**Communication**

**Novel Substrate Specificity of the Histone Acetyltransferase Activity of HIV-1-Tat Interactive Protein Tip60**

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Tohru Yamamoto and Masami Horikoshi  
From the Laboratory of Developmental Biology, Department of Cellular Biology, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

Tip60, originally isolated as an HIV-1-Tat interactive protein, contains an evolutionarily conserved domain with yeast silencing factors. We demonstrate here direct biochemical evidence that this domain of Tip60 has histone acetyltransferase activity. The purified recombinant effectively acetylates H2A, H3, and H4 but not H2B of core histone mixtures. This substrate specificity has not been observed among histone acetyltransferases analyzed to date. These results indicate that Tip60 is a histone acetyltransferase with a novel property, suggesting that Tip60 and its related factors may introduce a distinct alteration on chromatin.

Genetic material of eukaryotes is packaged into chromatin, of which the most fundamental structural unit is the nucleosome comprised of DNA and histones (1). Core histones are not only the primary proteins that fold DNA into chromatin but also play a key role in transcriptional regulation (reviewed in Ref. 2). Recent molecular cloning of histone acetyltransferase (HAT) (3) followed by identification of transcription factors that act as a HAT provided insights into understanding the mechanisms of transcriptional regulation through core histone acetylation at the molecular level (Refs. 4–7; reviewed in Refs. 8–11). Currently identified nuclear HATs are transcription factors such as GCN5-related factors, p300/CBP, and TAFI250. These findings further confirm the correlation between core histone acetylation and transcriptional activity of chromatin. In the case of transcriptional regulation of HIV-1, recent observations have shown that global hyperacetylation of histones induced by histone deacetylase inhibitors promotes nucleosome disruption at the transcriptional start site of the 5’ LTR to lead transcriptional activation of HIV-1 5’ LTR, suggesting a role of histone acetylation in transcriptional regulation of HIV-1 (12).

Tip60 was isolated as an HIV-1-Tat interactive protein and has been shown to modestly augment Tat-dependent transcriptional activation (13). It contains an evolutionarily conserved domain (about 250 amino acids in length; 40–50% identity) shared by several factors isolated by different genetic procedures (14–16). MOZ, a human member of this family, was found as a gene fused to CBP by a recurrent translocation associated with acute myeloid leukemia (14). mof, a related gene in Drosophila, was genetically isolated as a factor required for X-linked dosage compensation, the male-specific hypertranscription of X-linked genes (15). SAS2, a related gene in yeast, was isolated as a positive effector of transcriptional silencing and seems to be incorporated in a process of transcriptional repression together with its related factor SAS3 (16). The domain contains a short structural motif that is found in acetyltransferases and is speculated to be an acetyl-CoA binding site (14–16). It is an attractive hypothesis that Tip60 is an acetyltransferase whose substrates are histones; however, the primary structure of that domain, besides the short structural motif consisting of 20 amino acids in length, is totally unrelated to known acetyltransferases, and no biochemical evidence has been reported on the acetyltransferase activity to date (14–16). These results lead us to ask the following questions. First, whether the conserved domain among Tip60 and its related genes has acetyltransferase activity that modifies histones. Second, if it should have HAT activity, which histone species are acetylated, because the primary structure of the conserved domain other than the 20-amino acid structural motif is totally unrelated to known HATs. To address these questions, we investigated the histone acetyltransferase activity of the evolutionarily conserved domain of Tip60.

**EXPERIMENTAL PROCEDURES**

**Production and Purification of Recombinant Tip60**—Isolation of cDNA fragments encoding Tip60 as a potential interacting factor with a native human transcription factor will be published elsewhere. General methods for DNA manipulation were as described (17). NdeI recognition sequence was introduced at the first in-frame methionine codon (nucleotide position 605) of the Tip60 cDNA with the mutagenic oligonucleotide TCTGATGGAATACCGTCAGGATCCCATATGACTGTCGCGACCGCTG (18). Note that the proposed translation start site of Tip60 is not methionine but leucine headed by no in-frame stop codon (13).2 The NdeI (created) BamHI fragment of the resultant plasmid was introduced into the His6-T-PET11d (19). The resultant plasmid was transfected into Escherichia coli strain BL21(DE3)/LexA (20), and the transformed E. coli were grown at 27 °C. Expression of the transfected gene was induced by adding isopropyl β-D-thiogalactopyranoside to 0.4 mM when the A600 of the culture reached 0.8. 3 h after induction, the bacteria were collected and washed with buffer containing 20 mM Tris-HCl (pH 7.9 at 4 °C) and 0.2 mM NaCl. The washed bacteria cells were suspended in buffer containing 20 mM Tris-HCl (pH 7.9 at 4 °C), 0.5 mM NaCl, 50 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride  

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2 To whom correspondence should be addressed. Tel.: 81-3-5802-3388; Fax: 81-3-5864-8341; E-mail: horikoshi@imcbns.iam.u-tokyo.ac.jp.

3 The abbreviations used are: HAT, histone acetyltransferase; CBP, Coomasie Brilliant Blue; CoA, coenzyme A; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; LTR, long terminal repeat; HIV-1, human immunodeficiency virus, type 1.
First we tested the histone acetyltransferase activity of Tip60C. Puriﬁed recombinant Tip60C was incubated with calf thymus histones and [3H]acetyl-CoA. Acetylation of histones was detected by spotting onto P81 phosphocellulose ﬁlter paper (Whatman) and washed with 0.2 M sodium carbonate (pH 9.2) for 10 min at room temperature. The ﬁlter paper was successively washed with the same buffer for 10, 5, and 5 min at room temperature, respectively. The washed ﬁlter paper was dried for 30 min at room temperature and counted in a liquid scintillation counter.

PAGE analysis was performed as described above except that 90 pmol of [14C]acetyl-CoA (NEL Life Science Products; 37 GBq/mmol) and 0.1 mg/ml bovine serum albumin (Seikagaku Co.) were used as substrates. Their purity was veriﬁed by CBB staining the image of SDS-PAGE (see Fig. 4). Core histones and nucleosomes of HeLa cell nuclei were prepared as described (6).

RESULTS AND DISCUSSION

Preparation of Recombinant Tip60 Protein—To test the biochemical nature of Tip60, a recombinant Tip60 C-terminal fragment was prepared. The C-terminal fragment of Tip60, depicted in Fig. 1A, was introduced into 6HisT-pET11d, which expresses proteins in E. coli with six histidine residues appended to the N termini (19). This fragment composed of the highly conserved region shared with MOZ, mof, SAS2, and SAS3 was designated Tip60C (Fig. 1A). Although most of the expressed protein become insoluble in E. coli, a fraction of Tip60C remained soluble and efﬁciently bound heparin-Sepharose resin. Recombinant Tip60C was further puriﬁed by nickel-agarose chromatography, around 90% purity estimated from CBB staining (Fig. 1B). This fraction was used in the following biochemical experiments.

Tip60C Has Histone Acetyltransferase Activity—First we tested the histone acetyltransferase activity of Tip60C. Puriﬁed recombinant Tip60C was incubated with calf thymus histones and [3H]acetyl-CoA. Acetylation of histones was detected by retained 3H radioactivity on P81 ﬁlter paper (6). Histone-dependent radioactivity retained on the ﬁlter paper was observed in the presence of Tip60C as well as in the presence of the recombinant p300 fragment (6), which has been previously reported to have HAT activity (Fig. 2). The nickel-agarose bound fraction of the extract prepared from E. coli harboring only 6HisT-pET11d vector plasmid showed negligible activity in comparison to the background. These results indicate that Tip60C readily acetylates histones and provide the ﬁrst biochemical evidence of HAT activity of Tip60 and its related factors.

Tip60 Has Potential to Locate at Nucleus—HAT has a species localized in cytoplasm as HAT1 in yeast (4), and the
putative acetyl-CoA binding motif found in Tip60 resembles more closely that of HAT1 than that of Gcn5-related nuclear HATs (Fig. 1A). Lack of direct evidence of subcellular localization of Tip60 and its related factors leads us to determine where Tip60 can be distributed in a cell. GFP was appended to the N-terminus of the C-terminal region of Tip60 started from the 132nd residue from the putative translation start site, and the resultant fusion protein was transiently expressed in COS cells. GFP fluorescence of the fusion protein was primarily detected in the nucleus with some speckled images, whereas GFP itself was plainly distributed in the cells (Fig. 3), indicating that Tip60 has the potential to be readily transported into the nucleus. These findings strongly suggest that Tip60 and its related factors form a new family of nuclear HAT whose primary structure is unrelated to other HATs reported so far. Subcellular localization of GFP-Tip60 fusion protein in nucleus with speckles suggests that Tip60 may have the potential to be incorporated in a distinct complex in the nucleus.

**Substrate Specificity of Tip60C Histone Acetyltransferase Activity**—Difference of the primary structure of the conserved domain of Tip60 to other known HATs leads us to speculate that its substrate specificity for histones might be different from others. To determine the substrate specificity of the histone acetyltransferase activity of Tip60C, a core histone mixture prepared from HeLa cell nuclei were incubated with Tip60C and [14C]acetyl-CoA. The resultant acetylated histones were separated on SDS-polyacrylamide gel. Autoradiogram of the separated histones clearly indicated that Tip60C acetylated core histones H2A, H3, and H4 but that no acetylation was observed for H2B (Fig. 4). When all four species of core histone mixture were used as substrates, CBP/p300 acetylates all histones and others (yGcn5p, hGCN5, P/CAF, and TAF II250) acetylate predominantly H3 and H4 (5–7, 22). Hat1p, a cytoplasmic histone acetyltransferase, acetylates H4 (4). No histone acetyltransferase analyzed to date acetylates histones with this specificity, indicating that Tip60 is a nuclear HAT with novel substrate specificity. The biological significance of this substrate specificity is currently under investigation; however, this specificity may reflect the functional characteristics of Tip60-related factors. One possibility is that Tip60 and probably its related factors may distinctly mark a specific position in chromatin by this unique acetylation of histones, which could be a clue for subsequent molecular association. It should be pointed out that nuclear histone acetyltransferases analyzed to date, such as GCN5, P/CAF, CBP, and TAF II250, are primarily considered to play a role in transcriptional activation, whereas Tip60-related factors may differently contribute to regulation of transcription. Tip60 is suggested to augment Tat-dependent transcriptional activation (13), and 
mof was genetically isolated as a factor required for X-linked dosage compensation (15). These factors appear to be involved in transcriptional activation. On the contrary, SAS2 and SAS3 were isolated as positive regulators of transcriptional silencing (16), and they seem to be involved in the repression of transcription. It would be plausible that specific molecular assembly directing unique acetylation of histones modified by Tip60 and its related factors subsequently causes a distinct effect on transcription together with their interacting factors. HIV-1 Tat might affect these processes through interaction with Tip60 to augment the transcriptional activation of HIV 5′ LTR.

Tip60C readily acetylates histones; however, the activity was greatly reduced when nucleosomes were used as substrates (data not shown), suggesting that Tip60 might function as a member of a multi-protein complex in vivo, as in the case of GCN5 (23, 24). Alternatively, because Tip60C is a fragment of Tip60 composed of an evolutionarily conserved region in this family, it might be possible that the N-terminal region of Tip60 might be necessary to acetylate nucleosomal histones. The amino acid sequences flanking the conserved region are different among the members of this family (14–16), leading to the attractive hypothesis that their potential HAT activity might be regulated by their flanking region and/or associating factor(s) to these regions, which might produce functional differences observed in Tip60 and its related factors.

We have directly proven that Tip60 is a nuclear histone acetyltransferase with a novel substrate specificity. Our observations should be valuable in understanding the molecular mechanisms of transcriptional regulation governed by Tip60 and its related factors.

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