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# Application of Mass Spectrometry to the Identification and Quantification of Histone Post-Translational Modifications

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**Abstract** The core histones are the primary protein component of chromatin, which is responsible for the packaging of eukaryotic DNA. The $NH_2$-terminal tail domains of the core histones are the sites of numerous post-translational modifications that have been shown to play an important role in the regulation of chromatin structure. In this study, we discuss the recent application of modern analytical techniques to the study of histone modifications. Through the use of mass spectrometry, a large number of new sites of histone modification have been identified, many of which reside outside of the $NH_2$-terminal tail domains. In addition, techniques have been developed that allow mass spectrometry to be effective for the quantitation of histone post-translational modifications. Hence, the use of mass spectrometry promises to dramatically alter our view of histone post-translational modifications. J. Cell. Biochem. 92: 691–700, 2004. © 2004 Wiley-Liss, Inc.

**Key words:** chromatin; mass spectrometry; histone; post-translational modification

The study of histone post-translational modifications began in 1964 with the identification by K. Murray of ε-methyllysine in acid hydrolysates of calf thymus histones [Murray, 1964]. Soon thereafter, ε-acetyllysine and phosphoserine residues were also found in the core histones [Kleinsmith et al., 1966; Ord and Stocken, 1967; DeLange et al., 1968; Gershey et al., 1968; Vidali et al., 1968]. Later, the repertoire of modifications was extended to include ubiquitylation, ADP-ribosylation and, most recently, sumoylation [Goldknopf et al., 1975; Ueda et al., 1975; Hunt and Dayhoff, 1977; Shiio and Eisenman, 2003]. Until recently, the precise positions of these modifications were ascertained, in most instances, by Edman degradation sequencing of either intact proteins or proteolytic fragments. These analyses indicated that the vast majority of histone modifications reside in the $NH_2$-terminal tail domains. While a number of excellent studies have detailed their functional significance, this study will focus on recent advances in the analytical characterization of histones [Berger, 2002; Goll and Bestor, 2002; Turner, 2002; Iizuka and Smith, 2003]. These advances have significantly expanded our picture of the extent to which histones are dynamically modified.

## IDENTIFICATION OF SITES OF HISTONE MODIFICATION BY MASS SPECTROMETRY

Protein microsequencing proved to be highly successful in the identification of histone post-translational modifications. However, it is a fairly cumbersome technique that requires relatively large and highly purified samples. In addition, the presence of a blocked $NH_2$-terminus on many histone species complicates protein sequencing efforts. To circumvent the limitations of protein sequencing, researchers have recently been turning to mass spectrometry for the characterization of histone post-translational modifications.

Mass spectrometry is a chemical analysis technique that exploits the physical properties

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of ions to determine their mass to charge ratio (m/z). With the introduction of soft ionization techniques such as Matrix Assisted Laser Desorption Ionization (MALDI) [Karas and Hillenkamp, 1988; Tanaka et al., 1988; Karas et al., 1989] and ElectroSpray Ionization (ESI) [Whitehouse et al., 1985; Karas and Hillenkamp, 1988; Fenn et al., 1989], the mass of intact protein ions can easily be determined. If the primary amino acid sequence is known in advance, mass spectrometry can be used to detect the differences from the expected protein mass that are brought about by modifications of its primary structure. Thus mass spectrometry is a very adept tool for the direct identification of histone post-translational modifications. In addition, the sites of these modifications can be localized by either tandem mass spectrometry of the intact proteins, peptide mass fingerprinting following proteolysis, or a combination of these two techniques.

### Identification of Sites of Histone Modification by Mass Spectrometry

Edmonds et al. [1993] yielded the earliest report of the application of modern soft-ionization techniques to the analysis of histones. Later, Burlingame and colleagues were the first to successfully exploit mass spectrometry to pinpoint the location of histone modifications [Zhang et al., 2002a,b]. This group utilized high performance liquid chromatography to purify core histones. The HPLC fractions containing H3 were digested with trypsin and further fractionated by HPLC. The fractions were analyzed by MALDI-TOF and sequenced by post source decay (PSD) and collision-induced decay (CID). This group also used a similar approach to analyze the patterns of modification on the $NH_2$-terminus of H4. All of the previously identified sites of acetylation and methylation on histones H3 and H4 were observed. In addition, analysis of the peptides derived from the $NH_2$-terminal tail of histone H4 suggested an ordered progression of acetylation starting at lysine 16 and proceeding sequentially to lysines 12, 8, and 5.

Confirming sites of modification previously identified by protein sequencing methodologies provided proof of principle of the enormous potential for mass spectrometry to enhance our picture of the landscape of histone modifications. Allis and co-workers were the first to use mass spectrometry to add to this landscape through the identification of a novel site of histone methylation [Strahl et al., 2001]. Following the identification of mammalian protein arginine methyltransferases CARM1 and PRMT1 as transcriptional coactivators capable of methylating histones in vitro, mass spectrometry was used to determine whether histones isolated from human tissue culture cells contained methylarginine residues. Following reverse phase-HPLC purification, histones were digested with chymotrypsin and analyzed by microelectrospray ionization tandem mass spectrometry. This analysis confirmed the presence of arginine methylation in histones and specifically identified histone H4 arginine 3 as a site of monomethylation. Subsequent in vitro and in vivo experiments demonstrated that PRMT1 is the enzyme primarily responsible for this modification.

Mass spectrometry next played a critical role in the surprising revelation that methylation is not restricted to residues in the $NH_2$-terminal tails but can also be found in the globular core domains. Using histones purified from different sources (human tissue culture cells, calf thymus, chicken erythrocytes, and *Saccharomyces cerevisiae*) four groups used peptide mass fingerprinting to identify methylation on lysine residue 79 of histone H3 [Feng et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002; Zhang et al., 2002a]. The presence of methylation on histone H3 lysine 79 in this divergent collection of organisms indicates that this is a highly conserved modification. However, the degree of methylation at this residue appeared to differ from organism to organism. While yeast H3 lysine 79 was primarily tri-methylated, analysis of histone H3 from human and bovine tissue found ions indicative of only a single methyl group and avian H3 possessed mono- and di-methylation. It is unclear whether these are real differences or whether the absence of evidence for tri-methylation in the vertebrate species is simply an issue of detection sensitivity as enzymes (DOT1-like) responsible for the generation of this modification are highly, evolutionarily conserved. Indeed, a subsequent analysis of mouse histone H3 identified peptides containing mono-, di-, and tri-methylated lysine 79 [Cocklin and Wang, 2003].

The technique of peptide mass fingerprinting was then applied in a systematic fashion to identify and map the post-translational modifications present on all four core histones

[Zhang et al., 2003]. In this study, histones were isolated from bovine thymus and fractionated by HPLC into fractions containing the core histones. The core histones were then digested and analyzed by high resolution Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR MS). The high resolving power and mass accuracy of FTICR MS allowed for identification of the peptides without the need for separation by HPLC and sequencing by tandem mass spectrometry. More importantly it allowed for distinguishing between tri-methylated and acetylated peptides that differ in mass by 34 ppm. This study identified more than 20 novel sites of modification. Intriguingly, many of these previously uncharacterized modifications reside in the histone globular core domains. While there is obviously much work to do to decipher the in vivo relevance of these modifications, mapping their positions on the crystal structure of the nucleosome may provide clues to their function.

The newly identified modifications on histone H2A that are on residues visible in the crystal structure of the nucleosome cluster in three regions (Fig. 1A). Two of these clusters are near the contact points between histone H2A and DNA. The first cluster (K13, K15, and K36) is partially located in the $NH_2$-terminal tail domain that is projecting out from the nucleosome through a minor groove in the DNA duplex. These sites of modification are also in a region of the protein shown to be required for normal centromere function in yeast [Pinto and Winston, 2000]. The second cluster (K74, K75, and K77) is located in the L2 loop which is another contact point between H2A and DNA. The final novel site of H2A modification, methylation of K99, may be particularly interesting. Located in the very center of the nucleosome core particle, K99 is one of the few sites of modification found in bovine histones that is not conserved from bovine to yeast (these are restricted to histones H2A and H2B) [Luger et al., 1997]. Intriguingly, a phylogenetic comparison indicates that the presence of a lysine or arginine residue at this position is restricted to mammals. Hence, this modification may be performing a function that is unique to mammalian cells.

On histone H2B, patches of novel modification sites are found on the surface of the nucleosome (Fig. 1B). R99 and K108 are in close proximity near the center of the nucleosome while K85, K116, and K120 cluster on the outer face of the nucleosome. Novel H2B modifications are also found near regions of H2B-DNA interaction. K43 and K85 are in the L1 and L2 loops, respectively, that contact the DNA while K122 is on the COOH-terminal tail that projects out into the DNA helix [Luger et al., 1997].

The locations of the newly identified sites of modification on histone H3 suggest that they may be involved in regulating histone–DNA interactions (Fig. 1C). K52, K53, and K56 (K56 is shown in Fig. 1C) are in the αN helix which is involved in binding the final turn of DNA at the terminus of the superhelix. K115, T118, and K122, the latter of which was also identified as a site of methylation in a separate study, are all in the L2 loop which contacts the DNA helix and is in the region of interaction between the two molecules of histone H3 that stabilize the H3/H4 tetramer [Luger et al., 1997; Cocklin and Wang, 2003]. Strikingly, mutations at T118, identified as a site of phosphorylation, have previously been demonstrated to generate a SIN phenotype in yeast [Kruger et al., 1995]. SIN mutations (SWI/SNF Independent) bypass the need for the ATP-dependent chromatin remodeling activity SWI/SNF for activation of a pleiotropic set of genes including SUC2 and HO [Sternberg et al., 1987]. These mutations are thought to act by either loosening histone–DNA interactions or altering higher order chromatin structure [Kurumizaka and Wolffe, 1997; Wechser et al., 1997; Horn et al., 2002]. In addition to T118, a second residue in the L2 loop, R116, has also been identified as the site of a SIN mutation [Kruger et al., 1995]. Hence, the L2 loop is a highly modified region of histone H3 that plays a critical role in transcriptional regulation.

A number of novel modifications on histone H4 may play roles in regulating higher order chromatin structure. For example, K59 is a site of methylation found on the face of the nucleosome (Fig. 1D). Intriguingly, H4 K59 is in close proximity to histone H3 K79 whose identification as a site of methylation was described above. The methylation of H3 lysine 79 has been shown in vivo to play an important role in establishing euchromatic regions of the genome by preventing the promiscuous association of factors involved in the formation of heterochromatin. As a result, when histone H3 K79 is mutated in yeast, these heterochromatin associated proteins are mislocalized with the concomitant loss of heterochromatin-mediated gene silencing

[Feng et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002]. Consistent with the proximity of H4 K59 to H3 K79, mutations that alter H4 K59 have also been shown to disrupt yeast silent chromatin structure [Zhang et al., 2003].

There are several other novel sites of modification on histone H4 that may be playing related roles (Fig. 1D). K79 and K77 lie in the L2 loop of H4. K79 was identified in a large scale genetic screen that isolated point mutations in histones H3 and H4 that disrupt silent chromatin structure in yeast [Park et al., 2002]. Many of these mutations cluster in a region on the surface of the nucleosome that includes histone H3 K79 [Ng et al., 2002; van Leeuwen et al., 2002]. While H4 K77 was not identified in this

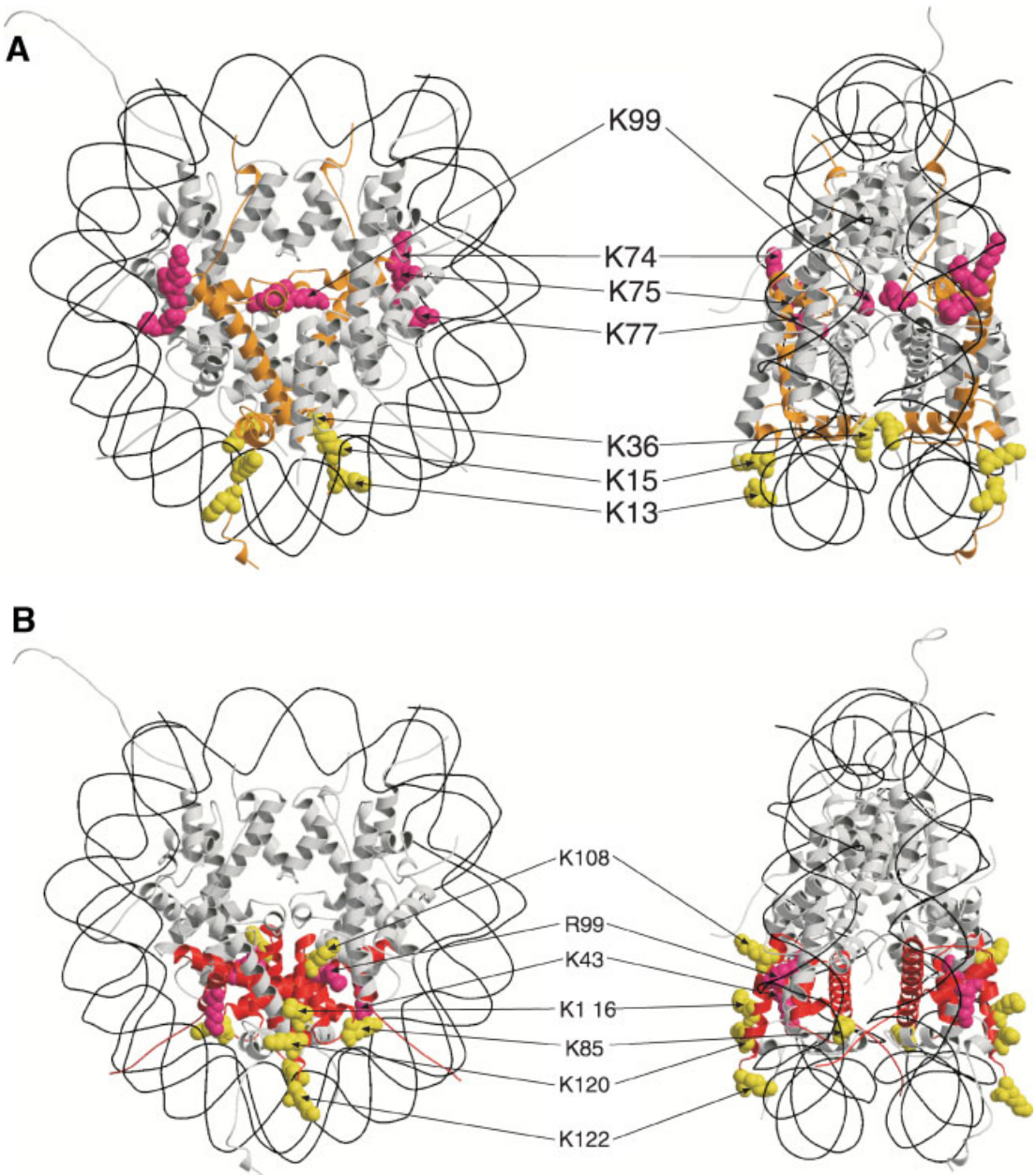


**Fig. 1.** Localization of novel sites of histone modification identified by mass spectrometry on the nucleosome crystal structure. Each panel shows a front and side view of the nucleosome with novel sites of modification highlighted on one of the core histones [(**A**) H2A-orange, (**B**) H2B-red, (**C**) H3-blue, (**D**) H4-green]. Specific residues are indicated by numbers and arrows with sites of acetylation highlighted in blue, sites of methylation highlighted in red and sites of phosphorylation highlighted in green. Structures were generated using MOLSCRIPT and RASTER 3D with atomic coordinates from PDB code 1ID3 [Kraulis, 1991; Merritt and David, 1997; White et al., 2001].

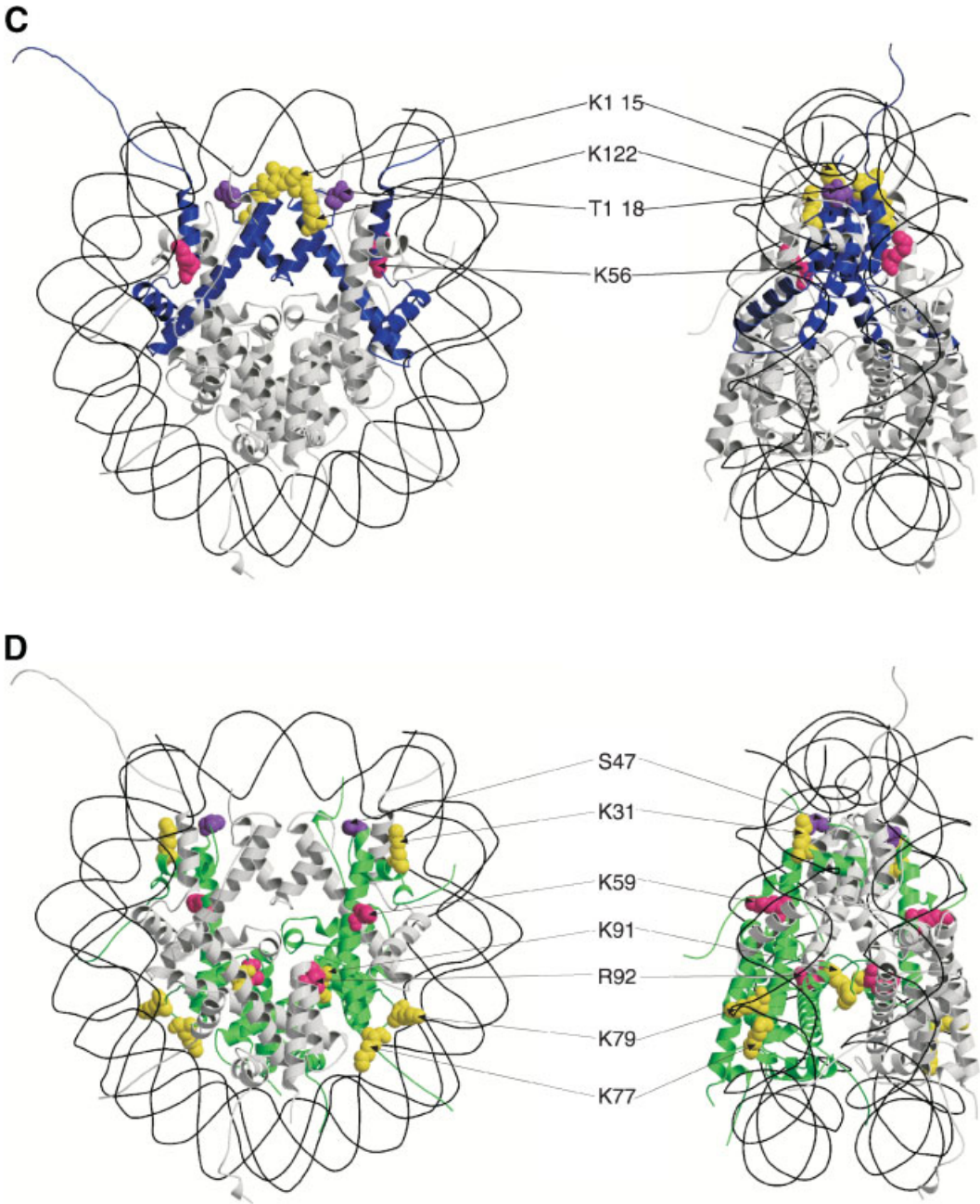


**Fig. 1.** (*Continued*)

screen, its proximity to this surface suggests that it may also influence silent chromatin structure. Hence, there may be multiple sites of modification in this critical region of the nucleosome whose modification is required for maintaining the proper regulation of higher order chromatin structure.

Histone H4 K91 and R92 were identified as sites of acetylation and methylation, respectively. These sites of modification are near the COOH-terminus of H4. Although these residues appear to be buried in the interior of the nucleosome structure, closer inspection reveals that they are in the region of histone H4 that is important for the docking of the H2A/H2B dimers with the H3/H4 tetramer [Santisteban et al., 1997]. In addition to histone H3 T118, the only other site of core domain phosphorylation

identified was histone H4 S47. Strikingly, these residues are in very close proximity in the three dimensional structure of the nucleosome, suggesting that these residues are capable of producing an acidic patch on the surface of the nucleosome. The generation of an acidic patch near a contact point with DNA may alter the stability of the octamer/helix interaction. H4 K31, which was found to be acetylated, is at the beginning of the $\alpha$1 helix of H4, again near the DNA, suggesting that modification of this site may also effect the interaction of the nucleosome with DNA [Luger et al., 1997].

The systematic application of peptide mass fingerprinting to the core histones fundamentally changes our view of the post-translational modifications on these proteins. Rather than being predominantly localized to the mobile $NH_2$-terminal tails, these modifications also decorate many areas on the core of the nucleosome. These sites are primarily localized either in several patches on the surface of the nucleosome or at sites of histone–DNA interaction. In fact, amino acids in the L2 loop of all four core histones, which are contact points with DNA, have been identified as sites of histone modification. While the application of mass spectrometry to the characterization of bovine thymus core histones has identified a wealth of novel modifications, the analysis of histones isolated from different tissue types or from cells grown under specific conditions (such as cell cycle arrest) is certain to expand this repertoire.

The histone modifications discussed thus far are the result of specific enzymatic reactions. The non-enzymatic modification of histones has also been detected by mass spectrometry. Reactive oxygen species generated from nitric oxide can cause cellular damage through their modification of proteins, DNA and lipids. Using a mouse tumor model, Haqqani, et al., identified a series of low molecular weight proteins that are preferentially modified by nitration. MALDI-TOF analysis of tryptic peptides isolated from these proteins identified them as core histones. Tandem mass spectrometry indicated that nitration occurred on only a limited number of histone residues. Specifically, H2B T37, T40, and T42, H3 T41 and H4 T72, T98 and H75 were found to be nitrated. How the nitration of specific residues in the core histones relates to the cytotoxic and mutagenic effects of reactive oxygen species remains to be determined.

In addition to identifying sites of histone modification in vivo, mass spectrometry has also proven useful in the identification of the target sites for histone modifying enzymes in vitro. It is a relatively straightforward matter to determine the specificity of enzymes such as histone acetyltransferases and histone methyltransferases that add modifications to a histone protein. This typically involves the post-reaction sequencing of radio-labeled histones or synthetic peptides. As these methods rely on protein sequencing they are subject to the same limitations discussed above. Accordingly, mass spectrometry was used to circumvent the use of protein sequencing methodologies and successfully identified the sites of action for the histone acetyltransferase Tip60 and the histone methyltransferases CARM1 and Dot1p [Kimura and Horikoshi, 1998; Schurter et al., 2001; van Leeuwen et al., 2002]. Intriguingly, for CARM1 a significant level of methylation was localized to arginine residues outside of the $NH_2$-terminal tail domain.

Mass spectrometry is likely to play a more important role in characterizing the substrate specificity of enzymes, such as histone deacetylases (HDACs), which are involved in the removal of histone modifications. As measuring the loss of histone modifications is a complex task, the substrate specificities of most HDACs are not well characterized. Grunstein and colleagues developed a mass spectrometry-based system to determine the site specificity of deacetylation by the yeast HDAC Hos3p [Carmen et al., 1999]. Recombinant Hos3p was incubated with peptides representing the $NH_2$-terminal tail domains of histones H3 and H4 that were synthesized with acetyllysine at each of the sites of reversible acetylation. Following the reactions, the total acetylation level of the peptides was determined by MALDI-TOF and the acetylation state of the individual lysine residues was determined by either MS/MS or peptide mapping. While this study represents a promising start, a major challenge will be to extend this system for use with full length histone substrates.

## QUANTIFICATION OF HISTONE POST-TRANSLATIONAL MODIFICATIONS BY MASS SPECTROMETRY

Once they have been identified, the ability to quantitate histone modifications is a critical

component of their functional characterization. Typically, monitoring changes in histone modifications relies on the use of modification-specific antibodies. However, the use of these immunological reagents has serious limitations. First, it is very difficult to precisely define the specificity of these antibodies [Suka et al., 2001]. They are generally produced using synthetic peptides as antigens and it is often not known whether their specificity is altered in the context of the full-length protein or in different experimental contexts (e.g., Western blotting, chromatin immunoprecipitation, immunofluorescence). Second, the influence of multiple modifications on a single histone is likely to impact the specificity of antibodies directed at each individual modification. Given their number, it is virtually impossible to completely characterize the complex physical interactions between all of the modifications.

Mass spectrometry is likely to be an important alternative method for quantitating histone modifications. Depending on the techniques used, mass spectrometry can provide two, complementary types of information. Liquid chromatography/mass spectrometry (LC/MS) analysis involves the initial separation of intact histones by reverse phase HPLC. Each histone peak is comprised of a population of molecules that can differ in the type and number of post-translational modifications. Coupling of HPLC to a mass spectrometer allows for the determination of the masses of the constituent isoforms. The modification state of each isoform can be deduced by comparison with the predicted mass of the unmodified protein. For example, increases in mass that are multiples of 14, 42, or 80 (or combinations thereof) indicate the presence of methylation, acetylation or phosphorylation, respectively. Changes in the proteins ability to ionize in the electrospray should be small for a family of isoforms. Thus, this technique can be semi-quantitative in that the relative abundance of each isoform is also determined. Hence, LC/MS can provide a snapshot view of the distribution of the differentially modified isoforms of each histone.

There are two important caveats that need to be considered when analyzing histone modifications by LC/MS. First, the accuracy of mass determination is not sufficient to distinguish between trimethylation ($\Delta$42.04 mass units) and acetylation ($\Delta$42.01 Mass units). Thus, peaks that differ by 42 mass units (or multiples thereof) can not be unambiguously assigned. Second, the presence of histone variants can complicate the assignment of peaks detected by LC/MS. For example, the unmodified forms of histones H2B.2 and H2B.F have predicted masses that differ by 43 mass units (13,776 and 13,816, respectively). Hence, an LC/MS peak at 13,816 can be either unmodified H2B.F or mono-acetylated H2B.2. Further characterization, by ultra high resolution Fourier Transform Ion Cyclotron Resonance Mass Spectrometry, MS/MS or peptide mass mapping would be required to discriminate between these possibilities.

The ability to characterize the distribution of histone isoforms provides an opportunity to correlate histone modification profiles with specific cellular parameters. This technique could be used to identify differential patterns of histone modification that may exist between different tissue types or between normal and malignant cells. For example, comparison of the LC/MS profiles of histones isolated from CD19-selected lymphocytes, both from normal individuals and patients with chronic lymphocytic leukemia (CLL), indicated that CLL may be associated with a marked decrease in the mono-acetylated isoform of histone H2A [Zhang et al., 2004].

Another important application of LC/MS is in defining changes in histone modifications that result from the treatment of cells with therapeutic agents that influence chromatin structure. LC/MS was used to identify interesting changes in histone modification that resulted from the treatment of K562 culture cells with the phosphatase inhibitor okadaic acid (OA). As expected, phosphorylated isoforms of histones H2A, H3, and H4 increased following OA treatment. However, a dramatic decrease in an isoform of histone H4 that represents a combination of mono-acetylation and di-methylation preceded the appearance of phosphorylated H4 isoforms suggesting that OA can also effect histone acetylation [Galasinski et al., 2002].

Several small molecule inhibitors of HDACs have been identified that have shown promise as chemotherapeutic agents. An important aspect of deciphering these compounds mechanism(s) of action is to characterize, in detail, the changes in histone modifications they induce. Treatment of the leukemia cell line Kasumi-1 with the HDAC inhibitor depsipeptide was shown by LC/MS to induce changes in acetylation that were isoform specific. Surprisingly,

increases in acetylation only occurred on the isoforms of histone H4 that were also dimethylated. The specificity of the effect of depsipeptide on dimethylated histone H4 isoforms was not a tissue culture artifact as identical results were seen with histones isolated from leukemia patients treated in a phase I clinical trial of depsipeptide [Zhang et al., 2004]. Importantly, these studies demonstrate that LC/MS has the sensitivity to be applied to primary tissue samples obtained in a clinical setting.

While LC/MS can provide valuable information concerning the dynamic nature of histone modifications on a global scale, it cannot quantitate the changes occurring at specific residues. Peptide mapping or MS/MS (or a combination of the two) can be used to monitor modifications at the level of individual residues. With peptide mapping, peptides containing the residue of interest are analyzed and the relative abundance of the unmodified and modified forms is determined. This technique was successfully used to characterize the fraction of yeast histone H3 lysine 79 present in the un-, mono-, di-, and trimethylated states [van Leeuwen et al., 2002].

As previously mentioned, the drawback of peptide mapping is that it is semi-quantitative. The addition of post-translational modifications to peptides alters their chemistry and, as a consequence, may change the efficiency with which they are proteolyzed or with which they are ionized during mass spectrometry. Hence, the observed relative abundances of differentially modified peptides may not accurately reflect their levels in vivo.

These issues were elegantly addressed in studies quantitating the levels of acetylation at each of the four lysine residues in the histone H4 $NH_2$-terminal tail (at positions 5, 8, 12, and 16) [Smith et al., 2002, 2003]. Taking advantage of the inability of trypsin to cleave acetylated lysine residues, Smith, et al, chemically acetylated purified histone H4 to block the cleavage of all lysine residues regardless of the degree to which they were acetylated in vivo. Therefore, trypsin cleavage only occurred at arginine residues allowing all four lysine residues to be recovered on a single, chemically homogeneous, peptide (spanning residues 4–17). To distinguish acetylation events that occurred in the cell from those added in the test tube, the chemical acetylation was performed with deuterated acetic anhydride which adds 3 mass units to the in vitro acetylation events. MS analysis of the 4–17 peptide revealed a set of 5 peaks that represent peptides containing from 0 to 4 deuterated acetyl moieties. As the cleavage and ionization of these peptides is equivalent, their relative abundance is a quantitative measure of the in vivo distribution of tetra-, tri-, di-, mono-, and un-acetylated histone H4. The degree of in vivo acetylation present at each of the four lysine residues was then calculated from the ratios of the protonated and deuterated b and y fragment ions produced during MS/MS. This analytical method was successfully used to quantify the effects, of mutations in histone H4 that influence silent chromatin structure, on the level of acetylation at each of the lysine residues in the H4 $NH_2$-terminal tail. These results indicated that acetylation of H4 lysine 12 may serve as a mark that functions in the inheritance of chromatin states [Smith et al., 2002].

A similar approach was used to investigate the methylation of lysine residues in the histone H3 $NH_2$-terminal tail [Peters et al., 2003]. While di- and tri-methylation prevent cleavage by trypsin, mono-methylation does not. This complicates the quantification of these differential states of methylation. This problem was overcome by the in vitro propionylation of un- and mono-methylated lysine residues in purified histone H3 to prevent trypsin cleavage at all lysine residues. Trypsin digestion could then be used to generate equal length peptides for MS analysis. While this system was used to provide evidence implicating the mammalian histone methyltransferase G9a in the mono- and dimethylation of histone H3 lysine 9 and the Svu39 enzyme as responsible for the trimethylation of this residue, there is an important caveat with respect to its use as a quantitative tool. While in vitro propionylation provides for the production of peptides of equal length regardless of lysine methylation status, all of the peptides generated are not chemically equivalent. Thus, the unequal distribution of the propionyl groups may have influences on ionization that bias the results.

Mass spectrometry has clearly emerged as a powerful technique for the analytical characterization of protein structures. The recent surge in the use of mass spectrometry to analyze the core histones is not surprising given the complexity inherent in their structure that results from the numerous post-translational modifications to which they are subject. The success of

these initial studies in identifying and quantitating histone post-translational modifications indicates that modern analytical techniques will play an important role in advancing our understanding of the regulation of chromatin structure.

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