Published in final edited form as:

Anal Biochem. 2007 April 1; 363(1): 22–34.

Histone H4 Acetylation Dynamics Determined by Stable Isotope Labeling with Amino Acids in Cell Culture and Mass Spectrometry

Xiaodan Su¹, Liwen Zhang^{2,*}, David M. Lucas³, Melanie E. Davis³, Amy R. Knapp⁴, Kari B. Green-Church², Guido Marcucci³, Mark R. Parthun⁴, John C. Byrd³, and Michael A. Freitas^{1,*}

1 Department of Chemistry, The Ohio State University, Columbus, OH 43210, USA

2 Mass Spectrometry and Proteomics Facility, The Ohio State University, Columbus, OH 43210, USA

3 Department of Internal Medicine, The Ohio State University, Columbus, OH 43210, USA

4 Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH 43210, USA

Abstract

This paper describes an integrated approach that couples Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC) to Acetic acid-Urea Polyacrylamide Gel Electrophoresis (AU-PAGE) and Matrix Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) for the quantitation and dynamics of histone H4 acetylation. The 697 acute lymphoblastic cell lines were grown in regular media (Lys-D0) and media in which lysine was substituted with deuterium-labeled lysine (Lys-D4). HDAC activity was inhibited by addition of the HDAC inhibitor depsipeptide to the culture media for different exposure times. Histones were extracted from cells pooled from unlabeled, untreated cells and labeled, treated cells, followed by AU-PAGE separation. Gel bands corresponding to different acetylation states of H4 were excised, in-gel digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. Detailed information was obtained for both the change of histone H4 acetylation specific to N-terminus and global transformation of H4 from one acetylation state to another following treatment with the HDAC inhibitor depsipeptide. The kinetics of H4 acetylation was also assessed. The current study provides quantitative basis for developing potential therapies by using epigenetic regulation and the developed methodology can be applied to quantitation of change for other histone modifications induced by external stimuli.

Introduction

Histones, rich in lysine (K) and arginine (R) residues, are an intensively studied group of basic nuclear proteins and are of great importance in the organization of chromatin structure and control of gene activity [1]. The core histones (H2A, H2B, H3 and H4) electrostatically interact with DNA molecules to form nucleosomes that are the fundamental packaging units of chromatin. A variety of physiologically related post-translational modifications such as methylation, acetylation, phosphorylation, ubiquitination and sumoylation are localized mainly on the N-terminal tail of core histones [2]. Among all of the post-translational modifications of histones, acetylation has been studied most extensively because aberrant

* To whom correspondence should be addressed: Department of Chemistry, The Ohio State University, 100 West 18th Avenue, Columbus, OH 43210, USA. Phone (614) 688-8432, Fax (614) 292-0559, e-mail freitas.5@osu.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

acetylation has been shown to be related to various human diseases, including lupus, leukemia, breast and ovarian cancer, and Huntington disease [3–6].

It has long been appreciated that the reversible acetylation and deacetylation of core histones can regulate transcription, DNA replication and the remodeling of chromosome structures [7]. This dynamic process is mediated by enzyme activities known as histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyze ϵ -N-lysine acetylation resulting in the removal of one positive charge, which alters the electrostatic interaction between neighboring molecules, *eg.* histone-histone or histone-DNA contacts. Thus, in regions of the genome where the histones are largely acetylated, the chromatin structure is relaxed and the DNA molecules are more accessible to regulatory factors including transcription activators, DNA repair machinery and other histone/DNA modifying enzymes. Conversely, the deacetylation of histones by HDACs reinstates the positive charge and thus promotes condensation of chromatin and repression of gene transcription. Recently, HDAC inhibitors have shown marked promise as chemotherapeutic targets in clinical studies [8]. Interestingly, these drugs may work by altering the transcription of a relatively small number of genes abnormally silenced in some genetic subtypes of cancer, rather than non-specific gene activation through chromatin relaxation [9].

A number of HDAC inhibitors have been evaluated for inhibition of tumor growth *in vitro* and *in vivo* with dosages that produce little or no toxicity to normal cells [10–13]. The HDAC inhibitor depsipeptide (DDP, FK228) is one of these agents. DDP has been shown to be cytotoxic to various human tumor cells such as neuroblastomas at submicromolar concentration, with little toxicity against human fibroblasts [3]. In previous work by our group, it has been demonstrated to enhance histone acetylation by inhibiting HDACs in chronic lymphocytic leukemia (CLL) cells concurrent with activation of caspase 8-mediated apoptosis [14,15]. However, the mechanism of action of HDAC inhibitors is not understood and the precise changes in the histones produced by treatment with these agents have not been comprehensively characterized. Previous attempts to examine post-translational modifications of histones relied on antibodies, and these studies were severely limited by reagent availability and specificity. Furthermore, this approach is unable to quantitatively assess the number of acetyl groups added to specific lysine tails of histone proteins. Due to the established importance of histone acetylation in gene regulation and the appearance of aberrant acetylation in tumor cells, as well as the development of therapeutic agents that target this, it is necessary to develop a new method to precisely quantify changes in histone acetylation induced by either disease or drug treatment [16].

Pulse-chase methods have also been used to determine the dynamics of histone acetylation [17]. These methods use radioactive acetate to label histone acetylation sites and can selectively label transcriptionally active regions of chromatin. However, these methods require careful control of pulse time and dose, and are not useful to determine precisely which site is acetylated. More recently, Smith reported a method for quantitation of H4 acetylation using stable isotope labeling and mass spectrometry [18,19]. In their study, the labeling was carried out *in vitro* by acetylating unmodified lysine residues with deuterated acetic anhydride. Each deuterated acetyl group (+45 Da) added an additional 3 Da mass shift compared with endogenous acetylation (+42 Da).

Here we have evaluated another approach that uses stable isotope labeling and mass spectrometry to overcome some of the deficiencies inherent in previously employed methods. Stable Isotopic Labeling with Amino acids in Cell culture (SILAC) has been attracting significant interest recently in mass spectrometry (MS)-based quantitative proteomics since its development for this purpose in 2002 [20–22]. The method is based on substitution of an amino acid in the growth media for another amino acid labeled with a heavy stable isotope, such as

deuterium (^2H). Generally, complete incorporation of labeled amino acids can be achieved for most types of cells in a short period of time (typically after 4 or 5 cell cycles). Thus, differences in labeling efficiency between two samples can be eliminated or at least reduced significantly. The characteristic patterns of light versus heavy peptide pairs in mass spectra of digested proteins can be utilized to identify those peptides containing lysine residues. Therefore, by using SILAC coupled with MS, quick identification of peptides and their posttranslational modifications is made possible [22–26].

In this work, we employed SILAC to identify and quantify histone acetylation changes at the amino acid level in the malignant human lymphoblast cell line 697. In our study lysine was chosen for labeling, as this is the residue on which histone acetylation events occur. Our results demonstrate the efficacy of the integrated methodology (SILAC coupled to MALDI-TOF MS after AU-PAGE separation) for the quantitative determination of H4 acetylation changes induced by HDAC inhibitors and provide an outstanding tool to study in detail the mechanism of action of this promising class of drugs.

Materials and methods

Cell line preparation

The 697 lymphoblast cell line was obtained from DSMZ (Braunschweig, Germany). 697 cells stably transfected either with an empty vector (697-neo) or a vector containing the gene for the anti-apoptotic protein Bcl2 (697-Bcl2) have been described previously [27] and were provided by the laboratory of Dr. John Reed, The Burnham Institute, La Jolla, CA. Depsipeptide (DDP, FK228) was obtained from the National Cancer Institute (Bethesda, MD). Cells were first grown for at least four doubling times in lysine-free RPMI 1640 media (JRH Biosciences; Lenexa, KS) supplemented either with standard L-lysine (Sigma; St. Louis, MO) or deuterium-labeled lysine (L-lysine-4,4,5,5-D₄ HCl, Isotech Inc.; Miamisburg, OH). Media were also supplemented with L-glutamine (final concentration 1 mM) and with fetal bovine serum dialyzed to remove inherent lysine (final concentration 10%) (both from Gibco; Grand Island, NY). Cells were enumerated in triplicate using a Coulter Z1 automated cell counter (Fullerton CA). Cell viability was verified by trypan blue staining, and all samples were greater than 95% live by this test. Following labeling, the enumerated cells were brought to equal concentrations, and incubated with or without 3.0 nM DDP. This resulted in four separate incubations for each cell line: **A**) unlabeled (Lys-D0) and untreated; **B**) labeled (Lys-D4) and treated with DDP; **C**) labeled (Lys-D4) and untreated; **D**) unlabeled (Lys-D0) and treated with DDP. When sufficient cells were available to maintain growth, at least 2×10^8 cells were harvested for each time point and treatment. At the consecutive time points of 0, 4, 8, 12, 24 and 48 hours following addition of drug, equal volumes/cell numbers of **A** and **B** or **C** and **D** were combined as mixture (**A+B**) or (**C+D**), and immediately centrifuged. Media was aspirated off and the cell pellets were frozen in liquid nitrogen for later histone extraction. Also at 4 and 24 hours cells were evaluated by annexin-FITC/propidium iodide flow cytometry [28] to determine if there was significant cell death that would contribute to changes in peptide ratios.

In order to test whether cells containing highly acetylated H4 isoforms were more susceptible to apoptosis, both 697-neo and 697-Bcl2 cells were treated with or without Z-VAD-fmk to inhibit caspase activation and block apoptosis. These cells were then further treated with or without 1 μM 2-F-ara-A (9-beta-D-arabinofuranosyl-2-fluoroadenine) that is metabolically active form of fludarabine, a drug commonly used in CLL that induces caspase-dependent apoptosis.

Histone preparation

Histones were extracted by a standard procedure starting with nuclei isolation buffer (NIB) containing 10 mM Tris-HCl (pH 7.5), 10 mM ZnSO₄, 1.5 mM MgCl₂, 1.0 mM CaCl₂, 0.25 M sucrose, 0.5% Triton X-100, 0.2 mM PMSF, and 3 µg/mL aprotinin (Sigma; St. Louis, MO). The cells were washed with 1 mL NIB followed by resuspension in another 5 mL NIB. Cells were lysed with 20 gentle strokes in a Dounce homogenizer and nuclei were recovered by centrifugation at 4°C. The nuclei pellet was washed twice with 5 mL NIB without Triton X-100 and resuspended in 5 mL 0.4 N H₂SO₄ (ice cold), followed by 30 min incubation on ice. After centrifugation, trichloroacetic acid (TCA) was added to the supernatant with a final concentration of 20% to precipitate histones. The mixture was then incubated for 30 min. The TCA extracted pellet was washed with cold acetone and dried before resuspension in 100 µL 0.1% TFA water solution.

Acetic acid-Urea Polyacrylamide Gel Electrophoresis (AU-PAGE)

Histone isoforms were resolved by acetic acid-urea polyacrylamide gel electrophoresis (AU-PAGE) as described in the literature [29–31] and the protein bands were visualized by Coomassie Blue staining. The identity of proteins within each gel band was determined by MALDI-TOF MS and compared with histone standards extracted from bovine thymus. In brief, after the overnight pre-running of resolving gel made of 6 M urea, 15% (w/v) acrylamide (acrylamide:bisacrylamide = 37.5:1), and 10% (v/v) acetic acid (all from Sigma; St. Louis, MO), 15–25 µg histone mixture denatured at 95°C for 2 min was loaded into each lane of stacking gel composed of 8 M urea, 15% (v/v) acetic acid and 0.05 M potassium acetate (Sigma; St. Louis, MO). The gel separation was conducted with 5% (v/v) acetic acid running buffer at 200 volts for 2 hours and 300 volts for 18 hours in 4°C cold room. The gels were fixed in fixing solution (ethanol: acetic acid: water = 50:10:40) at 4°C for 4 hours, then washed twice (30min each) in washing solution (methanol: acetic acid: water = 50:5:45), and stained with Coomassie Blue for 30 min. After the gels were rinsed in destaining solution (methanol: acetic acid: water = 30:10:60), different bands of H4 acetylation isoforms were excised and cut into approximately 1 mm pieces prior to the in-gel digestion.

Tryptic in-gel digestion

Gel pieces were put in a 96-well ZipPlate (Millipore; Bedford, MA) and digested with porcine trypsin (modified sequencing grade, Promega; Madison, WI) by using the Montage In-Gel Digestion Kit (Millipore; Bedford, MA). The gels were immersed in the washing solution (methanol: acetic acid: water = 50:5:45) twice for one hour before two cycles of dehydration with acetonitrile and rehydration with 100 mM ammonium bicarbonate. After being dried in a Speedvac, the gel pieces were incubated for 10 min on ice in 50 mM ammonium bicarbonate with 300 ng trypsin per gel band. Another 20 µL of 50 mM ammonium bicarbonate was then added and the mixture was incubated at 37°C for 2 hours. With 30–40 µL extraction solution (acetonitrile: formic acid: water = 50:5:45), peptides were extracted from the gels three times. The solution was combined into one tube and concentrated down to 15–20 µL aliquot in a Speedvac.

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

The peptide solution obtained from in-gel digestion was mixed in the ratio of 1:5 with the matrix α -cyano-4-hydroxy cinnamic acid (HCCA) (Bruker; Billerica, MA) that was saturated in 50% ACN / 0.1% TFA / 50% H₂O. An aliquot of 1.0 µL mixture was then spotted on the target plate and air dried before MS analysis. The MALDI-TOF instrument (Bruker Reflex III, Bruker Daltonics Inc; Bremen, Germany) was operated in linear, positive ion mode powered with a N₂ laser. The laser was set at its lowest threshold level necessary to generate signal and the accelerating voltage was performed at 28 kV. The instrument was calibrated by peptide

standards covering molecular weights of the interested peptides in the sample. A mixture of bradykinin fragment 1–5, angiotensin I, [Glu¹]-Fibrinopeptide B and ACTH fragment 18–39 was utilized as a mass standard. Peak integration was carried out for the two groups of isotope distribution by using X-TOF data analysis software. Typically, the total peak area from the first three isotopic peaks was used to calculate the abundance ratio for the light:heavy peptide pairs. For those peptides whose light or heavy isotopic peaks are not observed at the current limit of detection, the corresponding ratio of relative abundance is reported as “not available (N/A).”

Liquid Chromatography Mass Spectrometry (LC-MS)

Histones mixtures were separated by reverse phase HPLC (Waters 2690, Waters; Milford, MA). HPLC separation was conducted on a C18 column (4.6 mm x 250 mm, flow rate 0.5 ml/min) with mobile phase A (0.1% TFA in water) and mobile phase B (0.1% TFA in acetonitrile). The gradient of mobile phase B increased linearly from 25% to 48% in 3 min, then to 60% until 16 min and maintained at 60% B for 6 min. Between each run, the column was washed at 100% B for 1 min and equilibrated at 25% B for 4 min. A Micromass LCT (Micromass; Wythenshawe, UK) mass spectrometer with an orthogonal electrospray source (Z-spray) was coupled to the outlet of the HPLC using a t-flow-splitter. Histones were infused into the electrospray source at the flow rate of about 20 μ L/min (split ratio = 25:1). ESI was performed at the optimal conditions of 3 kV capillary voltage, 100°C source temperature and 50 V cone voltage. Data were acquired in continuum mode at the rate of 1 spectrum sec⁻¹. All spectra were obtained in the positive ion mode. NaI was used for external mass calibration over the m/z range 500–2500.

Results

SILAC and complete Lys-D4 incorporation

Scheme 1 shows a flowchart for the SILAC experiment used to quantify H4 modifications [32]. Briefly, a cell line was grown either in a light media (no amino acid substitution) or in a heavy media (lysine substituted with deuterium-labeled lysine, Lys-D4). The labeled cells were treated with 3.0 nM DDP for 0, 4, 8, 12, 24 and 48 hours. This treatment schedule did not induce significant apoptosis at times up to 24 hours, but induced notable apoptosis at 48 hours as detected by flow cytometric analysis (data not shown). Treated cells were pooled with an equal number of cells from untreated, unlabeled controls at the same time points. This procedure was also reversed (*i.e.* unlabeled, treated cells were mixed with labeled, untreated cells) to verify that there were no effects due purely to the labeling procedure. Histones were extracted from the pooled mixtures and characterized by LC-MS, AU-PAGE and MALDI-TOF MS. It has been shown that SILAC poses no detrimental effects on cell morphology, life cycles, growth rates or biological activity [21,33]. We also observed no changes in the cell growth or apoptosis in the Lys-D4 labeled cells. Thus, we infer that any changes in the acetylation of H4 were due to drug treatment. The changes in acetylation were found by comparing the relative ratios of light and heavy acetylated peptides observed in the MALDI-TOF MS spectra. Peptide abundance ratios were determined by dividing the total area of the peaks for the treated peptide by that of the untreated peptide. To correct for experimental variations (e.g. adherent cells may introduce counting error), the treated: untreated ratio for each peptide pair was normalized to the ratio for a control sample in which no drug was added, *i.e.* the time point corresponding to 0-hour treatment (Scheme 1). Consequently, the ratio for each histone H4 acetylation isoform in the control sample was normalized to 1. The trend in histone acetylation was then plotted as the ratios of the treated to the untreated abundance versus exposure time.

The SILAC strategy is a straightforward labeling process with no additional purification steps required for removal of excess labeling reagent, and labeling efficiency can approach 100%. Preliminary experiments were performed to investigate the appropriate growth time for maximum incorporation of Lys-D4. At different growth times, equal numbers of cells (5×10^7) were removed from the labeled and unlabeled media. Histones were extracted and analyzed by LC-MS. The results showed that complete incorporation of labeled lysine was achieved after four cell doublings. At longer growth time, no significant difference in labeling was observed. A mass shift of 44 Da for histone H4 (11 lysine residues) was used as the indicator of complete Lys-D4 incorporation (Figure 1A).

Quantitation of differential H4 acetylation by MALDI-TOF MS

Histone isoforms in these cell lines were separated by AU-PAGE based on difference in their size and charge (Figure 1B) [29–31]. The stained gels show a slight increase in the abundance of di-, tri- and tetra-acetylated isoforms of H4 after exposure to DDP. The gel bands for each H4 acetylation isoform were excised, in-gel digested and analyzed by MALDI-TOF MS as described in “Materials and methods” section. For consistency, at each time point, a total of 5 gel bands corresponding to non-, mono-, di-, tri- and tetra-acetylated isoforms were excised even when the hyperacetylated H4 isoforms were not visualized, as was the case for the non-treated samples and the samples with shorter exposures to DDP. On average the amino acid sequence coverage was 65% for each H4 isoform. To obtain the highest precision in peptide abundances samples were spotted three times on the MALDI target plate and each spot was analyzed three times, resulting in nine data files per sample. After normalization, the ratios were plotted versus exposure time accordingly.

Acidic conditions were used throughout the histone isolation and analysis, which could raise concerns that the native acetylation state is altered. However, based on long-term observations of histone acetylation profiles, the acetylation patterns appear to be extremely stable once after cellular extraction. Protease inhibitors were added to the nuclei isolation buffer (NIB) to minimize protein degradation that may also affect the observed profiles. The effects of 0.1% TFA on hydrolysis was assessed by resuspending histone pellets in both pure HPLC-grade water and 0.1% TFA water solution before heat denaturation. No significant difference between the two resuspensions was observed (data not shown).

Figure 2 shows one example of the MALDI-TOF mass spectra, which corresponded to the diacetylated H4 isoform from the 697 cells (A+B) after 24-hour exposure to DDP. The three enlarged insets show the peptide pairs for $^{60}\text{VFLENVIR}^{67}$, $^{24}\text{DNIQGITKPAIR}^{35}$ and $^9\text{GLGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$. These peptides contain zero, one and two lysine residues, respectively. The light and heavy peak clusters facilitate identification of the presence and number of lysine residues within a peptide. For example, the labeled peptides for $^9\text{GLGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$ and $^{24}\text{DNIQGITKPAIR}^{35}$ are shifted 8 and 4 Da higher in mass, respectively. Furthermore, peptides that do not have an isotopic partner do not contain lysine (or partner is below detection limit) and hence were excluded from the quantitation analysis. For histone H4 several light/heavy peptide pairs were observed. The most abundant acetylated forms of these peptides corresponded to the N-terminal tail. Acetylation of K16, K12, K8 and K5 was determined from the peptide ratios for $^{13}\text{GGAK}(\text{Ac})\text{R}^{17}$ (530.44^+ Th), $^9\text{GLGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$ (927.96^+ Th), $^4\text{GKGK}(\text{Ac})\text{GLGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$ and/or $^4\text{GK}(\text{Ac})\text{GKGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$ (1397.26^+ Th), and $^4\text{GK}(\text{Ac})\text{GKGK}(\text{Ac})\text{GLGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$ (1439.25^+ Th). In the mixture of labeled and unlabeled samples, these peptides contained 1, 2, 4 and 4 lysine residues, resulting shifts in the mass spectra of 4, 8, 16 and 16 Th, respectively (Figure 3). For the sake of presentation, the spectra in Figures 3A–3D, for the 5 time points were uniformly scaled. In Figure 3D, the isotopic distribution for the unlabeled and untreated species is not visible due

to this scaling. However, the isotopic distribution is indeed present and clearly visible upon magnification.

Another interesting tryptic peptide is the fragment $^{24}\text{DNIQGITKPAIR}^{35}$. It was the only abundant lysine-containing but non-acetylated peptide present in *all* the MALDI-TOF mass spectra. The enzymatic cleavage at K31 is inhibited by the existence of a proline residue in position +1, immediately C-terminal to the cleavage site [34]. Although trypsin is highly specific for lysyl and arginyl bonds, peptide bonds like lysylproline are generally trypsin-resistant with rare exceptions [35]. No modified forms of this peptide (or other peptides containing K31) were observed. This peptide was thus used to determine the relative change in the total H4 content within each gel band.

Time dependent change in H4 acetylation influenced by apoptosis

Assessment of multiple DDP concentrations and treatment time points demonstrated a dramatic decrease in the number of higher acetylated isoforms after 24-hour treatment for all examined DDP concentrations relative to the media control. This relative decrease can be related to a reduction in the number of cells that exhibited hyperacetylation as a consequence of apoptosis induced by DDP, as we have shown previously [14,15]. This hypothesis is supported by corresponding annexin/propidium iodide (PI) flow cytometry data, which indicates that apoptosis is induced in 697 cells treated for extended times (*i.e.* 24 hours) with 3.0 nM DDP (not shown). Additionally, inhibition of apoptosis occurred in 697 cells that overexpress the anti-apoptotic protein Bcl-2 (697-Bcl2) [27,36,37]. Relative to 697 cells transfected with an empty vector (697-neo), 697-Bcl2 cells exhibit substantial resistance to apoptosis mediated by a variety of anticancer agents, including DDP. As an example, the comparison of annexin/PI flow cytometry results between these two cell lines after 24-hour DDP (1.9 nM) treatment is shown in Figure 4. Under these conditions, little apoptosis (9.2% positive for both annexin and PI) was noted in the 697-Bcl2 cells, while significant apoptosis (85.1% annexin and PI positive) was observed in 697-neo cells. Histone H4 had a greater abundance of highly acetylated isoforms (*i.e.* tri- and tetra-acetylated) in the DDP-treated 697-Bcl2 cells relative to 697-neo cells. This result suggests that the cells containing the highly acetylated H4 isoforms are more susceptible to apoptosis. To test this hypothesis, both cell lines were treated with Z-VAD-fmk to inhibit caspase activation and block apoptosis. The 697-neo cells pre-incubated with Z-VAD-fmk followed by subsequent treatment with 190 nM DDP for 24 hours, showed no significant increase in annexin positivity relative to untreated cells. However, the DDP-induced pattern of histone acetylation that was seen in 697-Bcl2 cells was then observed in these DDP-treated 697-neo cells. To show that changes in acetylation were due to DDP treatment and not simply to the process of apoptosis, cells from both 697 lines were incubated with and without Z-VAD-fmk. These cells were then treated with or without 1 μM 2-F-ara-A (9-beta-D-arabinofuranosyl-2-fluoroadenine), the metabolically active form of fludarabine, a drug commonly used in CLL that induces caspase-dependent apoptosis. The resulting data, shown in Figure 4, indicates that although Z-VAD-fmk almost completely blocks 2-F-ara-A-mediated apoptosis, the pattern and extent of H4 acetylation remains unchanged. Taken together, these data indicate that the observed histone acetylation changes following HDAC inhibitor treatment can be affected by apoptosis, requiring close attention to this variable in experimental design. Thus for our purposes we did not include data from a treatment at 48 hours due to the likelihood that data at this time point is severely affected by loss of cells due to apoptosis.

Dynamics of histone H4 acetylation at the N-terminal tail

Figure 5A shows the time-dependent changes in histone acetylation for the 697 cell line (A +B) (Tables containing the average and standard deviations for all the data are also provided in the supplementary material). The abundance of the K16 (Ac) increased slightly up to 4-hour exposure to DDP and then decreased at exposure times longer than 4 hours. The abundance

ratios were obtained from the peptide $^{13}\text{GGAK}(\text{Ac})\text{R}^{17}$. The decrease in the abundance of this peptide suggests acetylation may be occurring at K12 (or methylation of R17) resulting in the inhibition of trypsin cleavage [18]. It was noted that the decrease in abundance of this peptide coincides with an increase in the abundance of $^9\text{GLGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$.

In all cases the di-, tri- and tetra-acetylated isoforms increased relative to the untreated control until 24 hours of exposure. In nearly all cases the tri- and/or tetra-acetylated isoforms were present in low abundance in the untreated cells and were not detected by MALDI-TOF MS. As a result, some of the relative peptide ratios could not be calculated (e.g. tetra-acetylated peptides with 0 and 4 hour DDP exposure) and were not plotted in Figure 5. However, increases in the tri- and tetra-acetylated isoforms were observed after 4 hours when accumulation of diacetylation became significant. The origin of the delay is likely due to the kinetics of conversion of the K16 (Ac), K12 (Ac) isoform to the triacetylated species. However, because of the uncertainty in the absolute abundance of the peptides determined by MALDI TOF MS, a full kinetic analysis for the specific isoforms was not possible.

The quantitation of K16 and K12 acetylation was straightforward since a single peptide pair for each isoform was observed and used for quantitation. However, the assignment of the order of acetylation for K8 and K5 proved to be more challenging. Three peptides were present that suggested the appearance at least two different isoforms in which either K8 or K5 were acetylated following K16 and K12. The peptides $^6\text{GGK}(\text{Ac})\text{GLGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$ (1211.80+ *Th*) and $^9\text{GLGK}(\text{Ac})\text{GGAK}(\text{Ac})\text{R}^{17}$ (927.69+ *Th*), were observed at very low abundance or were undetectable in nearly all the triacetylated bands. In contrast, the triacetylated state of peptide $^4\text{GKGGKGLGKGGAKR}^{17}$ was present as a major peak in the mass spectrum. This peptide provides inconclusive evidence as to whether K8 or K5 is acetylated in addition to K16 and K12. The presence of the former low abundant peptides suggests that cleavage by trypsin can occur after either K8 or K5; thus, the triacetylated peptide 4–17 may be a mixture in which K5 or K8 is acetylated or may result from incomplete digestion. It is not possible to conclude from the MALDI TOF MS data which isoform is present in greater abundance, as they are isobaric. However, these results provide support for our previous work in which DDP was demonstrated to induce histone H3 and H4 acetylation, inhibit HDAC activity, and cause cell apoptosis *in vivo* at concentrations corresponding to an LC_{50} (concentration producing 50% cell death) in cultured CLL cells [14]. In this work that was based on immunological assays, increases in acetylation of H4 were shown to be specifically occurring on K5, K12 and to a lesser extent K8, but not K16. In the current study, the peptide fragment from 4–17 is present at a greater abundance than the fragment 9–17, which supports the earlier findings concluding that K5 acetylation is more predominant than K8. These results indicate that DDP induced increase of histone H4 acetylation occurred first on K12 and then on either K8 or K5, but with K5 most likely being the predominant species. The data herein (Figure 5) also show a minor increase in K16 acetylation (~10%) for monoacetylated H4 isoform after 4 hour DDP treatment. A change of this magnitude would be difficult to detect immunologically suggesting that mass spectrometry based approaches have superior sensitivity for quantitation of changes in histone modification. In addition, the techniques used here provide valuable information concerning the ground state modification level that is impossible to assess via Western blot.

The data in this current study also supports the results reported by other groups. Several investigators have shown by peptide mass mapping that the N-terminal tail of histone H4 consists primarily of multiple methylations at lysine 20 combined with acetylation of K16, K12, K8 and K5 [38–41]. Zhang *et al.* have proposed a model for the acetylation of the N-terminal tail of H4, in which K16 is acetylated first followed by K12, K8 and finally K5 [42]. However, our current report improves significantly upon these observations by separating the

isoforms prior to analysis and performing SILAC to elucidate relative changes in the isoforms upon treatment with the HDAC inhibitor DDP [40,43].

Global change of H4 acetylation

Quantitative results based on the fragment $^{24}\text{DNIQGITKPAIR}^{35}$ pair are shown in Figure 6. The peptide abundance ratios for the $^{24}\text{DNIQGITKPAIR}^{35}$ peptide pair showed similar trends to those of the acetylated N-terminus peptides (Figure 5). However, the ratios for this peptide were smaller. For instance, after 24-hour DDP exposure, the 697 cells showed a 7-fold increase in abundance of the peptide $^9\text{GLGK(Ac)GGAK(Ac)R}^{17}$ from the diacetylated gel band. In contrast, only a 2-fold increase was observed for $^{24}\text{DNIQGITKPAIR}^{35}$ from the same gel band. Thus the turnover of diacetylated K16 and K12 isoform does not solely account for the total turnover of H4 in this gel band. This discrepancy may be due to the difference in turnover of histones in transcriptionally inactive heterochromatin that undergo acetylation induced by DDP treatment at a slower rate. Another possibility is that that DDP selectively targets HDACs with specificity for lysines located within the N-terminal tail. The effect could also be due to a combination of the two aforementioned scenarios.

Discussion

The quantitative investigation of histone acetylation dynamics, both under steady-state and non-steady-state conditions, has been ongoing for more than 30 years. Previous acetylation turnover measurements were based on pulse-chase or continuous-label techniques. As indicated by Waterborg, histone acetylation follows an exponential trend (either increasing or decreasing) due to a respective imbalance in HDAC or HAT activity [17]. Typically fast and slow turnover rates are observed, where the faster rate of acetylation is attributed to transcriptionally active chromatin. Both pulse-chase and continuous-label techniques rely on the incorporation of radiolabeled acetyl-CoA. Low concentrations of radioactive acetate must be used to prevent acidification of the cytoplasm, reducing sensitivity. In addition, the residual radioactive acetate must be removed from the cytoplasm in pulse-chase experiments prior to determination of acetylation dynamics. Furthermore, the length of the initial pulse of radioactive acetate can have an influence on the population of histones that are modified, *i.e.* those that are located in transcriptionally active sites of chromatin. These shortcomings have the potential to produce bias in the calculated results. With the SILAC approach presented here, no acetate is being added to the cells and all acetylated peptides are analyzed directly. In principle, residues both in transcriptionally active and silent chromatin will be labeled and a true rate of histone turnover can be determined.

In these experiments, we observed an imbalance in H4 acetylation due to HDAC inhibition by DDP. Since the global change of H4 acetylation within each gel band can be observed for the $^{24}\text{DNIQGITKPAIR}^{35}$ peptide pair, the corresponding kinetics of transformation of this pair could thus be determined and related to global transformation. To calculate the rate, the time-dependent change in the peptide ratios was modeled to fit parallel pseudo-first order reaction by use of Equation 1 (Eq.1, Figure 7). Rates constants were calculated by fitting the observed decrease in acetylation to two parallel first order rate equations. We fitted the rate equation to the rate constants K_1 , K_2 and the percentage of H4 in a given population. The best fit was determined by use of maximum likelihood where Chi^2 was calculated and minimized. Multiple sets of initial conditions were used to find the reported global minimum. The two reaction rates observed demonstrated that acetylation of H4 in 697 cells behaved as if two distinct populations were present. One population (57.2% of the total nonacetylated H4) was acetylated at a relatively fast rate, $t_{1/2} = 4.1$ h. The second population, making up the remainder of H4, gave a much slower rate, $t_{1/2} > 27$ h. These trends in acetylation are consistent with those observed

in pulse-chase studies and lend credibility of SILAC as an unbiased approach to monitor histone acetylation dynamics.

$$\ln \left(\frac{N_0}{N} \right) = kt, \quad t_{1/2} = \frac{0.693}{k} \quad \text{Eq. 1}$$

(Where N_0 is the original relative abundance, N is the changed relative abundance, $\ln$ is natural log function, and t is period of time).

The *in vivo* relevance of the faster and slower populations of histone hyperacetylation remains unclear, but related work sheds light on this issue. Ip *et al.* [44] demonstrated that the higher level of H4 acetylation occurs faster in rat hepatoma when the chromatin was actively transcribed, but the slower rate of hyperacetylation of H4 was independent of DNA transcription. Other studies on histones from maturing chicken erythrocytes [45] and from newly synthesized chromatin in rat hepatoma [46] gave the same conclusions. One possible explanation to the slower hyperacetylation could be that it would take time to convert an inactive gene domain to an active or potentially active one [17]. This inactive to active gene transition is controlled by the slow response of chromatin to the change of HAT/HDAC net activity, induced externally. This explanation is consistent with the results presented herein.

Accuracy and precision are important issues in the application of quantitative analysis. In our study, the accuracy was confirmed by use of an inverse labeling strategy. In these experiments, the treated to untreated abundance ratios from (C+D) groups gave consistently comparable results (supplementary material) with 10–15% relative deviation to those from (A+B) groups (Figure 5A). As shown in Equation 2 (Eq. 2), if the experimental procedures are reproducible, the product of the ratio (A/B) and ratio (D/C) should be “1”.

$$\frac{\text{Unlabeled Untreated}}{\text{Labeled Treated}} * \frac{\text{Unlabeled Treated}}{\text{Labeled Untreated}} = 1 \quad \text{Eq. 2}$$

The products of A/B and D/C (as shown in Eq. 2) ratios were plotted for the different exposure times for the diacetylated isoforms (Figure 5B). The average product of the ratios was 1.0 with a relative standard deviation (RSD) of less than 20%, further validating the efficiency of our quantitation methodology. The RSD (%) for the ratios for mono- and diacetylation were less than 10. The RSD (%) for tri- and tetra-acetylation isoforms were much larger (eg. at 24 hours, (A+B) ratios for tetra-acetylation varied from 99.24 to 148.8, with a RSD = 28.23%). This large range of ratios was the result of the low abundance of hyperacetylated peptides in the untreated samples. Even small signal fluctuations in low abundant peptides result in significant changes in the relative abundance ratios. Thus, higher accuracy of quantitation can only be achieved if signals selected for quantitation are well above background and the two isotopic envelopes are fully resolved [21].

The application of our methodology points to the limitation of studying histone modifications using antibody-based methods. Specifically, previous studies with DDP using antibody-based approaches have not shown dramatic changes in H4 K16 acetylation. This might be a consequence of the time kinetics of this modification that is influenced by apoptosis, or the lack of sensitivity of available antibody reagents to identify changes described herein. This work provides evidence that the SILAC method is a strong tool to study post-modification changes in histone proteins.

In conclusion, HDAC inhibitors are potentially important therapeutic agents and a detailed characterization of the specific molecular alterations caused by these agents is necessary for understanding their mechanism of action and for efforts to develop improved compounds. To this end, we have combined stable isotopic labeling with AU-PAGE and MALDI-TOF mass

spectrometry to create an effective means of quantifying the precise changes in histone acetylation that result from the treatment of cells with an HDAC inhibitor. This approach provides much more detail than is currently available using conventional antibody-based techniques. These studies also reveal modifications not previously detectable by immunoblot analysis, and demonstrate the importance of controlling for apoptosis in biologic studies of HDAC inhibitors. In addition, the basic strategies described here are flexible and can be applied to the analysis of other potential targets of HDAC inhibitors, such as Hsp90, or extended through the use of double labeling to analyze the interplay between different types of modifications (*e.g.* acetylation and methylation). Such detailed assessment will be important to understanding the importance (or lack thereof) of post-transcriptional histone modifications induced by HDAC inhibitors on gene re-expression and apoptosis.

We successfully applied the SILAC method, coupled to AU-PAGE and MALDI-TOF MS, to the quantitation of histone H4 acetylation in response to DDP treatment. The data showed interesting trends of histone acetylation in a lymphoid leukemia cell line: after 24-hour treatment, monoacetylation of K16 decreased to 0.60; diacetylation of K12 and K16 increased by a factor of 6.0; triacetylation of K16, K12 and either K8 or K5 increased by a factor of more than 30; tetraacetylation of K16, K12, K8 and K5 increased by a factor of more than 100. Global transformation of histone H4 from one acetylation state to the next was also determined by monitoring the unmodified peptide ²⁴DNIQGITKPAIR³⁵. From these data the reaction rates for H4 acetylation was determined under these conditions. The fast rate of loss of nonacetylated H4 in 697 cells was $t_{1/2} = 4.08$ hours corresponding to 57.2% of total histones; the slow rate was $t_{1/2} > 27$ hours observed for the remaining histone population. The method was validated by use of inverse labeling, which revealed good quantitative precision.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Chen Ren for assistance with AU-PAGE, Dr. Kalpana Ghoshal and Samson T Jacob for access to laboratory equipment, and Nanette Kleinholz, Rhonda Pitsch, Ben Jones and Kari Green-Church at Campus Chemical Instrumentation Center for assistance with in-gel digestion, MALDI-TOF MS and LC-MS. The study was funded by the Ohio State University, the Camille and Henry Dreyfus Foundation, the American Society for Mass Spectrometry and National Institutes of Health CA107106 (to M.A.F), National Institutes of Health GM62970 (to M.R.P.) and National Institutes of Health CA102031 (to GM). The authors are recipients of a V Foundation/American Association for Cancer Research Translational Cancer Research Grant and are also supported by National Institutes of Health R21 CA110496. JCB is supported by the Leukemia & Lymphoma Society and the D. Warren Brown foundation.

References

1. Lindner H, Sarg B, Meraner C, Helliger W. Separation of acetylated core histones by hydrophilic-interaction liquid chromatography. *J Chromatogr A* 1996;743:137–44. [PubMed: 8817877]
2. Freitas MA, Sklenar AR, Parthun MR. Application of mass spectrometry to the identification and quantification of histone post-translational modifications. *J Cell Biochem* 2004;92:691–700. [PubMed: 15211567]
3. Marks PA, Richon VM, Rifkind RA. Histone deacetylase inhibitors: Inducers of differentiation or apoptosis of transformed cells. *J Natl Cancer Inst* 2000;92:1210–6. [PubMed: 10922406]
4. Arts J, de Schepper S, Van Emelen K. Histone deacetylase inhibitors: From chromatin remodeling to experimental cancer therapeutics. *Curr Med Chem* 2003;10:2343–50. [PubMed: 14529477]
5. Richon VM, Sandhoff TW, Rifkind RA, Marks PA. Histone deacetylase inhibitor selectively induces p21waf1 expression and gene-associated histone acetylation. *Proc Natl Acad Sci U S A* 2000;97:10014–9. [PubMed: 10954755]
6. Zhang L, Freitas MA, Wickman J, Parthun MR, Klisovic MI, Marcucci G, Byrd JC. Differential expression of histone post-translational 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. [PubMed: 14698558]
7. Allfrey VG, Faulkner R, Mirsky AE. Acetylation and methylation of histones and their possible role in the regulation of rna synthesis. *Proc Natl Acad Sci U S A* 1964;51:786–94. [PubMed: 14172992]
 8. Mei S, Ho AD, Mahlknecht U. Role of histone deacetylase inhibitors in the treatment of cancer (review). *Int J Oncol* 2004;25:1509–19. [PubMed: 15547685]
 9. Chiurazzi P, Neri G. Reactivation of silenced genes and transcriptional therapy. *Cytogenet Genome Res* 2003;100:56–64. [PubMed: 14526164]
 10. Kwon HJ, Owa T, Hassig CA, Shimada J, Schreiber SL. Depudecin induces morphological reversion of transformed fibroblasts via the inhibition of histone deacetylase. *Proc Natl Acad Sci U S A* 1998;95:3356–61. [PubMed: 9520369]
 11. Yoshida M, Kijima M, Akita M, Beppu T. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin a. *J Biol Chem* 1990;265:17174–9. [PubMed: 2211619]
 12. Kijima M, Yoshida M, Sugita K, Horinouchi S, Beppu T. Trapoxin, an antitumor cyclic tetrapeptide, is an irreversible inhibitor of mammalian histone deacetylase. *J Biol Chem* 1993;268:22429–35. [PubMed: 8226751]
 13. Newmark HL, Lupton JR, Young CW. Butyrate as a differentiating agent: Pharmacokinetics, analogues and current status. *Cancer Lett* 1994;78:1–5. [PubMed: 8180951]
 14. Aron JL, Parthun MR, Marcucci G, Kitada S, Mone AP, Davis ME, Shen T, Murphy T, Wickham J, Kanakry C, Lucas DM, Reed JC, Grever MR, Byrd JC. Depsipeptide (fr901228) induces histone acetylation and inhibition of histone deacetylase in chronic lymphocytic leukemia cells concurrent with activation of caspase 8-mediated apoptosis and down-regulation of c-flip protein. *Blood* 2003;102:652–8. [PubMed: 12649137]
 15. Byrd JC, Shinn C, Ravi R, Willis CR, Waselenko JK, Flinn IW, Dawson NA, Grever MR. Depsipeptide (fr901228): A novel therapeutic agent with selective, in vitro activity against human b-cell chronic lymphocytic leukemia cells. *Blood* 1999;94:1401–8. [PubMed: 10438728]
 16. Wang YK, Ma Z, Quinn DF, Fu EW. Inverse 18o labeling mass spectrometry for the rapid identification of marker/target proteins. *Anal Chem* 2001;73:3742–50. [PubMed: 11510843]
 17. Waterborg JH. Dynamics of histone acetylation in vivo. A function for acetylation turnover? *Biochem Cell Biol* 2002;80:363–78. [PubMed: 12123289]
 18. Smith CM, Gafken PR, Zhang Z, Gottschling DE, Smith JB, Smith DL. Mass spectrometric quantification of acetylation at specific lysines within the amino-terminal tail of histone h4. *Anal Biochem* 2003;316:23–33. [PubMed: 12694723]
 19. Smith CM. Quantification of acetylation at proximal lysine residues using isotopic labeling and tandem mass spectrometry. *Methods* 2005;36:395–403. [PubMed: 16098763]
 20. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. Stable isotope labeling by amino acids in cell culture, silac, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 2002;1:376–86. [PubMed: 12118079]
 21. Ong SE, Foster LJ, Mann M. Mass spectrometric-based approaches in quantitative proteomics. *Methods* 2003;29:124–30. [PubMed: 12606218]
 22. Pan S, Gu S, Bradbury EM, Chen X. Single peptide-based protein identification in human proteome through maldi-tof ms coupled with amino acids coded mass tagging. *Anal Chem* 2003;75:1316–24. [PubMed: 12659191]
 23. Gu S, Pan S, Bradbury EM, Chen X. Use of deuterium-labeled lysine for efficient protein identification and peptide de novo sequencing. *Anal Chem* 2002;74:5774–85. [PubMed: 12463361]
 24. Gu S, Pan S, Bradbury EM, Chen X. Precise peptide sequencing and protein quantification in the human proteome through in vivo lysine-specific mass tagging. *J Am Soc Mass Spectrom* 2003;14:1–7. [PubMed: 12504328]
 25. Zhu H, Hunter TC, Pan S, Yau PM, Bradbury EM, Chen X. Residue-specific mass signatures for the efficient detection of protein modifications by mass spectrometry. *Anal Chem* 2002;74:1687–94. [PubMed: 12033261]
 26. Zhu H, Pan S, Gu S, Bradbury EM, Chen X. Amino acid residue specific stable isotope labeling for quantitative proteomics. *Rapid Commun Mass Spectrom* 2002;16:2115–23. [PubMed: 12415544]

27. Miyashita T, Reed JC. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood* 1993;81:151–7. [PubMed: 8417786]
28. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin v. *J Immuno Methods* 1995;184:39–51.
29. Panyim S, Chalkley R. High resolution acrylamide gel electrophoresis of histones. *Arch Biochem Biophys* 1969;130:337–46. [PubMed: 5778650]
30. Pastink A, Berkhout TA, Mager WH, Planta RJ. Analysis of histones from the yeast *saccharomyces carlsbergensis*. *Biochem J* 1979;177:917–23. [PubMed: 220954]
31. Waterborg JH, Harrington RE. Western blotting of histones from acid-urea-triton- and sodium dodecyl sulfate-polyacrylamide gels. *Anal Biochem* 1987;162:430–4. [PubMed: 3605607]
32. MacCoss MJ, Wu CC, Liu H, Sadygov R, Yates JR 3rd. A correlation algorithm for the automated quantitative analysis of shotgun proteomics data. *Anal Chem* 2003;75:6912–21. [PubMed: 14670053]
33. Ong SE, Kratchmarova I, Mann M. Properties of ¹³C-substituted arginine in stable isotope labeling by amino acids in cell culture (silac). *J Proteome Res* 2003;2:173–81. [PubMed: 12716131]
34. Nilsson I, Heijne Gv. A signal peptide with a proline next to the cleavage site inhibits leader peptidase when present in a sec-independent protein. *Federation of European Biochemical Societies* 1992;299:243–246.
35. Kieliszewski MJ, Leykam JF, Lampion DT. Trypsin cleaves lysylproline in a hydroxyproline-rich glycoprotein from *zea mays*. *Pept Res* 1989;2:246–8. [PubMed: 2520761]
36. Lucas DM, Davis ME, Parthun MR, Mone AP, Kitada S, Cunningham KD, Flax EL, Wickham J, Reed JC, Byrd JC, Grever MR. The histone deacetylase inhibitor ms-275 induces caspase-dependent apoptosis in b-cell chronic lymphocytic leukemia cells. *Leukemia* 2004;18:1207–1214. [PubMed: 15116122]
37. Tang C, Willingham MC, Reed JC, Miyashita T, Ray S, Ponnathpur V, Huang Y, Mahoney ME, Bullock G, Bhalla K. High levels of p26bcl-2 oncoprotein retard taxol-induced apoptosis in human pre-b leukemia cells. *Leukemia* 1994;8:1960–9. [PubMed: 7526093]
38. Schotta G, Lachner M, Peters AH, Jenuwein T. The indexing potential of histone lysine methylation. *Novartis Found Symp* 2004;259:22–37. 37–47, 163–9. [PubMed: 15171245]
39. Zhang Y, Reinberg D. Transcription regulation by histone methylation: Interplay between different covalent modifications of the core histone tails. *Genes Dev* 2001;15:2343–60. [PubMed: 11562345]
40. Bannister AJ, Schneider R, Kouzarides T. Histone methylation: Dynamic or static? *Cell* 2002;109:801–6. [PubMed: 12110177]
41. Zhang L, Eugeni EE, Parthun MR, Freitas MA. Identification of novel histone post-translational modifications by peptide mass fingerprinting. *Chromosoma* 2003;112:77–86. [PubMed: 12937907]
42. Zhang K, Williams KE, Huang L, Yau P, Siino JS, Bradbury EM, Jones PR, Minch MJ, Burlingame AL. Histone acetylation and deacetylation: Identification of acetylation and methylation sites of *hela* histone h4 by mass spectrometry. *Mol Cell Proteomics* 2002;1:500–8. [PubMed: 12239278]
43. Ren C, Zhang L, Freitas MA, Ghoshal K, Parthun MR, Jacob ST. Peptide mass mapping of acetylated isoforms of histone h4 from mouse lymphosarcoma cells treated with histone deacetylase (hdacs) inhibitors. *J Am Soc Mass Spectrom* 2005;16:1641–53. [PubMed: 16099169]
44. Ip YT, Jackson V, Meier J, Chalkley R. The separation of transcriptionally engaged genes. *J Biol Chem* 1988;263:14044–52. [PubMed: 3170538]
45. Zhang DE, Nelson DA. Histone acetylation in chicken erythrocytes. Rates of acetylation and evidence that histones in both active and potentially active chromatin are rapidly modified. *Biochem J* 1988;250:233–40. [PubMed: 2451508]
46. Cousens LS, Alberts BM. Accessibility of newly synthesized chromatin to histone acetylase. *J Biol Chem* 1982;257:3945–9. [PubMed: 7061519]

Keyword

SILAC

stable isotope labeling with amino acids in cell culture

AU-PAGE

acetic acid-urea polyacrylamide gel electrophoresis

MALDI-TOF MS

matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry

LC-MS

liquid chromatography-mass spectrometry

HDAC

histone deacetylase

DDP

depsipeptide

Lys-D4

deuterium-labeled lysine

Figure 1.

(1A) Complete incorporation of Lys-D4 into histone H4: A mass shift of 44 Da for histone H4 (11 lysine residues) was used as the indicator of complete Lys-D4 incorporation. **(1B)** AU-PAGE pattern of histone H4 acetylation in the 697 cell line treated for various times with 3.0 nM depsipeptide. The gel was stained by Coomassie Blue. AcX refers to the number of acetylation events.

Figure 2.

MALDI-TOF mass spectrum for the diacetylated H4 isoform from the 697 cells (A+B) after 24 hour exposure to DDP. The three insets show the peptides pairs for ⁹GLGK(Ac)GGAK(Ac)R¹⁷, ⁶⁰VFLENVIR⁶⁷, and ²⁴DNIQGITKPAIR³⁵.

(3A) Monoacetylation **(3B) Diacetylation**

Figure 3.

Characteristic patterns of isotope distribution of peptides specific to different H4 acetylation isoforms in MALDI-TOF mass spectra. The dynamics of H4 acetylation in 697 cell line is shown in (3A) through (3D) in the following order: mono-, di-, tri- and tetra-acetylated.

Figure 4.

Effect of apoptosis on distribution of acetylated H4 isoform: 697 cells transfected with an empty vector (neo) or a vector containing the bcl-2 gene (Bcl2) were incubated for 24 hours with depsipeptide (DDP) (1.9 nM), the metabolically active form of fludarabine (F-ara A) (1.0 μ M), or either drug in combination with the pancaspase inhibitor Z-VAD-fmk (150 μ M). Cells were analyzed for apoptosis by annexin/PI flow cytometry, and histones were extracted and separated by LC-MS. The mass of unmodified H4 is 11236 Da. Addition of an acetylation at the N-terminal serine (H4(N-Ac)) shifts its mass to 11278 Da. DiMe and Ac represent dimethylation and acetylation, respectively. The % values refer to the percentiles of cells that have undergone apoptosis in response to different treatment.

Figure 5.

Statistical analysis of time-dependent change in acetylation for the different H4 isoforms in of 697 (A+B) cells (**5A**). **5B** (upper) shows the results of reverse labeling for the diacetylated peptides and 5B (bottom) shows the product of ratio (A/B) and ratio (D/C).

Figure 6. Global transformation of H4 acetylation isoforms as a function of exposure period to DDP. The quantitative results were obtained from the SILAC data of fragment $^{24}\text{DNIQGITKPAIR}^{35}$.

Figure 7. Dynamics of Histone H4 acetylation. Two rates for H4 turnover in 697 cells were determined. A fast rate ($t_{1/2} = 4.08$ hours) was observed corresponding to 57.2% of H4 and a slow rate ($t_{1/2} > 27$ hours) was observed for the remaining H4.

Scheme 1.

Schematic flowchart for quantitation of histone H4 modifications by SILAC.