Communication

Overlapping but Distinct Patterns of Histone Acetylation by the Human Coactivators p300 and PCAF within Nucleosomal Substrates*

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A number of transcriptional coactivators possess intrinsic histone acetylase activity, providing a direct link between hyperacetylated chromatin and transcriptional activation. We have determined the core histone residues acetylated in vitro by recombinant p300 and PCAF within mononucleosomes. p300 specifically acetylates all sites of histones H2A and H2B known to be acetylated in bulk chromatin in vivo but preferentially acetylates lysines 14 and 18 of histone H3 and lysines 5 and 8 of histone H4. PCAF primarily acetylates lysine 14 of H3 but also less efficiently acetylates lysine 8 of H4. PCAF in its native form, which is present in a stable multimeric protein complex lacking p300/CBP, primarily acetylates H3 to a monoaCetylated form, suggesting that PCAF-associated polypeptides do not alter the substrate specificity. These distinct patterns of acetylation by the p300 and PCAF may contribute to their differential roles in transcriptional regulation.

The association of DNA with histones in chromatin antagonizes transcription in vitro (1, 2) and molecular genetic analyses in yeast have demonstrated roles for specific, evolutionarily conserved lysine residues within the N termini of the core histones in transcriptional regulation in vivo (for review see Ref. 3). Several studies have demonstrated an enrichment of hyperacetylated histones within transcriptionally active/competent chromatin in vivo (for review see Refs. 4–7). Strong support for the notion that histone acetylation facilitates transcription is supported by the discovery that transcriptional regulatory proteins, including GCN5 (8, 9), PCAF (10), p300 (11), CBP (12), TAF1 (13), and the nuclear hormone receptator coactivators ACTR (14) and SRC-1 (15), possess intrinsic histone acetyltransferase (HAT) activity (for review see Refs. 16 and 17). Moreover, mutational analyses of yeast GCN5 indicate that GCN5 HAT activity in vivo is correlated with histone acetylation at promoter regions and transcriptional activation of target genes in vivo (18, 19). These results are further supported by in vitro transcription experiments from nuclease templates showing acetylCoA-dependent activation by the GCN5-containing SAGA complex (20). These lines of evidence demonstrate the requirement of the HAT activity of GCN5 for transcriptional activation in a nucleosomal context.

The multiplicity of HATs identified suggests they may serve distinct functions. CBP and p300 are highly homologous coactivator proteins (21) that bind a number of sequence-specific transcriptional activators and have been suggested to be central integrators of transcriptional signals from various signal transduction pathways (22). Both CBP and p300 interact with the adenoviral E1A oncoprotein, and this interaction results in transcriptional repression of some CBP- and p300-regulated genes (23–25). We have previously described a cellular factor, PCAF (p300/CBP-associated factor), which competes both in vitro and in vivo with E1A for binding to p300 and CBP (10).

The C-terminal half of PCAF bears a high degree of sequence homology to the yeast GCN5 nuclear HAT. We have shown that PCAF (10) and p300 (11) have intrinsic HAT activity. Thus, p300 and PCAF form a histone acetylase complex in vivo. The functional requirements for the HAT activities of PCAF and p300/CBP have recently been examined in the regulation of myogenic differentiation (26), nuclear receptor-mediated transcriptional activation (27), as well as in the cAMP and growth factor-induced signaling pathways (28). The HAT activity of PCAF is required for myogenic, nuclear receptor- and growth factor-induced signaling pathways, whereas that of p300/CBP is dispensable. However, the intrinsic HAT activity of p300/CBP is required for cAMP-induced transcriptional activation (27, 28). These findings indicate distinct functional differences between the HAT activities of PCAF and p300/CBP.

To further characterize the functional differences between PCAF and p300, we have examined the specificity of human p300 and PCAF for core histones in mononucleosomes isolated from HeLa cells. To date, the specific residues within core histones targeted by a nuclear HAT have been determined only for yeast GCN5 (8). In this report, we show that p300 specifically targets all four core histones at sites known to be acetylated in vivo. These sites both overlap and extend the sites used by GCN5 (8). PCAF, which is homologous to GCN5, targets the same free histones H3 and H4 (8). Significantly, even though numerous lysine residues are present in the N termini of core histones, only residues known to be acetylated in vivo are acetylated by p300 and PCAF, suggesting that histones are a physiological substrate for these enzymes.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant p300 and PCAF—A cDNA fragment containing the entire open reading frame of p300 was subcloned into the baculovirus expression vector, pAC5G2 (PharMingen), downstream and in-frame with a DNA sequence encoding the FLAG epitope. Individual plaques of recombinant virus that express high levels of FLAG-p300 were isolated. Recombinant FLAG-p300 and FLAG-PCAF were

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1 The abbreviations used are: HAT, histone acetyltransferase; HPLC, high pressure liquid chromatography.
purified as described previously (10).

Acetylation of Nucleosomal Histones in Vitro—HeLa mononucleosomes were prepared as described previously (29). Sucrose density gradient-purified HeLa mononucleosomes (16 μg with respect to DNA) were labeled in a 150-μl reaction containing 50 mM Tris-HCl, pH 8.0, 10% glycerol, 10 mM sodium butyrate, 0.1 mM EDTA, 1.0 mM dithiothreitol, and 1.0 mM phenylmethylsulfonyl fluoride. Purified recombinant FLAG-p300 (600 fmol) or FLAG-PCAF (50 pmol) and [3H]acetyl-CoA (1.3 nmol at 50 nCi/threitol, and 1.0 mM phenylmethylsulfonyl fluoride. Purified recombinant FLAG-p300 (600 fmol) or FLAG-PCAF (50 pmol) and [3H]acetyl-CoA (1.3 nmol at 50 nCi/threitol, and 1.0 mM phenylmethylsulfonyl fluoride. Purified recombinant FLAG-p300 (600 fmol) or FLAG-PCAF (50 pmol) and [3H]acetyl-CoA (1.3 nmol at 50 nCi/threitol, and 1.0 mM phenylmethylsulfonyl fluoride.

Acid Urea Gel Analysis of Nucleosomal Histones Acetylated by the PCAF Complex—FLAG epitope-tagged PCAF was expressed in HeLa cells, and a stable high molecular weight PCAF complex was purified as described previously (31). Nucleosomes and free histones were acetylated as described (31), except that the incubation time of the reaction was increased to 1 h to maximize acetylation. The products of these reactions were analyzed on an acid-urea gel as described (32), except that Triton X-100 was omitted to avoid separation of H3 isofoms.

RESULTS AND DISCUSSION

To date, the specific lysine residues within the core histones targeted by a nuclear histone acetyltransferase have only been examined in detail for yeast GCN5 (8). Recombinant yeast GCN5 acetylates Lys14 of H3 and Lys8 and Lys16 of H4 when free (non-nucleosomal) histones are used as substrate. This pattern of acetylation is distinct and nonoverlapping with the acetylation pattern of the cytoplasmic HAT B involved in histone deposition (Refs. 33 and 34 and for review see Refs. 4 and 5). This suggests that acetylation of these residues may correlate with increased transcription.

All reports of acetylation site usage by specific HATs to date have utilized free (non-nucleosomal) histone or synthetic peptide substrates (8, 13, 35). Given their nuclear localization, it seems likely that the coactivators PCAF and p300 acetylate nucleosomal substrates in vivo. Because functional differences between PCAF and p300 have been reported (26–28), we sought to determine whether the acetylation site profiles of PCAF and p300 on a chromatin substrate were different. Recombinant human p300 and PCAF were used to label HeLa mononucleosomes with [3H]acetyl-coenzyme A. Individual core histones were then separated by reverse-phase HPLC and analyzed by N-terminal microsequencing. Radioactivity eluted in each cycle of the microsequencer was quantitated by scintillation counting for a direct measure of acetylation site usage. Previous studies have shown that the steady-state level of acetylation in unsynchronized growing HeLa cells is relatively low with the majority of the core histones being unacetylated or monoacetylated (36). Moreover, these studies have determined non-random site occupancy on H3 and H4. Lys14 of H3 and Lys8 and Lys16 of H4 are the residues most frequently acetylated in the monoacletylated forms of these histones in bulk chromatin (36). Thus, although the mononucleosome substrates used in these experiments were not completely devoid of acetylated groups, the majority of the core histones were unacetylated or monoacetylated. In interpreting our acetylation data, we have considered that a small fraction of H3 and H4 was pre-acetylated at Lys14 and Lys16, respectively.

Acetylation of mononucleosomes with PCAF results in strong acetylation of H3 and weak but reproducible acetylation of H4 (Fig. 1). The yeast homolog of PCAF, GCN5, is unable to acetylate mononucleosomes in vitro (37) but acetylates H3 and H4 when presented as free histones (8). Our results indicate that Lys14 of H3 (Fig. 2A) and Lys8 of H4 (Fig. 2B) are acetylated when purified from a PCAF-acetylated mononucleosome preparation. Little, if any, acetylation occurred at other known sites of in vivo acetylation. In the case of H3, the lack of acetylation at other residues is unlikely to be due to prior acetylation in vivo, because Lys14 is the preferred steady-state site in vivo, and this residue is strongly preferred by PCAF under our assay conditions.

We expected that Lys16 of H4 would be acetylated by PCAF because this residue is acetylated in free histone H4 by yeast GCN5 in vitro (8). We considered that Lys8 may have been unavailable for acetylation in our experiments, because this is a known preferred acetylation site in vivo in HeLa cells (37). We measured the amounts of bulk acetyllysine and lysine eluted in cycles 5, 8, 12, and 16 from the microsequencer. Less than 5% of the lysine residues in cycles 5 and 12 were acetylated (data not shown), indicating that the lack of acetylation at these residues by PCAF was not due to in vivo acetylation at these sites. We found that Lys16 of H4 was 59% acetylated (data not shown), even though little, if any, [3H]acetate was incorporated at this position (Fig. 2C). Thus site occupancy may have been a contributing factor to our inability to acetylate this site. Alternatively, access to Lys16 of H4 in nucleosomes by HATs may be restricted either due to the association of the H4 tail with nucleosomal DNA (38) or its proximity to the globular domain of H4 (38, 39). In this regard, it would be interesting to determine whether acetylation site usage is altered in the presence of ATP-dependent chromatin remodeling complexes, such as Swi/SNF, NURF, and CHRAC (for review see Ref. 40).

We have reported that PCAF in its native form is present in a multisubunit complex containing more than 20 polypeptides (31). Although not all of these subunits have been identified, the PCAF complex contains human counterparts of the yeast
ADA2, ADA3, and SPT3 proteins; a subset of TAFs (TBP-associated factors); and TAF-related factors. Consistent with the observation that the interaction of p300 and CBP with PCAF in vivo is not stoichiometric or stable (10), the PCAF complex contains no p300 or CBP (31). Importantly, the N-terminal half of PCAF, the region required for p300 and CBP interaction, is apparently dispensible for complex formation, because the short form of hGCN5, which lacks sequences homologous to the N terminus of PCAF, is found in an indistinguishable complex (31). This finding is consistent with the notion that the N terminus of PCAF may be involved in transient interactions with p300/CBP, other coactivators, or sequence-specific transcription factors.

The PCAF complex, like recombinant PCAF, preferentially acetylates H3 but weakly acetylates H4 (31). We examined whether the PCAF complex and recombinant PCAF exhibit a similar pattern of acetylation of H3 and H4. Mononucleosomes and free core histones were acetylated by the native PCAF complex or recombinant PCAF, and the degree of acetylation was determined by acid-urea gel analysis followed by autoradiography. In contrast to p300, which acetylates H3 and H4 on multiple lysine residues (Fig. 3, A, lane 2, and B, lane 2), both recombinant PCAF and the PCAF complex primarily acetylate H3 to a monoacetylated form when either free histones (Fig. 3A, lanes 3 and 4, respectively) or nucleosomes (Fig. 3A, lanes 6 and 7, respectively) are used as substrates. Histone H4 is also primarily monoacetylated; however, the complex does yield a significant level of diacetylated H4 on free histone H4 (Fig. 3B, lane 4). Because the steady-state level of H4 monoacetylated at Lys\(^{16}\) is approximately 60%, it is likely that this diacetylated form is derived from modification of endogenously monoacetylated H4 at a residue other than Lys\(^{16}\). Thus, we conclude that the PCAF complex and the recombinant PCAF catalytic subunit have a similar substrate specificity in that they both preferentially acetylate a single residue of H3 and are only able to weakly acetylate a single residue of H4 within nucleosomal substrates in vitro.

We have previously shown that p300 is capable of acetylating all four core histones in HeLa mononucleosomes (Ref. 11 and Fig. 1, lane 2). Sequence analysis of nucleosomal H3 acetylated by p300 revealed a strong preference for Lys\(^{14}\) and Lys\(^{18}\) with a significant amount of labeling on Lys\(^{20}\) (Fig. 4C). A low level of \[^3H\]acetyl cpm was also detected at Lys\(^{4}\). Thus, p300 is capable of acetylating four of the six lysines known to be acetylated in vivo but has a preference for Lys\(^{14}\) and Lys\(^{18}\). Significantly, p300 did not detect acetyl residues Lys\(^{9}\) and Lys\(^{27}\) of H3. Acetylation at these sites has been correlated with deposition of H3 in replicating chromatin (33, 34), and the lack of acetylation at these sites by p300 is strong evidence for the involvement of a distinct activity in deposition-related acetylation of H3. Interestingly, both PCAF and p300, which can associate under certain conditions in vivo (10), demonstrate a strong preference for Lys\(^{14}\) of H3. The combined action of both of these HATs at this residue may contribute to the high steady-state level of acetylation of this residue observed in vivo (36).

When nucleosomal H4 was acetylated by p300, a strong preference for Lys\(^{5}\) and Lys\(^{8}\) was detected (Fig. 4D). Considerably less acetylation occurred at Lys\(^{12}\) and Lys\(^{16}\). As mentioned above, relatively high steady-state levels of acetylation at Lys\(^{12}\) and Lys\(^{16}\) or restricted accessibility of this residue within nucleosomes may result in low levels of acetylation at this site in vivo.
Acetylation of Lys\(^6\) and Lys\(^{12}\) has been correlated with histone deposition (33, 34); however, the results presented here suggest that acetylation of these sites may also play a role in transcriptional activation. The role of these particular acetylation events within these distinct processes may be distinguished by the context in which they occur (e.g. nucleosomal and nuclear versus non-nucleosomal and cytoplasmic) and also the potential for additional acetylations in combinatorial fashion by transcription-associated HATs.

Sequence analysis of H2A demonstrated an absolute specificity of p300 for Lys\(^6\), the predominant site acetylated in H2A \textit{in vivo} (36), despite the presence of three additional lysines (Fig. 4A). This single residue specificity was in stark contrast to the multi-residue specificity of p300 for H2B. H2B contains 10 lysine residues within its first 30 amino acids, 4 of which (Lys\(^3\), Lys\(^{12}\), Lys\(^{15}\), and Lys\(^{20}\)) have been reported to be acetylated \textit{in vivo} (Ref. 36 and Table I). All four of these sites are acetylated by p300 \textit{in vitro} with an apparent preference of p300 for Lys\(^{12}\) and Lys\(^{15}\) (Fig. 3B). Significantly, acetylation was not detected at lysine residues that are not known to be acetylated \textit{in vivo}. (Note that we attribute the [\(^3\)H]acetyllysine signal detected in cycle 16 of this analysis to carryover of a portion of the strong signal at Lys\(^{15}\) due to sequencing lag). These findings indicate that the HAT activity of p300 is highly selective for known in \textit{vivo} acetylation sites and acetylates essentially all the lysines in H2A and H2B that are acetylated \textit{in vivo}. The acetylation site specificity of p300 and PCAF with nucleosomal substrates under our assay conditions is summarized in Table 1.

The results presented here demonstrate that both PCAF and p300 acetylase known \textit{in vivo} acetylation sites. Therefore, although it has been reported that these enzymes are able to acetylate and modulate the activities of proteins other than histones \textit{in vitro} (41–43), our data lend strong support to the idea that core histones are bona fide substrates of p300 and PCAF \textit{in vivo}, possibly in addition to other proteins. Note that histones were recently shown to be targeted by the HAT activity of yeast GCN5 \textit{in vivo} (18).

We show striking differences in the acetylation sites preferred by p300 and PCAF in nucleosomal histones, with PCAF showing specificity for fewer sites than p300 under the conditions employed here. Recent studies have demonstrated that the HAT activity of PCAF is required for myogenic differentiation (26), as well as nuclear hormone receptor (27) and growth factor-dependent signaling (28), whereas that of p300 is dispensable. One hypothesis consistent with our data and these unique requirements for the HAT activity of PCAF over that of p300 is that acetylation site-specific phenomena are involved in transcriptional regulation. The mechanism(s) by which acetylation facilitates transcription remains to be defined, yet current data are consistent with the possibility that acetylation at sites preferred by PCAF in nucleosomes positioned at promoters may serve to recruit factors required for transcription that are not recruited by acetylation at sites preferred by p300. Alternatively it is conceivable that differences in the manner in which PCAF and p300 themselves are recruited to promoters underlie the differential functional requirements. For example, PCAF may be recruited to promoters through a transient interaction with p300/CBP and, once recruited, may associate with other factors that allow more extensive accessibility to nucleosomes beyond the immediate transcription factor binding site. That is, the HAT activity of p300/CBP may be unable to direct extensive acetylation within the promoter region or throughout the coding region of the gene due to its sequestration at the transcription factor binding site. Identification of the sites acetylated by these HATs in nucleosomal substrates, as reported here, should facilitate experimental investigation of the mechanisms of activation by these and other transcriptional regulators that possess HAT activity.

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