–33]. Purification and separation of histone isoforms also play a critical role in the accurate and fast identification of histone posttranslational modifications. Core histones can be separated by reverse phase high-pressure liquid chromatography but without resolution of the different isoforms [26, 27]. Hydrophilic-interaction liquid chromatography (HILIC) uses a weak cation exchange column and an increasing salt concentration gradient to elute different acetylation, phosphorylation and methylation isoforms [34, 35]. However, in HILIC the high organic and salt concentrations are not directly amenable to online MS analysis without extensive workup.

Acetic acid urea-polyacrylamide gel electrophoresis (AU-PAGE) separates proteins based on their size and effective charge. Acetylation of the positively charged ϵ -amino lysine side-chain residues causes a decrease of the effective charge and the mobility of histones in gels. It has been shown that different acetylation isoforms of histone H4 and H3 can be separated by AU-PAGE and only micrograms of the whole histone mixture are

required for further MS analysis [36]. The enzymatic in-gel digests can be used to identify the sites of posttranslational modifications by mass spectrometry. Person used MALDI-TOF MS and LC-MS/MS to identify histone H4 modifications from in-gel chymotrypsin digestion with H4 separated on Triton AU gels [37]. We modified their approach for the use of trypsin to examine the acetylation profiles of histone H4 from mouse lymphosarcoma cells treated with TSA and depsipeptide. Trypsin digests are much easier to obtain reproducible digestion profiles than chymotrypsin. Since lysine acetylation inhibits cleavage by trypsin, this enzyme also provides additional diagnostic information useful in sequencing the sites of modification. Here we present this approach as a rapid, robust and highly reproducible method to profile acetylation on the NH_2 -terminal tails of histone H4 for cancer cells treated with HDAC inhibitor chemotherapies.

Experimental

Materials

HDAC inhibitor trichostatin A (TSA) was purchased from Sigma Chemical Company (St. Louis, MO) and depsipeptide (DDP) was obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Modified sequencing grade trypsin (V511) was purchased from Promega (St. Louis, MO). The MALDI matrix, α -cyano-4-hydroxy-cinnamic acid (HCCA), was purchased from Bruker Daltonics (Billerica, MA). All other chemicals were analytical or HPLC grade.

Cell Culture and Treatment of Cells with HDAC Inhibitors TSA, and DDP

Lymphosarcoma cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 20 mM Hepes, 2% sodium bicarbonate, 1 mM glutamine, 10 nM 2-mercaptoethanol, and 5% FBS at 37 °C in the presence of 5% CO_2 [38]. Cells were treated for 12 h with TSA (300 nM) or DDP (10 nM) from a 1000X stock dissolved in methanol before isolation of histones. Control cells were treated with the same volume of methanol.

Histone Purification

Histones were isolated from lymphosarcoma cells as described previously [39]. Briefly, cells (5×10^7) were harvested by centrifugation at 4 °C at $2000 \times g$ for 5 min, washed with PBS, and resuspended in 5 ml of nuclear isolation buffer consisting of 0.25 M sucrose, 10 mM Tris HCl (pH 7.5), 1.5 mM MgCl_2 , 0.5% Triton X-100, 0.5 mM PMSF, protease inhibitor cocktail (Sigma). Cells were either homogenized in a dounce homogenizer on ice with tight pestle or vortexed until 90 to 95% of plasma membrane of the cells were broken. Nuclei were isolated by spinning at $3000 \times g$ for 5 min

at 4 °C and washed twice with the same buffer without Triton X-100. Histones were extracted from the nuclei with 0.4 N ice-cold H₂SO₄ for 30 min and insoluble particles were removed by centrifugation at 15,000 × g for 10 min. Histones were precipitated from the supernatant with 10× volumes of acetone and collected by centrifugation at 22,000 × g for 30 min. The pellet was washed twice with ice-cold acetone, dissolved in water, and stored at –80 °C in small aliquots.

Acetic Acid-Urea Polyacrylamide Gel Electrophoresis (AU-PAGE) Separation of Acetylated Histone Isoforms

Acetic acid urea-polyacrylamide gel electrophoresis (AU-PAGE)° was° performed° as° described° earlier° [39] with some modifications. 50 µg of histones from control, TSA, and DDP treated cells were separated by AU-PAGE (15% acrylamide, acrylamide:bisacrylamide: 38.7:1.3) at 300 V (constant voltage) overnight (20 h) at 4 °C using 5% acetic acid as the running buffer. The gels were fixed in 50% ethanol/10% acetic acid at room temperature for 4 h, then washed twice (30 min each) with 50% methanol/5% acetic acid, stained with Coomassie Brilliant Blue for 30 min and then destained in 30% methanol/10% acetic acid until the background was clear.

Trypsin In-Gel Digestion

Each of the gel bands was excised into small pieces and two bands of the same isoforms combined. The gel pieces were washed twice (one h each) with freshly made 50% methanol/5% acetic acid solution. Gel pieces were dehydrated in 200 µl ACN for 5 min followed by a 5 min rehydration in 200 µl 100 mM NH₄HCO₃ twice. Gels were dehydrated in ACN again for 5 min and dried in speed vacuum for 2 to 3 min. Thirty µl of freshly prepared trypsin (20 ng/µl in 50 mM NH₄HCO₃) were added to each sample and rehydrated on ice for 10 min, then digested at 37 °C for 1 or 2 h. Tryptic digested peptides were extracted with 50% ACN/5% formic acid three times and were dried to about 10 µl in the speed vacuum.

Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) MS

MALDI-TOF MS was performed on a Bruker Reflex III (Bruker, Bremen, Germany). The mass spectrometer was operated in linear, positive ion mode. The N₂ laser power was adjusted to the minimum intensity necessary to obtain signal. The accelerating voltage was maintained at 28 kV. The instrument was calibrated with protein standards bracketing the molecular weights of the protein samples (typically mixtures of bradykinin fragment 1-5 and ACTH fragment 18-39 as appropriate). Saturated α-cyano-4-hydroxy-cinnamic

acid (HCCA) matrix solution in 0.1% TFA/50% ACN was mixed with the sample in 5:1 ratio and 1 µl of the mixture was spotted on a ground steel MTP384 target (Bruker) and allowed to air dry. Samples were further desalted by washing the dried spots with 1 µl 0.1% TFA.

Nano-Liquid Chromatography Tandem Mass Spectrometry (Nano-LC-MS/MS)

Nano-LC-MS/MS were performed on a hybrid quadrupole time-of-flight Q-TOF II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal nanospray source from New Objective, Inc. (Woburn, MA) operated in positive ion mode. The LC system was a Waters Capillary LC (Waters, Milford, MA). The mobile phase A was water containing 50 mM acetic acid and the mobile phase B was acetonitrile. Samples dried in the speed vacuum were dissolved in 15 µl acetic acid (50 mM). 2.5 µl of the samples were injected onto the trapping column (LC Packings, Sunnyvale, CA) and washed with 50 mM acetic acid. The injector port was switched to inject and the peptides were eluted off the trap onto the column. A 5 cm 75 µm i.d. ProteoPep C18 column (New Objective) packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the Q-TOF system using a gradient of 2 to 80% B over 30 min, with a flow rate of 500 nl/min (precursor split from 40 µl/min). The total run time was 55 min. The nanospray capillary voltage was set at 3.0 kV and the cone voltage at 55 V. The source temperature was maintained at 100 °C. Mass spectra were recorded using MassLynx 4.0 with automatic switching functions and were acquired from mass 400 to 2000 Da every second with a resolution of 8000 (FWHM). When a desired peak was detected at a minimum of 15 ion counts, the mass spectrometer automatically acquired CID MS/MS spectrum of the individual peptide. Collision energy was set dependent on charge state recognition properties. Sequence information from the MS/MS data were processed using Mascot Distiller. Database searches were performed using Mascot MS/MS ion search (Matrix Science, Boston°MA)°[40].

Micro-Liquid Chromatography Tandem Mass Spectrometry (Micro-LC-MS/MS)

In-gel trypsin digests were also run on micro-LC coupled with a Thermo Finnigan LCQ DECA XP+ ion trap (San Jose, CA). MS/MS data were obtained from *m/z* 200 to 2000 to compensate for low *m/z* ions that may have been missed in the nano-LC-MS/MS experiments. Ten µl of the in-gel digests were injected into a supelco (Bellefonte, PA) biowide pore C18 column (15 cm × 1 mm, 5 µm) connected to a Shimadzu capillary LC (Columbia, MD). The mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. Peptides were separated using a

Figure 1. AU-PAGE separation of control, TSA, and DDP treated histones.

gradient of 5 to 75% B over 60 min. The total run time was 80 min. Peptides eluted off the column flowed into the ESI interface of the mass spectrometer. The capillary voltage was maintained at 4.5 KV and the sheath gas

flow was set to 40. A full mass scan was obtained from 200 to 2000 Da and data-dependent MS/MS spectra were obtained for the three most intense precursor ions. Database searches on the product ions were performed the same as for the nano-LC-MS/MS data.

Results and Discussion

AU-PAGE Separation and Peptide Mass Mapping

Histone variants were separated by AU-PAGE based on their acetylation state. The acetylated isoforms of histones H4 and H3 were well separated by AU-PAGE unlike the acetylated isoforms of histones H2A and H2B, which overlapped with other bands (Figure 1). Acetylation of H4 increased upon treatment with both TSA and DDP. The NH₂-terminal serine is assumed to be acetylated in all isoforms. Three gel bands were clearly observed for the untreated control corresponding to the non-, mono-, and diacetylated isoforms (note: to avoid confusion, this discussion keeps with the tradition of labeling the first gel band as 0-Ac. Furthermore, this band is referred to as nonacetylated since it has no acetylated lysines). The number of visible bands

Table 1. MALDI-TOF Data for TSA treated histone H4 acetylation isoforms

Isoform/coverage	Measured (MH ⁺)	Rel. abund.	Calculated (MH ⁺)	Possible sequence	Error (ppm)
0 Ac	515.30	1.00	515.34	³⁶ RLAR ³⁹	-77.62
	543.36	0.60	543.40	²⁰ K _{Me2} VLR ²³	-73.61
	989.54	0.12	989.58	⁶⁰VFLENVIR⁶⁷	-40.42
	1180.56	0.040	1180.62	⁴⁶ISGLIYEETR⁵⁵	-50.82
	1325.68	0.11	1325.75	²⁴DNIQGITKPAIR³⁵	-52.80
1 Ac	515.34	1.0	515.34	³⁶ RLAR ³⁹	0.00
	530.32	0.50	530.30	¹³ GGAK _{Ac} R ¹⁷	37.71
	543.41	0.73	543.40	²⁰ K _{Me2} VLR ²³	18.40
	989.62	0.17	989.58	⁶⁰VFLENVIR⁶⁷	40.42
	1180.65	0.09	1180.62	⁴⁶ISGLIYEETR⁵⁵	25.41
2 Ac	1325.79	0.20	1325.75	²⁴DNIQGITKPAIR³⁵	30.17
	515.44	1.0	515.34	³⁶ RLAR ³⁹	194.05
	530.43	0.24	530.30	¹³ GGAK _{Ac} R ¹⁷	245.14
	543.50	0.53	543.40	²⁰ K _{Me2} VLR ²³	184.03
	927.72	0.68	927.54	⁹GLGK_{Ac}GGAK_{Ac}R¹⁷	194.06
3 Ac	989.77	0.38	989.58	⁶⁰VFLENVIR⁶⁷	192.00
	1180.83	0.34	1180.62	⁴⁶ISGLIYEETR⁵⁵	177.87
	1325.96	0.34	1325.75	²⁴DNIQGITKPAIR³⁵	158.40
	515.50	1.00	515.34	³⁶ RLAR ³⁹	306.98
	543.54	0.35	543.40	²⁰ K _{Me2} VLR ²³	260.95
4 Ac	927.69	0.75	927.54	⁹GLGK_{Ac}GGAK_{Ac}R¹⁷	164.31
	989.70	0.20	989.58	⁶⁰VFLENVIR⁶⁷	122.88
	1180.70	0.11	1180.62	⁴⁶ISGLIYEETR⁵⁵	66.58
	1211.79	0.037	1211.69	⁶GGK_{Ac}GLGK_{Ac}GGAK_{Ac}R¹⁷	85.83
	1325.82	0.33	1325.75	²⁴DNIQGITKPAIR³⁵	49.71
5 Ac	1396.85	0.87	1396.80	⁴GK_{Ac}GGKGLGK_{Ac}GGAK_{Ac}R¹⁷	34.01
				⁴GKGGK_{Ac}GLGK_{Ac}GGAK_{Ac}R¹⁷	
	515.39	1.0	515.34	³⁶ RLAR ³⁹	97.02
	543.46	0.30	543.40	²⁰ K _{Me2} VLR ²³	110.42
	989.73	0.11	989.58	⁶⁰VFLENVIR⁶⁷	151.58
6 Ac	1180.76	0.046	1180.62	⁴⁶ISGLIYEETR⁵⁵	118.58
	1325.96	0.12	1325.75	²⁴DNIQGITKPAIR³⁵	158.40
	1439.04	0.54	1438.81	⁴GK_{Ac}GGK_{Ac}GLGK_{Ac}GGAK_{Ac}R¹⁷	159.85

Me2: Dimethylation; Me: Monomethylation; Ac: Acetylation; Rel. Aund.: Relative Abundance.

Note: The bolded peaks are confirmed by nano-LCMS/MS run on quadrupole Time-of-flight Q-ToF II.

The others were verified by micro-LC-MS/MS run on LCQ DECA XP+ ion trap.

Figure 2. MALDI-TOF MS spectra of trypsin in-gel digests of different acetylation isoforms isolated by AU-PAGE from TSA treated histones. Signals attributable to the acetylation of the NH_2 -terminus are italicized. Trypsin autolysis products (842.7^+ Th) are indicated by two asterisks and signals attributable to the coomassie stain (825.1^+ Th) are indicated by one asterisk.

increased to five after treatment with TSA and DDP (non-, mono-, di-, tri-, and tetraacetylated isoform of histone H4). The gel bands were excised separately and in-gel digested with trypsin to determine the posttranslational modifications for each of the isoforms. The masses for the peptides were determined from the MALDI-TOF MS and were compared with theoretical masses obtained by use of MS-Digest on the Protein Prospector website (prospector.ucsf.edu) [41]. Modifications that were allowed in the theoretical digest included: S/T/Y phosphorylation; NH_2 -terminus acetylation; lysine mono-, di-, and trimethylation, lysine acetylation, and oxidation of methionine (methylation at arginine was not considered at this time). On average, the dynamic range between the highest and lowest abundance peptides was 140.

Acetylation Patterns for TSA Treated Cells

To identify the modifications of H4 in the control cells and in cells treated with HDAC inhibitors, a systematic strategy was developed for the analysis of H4 isoforms separated by AU-PAGE using mass spectrometry after in-gel trypsin digestion. Arg-C digestion was also performed but the results exhibited poorer reproducibility than trypsin due to nonspecific cleavage at lysine residues (data not shown). In-gel digestion procedures for Arg-C are still being developed as selective cleavage at arginine is highly desirable. The peptides shown in Table 1 were observed in the MALDI-TOF MS data from

the TSA treated histones and confirmed by LC-MS/MS. A full list of the MALDI-TOF MS masses is provided in the Supplementary Material with the online version of this article, along with their potential assignments as generated by protein prospector [41]. However, **only the assignments confirmed by MS/MS** are discussed in the following sections. The MALDI-TOF MS peptide mapping data in Figures 2 and 3 are discussed separately from the supporting MS/MS data in Figures 4, 5, 6, 7, 8, and 9.

Several peptides were observed in all gel bands in MALDI-TOF MS. These included: the dimethylation of K20 in fragment 20-23 (543.40^+ Th) and the unmodified fragments 36-39 (515.30^+ Th), 60-67 (989.58^+ Th), 46-55 (1180.62^+ Th), 24-35 (1325.75^+ Th). In the first gel band (Figure 2a), no lysine acetylation was observed. This is not unexpected since the NH_2 -terminal serine is believed to be predominantly acetylated. The only modification observed was dimethylation of K20 as was shown by the strong peak at 543.36^+ Th. However, fewer peaks were observed compared with the other isoforms. This is because the tails of histone H4 are arginine- and lysine rich, resulting in small fragments (<500 Th) that were likely obscured by strong signals from the matrix.

In the second gel band, K16 was found to be acetylated as indicated by the presence of the peptide at 530.32^+ Th corresponding to one acetylation in fragment 13-17, in addition to the previously discussed dimethylation of K20 (Figure 2b). For the third gel band, a peptide at 927.72^+ Th was observed (Figure 2c). This peptide corresponded to

Figure 3. MALDI-TOF MS spectra of trypsin in-gel digests (1 h) of different acetylation isoforms isolated by AU-PAGE from DDP treated histones. Signals attributable to the acetylation of the NH₂-terminus are italicized. Trypsin autolysis products (842.7⁺ Th) are indicated by two asterisks and signals attributable to the coomassie stain (825.1⁺ Th) are indicated by one asterisk.

the addition of two acetyl groups to K12 and K16 in fragment 9-17. The peak at 530.43⁺ Th (K16 acetylation in fragment 13-17) was still present although in lower abun-

dance as compared with the previous gel band. Since trypsin is inhibited by lysine acetylation, the presence of fragment 13-17 indicated acetylation somewhere else on

Figure 4. Nano-LC-MS/MS spectrum of the precursor ion at 464.382⁺ Th. These data were obtained from TSA treated diacetylation band. The same fragmentation pattern of this precursor ion was also observed in both the TSA and DDP treated triacetylation band.

Figure 5. Nano-LC-MS/MS spectrum of the precursor ion at 606.53²⁺ Th (data obtained from TSA treated triacetylation band).

the protein that at this time is still undetermined. Thus, this band contains a mixture of isoforms in which (1) K12 is acetylated after K16 and (2) K16 is acetylated along with another undetermined site on the molecule.

As was shown in Figure 2d for the fourth gel band, two peptides were observed that correlated with the acetylation of three lysine residues. The first of these was a peptide (1211.79⁺ Th) that corresponded to

Figure 6. Nano-LC-MS/MS spectrum of precursor ion at 698.94²⁺ Th. K8, K12, and K16 acetylation in fragment 4-17 was observed. The presence of mass shift of 42 Da in the b ions near the NH₂-terminus and y₁₂' indicated an additional pattern of K5, K12, and K16 acetylation (data obtained from DDP treated triacetylation band).

acetylated K8, K12, and K16 in fragment 6-17. The second peptide (1396.85⁺ Th) also corresponded to three acetylated lysines in fragment 4-17. This fragment contains 4 lysine residues, K5, K8, K12, and K16. It was assumed that K12 and K16 remained acetylated leaving either K5 or K8 as the next likely location for modification. Nano-LC-MS/MS of the peptides in this digested gel band showed the presence of two peptide sequences in which K16, K12, and either K8 or K5 were acetylated based on the observance of two y_{12} ions (1211.96⁺ and 1169.93⁺, respectively). The y ions corresponded to acetylation of K16, K12, and K8 were present at higher abundance. The result is partially corroborated by the presence of the diacetylated fragment 9-17. It was inferred that K8 must be at least partially unmodified to allow trypsin cleavage after K8, leading to the conclusion that an additional acetylation was located on K5. The abundance for the peptide at 1211.79⁺ Th was very low in the one h trypsin digestion (data not shown). Its abundance increased as the digestion time increased to two h, whereas that of the peptide at 1396.85⁺ Th decreased. This observation supports the presence of a missed cleavage at K5, which would result in a lower abundance of the peptide at 1211.79⁺ Th. However, little additional benefit was noted between the digestion time of one h and two h.

The MALDI-TOF MS of the fifth gel band showed an abundant peptide (1439.04⁺ Th) that corresponded to the fully acetylated fragment 4-17 (Figure 2e). No other acetylated peptides described above were observed indicating complete acetylation of the NH₂-terminal lysine residues of histone H4.

Acetylation Patterns of DDP Treated Cells

The histone H4 modifications of DDP treated lymphosarcoma cells are summarized in Table 2. As indicated above, **only the assignments confirmed by MS/MS** are discussed in the following. The pattern of peptides observed for the DDP treated cells is virtually identical to that observed for the TSA treated cells (Figure 3). Some low abundance peptides observed in the TSA treated cells were not observed in DDP treated cells likely because of lower protein concentration and experimental variance. However, the pattern of modification follows the same trend observed for TSA. K16 is initially acetylated, followed by K12. The triacetylation isoform contains the additional acetylation of K5 or K8. Thus, the experiments revealed no major differences in the NH₂-terminal specificity between TSA and DDP with the exception that DDP is effective at much lower concentrations (10 nM DDP compared with 300 nM TSA). No differences in the peptide identity and locations of modifications were observed when compared with the peptides from the gel bands of the untreated control except that only three isoforms were observed in the control (Table 3). For comparison, the MALDI-TOF MS data are provided in the Supplementary Material available online (Tables A1, A2, A3, Figures 1, 2, 3).

Verification of Acetylation Patterns by LC-MS/MS

Since MALDI-TOF MS does not have the mass accuracy to differentiate trimethylation and acetylation

Figure 7. Nano-LC-MS/MS spectrum of precursor ion at 719.952⁺ Th (data obtained from DDP treated tetraacetylation band).

Figure 8. Micro-LC-MS/MS spectrum of precursor ion at 265.91²⁺ Th. K16 was verified to be acetylated in fragment 13 to 17 (data obtained from DDP treated diacetylation band).

(42.0470 versus 42.0106 Da), LC-MS/MS experiments were also performed to verify the sequences and modification patterns for each of the in-gel digests. In addition to direct sequence information, MS/MS generates diagnostic fragments that aid in the assignment. For example, trimethylated peptides yield y-59

ions° and/or b-59° ions° [29].° If the° peptide° is° acetylated, y and b ions will be observed in addition to an immonium° ion° 126° Th° [29].

The precursor ion at 464.38²⁺ Th was assigned unambiguously by nano-LC-MS/MS to be acetylation at K12 and K16 in fragment 9-17. The immonium ion at 126.14⁺

Figure 9. Micro-LC-MS/MS spectrum of precursor ion at 272.64²⁺ Th. K20 was verified to be dimethylated in fragment 20 to 23 (data obtained from DDP treated diacetylation band).

Table 2. MALDI-TOF Data for depsiptide treated histone H4 acetylation isoforms

Isoform/coverage	Measured (MH ⁺)	Rel. abund.	Calculated (MH ⁺)	Sequence	Error (ppm)
0 Ac	515.40	1.00	515.34	³⁶ RLAR ³⁹	112.93
	543.47	0.65	543.40	²⁰ K _{Me2} VLR ²³	132.13
	989.74	0.13	989.58	⁶⁰VFLENVIR⁶⁷	163.30
	1180.79	0.033	1180.62	⁴⁶ISGLIYEETR⁵⁵	142.81
	1325.98	0.088	1325.75	²⁴DNIQGITKPAIR³⁵	170.39
1 Ac	515.36	0.35	515.34	³⁶ RLAR ³⁹	38.81
	530.33	0.18	530.30	¹³ GGAK _{Ac} R ¹⁷	56.57
	543.42	0.27	543.40	²⁰ K _{Me2} VLR ²³	40.12
	989.65	0.18	989.58	⁶⁰VFLENVIR⁶⁷	70.74
	1180.74	0.004	1180.62	⁴⁶ISGLIYEETR⁵⁵	101.64
2 Ac	1325.82	0.010	1325.75	²⁴DNIQGITKPAIR³⁵	52.80
	515.40	0.46	515.34	³⁶ RLAR ³⁹	112.93
	530.39	0.078	530.30	¹³ GGAK _{Ac} R ¹⁷	169.72
	543.47	0.29	543.40	²⁰ K _{Me2} VLR ²³	132.13
	927.65	0.16	927.54	⁹GLGK_{Ac}GGAK_{Ac}R¹⁷	121.18
3 Ac	989.67	0.031	989.58	⁶⁰VFLENVIR⁶⁷	92.56
	1180.71	0.015	1180.62	⁴⁶ISGLIYEETR⁵⁵	92.56
	1325.87	0.039	1325.75	²⁴DNIQGITKPAIR³⁵	87.42
	515.45	1.00	515.34	³⁶ RLAR ³⁹	161.24
	543.52	0.38	543.40	²⁰ K _{Me2} VLR ²³	224.15
4 Ac	927.73	0.58	927.54	⁹GLGK_{Ac}GGAK_{Ac}R¹⁷	207.43
	989.80	0.23	989.58	⁶⁰VFLENVIR⁶⁷	223.93
	1180.87	0.14	1180.62	⁴⁶ISGLIYEETR⁵⁵	210.57
	1211.92	0.037	1211.69	⁶GGK_{Ac}GLGK_{Ac}GGAK_{Ac}R¹⁷	189.82
	1326.01	0.18	1325.75	²⁴DNIQGITKPAIR³⁵	193.02
5 Ac	1397.06	0.048	1396.80	⁴GK_{Ac}GGKGLGK_{Ac}GGAK_{Ac}R¹⁷	184.35
				⁴GKGGK_{Ac}GLGK_{Ac}GGAK_{Ac}R¹⁷	
	515.44	0.37	515.34	³⁶ RLAR ³⁹	190.55
	543.51	0.30	543.40	²⁰ K _{Me2} VLR ²³	205.74
	989.74	0.040	989.58	⁶⁰VFLENVIR⁶⁷	163.30
6 Ac	1180.86	0.017	1180.62	⁴⁶ISGLIYEETR⁵⁵	202.10
	1325.99	0.027	1325.75	²⁴DNIQGITKPAIR³⁵	177.94
	1439.06	0.54	1438.81	⁴GK_{Ac}GGK_{Ac}GLGK_{Ac}GGAK_{Ac}R¹⁷	182.10

Me2: Dimethylation; Me: Monomethylation; Ac: Acetylation; Rel. Abund.: Relative Abundance.

Note: The bolded peaks are confirmed by nano-LCMS/MS run on quadrupole Time-of-flight Q-Tof II.

The others were verified by micro-LC-MS/MS run on LCQ DECA XP+ ion trap.

Th and the abundant y and b ions confirm the MALDI-TOF MS peak at 927.76⁺ Th as ⁹GLGK_{Ac}GGAK_{Ac}R¹⁷ (Figure 4). Using the same strategy, the monoisotopic peak at 1211.79⁺ Th from MALDI-TOF MS was identified as ⁶GGK_{Ac}GLGK_{Ac}GGAK_{Ac}R¹⁷ from the nano-LC-MS/MS data of precursor ion 606.53²⁺ Th (Figure 5). This assignment confirms the absence of the isobaric unmodified fragment 92-102 that could also occur at 1211.63⁺ Th. The monoisotopic peak at 1396.85⁺ Th has three lysines acetylated among four lysines in fragment 4-17 giving rise to four possible isoforms. Nano-LC-MS/MS showed that K12 and K16 were acetylated. However, closer inspection of the data revealed two sets of NH₂-terminal b and y ions differing in mass by 42 Da. Although the product ion for K5 acetylation b₂'(228.20⁺), b₃'(285.23⁺), b₄'(342.27⁺) might also be assigned as internal fragment ions, the unique y₁₂' (1169.93⁺) supports K5 as another site of acetylation. Thus, this peak is from a mixture of peptides where either K5 or K8 were acetylated. The sequences for these monoisotopic peaks were ⁴GKGGK_{Ac}GLGK_{Ac}GGAK_{Ac}R¹⁷ and ⁴GK_{Ac}GGKGLGK_{Ac}GGAK_{Ac}R¹⁷ (Figure 6). The peptide with monoisotopic mass 1439.04⁺ Th was established by nano-LC-MS/MS to be the acetylation of

K5, K8, K12, and K16 in fragment 4-17 from the precursor ion at 719.95²⁺ Th (Figure 7) excluding assignment to the unmodified isobaric fragment 79-91 (1438.80⁺ Th).

For the low mass precursor ions, micro-LC-MS/MS data were obtained to verify their sequence. For the peptide at 515.30⁺ Th, there are three possible sequences based on the monoisotopic mass: ³⁷LARR⁴⁰, ³⁶RLAR³⁹, or ²⁰KVLR²³. MS/MS of the precursor ion at 258.37²⁺ Th revealed its sequence as ³⁶RLAR³⁹ (see supplementary data). Using the same method, the precursor ion at 265.91²⁺ corresponded to acetylation of K16 in fragment 13-17 (Figure 8) and the precursor ion at 272.64²⁺ confirmed the dimethylation of K20 in ²⁰K_{Me2}VLR²³ (Figure 9). A singly charged precursor ion at 543.30⁺ was also fragmented and further verified the dimethylation of K20 in fragment 20-23 (see Supplementary Material).

Acetylation Patterns of Histone H4

Histone acetylation has been widely studied due to its significant role in nucleosome assembly. More importantly acetylation does not appear to be ran-

Table 3. MALDI-TOF Data for control histone H4 acetylation isoforms

Isoform/coverage	Measured (MH ⁺)	Rel. abund.	Calculated (MH ⁺)	Sequence	Error (ppm)
0 Ac	515.43	1.00	515.34	³⁶ RLAR ³⁹	171.15
	543.50	0.73	543.40	²⁰ K _{Me2} VLR ²³	187.34
	989.79	0.32	989.58	⁶⁰ VFLENVIR ⁶⁷	213.83
	1180.87	0.17	1180.62	⁴⁶ ISGLIYEETR ⁵⁵	210.57
	1326.03	0.12	1325.75	²⁴ DNIQGITKPAIR ³⁵	208.11
1 Ac	515.46	0.74	515.34	³⁶ RLAR ³⁹	180.65
	530.45	0.48	530.30	¹³ GGAK _{Ac} R ¹⁷	273.24
	543.53	0.52	543.40	²⁰ K _{Me2} VLR ²³	242.55
	989.86	0.23	989.58	⁶⁰ VFLENVIR ⁶⁷	284.57
	1180.95	0.17	1180.62	⁴⁶ ISGLIYEETR ⁵⁵	265.03
2 Ac	1326.11	0.43	1325.75	²⁴ DNIQGITKPAIR ³⁵	278.33
	515.43	0.78	515.34	³⁶ RLAR ³⁹	171.15
	530.42	0.14	530.30	¹³ GGAK _{Ac} R ¹⁷	216.67
	543.48	0.14	543.40	²⁰ K _{Me2} VLR ²³	150.53
	927.75	0.13	927.54	⁹ GLGK _{Ac} GGAK _{Ac} R ¹⁷	228.99
	989.81	0.035	989.58	⁶⁰ VFLENVIR ⁶⁷	234.04
	1180.88	0.020	1180.62	⁴⁶ ISGLIYEETR ⁵⁵	219.04
	1326.06	0.044	1325.75	²⁴ DNIQGITKPAIR ³⁵	230.74

Me2: Dimethylation; Me: Monomethylation; Ac: Acetylation; Rel. Abund.: Relative Abundance.

Note: The bolded peaks are confirmed by nano-LCMS/MS run on quadrupole Time-of-flight Q-Tof II.

The others were verified by micro-LC-MS/MS run on LCQ DECA XP+ ion trap.

dom configuration. Nascent histone H4 was found to be diacetylated exclusively on K5 and K12 by histone acetyltransferase[°]B[°](HAT[°]B)[°][42,43]. This[°]was[°]confirmed[°]by[°]the[°]acetylation[°]patterns[°]of[°]*Drosophila* [42] and *Tetrahemena* [44]. Coupez[°]showed[°]that[°]K12[°]was[°]acetylated[°]first, then K5 and K16 in cuttlefish testis [45]. They[°]also[°]showed[°]that[°]K16[°]was[°]primarily[°]acetylated[°]followed[°]by[°]K12, K5[°]or[°]K8[°]in[°]calf[°]thymus[°][45]. K16 was predominately acetylated in histone H4 of both pig thymus and HeLa cells, but the progressive addition of acetyl groups to K12, K8 and K5 was less specific [46]. Turner[°]studied[°]histone[°]H4[°]acetylation[°]in human cells by immunolabeling with site-specific antibodies and found out that K16 was the preferred site[°]of[°]acetylation, followed[°]by[°]K12, K8[°]and[°]K5[°][47]. Zhang proposed a “zip” model for histone acetylation and deacetylation based on their study of histone H4 in HeLa S3 cells treated with sodium butyrate by use[°]of[°]mass[°]spectrometry[°][24]. They[°]suggested[°]that histone H4 in HeLa S3 cells was primarily acetylated at K16, followed by K12, K8 and K5 in that strict order. Histone deacetylation occurred in the reverse direction: K5 first, then K8, K12 and K16. K20 was predominantly dimethylated in all isoforms, which may have some effect on histone acetylation and deacetylation. In their work they examined the crude mixture of histone H4 in which all isoforms were present. It was thus hard to distinguish which modifications were associated with a particular acetylation isoform.

In our study, the acetylation isoforms of histone H4 were separated by AU-PAGE and the resolved isoforms were in-gel digested and mass analyzed. Our data showed that K16 is acetylated first in mouse lymphosarcoma cells followed by K12. However, we observed that both K8 and/or K5 may be acetylated

next. In the triacetylated gel bands we observed a mixture of K5 and K8 acetylation that was confirmed by tandem mass spectrometry. More importantly, the pattern of acetylation upon treatment with TSA and DDP was conserved and indicates that these drugs may induce similar, if not the same, physiological response. In all of the acetylated isoforms of histone H4 with or without treatment of HDAC inhibitor, K20 was always predominantly dimethylated. K20 monomethylation in fragment 20-23 was also observed as was indicated by the peptide at 529.4⁺ Th (see supplementary data). However no MS/MS data were obtained due to its low abundance. The monoisotopic mass of the same peptide with unmodified K20 overlapped with that of fragment of 36-39 and fragment 27-40. Micro-LC-MS/MS data confirmed that peptide to be unmodified fragment of 36-39. Lindner et al. reported an increased trimethylation of K20 in histone H4 in hypoacetylated isoforms [48]. In our study, trimethylation of K20 was not observed or buried under background.

Finally, interplay among different acetylation, methylation, and phosphorylation sites of histone is important. For example, such interplay between site on[°]H3[°]has[°]been[°]reported[°]in[°]*Xenopus* oocytes[°][49]. Western blot analysis revealed an increase in methylation of K4, increase in dual modification of S10-P/K14-Ac, and decrease in methylation of H3-K9 following treatment with TSA. It will be of interest to investigate whether there is cross talk among different posttranslational modifications of H3 induced by HDAC inhibitors in mammalian cells. We want to pursue this by employing mass spectrometry instead of western blot analysis, which may not provide an accurate picture if the antibody is not highly specific.

Conclusions

The present study was undertaken to explore the acetylation patterns of histone H4 after treatment with HDAC inhibitors. To address this question, different acetylation isoforms of histone H4 were separated first by AU-PAGE; then MALDI-TOF MS and nano- or micro-LC-MS/MS was performed to give a systematic analysis. Our study demonstrates the successful application of peptide mass fingerprinting on tryptic in-gel digests after the separation of acetylation isoforms by AU-PAGE. MALDI-TOF MS data identified the trend of acetylation for the HDAC inhibitor TSA and DDP treated histones on the NH₂-terminal residues. In histone H4 of mouse lymphosarcoma cells, K16 is preferentially acetylated, followed by K12, K8, and/or K5. Nano- or micro-LC-MS/MS of the in-gel digests verified the modification patterns.

HDAC inhibitors also play an important role in the reversible acetylation of nonhistone DNA binding proteins such as p53[50] and NF- κ B[51]. Likely, many key cellular proteins are hyperacetylated in cells treated with TSA or DDP. It will be important to identify these proteins specifically in cancer cells that are sensitive or resistant to HDAC inhibitors by employing whole cell proteomics. Studies along these lines are our goals in the future.

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References

- Luger, K.; Mader, A. W.; Richmond, R. K.; Sargent, D. F.; Richmond, T. J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **1997**, *389*, 251–260.
- Luger, K.; Richmond, T. J. The histone tails of the nucleosome. *Curr. Opin. Genet. Dev.* **1998**, *8*, 140–146.
- Strahl, B. D.; Allis, C. D. The language of covalent histone modifications. *Nature* **2000**, *403*, 41–45.
- Marks, P.; Rifkind, R. A.; Richon, V. M.; Breslow, R.; Miller, T.; Kelly, W. K. Histone deacetylases and cancer: Causes and therapies. *Nat. Rev. Cancer* **2001**, *1*, 194–202.
- Strahl, B. D.; Ohba, R.; Cook, R. G.; Allis, C. D. Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in tetrahymena. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14967–14972.
- Jenuwein, T.; Allis, C. D. Translating the histone code. *Science* **2001**, *293*, 1074–1080.
- Sif, S. ATP-dependent nucleosome remodeling complexes: Enzymes tailored to deal with chromatin. *J. Cell. Biochem.* **2004**, *91*, 1087–1098.
- Zhang, Y.; Reinberg, D. Transcription regulation by histone methylation: Interplay between different covalent modifications of the core histone tails. *Genes Dev.* **2001**, *15*, 2343–2360.
- Xu, W.; Cho, H.; Evans, R. M. Acetylation and methylation in nuclear receptor gene activation. *Methods Enzymol.* **2003**, *364*, 205–223.
- Santos-Rosa, H.; Schneider, R.; Bannister, A. J.; Sherriff, J.; Bernstein, B. E.; Emre, N. C.; Schreiber, S. L.; Mellor, J.; Kouzarides, T. Active genes are trimethylated at K4 of histone H3. *Nature* **2002**, *419*, 407–411.
- Bannister, A. J.; Schneider, R.; Kouzarides, T. Histone methylation: Dynamic or static? *Cell* **2002**, *109*, 801–806.
- Shi, Y.; Lan, F.; Matson, C.; Mulligan, P.; Whetstone, J. R.; Cole, P. A.; Casero, R. A. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **2004**, *119*, 941–953.
- Wang, Y.; Wysocka, J.; Sayegh, J.; Lee, Y. H.; Perlin, J. R.; Leonelli, L.; Sonbuchner, L. S.; McDonald, C. H.; Cook, R. G.; Dou, Y.; Roeder, R. G.; Clarke, S.; Stallcup, M. R.; Allis, C. D.; Coonrod, S. A. Human PAD4 regulates histone arginine methylation levels via demethylation. *Science* **2004**, *2*, 2.
- Cuthbert, G. L.; Daujat, S.; Snowden, A. W.; Erdjument-Bromage, H.; Hagiwara, T.; Yamada, M.; Schneider, R.; Gregory, P. D.; Tempst, P.; Bannister, A. J.; Kouzarides, T. Histone demethylation antagonizes arginine methylation. *Cell* **2004**, *118*, 545–553.
- Mahadevan, L. C.; Clayton, A. L.; Hazzalin, C. A.; Thomson, S. Phosphorylation and acetylation of histone H3 at inducible genes: Two controversies revisited. *Novartis Foundation Symp.* **2004**, *259*, 102/11; 111/4; Discussion; 163–169.
- Crane-Robinson, C.; Hebbes, T. R.; Clayton, A. L.; Thorne, A. W. Chromosomal mapping of core histone acetylation by immunoselection. *Methods* **1997**, *12*, 48–56.
- Suka, N.; Suka, Y.; Carmen, A. A.; Wu, J.; Grunstein, M. Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin. *Mol. Cell* **2001**, *8*, 473–479.
- Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. Electrospray ionization for mass spectrometry of large biomolecules. *Science* **1989**, *246*, 64–71.
- Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F. Electrospray. *Mass Spectrom. Rev.* **1990**, *9*, 37–70.
- Whitehouse, C. M.; Dreyer, R. N.; Yamashita, M.; Fenn, J. B. Electrospray. *Anal. Chem.* **1985**, *57*, 675–679.
- Karas, M.; Bahr, U.; Hillenkamp, F. UV laser matrix desorption/ionization mass spectrometry of proteins in the 100,000 Da range. *Int. J. Mass Spectrom. Ion Processes* **1989**, *92*, 231–242.
- Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T. Protein and polymer analyses up to *m/z* 100,000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 151–153.
- Hillenkamp, F.; Karas, M.; Beavis, R. C.; Chait, B. T. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal. Chem.* **1991**, *63*, 1193A–1203A.
- Zhang, K.; Williams, K. E.; Huang, L.; Yau, P.; Siino, J. S.; Bradbury, E. M.; Jones, P. R.; Minch, M. J.; Burlingame, A. L. Histone acetylation and deacetylation: Identification of acetylation and methylation sites of HeLa histone H4 by mass spectrometry. *Mol. Cell. Proteomics* **2002**, *1*, 500–508.
- Zhang, K.; Tang, H.; Huang, L.; Blankenship, J. W.; Jones, P. R.; Xiang, F.; Yau, P. M.; Burlingame, A. L. Identification of acetylation and methylation sites of histone H3 from chicken erythrocytes by high-accuracy matrix-assisted laser desorption ionization-time-of-flight, matrix-assisted laser desorption ionization-postsource decay, and nanoelectrospray ionization tandem mass spectrometry. *Anal. Biochem.* **2002**, *306*, 259–269.
- Zhang, K.; Tang, H. Analysis of core histones by liquid chromatography-mass spectrometry and peptide mapping. *J. Chromatogr. B* **2003**, *783*, 173–179.

27. Zhang, L.; Freitas, M. A.; Wickman, J.; Parthun, M. R.; Klisovic, M. I.; Marcucci, G.; Byrd, J. C. Differential expression of histone posttranslational modifications in acute myeloid and chronic lymphocytic leukemia determined by high-pressure liquid chromatography and mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 77–86.
28. Zhang, L.; Eugeni, E. E.; Parthun, M. R.; Freitas, M. A. Identification of novel histone posttranslational modifications by peptide mass fingerprinting. *Chromosoma* **2003**, *112*, 77–86.
29. Zhang, K.; Yau, P. M.; Chandrasekhar, B.; New, R.; Kondrat, R.; Imai, B. S.; Bradbury, M. E. Differentiation between peptides containing acetylated or trimethylated lysines by mass spectrometry: An application for determining lysine 9 acetylation and methylation of histone H3. *Proteomics* **2004**, *4*, 1–10.
30. Galasinski, S. C.; Resing, K. A.; Ahn, N. G. Protein mass analysis of histones. *Methods* **2003**, *31*, 3–11.
31. Freitas, M. A.; Sklenar, A. R.; Parthun, M. R. Application of mass spectrometry to the identification and quantification of histone posttranslational modifications. *J. Cell. Biochem.* **2004**, *92*, 691–700.
32. Medzihradsky, K. F.; Zhang, X.; Chalkley, R. J.; Guan, S.; McFarland, M. A.; Chalmers, M. J.; Marshall, A. G.; Diaz, R. L.; Allis, C. D.; Burlingame, A. L. Characterization of tetrahymena histone H2B variants and posttranslational populations by electron capture dissociation (ECD) Fourier transform ion cyclotron mass spectrometry (FT-ICR MS). *Mol. Cell. Proteomics* **2004**, *3*, 872–886.
33. Galasinski, S. C.; Louie, D. F.; Gloor, K. K.; Resing, K. A.; Ahn, N. G. Global regulation of posttranslational modifications on core histones. *J. Biol. Chem.* **2002**, *277*, 2579–2588.
34. Lindner, H.; Sarg, B.; Meraner, C.; Helliger, W. Separation of acetylated core histones by hydrophilic-interaction liquid chromatography. *J. Chromatogr. A* **1996**, *743*, 137–144.
35. Lindner, H.; Sarg, B.; Helliger, W. Application of hydrophilic-interaction liquid chromatography to the separation of phosphorylated H1 histones. *J. Chromatogr. A* **1997**, *782*, 55–62.
36. Reinhold, B. B.; Chan, S.-Y.; Reuber, L.; Walker, G. C.; Reinhold, V. N. Detailed structural characterization of the exopolysaccharide from *Rhizobium meliloti*. *J. Bacteriol.* **1994**, *176*, 1997–2002.
37. Person, M. D.; Monks, T. J.; Lau, S. S. An integrated approach to identifying chemically induced posttranslational modifications using comparative MALDI-MS and targeted HPLC-ESI-MS/MS. *Chem. Res. Toxicol.* **2003**, *16*, 598–608.
38. Majumder, S.; Ghoshal, K.; Li, Z.; Bo, Y.; Jacob, S. T. Silencing of metallothionein-I gene in mouse lymphosarcoma cells by methylation. *Oncogene* **1999**, *18*, 6287–6295.
39. Ghoshal, K.; Datta, J.; Majumder, S.; Bai, S.; Dong, X.; Parthun, M.; Jacob, S. T. Inhibitors of histone deacetylase and DNA methyltransferase synergistically activate the methylated metallothionein I promoter by activating the transcription factor MTF-1 and forming an open chromatin structure. *Mol. Cell. Biol.* **2002**, *22*, 8302–8319.
40. Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20*, 3551–3567.
41. Clauser, K. R.; Baker, P.; Burlingame, A. L. Role of accurate mass measurement (± 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal. Chem.* **1999**, *71*, 2871–2882.
42. Sobel, R. E.; Cook, R. G.; Perry, C. A.; Annunziato, A. T.; Allis, C. D. Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1237–1241.
43. Chang, L.; Loranger, S. S.; Mizzen, C.; Ernst, S. G.; Allis, C. D.; Annunziato, A. T. Histones in transit: Cytosolic histone complexes and diacetylation of H4 during nucleosome assembly in human cells. *Biochemistry* **1997**, *36*, 469–480.
44. Chicoine, L. G.; Schulman, I. G.; Richman, R.; Cook, R. G.; Allis, C. D. Nonrandom utilization of acetylation sites in histones isolated from tetrahymena. Evidence for functionally distinct H4 acetylation sites. *J. Biol. Chem.* **1986**, *261*, 1071–1076.
45. Couppez, M.; Martin-Ponthieu, A.; Sautiere, P. Histone H4 from cuttlefish testis is sequentially acetylated. Comparison with acetylation of calf thymus histone H4. *J. Biol. Chem.* **1987**, *262*, 2854–2860.
46. Thorne, A. W.; Kmiecik, D.; Mitchelson, K.; Sautiere, P.; Crane-Robinson, C. Patterns of histone acetylation. *Eur. J. Biochem.* **1990**, *193*, 701–713.
47. Turner, B. M.; O'Neill, L. P.; Allan, I. M. Histone H4 acetylation in human cells. Frequency of acetylation at different sites defined by immunolabeling with site-specific antibodies. *FEBS Lett.* **1989**, *253*, 141–145.
48. Sarg, B.; Helliger, W.; Talasz, H.; Koutzamani, E.; Lindner, H. H. Histone H4 hyperacetylation precludes histone H4 lysine 20 trimethylation. *J. Biol. Chem.* **2004**, *279*, 53458–53464.
49. Li, J.; Lin, Q.; Yoon, H. G.; Huang, Z. Q.; Strahl, B. D.; Allis, C. D.; Wong, J. Involvement of histone methylation and phosphorylation in regulation of transcription by thyroid hormone receptor. *Mol. Cell. Biol.* **2002**, *22*, 5688–5697.
50. Luo, J.; Li, M.; Tang, Y.; Laszkowska, M.; Roeder, R. G.; Gu, W. Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 2259–2264.
51. Greene, W. C.; Chen, L. F. Regulation of NF- κ B action by reversible acetylation. *Novartis Foundation Symp.* **2004**, *259*, 208/17; Discussion; 218–225.