The Histone Acetyltransferase Activity of Human GCN5 and PCAF

Is Stabilized by Coenzymes*

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Julio E. Herrera‡§, Michael Bergel‡, Xiang-Jiao Yang‡, Yoshihiro Nakatani‡, and Michael Bustin‡

From the ‡Laboratory of Molecular Carcinogenesis, Division of Basic Sciences, National Cancer Institute and the §Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

Here we report that PCAF and human GCN5, two related type A histone acetyltransferases, are unstable enzymes that under the commonly used assay conditions are rapidly and irreversibly inactivated. In addition, we report that free histone H1, although not acetylated in vivo, is a preferred and convenient in vitro substrate for the study of PCAF, human GCN5, and possibly other type A histone acetyltransferases. Using either histone H1 or histone H3 as substrates, we find that preincubation with either acetyl-CoA or CoA stabilizes the acetyltransferase activities of PCAF, human GCN5 and an enzymatically active PCAF deletion mutant containing the C-terminal half of the protein. The stabilization requires the continuous presence of coenzyme, suggesting that the acetyltransferase-coenzyme complexes are stable, while the isolated apoenzymes are not. Human GCN5 and the N-terminal deletion mutant of PCAF are stabilized equally well by preincubation with either CoA or acetyl-CoA, while intact PCAF is better stabilized by acetyl-CoA than by CoA. Intact PCAF, but not the N-terminal truncation mutant or human GCN5, is autoacetylated. These findings raise the possibility that the intracellular concentrations of the coenzymes affect the stability and therefore the nuclear activity of these acetyltransferases.

Post-translational modification of chromosomal proteins, in particular the specific acetylation of histones, is correlated with various nuclear activities such as replication, chromatin assembly, and transcription (1–10). The acetylation state of specific lysine residues in the evolutionarily conserved tails of the core histones can alter the interaction between these histones and the DNA and perhaps also the interactions between the histone tail domains in adjacent nucleosomes. These interactions affect the state of chromatin compaction and therefore may be involved in regulating access to the underlying DNA sequence. Increased histone acetylation promotes chromatin decompaction (11) and is associated with gene activation, while loss of acetyl from histone tails is associated with gene silencing (12). For example, in the inactive X chromosome of female cells the core histones are underacetylated, while in the active X chromosome the histones are acetylated (13).

The recent isolation and characterization of several enzymes involved in the acetylation and deacetylation of specific histone residues provide additional insights into the possible relation between histone acetylation and gene activation (8, 14, 15). The Tetrahymena histone acetyltransferase is homologous to the yeast protein GCN5, which is a putative transcription activator and contains intrinsic acetyltransferase activity (8). We have recently demonstrated that human PCAF, a protein that competes with oncoprotein E1A for binding to the gene products of the p300/CBP and retinoblastoma gene families, has intrinsic acetyltransferase activity and is in part homologous to GCN5 protein. More recently, Ogryzko et al. demonstrated that the P300 protein has intrinsic histone acetyltransferase activity (15). Taken together, these results suggest that some transcription activators may be able to acetylate histone tails, thereby disrupting the nucleosomal structure and promoting transcription from chromatin templates.

Although the genes coding for PCAF and GCN5 have been characterized and recombinant proteins have been produced in several systems, the enzymatic properties of these enzymes have not been extensively studied. In this article, we report that human GCN5, the catalytic subunit of a type A nuclear histone acetyltransferase (14, 16), and PCAF, a histone acetyltransferase containing a domain that is highly homologous to the human GCN5 (14), are highly unstable enzymes that rapidly and irreversibly lose histone acetyltransferase activity. We demonstrate that histone acetyltransferase activity is stabilized by the continuous presence of the coenzymes acetyl-CoA or CoA and that PCAF, but not hGCN51 is autoacetylated, a modification that may further stabilize this enzyme. In addition, we demonstrate that both PCAF and hGCN5 catalyze the transfer of acetyl groups into free but not chromatin-bound histone H1. These results provide new information on the properties of these histone acetyltransferases and are pertinent to studies on the cellular function and mechanism of action of these, and perhaps other, histone acetyltransferases.

MATERIALS AND METHODS

Substrates and Enzymes—Recombinant human GCN5 and PCAF were prepared as described previously (14). Mutant PCAF having an internal deletion of amino acid residues 61–465 (see Ref. 14) and containing the Flag tag (Kodak) was expressed and purified by the same approach as described for the intact PCAF (14). Histone H1 was extracted from calf thymus with 5% trichloroacetic acid and purified by chromatography on Amberlite IRC-50 (17). Histone H3 was extracted from calf thymus with 0.1 M HCl, oxidized, and purified on Sephacryl G-100 (18). Chromosomal protein HMG-1 was isolated from calf thymus by extraction with 0.35 M NaCl and purified by ion exchange chromatography (19). Lysozyme, cytochrome c, acetyl-CoA, and CoA were obtained from Sigma. [1-14C]Acetyl-CoA (55 mCi/mmol) was obtained from New England Nuclear, and 2H]acetate was obtained from ICN.

The abbreviations used are: hGCN5, human GCN5; PCA, perchloric acid.

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‡ To whom correspondence and requests for reprints should be addressed; Bldg. 37, Room 3D-12, National Cancer Institute, NIH, Bethesda, MD 20892. Tel.: 301-496-2885; Fax: 301-496-8419; E-mail: herr@helix.nih.gov.

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Enzymatic Assay of Histone Acetyltransferases—All assays were performed in Buffer A (50 mM Tris-HCl (pH 8.0), 10% glycerol (v/v), 1 mM dithiothreitol, 0.1 mM EDTA, 10 mM butyric acid) (20). Substrate concentrations were 0.1–0.25 mg/ml and [1-14C]acetyl-CoA concentrations were at 9 μM (unless otherwise indicated). The assay was performed at 37 °C and initiated by addition of the protein substrate to a mixture containing the acetyltransferase and acetyl-CoA in buffer A. The radioactivity incorporated into the protein substrates was detected by either a filter, or a polyacrylamide gel assay. For the filter assay the reaction was stopped at given times by spotting onto filter papers (Whatman F81), which were then washed and counted as described previously (21). In the polyacrylamide gel assays, the reaction was stopped by the addition of an equal volume of an SDS-gel sample buffer (100 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 20% glycerol), boiled for 5 min, and loaded onto either a 10 or 15% polyacrylamide-SDS gel (22) and electrophoresed at 15 V/cm until the bromphenol blue reached the bottom of the gel. The gels were stained with Coomassie Blue for estimation of protein quantities and then soaked in Enlightening Enhancer solution (Dupont) for 30 min and vacuum dried; the radioactivity incorporated into the protein bands was then visualized and quantified with a PhosphorImager (Molecular Dynamics) using Imagequant software. Acetylation of H1 in the presence of DNA was performed with DNA isolated from mononucleosomes (average length, 200 base pairs). The ratio was adjusted to 1 molecule of H1 per 200 base pairs of DNA.

Estimates of the Ks and Vmax of PCAF for H1 were determined using Lineweaver-Burk plots. The kinetic parameters were estimated for enzyme that had been preincubated with or without acetyl-CoA (9 μM) for 10 min at 37 °C. The concentration of histone H1 was varied from 0.02 to 2 mg/ml. The enzymatic reactions were initiated by the addition of the protein substrate and sufficient acetyl-CoA to bring its final concentration to 15 μM.

Labeling of Chromosomal Proteins in Tissue Culture Cells—HeLa cells were grown in suspension in minimal Eagle’s medium. Exponentially growing cells (200 ml, 5.0 × 10^5 cells/ml) were concentrated by centrifugation. The cells were resuspended in 8 ml of medium containing 200 μg/ml of cycloheximide (Sigma) and incubated at 37 °C for 10 min. After the incubation, one culture was brought to 0.5 μM trichostatin A (WAKO), and 2 μC of [3H]acetate (5 Ci/mmol, ICN) was added; the second culture was treated identically except no trichostatin A was added. The labeling was performed for 1 h at 37 °C with gentle rocking. Each culture was split into two, and the cells were harvested by centrifugation. One set of cells from each treatment was extracted with 5% perchloric acid (PCA) and precipitated with 6 volumes of acetone to measure incorporation into H1, and the second set was extracted with 0.25 M sulfurous acid and precipitated with 6 volumes of acetone to measure incorporation into the core histones. The incorporation of [3H]acetate into the given protein was measured by resolving the proteins on a 15% SDS/PAGE, staining the gel with Coomassie Blue to estimate the amount of each protein, and then excising the protein bands from the gel. The excised bands were digested at 65 °C overnight with 30% hydrogen peroxide, and the [3H]acetate incorporation into each band was determined by scintillation counting.

Mouse Thymus Oligonucleosomes—Mouse thymus nuclei were isolated by homogenization and sedimentation in sucrose as described previously (23). The nuclei (3 mg/ml in DNA) were mildly digested with micrococcal nuclease (100 units/ml, 5 min at 37 °C; Boehringer Mannheim). The digested nuclei were harvested and extracted in 0.25 mM EDTA, 1 mM phenylmethylsulfonfyl fluoride at 4 °C. The extract was centrifuged, and the supernatant was loaded onto a 12–50% sucrose gradient containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM NaCl. After 20 h of centrifugation at 28,000 rpm (Beckman rotor, SW28), the gradient was fractionated, and the DNA in each fraction was analyzed using agarose gels. The fractions corresponding to oligonucleosome lengths of 4–10 oligonucleosomes were pooled, dialyzed to remove the sucrose, and concentrated by centrifugation dialysis (Filtron).

RESULTS AND DISCUSSION

Human PCAF and GCN5 Acetylase Histone H1 in Vitro—PCAF and hGCN5, two related human histone acetyltransferases (14), exhibit identical substrate preferences (Fig. 1). In addition to the previously documented acetylation of both free and nucleosomal bound histone H3 (14, 24), the linker histone H1 is also an excellent substrate for both enzymes. Quantita-

**Table I**

| Protein | Specific activity |
|---------|------------------|
| Histone H4 | 100 |
| Histone H2 | 67 |
| Histone H2B | 51 |
| Histone H2A | 33 |
| Histone H1 | <3 |

**FIG. 1.** PCAF and hGCN5 acetylate histone H1. The ability of the two enzymes to incorporate acetate into the substrates is examined by autoradiography. A, C, protein gels; B, D, corresponding autoradiographs; A, B, PCAF; C, D, hGCN5. Substrates: H3, histone H3; NucL, nucleosome core particles; H1, histone H1; HMG1, nonhistone protein HMG1; Cyt C, cytochrome c; Lys, chicken lysozyme.

**FIG. 2.** PCAF cannot acetylate H1 in the presence of DNA or in the context of chromatin. H1 acetylation by PCAF was examined using the gel assay described under "Materials and Methods." A, Coomassie Blue stain; B, PhosphorImager scan for 14C incorporation. Lanes 1 and 3, histone H1 from calf thymus and mouse thymus, respectively; lanes 2 and 4, as lanes 1 and 3 except with the addition of free nucleosomal DNA (H1 to DNA weight ratio was 1 molecule of H1 per 200 base pairs of DNA); lanes 5 and 6, two mouse thymus oligonucleosome preparations containing endogenously bound H1.
tive analysis indicates that both PCAF and hGCN5 acetylate histone H1 2-fold more efficiently than histone H3 (see also Fig. 3). The acetylation of histone H1 is specific since other positively charged proteins containing numerous lysine residues (lysozyme, cytochrome c, HMG-1, nucleosomal histones H2A and H2B), similar to histone H1, were not substrates for either acetyltransferase (Fig. 1).

Histone H1 is known to be acetylated at its N terminus; however, numerous studies on histone acetylation failed to detect post-translational acetylation of this histone (for review, see Ref. 2). Since our studies suggested that H1 is an excellent substrate for human histone acetyltransferases, we reexamined whether histone H1 is acetylated in HeLa cells. The incorporation of [3H]acetate into histones was determined in the presence or absence of a histone deacetylase inhibitor (trichostatin A) after treatment of the cells with cycloheximide to inhibit protein synthesis. Quantitation of the specific radioactivity of each histone showed that the specific activity of histone H1 was <5% of that of histone H4 (Table I). Trichostatin A increased the level of acetylation of the core histones but not that of histone H1. Thus, our results are in agreement with previous findings (2) which indicated that in vivo, histone H1 is not acetylated post-translationally.

To explain the apparent discrepancy between the in vivo and in vitro acetylation of H1, we examined the ability of PCAF to acetylate H1 in the presence of DNA or in the context of purified oligonucleosomes containing endogenously bound H1. Fig. 2 shows that purified histone H1 either from calf or mouse thymus is efficiently acetylated by PCAF. However, acetylation of H1 is completely abolished either by addition of DNA or in the context of oligonucleosomes. In contrast, histone H3 in oligonucleosomes was well acetylated, indicating that the en-
Acetyl-CoA stabilizes the acetyltransferase activity of PCAF. PCAF was incubated, under the standard assay conditions, in either the presence (●) or absence (○) of 9 μM acetyl-CoA for the time indicated. At the end of the incubation, the remaining acetyltransferase activity was determined by the filter assay using histone H1 as substrate.

Stabilization of PCAF Histone Acetyltransferase Activity by Acetyl-CoA—During these studies, we noted that in the absence of acetyl-CoA, the acetylation activity of PCAF was rapidly lost (Fig. 3). In these assays, the enzyme was preincubated in the buffer used for acetylation in either the presence or absence of acetyl-CoA prior to activity measurements using histone H1, histone H3, or nucleosome cores as substrates. After 10 min of preincubation in the absence of acetyl-CoA, the acetylation activity of PCAF is rapidly and irreversibly lost and that this loss is not a result of degradation or proteolytic degradation during preincubation in the absence of acetyl-CoA. Conceivably, the presence of acetyl-CoA protects PCAF from proteolytic degradation. We used polyacrylamide gels to estimate the amount of PCAF protein present after 10 min of preincubation in either the presence or absence of radioactive acetyl-CoA. The amount of PCAF protein, as determined by the intensity of the Coomassie Blue band corresponding to the 83,000 protein (14) did not diminish under either condition (Fig. 5). After a 10-min preincubation in the absence of acetyl-CoA, the self-acetylating activity of the enzyme was lost, and subsequent addition of radioactive [1-14C]acetyl-CoA to this PCAF preparation did not result in autoacetylation (Fig. 5B). In contrast, in the presence of acetyl-CoA, a significant amount of radioactivity was incorporated into PCAF (Fig. 5B). We conclude that in the absence of acetyl-CoA the acetylation activity of PCAF is rapidly and irreversibly lost and that this loss is not a result of degradation of the enzyme. These results and the kinetic analysis suggest that in the absence of coenzyme PCAF is irreversibly inactivated.

Coenzymes Also Stabilize the Acetylation Activity of hGCN5—Because the amino acid sequence of C-terminal half of PCAF is 86% homologous to the human GCN5 (14) and the two proteins have identical acetyltransferase substrate specificity (Fig. 1), we tested whether preincubation with acetyl-CoA also protects hGCN5 from inactivation. In these experiments, the enzyme was preincubated in either the presence
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or absence of acetyl-CoA before to the addition of the histone substrate. The results indicate that in the absence of the coenzyme the ability of hGCN5 to incorporate acetyl groups into histone H1 or histone H3 is rapidly lost, suggesting that this enzyme undergoes a rapid inactivation in a manner indistinguishable from that of PCAF. As with PCAF, within 10 min of preincubation without the substrate, the $V_{\text{max}}$ of hGCN5 decreased 5-fold, from 12.4 pmol of acetyl/µg of enzyme/min for the enzyme incubated in the presence of coenzyme to 2.5 pmol of acetyl/µg of enzyme/min for the enzyme incubated in the absence of the coenzyme. The $K_m$ was not changed significantly.

PCAF, but Not GCN5 or the N-terminal Truncated PCAF, Require Acetyl-CoA for Full Stability—To better understand the factors associated with the stabilization, we examined the ability of both CoA and acetyl-CoA to stabilize the activity of PCAF, hGCN5, and an N-terminal truncation mutant of PCAF. The N-terminal truncation mutant of PCAF contains an internal deletion of amino acids 61–465 and retains the domain that is homologous to the human and yeast GCN5 (14) and is competent in both histone H1 and histone H3 acetylation. We tested whether the continuous presence of the coenzyme is required for stabilization. In the experiments depicted in Fig. 6, we incubated the intact enzyme, the N-terminal truncation mutant of PCAF, and hGCN5 for 5 min either with or without acetyl-CoA or CoA. After incubation, the unbound coenzyme was removed using a spin column (G50-Prebound column [Pharmacia]), and the enzymes were again incubated for various times in the absence of additional coenzymes prior to determination of acetyltransferase activity. The results (Fig. 6) indicate that both acetyl-CoA and CoA protect all three recombinant enzymes from inactivation albeit to differing degrees (2–10-fold). After removal of the unbound coenzymes, all three acetyltransferases exhibited a gradual decline in activity. This suggests that the stabilization is mediated by bound coenzyme and not a coenzyme-mediated modification of the structure of the enzymes. Acetyl-CoA stabilized hGCN5 and the N-terminal truncation mutant to the same extent as CoA (Fig. 6, B and C). However, with full-length PCAF, the enzymatic activity was stabilized by acetyl-CoA better than by CoA (6-fold above control and 2-fold above control, respectively), indicating that the presence of the N-terminal domain confers this preferential stabilization by acetyl-CoA.

PCAF Is Autoacetylated in the N-terminal Region—During these studies, we noted that PCAF incorporated counts during its preincubation with [1-14C]acetyl-CoA, raising the possibility that PCAF is autoacetylated. The incorporation of counts into PCAF could result from a catalytic intermediate formed at the active site of the enzyme, or alternatively, from a covalent modification. To distinguish between these possibilities, we tested whether the radioactivity incorporated into PCAF by a 10-min incubation with [1-14C]acetyl-CoA could be chased by either unlabeled acetyl-CoA, histone H1, or a mixture of both unlabeled acetyl-CoA and histone H1. As indicated by the data presented in Fig. 7, neither the addition of unlabeled acetyl-CoA nor the addition of unlabeled acetyl-CoA in conjunction with substrate affected the amount of 14C incorporated into the enzyme. Throughout these experiments, the acetyltransferase activity of PCAF was not diminished. Neither hGCN5 nor the N-terminal truncated PCAF mutant incorporates counts during incubation with acetyl-CoA, suggesting that the modification occurs in the N-terminal region of PCAF. In fact, a C-terminal truncated PCAF containing only the N terminal half of PCAF is devoid of intrinsic acetyltransferase activity yet can be acetylated by full-length PCAF. These results suggest that the autoacetylation of PCAF occurs by an intermolecular reaction in its N-terminal domain. The N terminus is required for PCAF to distinguish, with regard to stability, between acetyl-CoA and CoA. It is conceivable that autoacetylation may play a role in the ratio of CoA to acetyl-CoA, providing a potential means of regulating the enzymatic activity.

In summary, our experiments demonstrate that the human acetyltransferases PCAF and GCN5 are unstable enzymes that are rapidly and irreversibly inactivated. The enzymes are stable in the presence of their coenzymes. The stabilization appears to be mediated by binding of the coenzyme, suggesting that the stable species is an enzyme-coenzyme complex. Based on these findings, it is tempting to speculate that the intracellular activity of these enzymes may be regulated in part by the intracellular levels of these coenzymes.

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