Effects of Acetylation of Histone H4 at Lysines 8 and 16 on Activity of the Hat1 Histone Acetyltransferase*

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During nucleosome assembly in vivo, newly synthesized histone H4 is specifically diacetylated on lysines 5 and 12 within the H4 NH2-terminal tail domain. The highly conserved “K5/K12” deposition pattern of acetylation is thought to be generated by the Hat1 histone acetyltransferase, which in vivo is found in the HAT-B complex. In the following report, the activity and substrate specificity of the human HAT-B complex and of recombinant yeast Hat1p have been examined, using synthetic H4 NH2-terminal peptides as substrates. As expected, the unacetylated H4 peptide was a good substrate for acetylation by yeast Hat1p and human HAT-B, while the K5/K12-diacetylated peptide was not significantly acetylated. Notably, an H4 peptide previously diacetylated on lysines 8 and 16 was a very poor substrate for acetylation by either yeast Hat1p or human HAT-B. Treating the K8/K16-diacetylated peptide with histone deacetylase prior to the HAT-B reaction raised acetylation at K5/K12 to 70–80% of control levels. These results present strong support for the model of H4-Hat1p interaction proposed by Dutnall et al. (Dutnall, R. N., Tafrov, S. T., Sternglanz, R., and Ramakrishnan, V. (1998) Cell 94, 427–438) and provide evidence for the first time that site-specific acetylation of histones can regulate the acetylation of other substrate sites.

Chromatin replication involves both the transfer of parental histones to new DNA and the assembly of new nucleosomes de novo. To provide the histones required for de novo nucleosome assembly, histone synthesis in somatic cells is for the most part coupled to DNA replication (reviewed in Ref. 1). Newly synthesized H3 and H4 are selectively targeted to nascent DNA by the chromatin assembly factor CAP-1 (2–6), while new H2A, H2B, and H1 enter preexisting chromatin through a process of histone exchange (7, 8).

The core histones (H2A, H2B, H3, and H4) that comprise the histone octamer are subject to reversible posttranslational acetylation on their extended NH2-terminal “tail” domains. Acetylation occurs on specific, highly conserved lysine residues, e.g. lysines 5, 8, 12, and 16 of H4 and lysines 9, 14, 18, and 23 of H3 (also K27 in some organisms) (reviewed in Ref. 9). A remarkable feature of histone metabolism is that in species as divergent as humans, Drosophila, and Tetrahymena, newly synthesized H4 is specifically diacetylated on lysines 5 and 12 (or lysines 4 and 11 in Tetrahymena, due to a deletion of the typical arginine at position 3) (10, 11). New H4 is acetylated prior to its deposition onto DNA and is subsequently deacetylated following nucleosome assembly (12, 13). Although inhibiting deacetylation prevents the complete maturation of newly replicated chromatin (14, 15), the function of the acetylation of new H4 is unknown (reviewed in Refs. 16–18).

One histone acetyltransferase (or HAT)1 that may possibly catalyze the acetylation of newly synthesized H4 has been identified. This is the “type B” or HAT-B histone acetyltransferase, which is typically recovered in the cytosol following cellular disruption. HAT-B enzymes have been studied in a number of organisms and have been shown to specifically diacetylate free but not nucleosomal H4 (reviewed in Ref. 18). Moreover, native HAT-B acetyltransferases from humans (19, 20), maize (21), Xenopus (22), and Tetrahymena (23) are capable of generating the complete Lys5/Lys12 acetylation pattern of newly synthesized H4 in vitro (or Lys8/Lys12 acetylation for the Tetrahymena enzyme). HAT-B functions as a complex containing the Hat1 catalytic subunit and, in humans, Rbap46 (a small protein that also associates with the retinoblastoma protein, RB) (20). HAT-B enzymes from other species contain orthologues of p46 or of p48 (another RB-binding protein). In yeast, the p46 subunit is termed Hat2p (24, 25).

The crystal structure of the Hat1p acetyltransferase from Saccharomyces cerevisiae (24, 26) has been determined at 2.3-Å resolution (27). Hat1p has a curved shape; acetyl-CoA binds within a depression on the concave surface of the protein, with the acetyl group designating the active site. A model has been proposed for the enzyme-H4 interaction, based on the assumptions that the H4 tail has an extended conformation, that Lys12 is positioned adjacent to the acetyl group of acetyl-CoA, and that Hat1p itself undergoes no major conformational changes after binding the substrate. By applying these guidelines, it has been proposed that the region of the H4 tail between Lys8 and Lys16 binds along a channel that is long enough to accommodate 6–7 amino acids (27). When Lys12 is positioned adjacent to the acetyl group of acetyl-CoA, Lys8 and Lys16 are found to lie opposite two acidic patches at the ends of the channel, theoretically engaging in electrostatic interactions that hold the tail in place. Aligning Lys8 near the acetyl group of acetyl-CoA also yields a reasonable, though somewhat less favorable, fit. However, modeling Lys8 adjacent to acetyl-CoA produces a steric clash with the channel (27, 28).

As noted above, the present model for the binding of the H4

1 The abbreviations used are: HAT, histone acetyltransferase; HDAC, histone deacetylase; RB, retinoblastoma.
tail to Hat1 predicts that the lysine residues at positions 8 and 16 of H4 play a significant role in stabilizing the Hat1-histone tail interaction. It therefore may be postulated that abrogating the positive charge of Lys8 and/or Lys16 by acetylation should decrease the ability of the H4 tail to bind Hat1 and thus interfere with enzyme activity. In the following report we have tested this prediction directly, by performing HAT assays in vitro using several acetylated H4 NH2-terminal peptides. We find that the acetylation of Lys8 and/or Lys16 of H4 dramatically reduces the activity of recombinant yeast Hat1p and native human HAT-B in vitro.

EXPERIMENTAL PROCEDURES

Cell Culture; Preparation of HAT-B Extract—HeLa cells were grown in spinner culture at 37 °C (14). Cytosolic extracts (S100 extract) from HeLa cells were prepared as described previously (19, 29). The HeLa S100 cytosolic extract contains HAT-B as the sole HAT activity, which exclusively acetylates the H4 NH2 terminus on lysines 5 and 12 (19).

In Vitro Acetylation Assays—Human HAT-B activity was measured using HeLa cytosolic extracts and an in vitro peptide assay (30), as described previously (19). Synthetic 20-mer peptides (typically 0.1 μg/μl final concentration) corresponding to the first 18 amino-terminal residues (plus a C-terminal Gly-Cys coupling linker) of either unacetylated or variably acetylated NH2 termini of human histone H4 (19) were incubated in a reaction volume of 50 μl in HB buffer (20 mM Hepes, 5 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, adjusted to pH 7.5 with KOH) containing 40 μl of HeLa S100, an additional 60 mM Hepes (pH 8.0), 5 mM sodium butyrate, and 4–8 μM/ml [3H]acetyl-CoA (1–3 Ci/m mole, PerkinElmer Life Sciences), for the times indicated in the legends; in some cases the reaction volume was doubled. All peptides used in this study were quantitated by mass spectrometry, and all experiments included parallel reactions using the unacetylated peptide as a positive control. Reactions were stopped by spotting in duplicate onto Whatman P-81 filters as described above. Recombinant Hat1p was prepared as described previously (27).

Preparation of H4 Tail Acetate (H4TA) Extract—HeLa histone deacetylase extract was prepared as described by Yoshida et al. (31). HeLa cells (250 ml) were washed twice with HDAC buffer (15 mM potassium phosphate (pH 7.5), 5% glycerol, and 0.2 mM EDTA). Cells were allowed to swell on ice for 15 min and were homogenized with a Dounce homogenizer. Nuclei were collected by centrifugation (14,000 × g; 10 min; 4 °C) and resuspended (60 mg/ml; A260 measured in SDS) in HDAC buffer plus 1 mM (NH4)2SO4. After a second homogenization, samples were sonicated for 10 s and pelleted as described above. The supernatant, containing histone deacetylase activity, was brought to a final concentration of 3.5 mM (NH4)2SO4, kept on ice for 30 min, and centrifuged. The pellet was resuspended in 500 μl of HDAC buffer and dialyzed overnight to 1 liter of HDAC buffer. Samples were then stored at −70 °C. HDAC activity was monitored using [3H]acetate-labeled acetylated histones as substrates.

Treatment of Peptides with Histone Deacetylase—Following the procedure of Inoue and Fujimoto (32), 8 μg of peptide were added to 37 μl of HDAC extract and incubated for 1 h at 37 °C. Samples were boiled for 5 min and cooled. HDAC extracts treated in this manner contained no contaminating HAT activity, as determined by HAT assay. HDAC-treated peptides were then used in subsequent HAT-B reactions, containing 45 μl of deacetylated peptide/HDAC extract, 40 μl of HeLa S100 cytosolic extract, 60 mM Hepes (pH 8.0), 20 mM sodium butyrate, and 0.32 μM of [3H]acetate-CoA, adjusted to a final reaction volume of 100 μl with HB buffer.

RESULTS

Histone H4 NH2-terminal peptides were synthesized in various isoforms, representing the following acetylation states: unacetylated; monoacetylated at either Lys5, Lys8, Lys12, or Lys16 (termed Mono-5, Mono-8, Mono-12, and Mono-16, respectively); and diacetylated at either Lys5/Lys12, or Lys8/Lys16 (Di-5/12 and Di-8/16). The peptides were then used to examine the activity of recombinant yeast Hat1p, as measured by the transfer of radiolabeled acetate from [3H]acetyl-CoA. All acetylation reactions were performed in the presence of sodium butyrate, to inhibit possible deacetylation during incubation. The results are presented in Fig. 1.

As predicted, an H4 peptide already acetylated on lysines 5 and 12 (the sites acetylated by Hat1 enzymes) was an extremely poor substrate (Fig. 1). The depressed acetylation of the Di-5/12 peptide also verifies that no significant deacetylation is occurring under our experimental conditions. In contrast, both the unacetylated peptide and the peptide previously acetylated on Lys8 were readily acetylated by Hat1p. Labeling of the Mono-5 peptide was reduced relative to the unacetylated substrate (Figs. 1 and 2). This is as expected, since only one substrate lysine is available for radiolabeling; however, other factors may also be involved (see “Discussion”). Notably, the Mono-12 peptide was acetylated poorly (Figs. 1 and 2), consistent with the great preference of native and recombinant yeast Hat1p for Lys12 over Lys8 (24, 26).

The effect of prior acetylation Lys5 and Lys16 (which are not substrate lysines for Hat1p) on Hat1p activity was then examined (Fig. 1). As predicted from the proposed interaction of the H4 tail with Hat1p (27), acetylation at either Lys5 or Lys16 significantly reduced the ability of Hat1p to acetylate its substrate lysines, with acetylation at Lys16 having a somewhat greater effect. Strikingly, the Lys5/Lys16-diacetylated peptide was almost refractory to acetylation by Hat1p, yielding results close to those obtained with the 5/12-diacetylated peptide, in which the substrate lysines are already acetylated. A summary of the results from several independent experiments are presented in Fig. 2, in which incorporation of radiolabeled acetate into each of the substrates is presented relative to that of the unacetylated peptide, which was always tested as a positive control. The combined data demonstrate that lysines 8 and 16 of H4 are critical to Hat1p activity (at least in vitro).

The experiments were then repeated using the human Hat1 enzyme, which in vitro resides in the HAT-B complex (19, 20). One of the hallmark features of HAT-B enzymes is their fractionation into the cytosol following cell disruption. We have previously shown that HAT-B is the sole HAT activity present in a cytosolic extract prepared from HeLa cells ("S100" extract) and that human HAT-B acetylates H4 exclusively in the Lys5/Lys12 deposition pattern (19). Moreover, Hat-B in the S100 comprises a native complex of ~100 kDa (19), which contains Hat1 and p46 (20). It is therefore possible to study HAT-B activity using the S100 extract as a native enzyme source (19).
Unacetylated, as well as Mono-8, Mono-16, Di-5/12, and Di-8/16 acetylated peptides, were tested for their ability to be acetylated by human HAT-B; also tested was an H4 NH$_2$-terminal peptide with a deletion of Lys$_{16}$ (Fig. 3). Control experiments verified by immunoprecipitation of the acetylated peptides with site-specific anti-acetylated H4 antibodies (19) that HAT-B was acetylating lysines 5 and 12 of the H4 tail (data not presented), consistent with our previous description of human HAT-B activity (19).

Deleting or acetylating Lys$_{16}$ reduced the ability of HAT-B to acetylate the H4 peptide by $\frac{50\%}{H11011}$, and acetylating Lys$_{8}$ had a slightly greater effect (Fig. 3). The more pronounced effect of Lys$_{8}$ acetylation on the activity of recombinant Hat1p (Figs. 1 and 2), relative to human HAT-B (Fig. 3), may be due to the presence of the p46 subunit in the human enzyme; it has been demonstrated that the interaction of the H4 tail with yeast Hat1p is stabilized by Hat2p (24). Notably, as seen earlier with yeast Hat1p, the simultaneous acetylation of Lys$_{8}$ and Lys$_{16}$ severely reduced the acetylation of the H4 tail by the human enzyme (Fig. 3). Competition experiments further established that the Lys$_{8}$/Lys$_{16}$ peptide did not inhibit HAT-B activity, even when present in 10-fold excess over the unacetylated peptide (data not presented). Thus the Lys$_{8}$/Lys$_{16}$ peptide is itself a poor Hat1p/HAT-B substrate.

Additional experiments were then performed to ensure that acetylation per se was regulating HAT-B activity. For these experiments, a preparation of HDAC was obtained from isolated HeLa cell nuclei (see "Experimental Procedures"). Unacetylated and Lys$_{8}$/Lys$_{16}$-diacetylated H4 peptides were then pretreated with HDAC prior to using the peptides in HAT-B assays. Control experiments verified that no contaminating HAT activities from the HDAC extract were present during the subsequent HAT-B reactions.

Pretreating the Lys$_{8}$/Lys$_{16}$-diacetylated peptide with HDAC enabled HAT-B to acetylate the peptide at lysines 5 and 12, to 75–80% of control levels; buffer alone or BSA had no effect (Fig.
The acetylation of specific, nonsubstrate lysine residues (i.e., Lys\textsuperscript{8} and Lys\textsuperscript{12}) of the histone H4 NH\textsubscript{2} terminus reduces the acetylation of lysines 5 and 12 by both yeast and human Hat1 enzymes \textit{in vitro}. Our results provide strong support for the model of H4-Hat1p interaction proposed by Dutnall \textit{et al.} (27, 28), in which Lys\textsuperscript{8} and Lys\textsuperscript{12} are postulated to interact electrostatically with acidic domains within the Hat1p active site. In addition, this is the first demonstration that the activity of any histone acetyltransferase can potentially be regulated by the prior acetylation of nonsubstrate lysine residues within an individual histone tail.

Year Hat1p exhibits a strong preference for acetylating Lys\textsuperscript{12} of H4 over Lys\textsuperscript{8} (24, 26). In light of this, it might be expected that acetylation of the Mono-5 peptide (with Lys\textsuperscript{8} available for modification) would more closely approach that of the unacetylated peptide (Fig. 2). The presence of acetylation on Lys\textsuperscript{8} may therefore lower the ability of the H4 tail to interact with Hat-B. Notably, it has been shown by Lodil and colleagues (21) that maize Hat-B is incapable of using a native mixture of monocetyl H4 isoforms as a substrate, although in this case the specific lysines inhibiting acetylation were not identified. Thus in that study a significant percentage of input H4 was very likely acetylated on Lys\textsuperscript{5} or Lys\textsuperscript{12}.

It is now becoming clear that histone acetyltransferases may be controlled (either positively or negatively) by posttranslational modifications on residues that neighbor HAT-specific target lysines. For example, it has recently been shown that the Cen5 histone acetyltransferase strongly prefers phosphorylated over unphosphorylated H3 as a substrate and that prior phosphorylation of serine 10 of H3 can potentiate subsequent acetylation of lysine 14 (33, 34). Moreover, the methylation of H3 at lysine 9 inhibits the phosphorylation of H3 at serine 10 (35), and it has been proposed that histone methylation can help to regulate acetylation (and vice versa), at least in some instances (36). While our experiments have focused on the activity of Hat1 \textit{in vitro}, peptide assays have previously been shown to be excellent predictors of the interactions between histone modifications \textit{in vivo}, as seen with the enhancement of H3 acetylation by phosphorylation (33, 34) and the inhibition of H3 phosphorylation by prior methylation (35). The positive or negative interaction of histone modifications thus appears to be a general regulatory mechanism of histone metabolism, as detailed in the “histone code” hypothesis proposed by Strahl and Allis (37), in which histone modifications act in concert to regulate gene activity.

Hat1 is most likely involved in the acetylation of newly synthesized H4, which is not expected to be pre-acetylated at lysines 8 and/or 16. Nevertheless, our results have relevance beyond supporting the proposed model of H4-Hat1 interaction. For example, in human cells a nuclear CAC complex (comprising the chromatin assembly factor CAF-1 and histones H3 and H4) has been described, in which a fraction of H4 is acetylated at Lys\textsuperscript{8}, as well as at Lys\textsuperscript{5} and Lys\textsuperscript{12} (38). The CAC complex thus appears to represent a chromatin-bound intermediate in the deposition of new H4 onto chromatin, isolated prior to the deacetylation of nascent H4. Our results now suggest that the acetylation of new H4 at Lys\textsuperscript{8} should follow the acetylation of Lys\textsuperscript{8} and Lys\textsuperscript{12} by Hat-B, since prior acetylation of Lys\textsuperscript{8} could inhibit the activity of human Hat-B.

Although Hat1 is typically recovered from cytosolic extracts, there is evidence that the great majority of the human Hat1 is nuclear \textit{in vivo} (20). Nuclear as well as cytoplasmic Hat-B type enzymes have also been described in \textit{Tetrahymena} cells (23) and maize embryos (39). Interestingly, Hat1 in \textit{Xenopus} oocytes has been demonstrated to shuttle between nuclear and cytoplasmic compartments (22), and in yeast, Hat1p is found in two separate complexes, at least one of which is nuclear (25). It has been shown that the activity of HAT enzymes can be modulated by their association with other proteins within HAT-containing complexes (reviewed in Ref. 40), and it remains to be seen whether the Hat1 catalytic subunit can in some circumstances associate with proteins that expand its specificity to include nucleosomal histones \textit{in vivo}. If so, pre-existing acetylation patterns could also help to regulate nuclear Hat1 complexes that engage chromatin-bound substrates.
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