

Multiple Histone Deacetylases and the CREB-binding Protein Regulate Pre-mRNA 3'-End Processing*

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Trichostatin A (TSA), a specific inhibitor of histone deacetylases (HDACs), induces acetylation of various non-histone proteins such as p53 and α -tubulin. We purified several acetylated proteins by the affinity to an anti-acetylated lysine (AcLys) antibody from cells treated with TSA and identified them by mass spectrometry. Here we report on acetylation of CFIm25, a component of mammalian cleavage factor Im (CF Im), and poly(A) polymerase (PAP), a polyadenylating enzyme for the pre-mRNA 3'-end. The residues acetylated in these proteins were mapped onto the regions required for interaction with each other. Whereas CBP acetylated these proteins, HDAC1, HDAC3, HDAC10, SIRT1, and SIRT2 were involved in *in vivo* deacetylation. Acetylation of the CFIm25 occurred depending on the cleavage factor complex formation. Importantly, the interaction between PAP and CF Im complex was decreased by acetylation. We also demonstrated that acetylation of PAP inhibited the nuclear localization of PAP by inhibiting the binding to the importin α/β complex. These results suggest that CBP and HDACs regulate the 3'-end processing machinery and modulate the localization of PAP through the acetylation and deacetylation cycle.

Eukaryotic mRNA precursors (pre-mRNAs) are synthesized and processed in the nucleus prior to their export to the cytoplasm. Transcription is thought to be coupled spatially and temporally to the capping of the pre-mRNA at the 5'-end, splicing, and 3'-end formation (1, 2). The mature 3'-ends of most eukaryotic mRNAs are generated by endonucleolytic cleavage of the primary transcript followed by the addition of a poly(A) tail to the upstream cleavage product. In mammals, these reactions are catalyzed by a large multicomponent complex that is assembled in a cooperative manner. The cleavage and polyadenylation specificity factor (CPSF)² recognizes the highly con-

served hexanucleotide AAUAAA, whereas the cleavage stimulation factor (CstF) binds to GU- or U-rich elements downstream of the poly(A) site. In addition, the cleavage reaction requires mammalian cleavage factor I (CF Im), cleavage factor IIIm (CF IIIm), and poly(A) polymerase (PAP). After the first step of 3'-end processing, CPSF remains bound to the upstream cleavage fragment and tethers PAP to the 3'-end of the pre-mRNA. In the presence of the nuclear poly(A)-binding protein 1 (PABPN1), PAP elongates the poly(A) tail (reviewed in Ref. 3).

The cleavage factor Im (CF Im) is a heterodimer composed of a 25-kDa small subunit and one of the 59-, 68-, and 72-kDa large subunits, which is required for the first step in pre-mRNA 3'-end processing (4). The complex containing 25 and 68 kDa subunits has been shown to be sufficient for the *in vitro* CFIm activity (5). The binding of CF Im to the pre-mRNA is thought to be one of the earliest steps in the assembly of the cleavage and polyadenylation machinery and facilitates the recruitment of other processing factors. For example, it has been shown that the N-terminal region of CFIm25 was responsible for the interaction with the C-terminal region of PAP (6).

PAP is a key protein in the pre-mRNA 3'-end processing reaction in mammals because PAP catalytically participates in the polyadenylation reaction of pre-mRNA. PAP adds the poly(A) tails of 200–250 residues at the specific sites of pre-mRNAs introduced by CPSF, CstF, CF Im, and CF IIIm. The catalytic function of PAP resides in its N-terminal region, which has a sequence similarity with a nucleotidyl transferase family. The C-terminal region carries an RNA binding region, two nuclear localization signals (NLS1 and NLS2), and a serine/threonine-rich region. The activity of PAP is regulated by phosphorylation during the cell cycle (7). The serine/threonine-rich domain of PAP is responsible for the regulation by phosphorylation. The C-terminal region of PAP is also involved in the coupling of splicing and polyadenylation via protein-protein interactions with splicing factors U1A and U2AF65. The C-terminal region of PAP can also serve as a target of other regulatory proteins for protein-protein interactions such as CFIm25 and PABPN1 (6, 8).

Recently, a large number of non-histone proteins have been shown to be acetylated including transcription factors such as p53 (9, 10), cytoskeletal proteins such as α -tubulin (11, 12), molecular chaperone HSP90 (13), and viral protein such as Tat (14) and T-antigen (15, 16). Acetylation of not only histones but also these non-histone proteins are controlled by two enzyme families histone acetyltransferases (HAT) and deacetylases

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² The abbreviations used are: CPSF, cleavage and polyadenylation specificity factor; HA, hemagglutinin; PBS, phosphate-buffered saline; HDAC, histone deacetylase; TSA, trichostatin A; PAP, poly(A) polymerase; CREB, cAMP response element-binding protein; HAT, histone acetyltransferase.

(HDAC). Treatment of cells with HDAC inhibitors such as TSA have been shown to accumulate the acetylated proteins by blocking the *in vivo* deacetylation of these proteins.

During the course of our screening for novel acetylated protein using TSA, we identified CFIm25 as a 25-kDa acetylated protein. In addition, PAP that interacts with CFIm25 was also found to be acetylated and deacetylated by the same enzyme set as that for CFIm25. Functional analysis suggests that interaction between these proteins and/or localization of PAP is regulated by an acetylation/deacetylation cycle. These results provide a new insight into the role of protein acetylation in not only transcriptional initiation and elongation but also termination.

EXPERIMENTAL PROCEDURES

Materials, Cell Lines, and Antibodies—TSA was prepared as described previously (17, 18). Nicotinamide was purchased from Sigma. COS-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (Sigma). The monoclonal mouse antibody against the anti-FLAG M2 antibody, α -tubulin (B-5-1-2) and Ac- α -tubulin (6-11B-1) were purchased from Sigma. The monoclonal anti-HA (clone 16B12), anti-c-Myc (9E10), and anti-V5 antibodies were purchased from BAbCO, Santa Cruz Biotechnology, and Invitrogen, respectively. The rabbit polyclonal antibody against acetylated lysine (AcLys) was purchased from Cell Signaling Technology. A rabbit polyclonal anti-CFIm68 antibody was kindly supplied from Dr. Keller (5).

Plasmids and Transfection—For construction of the C-terminally FLAG-tagged CFIm25 (pcDNA3-CFIm25-FLAG), C-terminally FLAG₂-His₆-tagged CFIm68 (pcDNA3-CFIm68-FFH), N-terminally His₆-FLAG₂-tagged bovine PAP (pcDNA3.1-HFF-PAP) and N-terminally V5-tagged importin β , recombination-based cloning (Gateway, Invitrogen) was used. For CFIm25, PCR products amplified from the human kidney cDNA library (Invitrogen) were integrated into the pcDNA3-cFLAG-ccdB vector, which was constructed by inserting the *ccdB* cassette and the FLAG cassette into pcDNA3. The full-length cDNA sequence was identical to that of the CPSF5 (GenBankTM accession number NM_007006). The specific primer set was 5'-GGGGACAAGTTTGTACAAAAGCAGGCTCCGGTACCATGCTGTGTGGTACCGCCCAATCGC-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCGACGTTGTAAATAAAATTGAACCTGCTC-3'. For CFIm68 (5) and PAP α (GenBankTM accession number X61585) (19), each cDNA was amplified by PCR and integrated into pcDNA3.1-ccdB-FFH or pcDNA3.1-HFF-ccdB, which were constructed by inserting the *ccdB* cassette and the FFH or HFF cassette into pcDNA3.1. For importin β , PTAC97 (20, 21) was used as a PCR template, and the product was introduced into the pcDNA3.1/nV5-DEST vector (Invitrogen). Point mutants of these plasmids were generated by PCR using a QuikChangeTM site-directed mutagenesis kit (Stratagene). HA-tagged HDAC1–8 and HDAC10 were prepared as described previously (12). pcDNA3 β -CBP-CHA, NF-CBP-CHA-pcDNA3 β , NF-CBP-LD-CHA-pcDNA3 β , and the Myc-tagged SIRT1-SIRT5 expression plasmids were provided by Tso-Pang Yao. pCX-FLAG-PCAF was a gift from Xiang-Jiao Yang. The HA-tagged human TIP60 expression plasmid was supplied by Saadi Khochbin.

Bovine PAP α and human CFIm68 cDNA were provided by Walter Keller. Mouse importin β cDNA was supplied by Yoshihiro Yoneda. COS-7 cells were transiently transfected with each plasmid using the Lipofectamine2000 Reagent (Invitrogen) according to the manufacturer's protocol. For siRNA knockdown of CFIm68, COS-7 cells were transfected with CFIm68 siRNA oligonucleotides (Dharmacon, cat. 012334) using a DharmaFECT1 transfection reagent (Dharmacon).

Mass Spectrometry—Following Coomassie Blue staining, bands corresponding to the CFIm25 or PAP were excised, and the gel pieces were destained with 50% CH₃CN in 50 mM NH₄HCO₃ solution. After removal of the supernatant, cysteine residues were reduced with dithiothreitol, carbamidomethylated with IAA, and the proteins were digested with trypsin at 37 °C overnight. The tryptic peptides were recovered by sequentially adding 50% CH₃CN/1% trifluoroacetic acid, 20% HCOOH/25% CH₃CN/15% *i*-PrOH, and 80% CH₃CN solutions. The supernatants were collected and pooled into one tube, reducing the volume *in vacuo*. The dried tryptic peptides were suspended in 2% CH₃CN/0.1% trifluoroacetic acid and applied to the following LC-MS/MS system.

Chromatographic separation was accomplished with the MAGIC 2002 HPLC system (Michrom BioResources, Inc., Auburn, CA). Peptide samples were loaded onto a Cadenza C18 custom-packed column (0.2 \times 50 mm, Michrom BioResources, Inc.), and eluted using a linear gradient of 5–60% CH₃CN in 0.1% HCOOH for 30 min with a flow rate of 1 μ l/min. Samples were ionized with the Nanoflow-LC ESI, and MS/MS spectrum data were obtained with a LCQ-Deca XP ion trap mass spectrometer (Thermo Electron Corp., Waltham, MA). The Mascot data base-searching software (Matrix Science, Inc., London, UK) was used for the identification of acetylated proteins.

Immunoprecipitation and Immunoblotting—Cells were harvested and sonicated for 10 s twice in ice-cold Immunoprecipitation (IP) buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, and the CompleteTM protease inhibitor mixture (Roche Applied Science). The lysates were centrifuged at 15,000 \times *g* for 10 min at 4 °C. The supernatants were incubated with each primary antibody for 1 h at 4 °C using gentle agitation and with the protein A/G-agarose beads (Santa Cruz Biotechnology) for another 1 h. The agarose pellets were then washed three times with IP buffer, and the bound proteins were extracted with the SDS-PAGE loading buffer by heating at 95 °C for 5 min. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore) by electroblotting. After the membranes had been incubated with primary and secondary antibodies, the immune complexes were detected with an ECL Western blotting kit (Amersham Biosciences), and the luminescence was analyzed with a LAS-3000 image analyzer (Fujifilm).

Nonspecific Polyadenylation Assay—Each 10 μ g of the plasmids for HFF-PAP was introduced into COS-7 cells (10-cm dish). 24 h after transfection, cells were treated with TSA (3 μ M) plus NA (5 mM) and cultured for an additional 12 h. Cells were then harvested and PAP proteins were immunoprecipitated with the anti-FLAG antibody, and subsequently eluted with the FLAG peptide (Sigma). DIG-labeled RNA was synthesized from RsaI-digested pSP6L3 pre Δ 1 plasmids (22) by *in vitro* transcrip-

Acetylation of CFIm25 and PAP

tion using a DIG RNA Labeling kit (Roche Applied Science) according to the manufacturer's protocol. Purified PAP and 0.1 μ g of DIG-L3 pre Δ 1 were mixed in the reaction buffer containing 10 mM HEPES pH 7.9, 50 mM KCl, 0.05 mM EDTA, 2 mM dithiothreitol, 2.5% polyvinyl alcohol, 0.1 mg/ml tRNA, 20 mM creatine phosphate, 5% glycerol, 0.8 mM ATP, 0.5 mM MnCl₂, and 5 units of RNase inhibitor (Roche Applied Science). The reaction was performed at 37 °C, and then stopped at 30 min or 2 h by adding 2 \times TBE-urea sample buffer containing 3.5 M urea (Invitrogen). After the poly(A) addition reaction, the RNA was separated by 10% TBE-urea gel (Invitrogen) at 200 V for 60 min, and subsequently electroblotted to a nylon membrane (Pall Corp.). A DIG Luminescent Detection Kit (Roche Applied Science) was used for the detection of DIG-labeled L3pre Δ 1 RNA, and the luminescent was detected with a LAS-3000 image analyzer.

Immunofluorescence and Microscopy—Exponentially growing cells were plated on 18-mm coverslips and incubated overnight. After transfection and drug treatment, cells were washed in phosphate-buffered saline (PBS) and fixed with 3.5% formaldehyde/PBS for 10 min at room temperature. Coverslips were then rinsed three times with PBS, and the cells were permeabilized with 1% Triton X-100/PBS for 10 min at room temperature. After the coverslips had been incubated in blocking solution (5% calf serum/PBS) for 30 min, the primary antibody was added and incubated for 1 h at room temperature. The coverslips were washed three times with PBS, and the Alexa-fluor anti-mouse antibody (Molecular Probes) was used as the secondary antibody. After the coverslips had been washed three times with PBS, they were observed under an epifluorescence microscope (Zeiss Axiophot2).

RESULTS

Identification of CFIm25 as an Acetylated Protein in TSA-treated COS-7 Cells—To identify proteins of which acetylation is enhanced by TSA treatment, we carried out affinity purification of acetylated proteins using an acetylated lysine-specific (AcLys) antibody. COS-7 cells were treated with 300 nM TSA for 6 h; the lysates were immunoprecipitated with the anti-AcLys antibody, and the precipitates were separated by SDS-PAGE. These putative acetylated proteins were excised from the gel, digested by trypsin, and analyzed by LC-MS/MS. In this experiment, CFIm25 was identified as the 25-kDa acetylated protein besides known acetylated proteins such as p53, T-Ag, and histones (16). CFIm25 is a component of the mammalian cleavage factor Im (CF Im), which is thought to be required for the first step in pre-mRNA 3'-end processing, and interacts directly with mRNA or PAP (Fig. 1A).

LC-MS/MS suggested that the acetylation occurs at lysine 23 (Fig. 1B). To confirm the acetylation of CFIm25 at lysine 23, CFIm25 cDNA was cloned into the pcDNA3-cFLAG vector from a human kidney cDNA library. Point mutants at lysine 23 and lysine 29 (as a control) were subsequently generated, and COS-7 cells were transfected with these plasmids (Fig. 1C). Because the point mutation at lysine 23 (K23R) completely abolished the acetylation of CFIm25, we concluded that the acetylation site in CFIm25 is lysine 23 (compare Fig. 1C, lanes 4 and 6). Because TSA inhibits all class I and II HDAC enzymes,

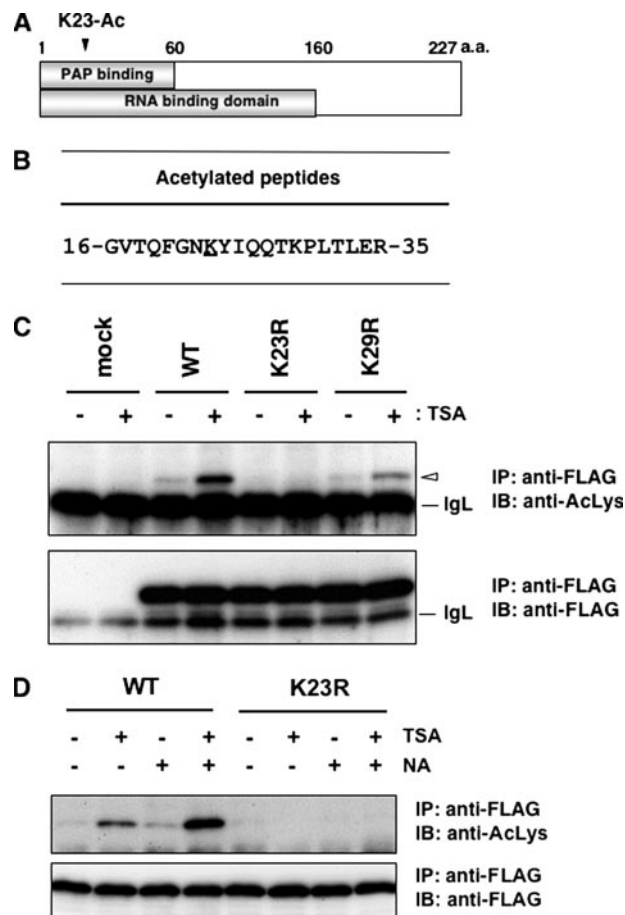


FIGURE 1. CFIm25 is an acetylated protein. A, schematic structure of the CFIm25. CFIm25 has a PAP-interacting domain (amino acids 1–60) and an RNA binding region (amino acids 1–160). It also binds to CFIm68 and forms the CF Im complex. B, peptide fragments of CFIm25 containing an acetylated lysine residue. Bands corresponding to acetylated proteins were excised and in-gel-digested with trypsin, then analyzed with LC-MS/MS. The underline shows acetylated residues on CFIm25 peptides. C, lysine 23 as the acetylation site in the CFIm25. COS-7 cells were transfected with the plasmids for FLAG-tagged wild-type CFIm25, K23R, or K29R and cultured for 24 h. Cells were treated with (+) or without (–) 300 nM TSA for an additional 6 h. The proteins in the cell lysates were immunoprecipitated with the anti-FLAG antibody and immunoblotted with the anti-AcLys (top) or the anti-FLAG (bottom) antibody. D, hyperacetylation of CFIm25 induced by TSA and NA. COS-7 cells overexpressing FLAG-tagged CFIm25 or K23R were treated with or without 3 μ M TSA and 5 mM NA for 6 h. The proteins in the cell lysates were immunoprecipitated with the anti-FLAG antibody and immunoblotted with the anti-AcLys (top) or the anti-FLAG (bottom) antibody.

some members of these families may catalyze the deacetylation at lysine 23.

We next examined whether class III HDACs that are NAD-dependent enzymes are also involved in the deacetylation of CFIm25 besides TSA-sensitive class I/II enzymes. Whereas the class III enzymes are resistant to TSA, they can be inhibited by nicotinamide (NA), a by-product of the catalytic reaction. COS-7 cells were treated with or without TSA and/or NA, and *in vivo* acetylation levels were determined. As shown in Fig. 1D, the combined treatment with TSA and NA remarkably increased the CFIm25 acetylation, suggesting that CFIm25 is deacetylated by both class I/II and class III HDACs. Even in this experiment, the K23R mutant was not acetylated, indicating that the acetylation site in CFIm25 is only lysine 23.

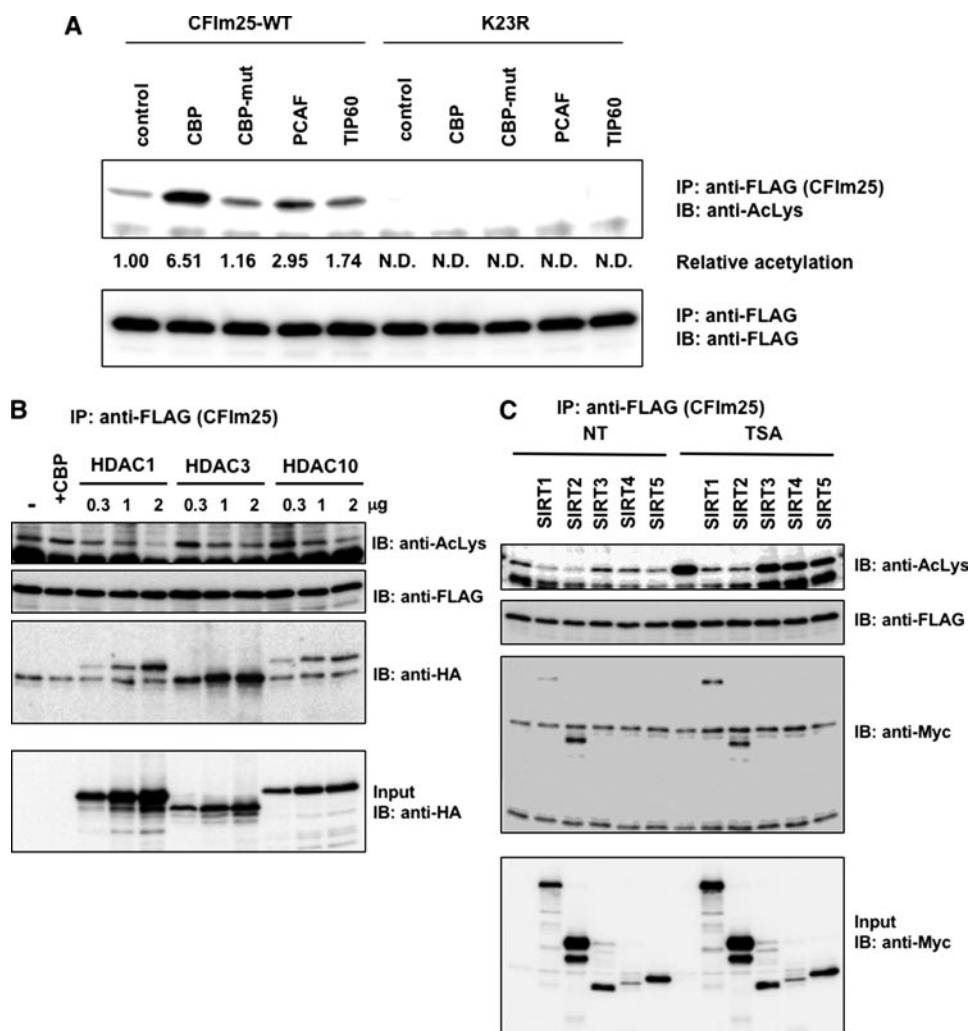


FIGURE 2. Enzymes responsible for acetylation and deacetylation of CFIm25. *A*, CBP acetylates lysine 23 on CFIm25. COS-7 cells were transfected with the plasmids for wild type or K23R of CFIm25 and CBP-HA (CBP), the catalytically inactive CBP mutant (CBP-mut), PCAF, or TIP60. The proteins in the cell lysates were immunoprecipitated with the anti-FLAG antibody and immunoblotted with the anti-AcLys (*top*) or the anti-FLAG (*bottom*) antibody. Relative acetylation was calculated from the intensity of anti-AcLys/anti-FLAG using a LAS-3000 image analyzer (N.D., not determined). *B*, HDAC1, -3, and -10 deacetylates CFIm25. COS-7 cells were transiently transfected with the plasmids for CFIm25-FLAG (1 μ g), CBP-HA (1 μ g), and various amounts of the indicated HA-HDACs (0.3, 1, and 2 μ g). Twenty-four hours after transfection, cells were harvested, and the proteins were immunoprecipitated with the anti-FLAG antibody, detected with the anti-AcLys, the anti-FLAG or the anti-HA antibody. The amounts of HDACs used in immunoprecipitation are shown in the *bottom panel* (5% input). *C*, SIRT1 and SIRT2 are class III deacetylases for CFIm25. COS-7 cells were transfected with the plasmids for CFIm25-FLAG (1 μ g) and the indicated Myc-tagged SIRTs (1 μ g each). Twenty-four hours after transfection, cells were treated with or without TSA. The proteins were immunoprecipitated with the anti-FLAG antibody, and detected with the anti-AcLys, the anti-FLAG or the anti-Myc antibody. 5% input of SIRTs is shown in the *bottom panel*.

Enzymes Responsible for Acetylation and Deacetylation of CFIm25—It has been shown that p300/CBP and PCAF acetylate many non-histone proteins. To determine which enzyme is responsible for the acetylation of CFIm25, COS-7 cells were transfected with the plasmids for FLAG-tagged CFIm25 together with CBP, PCAF, or TIP60. As shown in Fig. 2A, CBP could acetylate CFIm25 but not the K23R mutant, whereas catalytic-dead CBP (CBP-mut) failed to acetylate CFIm25. Whereas PCAF could weakly acetylate CFIm25 (2.95-fold), TIP60 could not. If there was any activity at all, it was very weak. These results suggest that CBP is the major HAT for the CFIm25 acetylation.

Thus far, 11 HDACs classified into class I and class II HDACs have been reported. To identify the HDACs involved in the

deacetylation of CFIm25, we first determined the enzymes that physically interact with CFIm25 by the co-immunoprecipitation assay. COS-7 cells were transfected with the plasmids for FLAG-tagged CFIm25, HA-tagged CBP, and HA-tagged HDAC1–8 and -10; the cell lysates were immunoprecipitated with the anti-FLAG antibody, and the CFIm25-binding proteins were detected with the anti-HA antibody. This experiment showed that HDAC1, -3, and -10 were detected as the proteins bound to CFIm25 (data not shown). As shown in Fig. 2B, these three enzymes were able to bind CFIm25, and their overexpression caused marked decreases in the *in vivo* acetylation of CFIm25.

We next investigated the physical interaction with CFIm25 and the deacetylating activity of human SIRT1–5 to determine the class III HDAC responsible for the deacetylation of CFIm25. To this end, Myc-tagged SIRT1–SIRT5 were co-expressed with the FLAG-tagged CFIm25 in COS-7 cells and the CFIm25-binding proteins were immunoprecipitated with an anti-FLAG antibody. As shown in Fig. 2C, SIRT1 and SIRT2 interacted with and deacetylated CFIm25 *in vivo*. These results imply that SIRT1 and SIRT2 are the CFIm25-deacetylating enzymes among the class III HDACs.

Acetylation of CFIm25 Is Dependent on Pre-mRNA Cleavage Factor Complex Formation—We next investigated how the CFIm25 acetylation is regulated in the cleavage factor complex. CFIm25 has been

shown to bind to CFIm68 and forms the CF Im heterodimer complex. First, we tested whether expression of complex members affects the acetylation of CFIm25. Surprisingly, CFIm25 could be acetylated even in the absence of TSA or NA treatment when CFIm68 was co-overexpressed (Fig. 3A, compare lanes 1 and 7). On the other hand, the acetylation level of CFIm25 was not affected by the PAP overexpression. Because CBP was the acetylase of CFIm25 (Fig. 2A), we next checked whether CBP interacted with CFIm25, CFIm68, or PAP (Fig. 3B). Importantly, whereas CFIm68 and PAP could bind to CBP, CFIm25 could not interact with CBP. This observation suggests that CFIm68 mediates acetylation of CFIm25 by recruiting CBP. To test this possibility, COS-7 cells were co-transfected with CFIm25, CFIm68, and CBP. As shown in Fig. 3C, acetylation of

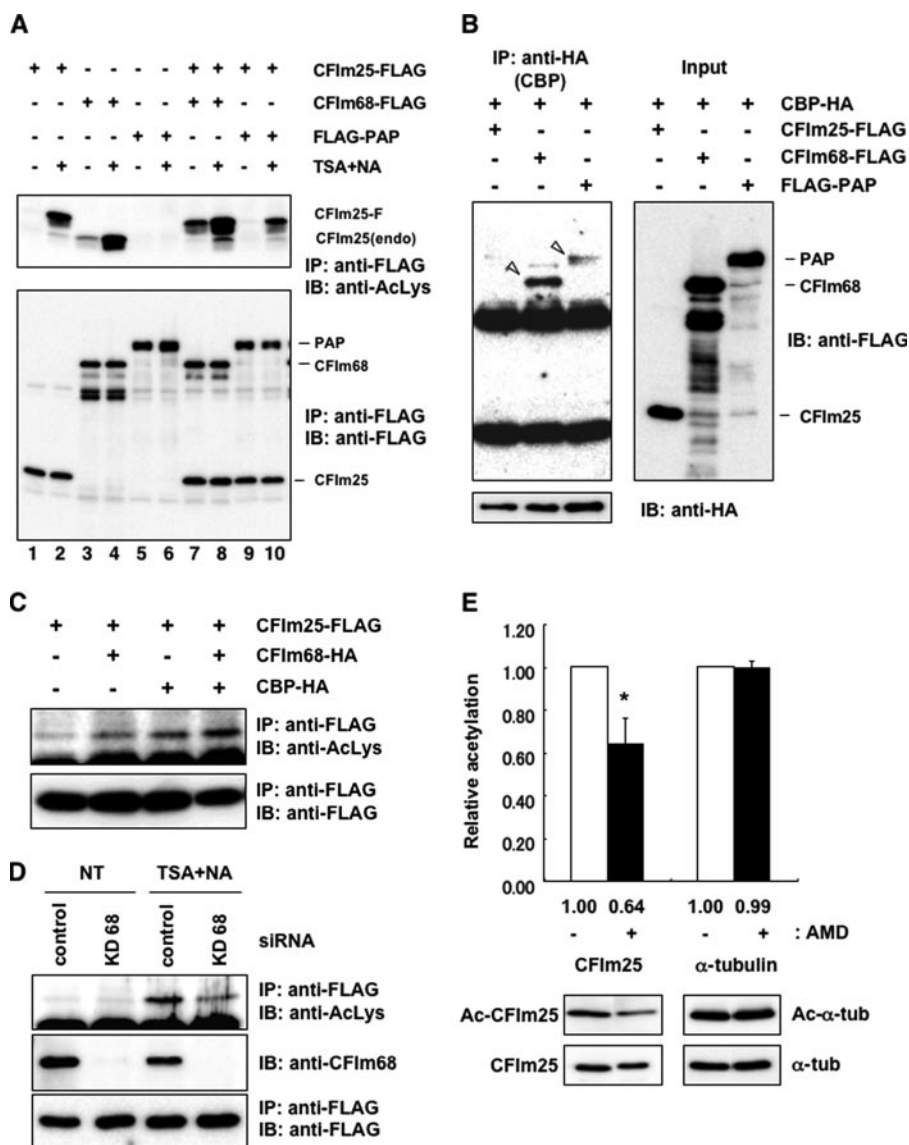


FIGURE 3. Cleavage complex formation facilitates acetylation of CFIm25. A, CFIm68 enhances acetylation of CFIm25. COS-7 cells were transfected with 1 μ g of indicated plasmids. Twenty-four hours after transfection, cells were treated with (+) or without (-) 3 μ M TSA and 5 mM NA. The immunoprecipitates with the anti-FLAG antibody were analyzed by immunoblotting with the anti-AcLys (top) or the anti-FLAG (bottom) antibody. B, CBP interacts with PAP and CFIm68, but not with CFIm25. Cells were transfected with HA-tagged CBP and indicated FLAG-tagged plasmids. The immunoprecipitates with the anti-HA antibody were analyzed by immunoblotting with the anti-FLAG or the anti-HA antibody. 5% input is shown in the right panel. The arrowheads show specific interactions between CBP and CFIm68 or PAP. C, CFIm68 enhances acetylation of CFIm25 by CBP. Cells were transfected with 2 μ g of FLAG-tagged CFIm25 together with 0.5 μ g of CFIm68-HA or 2 μ g of CBP-HA. The immunoprecipitates with the anti-FLAG antibody were immunoblotted with the anti-AcLys (top) or anti-FLAG (bottom) antibody. D, inhibition of CFIm25 acetylation by CFIm68 knockdown. COS-7 cells were transfected with or without siRNA for CFIm68 together with the plasmid for CFIm25-FLAG. Forty-eight hours after transfection, cells were treated with or without TSA + NA. The immunoprecipitates with the anti-FLAG antibody were immunoblotted with the anti-AcLys (top) or anti-FLAG (bottom) antibody. Synthesis of CFIm68 in the lysate was detected with an anti-CFIm68 antibody (middle). E, RNA-dependent acetylation of CFIm25. COS-7 cells were transfected with CFIm25-FLAG and treated with 3 μ M TSA and 5 mM NA, with (+) or without (-) 5 μ g/ml AMD for 9 h. The proteins in the cell lysates were immunoprecipitated with the anti-FLAG antibody and acetylation of CFIm25 was detected with the anti-AcLys antibody. Acetylation of α -tubulin (α -tub) in the crude lysates was detected with the anti-Ac- α -tubulin (Ac- α -tub) antibody. Relative acetylation level was calculated according to the intensity of anti-AcLys/anti-FLAG or anti-Ac- α -tub/ α -tub measured with a LAS-3000 image analyzer. Mean \pm S.D. are shown ($n = 3$). *, $p < 0.05$.

CFIm25 by CBP was enhanced when CFIm68 was simultaneously overexpressed. On the other hand, CFIm68 knockdown by siRNA remarkably reduced CFIm25 acetylation in the presence of TSA plus NA (Fig. 3D). Furthermore, acetylation of the CFIm25 was decreased when cells were treated with an

RNA synthesis inhibitor, actinomycin D (AMD) (Fig. 3E), suggesting that the acetylation of CFIm25 enhanced during active transcription, which requires the CFIm complex formation for polyadenylation of the transcripts.

Acetylation of PAP, Another Pre-mRNA Processing Complex Protein That Interacts with CFIm25—

The above results suggest that CBP is one of the components of the pre-mRNA processing complex, and that not only CFIm68 but also PAP are associated with CBP. Therefore, we examined whether CFIm68 or PAP can be acetylated by CBP. COS-7 cells were transfected with the plasmids for either FLAG-tagged CFIm25, CFIm68, or PAP. Twenty-four hours after transfection, cells were treated with or without TSA and NA. As shown in Fig. 4A, PAP, but not CFIm68, was acetylated when treated with TSA plus NA. LC-MS/MS analysis suggested the four potentially acetylated sites (Lys⁶³⁵, Lys⁶⁴⁴, Lys⁷³⁰, and Lys⁷³⁴) in PAP (Fig. 4B). All the acetylation sites were mapped in the C-terminal regulatory domain including lysine 644, one of the four lysine residues in the C-terminal NLS2 sequence (⁶⁴⁴KRTSSPH-KEESPKKTK⁶⁵⁹; Fig. 4C). To see if these sites are actually acetylated, we introduced point mutations into these sites, and tested whether they are acetylated in COS-7 cells treated with or without TSA plus NA. Among these lysine residues, lysine 644 was shown to be the major acetylation site because the single point mutation at lysine 644 resulted in a remarkable decrease in the acetylation (Fig. 4D, lane 6). Although a weak acetylation still remained in the double mutant of lysine 635 and lysine 644, the acetylation of the 4KR mutant was undetectable even when treated with TSA plus NA. Thus, we conclude that all four lysine residues of PAP identified by LC-MS/MS experiments can be acetylated. The C-terminal region of PAP is known to be required for various protein interactions, and contains multiple Ser phosphorylation sites. Our results indicate the presence of another modification in the C-terminal regulatory region of PAP.

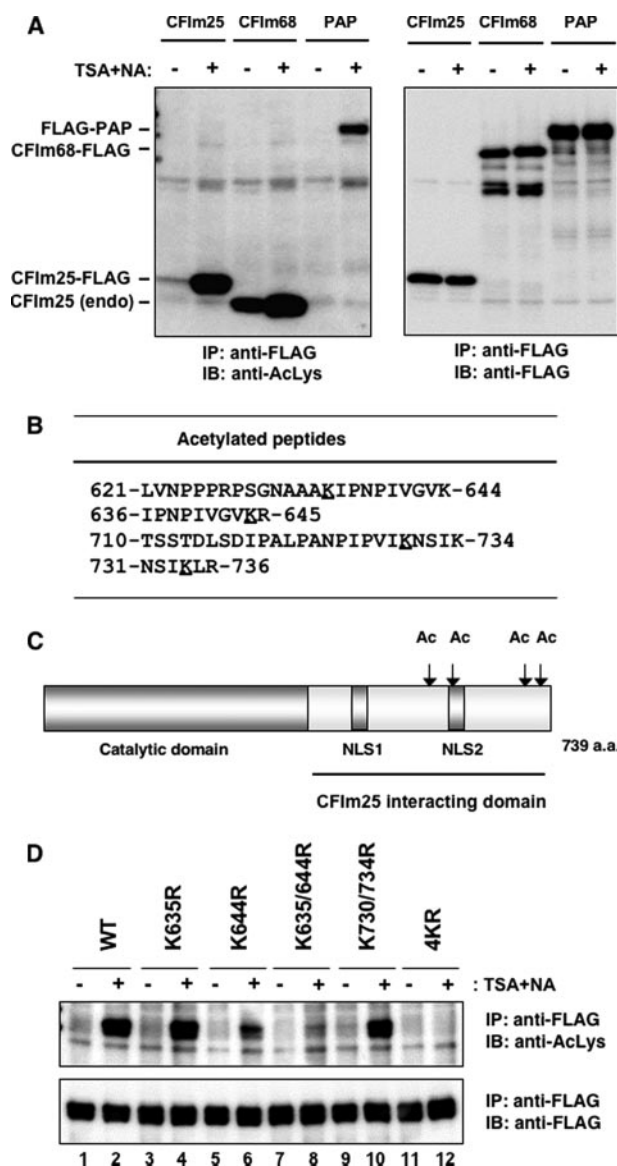


FIGURE 4. PAP is acetylated in its C-terminal region. *A*, PAP was another acetylated protein in the pre-mRNA cleavage complex. COS-7 cells were transfected with the plasmids for CFIm25-FLAG, CFIm68-FFH, or HFF-PAP. Twenty-four hours after transfection, cells were treated with (+) or without (-) TSA and NA for an additional 12 h. The proteins were immunoprecipitated with the anti-FLAG antibody, and detected by immunoblotting using the anti-AcLys (*left*) or the anti-FLAG (*right*) antibody. *B*, peptide fragments of PAP containing acetylated lysine residues. Bands corresponding to PAP were excised and in-gel-digested with trypsin, analyzed with LC-MS/MS. Underlines show acetylated residues in PAP peptides. *C*, schematic structure of PAP. PAP has the catalytic N-terminal domain, NLS1, and the C-terminal region required for protein interaction or phosphorylation. This C-terminal region has NLS2 (⁶⁴⁴KRTSSPHKEESP⁶⁵⁹), in which the first lysine could be acetylated. *D*, identification of multiple acetylation sites on PAP. COS-7 cells were transfected with the plasmids for indicated point mutants of HFF-PAP and cultured for 24 h. Cells were then treated with (+) or without (-) 3 μ M TSA and 5 mM NA for 12 h. The proteins were immunoprecipitated with the anti-FLAG antibody and immunoblotted with the anti-AcLys (*top*) or the anti-FLAG (*bottom*) antibody.

Enzymes Responsible for Acetylation and Deacetylation of PAP—To verify whether PAP is also acetylated by CBP like CFIm25, we examined the effect of HAT expression on the PAP acetylation. As shown in Fig. 5*A*, PAP was actively acetylated by CBP (6.31-fold), but was only weakly acetylated by other HATs, indicating that CBP is the major acetylating enzyme for PAP

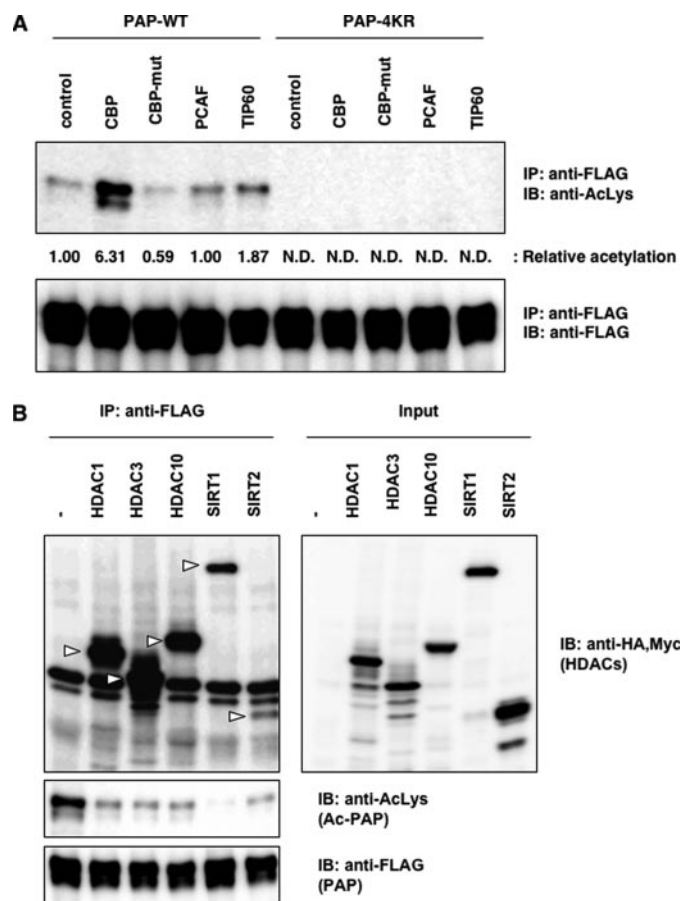


FIGURE 5. Enzymes responsible for acetylation and deacetylation of PAP. *A*, CBP acetylates PAP. COS-7 cells were transfected with the plasmids for HFF-PAP and CBP-HA (CBP), the catalytically inactive CBP mutant (CBP-mut), PCAF or TIP60. The proteins were immunoprecipitated with the anti-FLAG antibody and immunoblotted with the anti-AcLys (*top*) or the anti-FLAG (*bottom*) antibody. A relative acetylation level was calculated from the intensity of anti-AcLys/anti-FLAG measured using a LAS-300 image analyzer. *B*, PAP deacetylation by HDAC1, -3, -10, SIRT1 and SIRT2. COS-7 cells were transfected with the plasmids for 10 μ g of HFF-PAP and 4 μ g of indicated HDACs. Forty-eight hours after transfection, cells were harvested and sonicated. The proteins were immunoprecipitated with the anti-FLAG antibody, and detected with the anti-Myc, anti-HA (*top*), anti-AcLys (*middle*), or the anti-FLAG antibody (*bottom*). 5% input of HDACs is shown in the *right* panel.

like CFIm25. Similarly, all the HDACs (HDAC1, -3, -10, and SIRT1, 2) that interacted with CFIm25 could also associate with PAP (Fig. 5*B*). As shown in Fig. 5*B*, all these enzymes (HDAC1, 3, 10 and SIRT1, 2) could reduce the acetylation when co-expressed with PAP. These results indicate that the same enzymes as those for CFIm25 are responsible for the acetylation and deacetylation of PAP.

Acetylation Reduces the Interaction between PAP and CFIm—The N-terminal region of CFIm25 and the C-terminal region of PAP are known to be responsible for the protein interaction with each other (6). Because the acetylation sites in each protein are also located in these regions, it seems likely that acetylation influences the interaction between PAP and the CFIm complex. To test this possibility, we measured the interaction between FLAG-tagged PAP and HA-tagged CFIm25 and CFIm68 by co-immunoprecipitation assay. As shown in Fig. 6, the interaction between PAP and CFIm25 or CFIm68 was reduced compared with that of wild type or KR mutant (*lanes* 2

Acetylation of CFIm25 and PAP

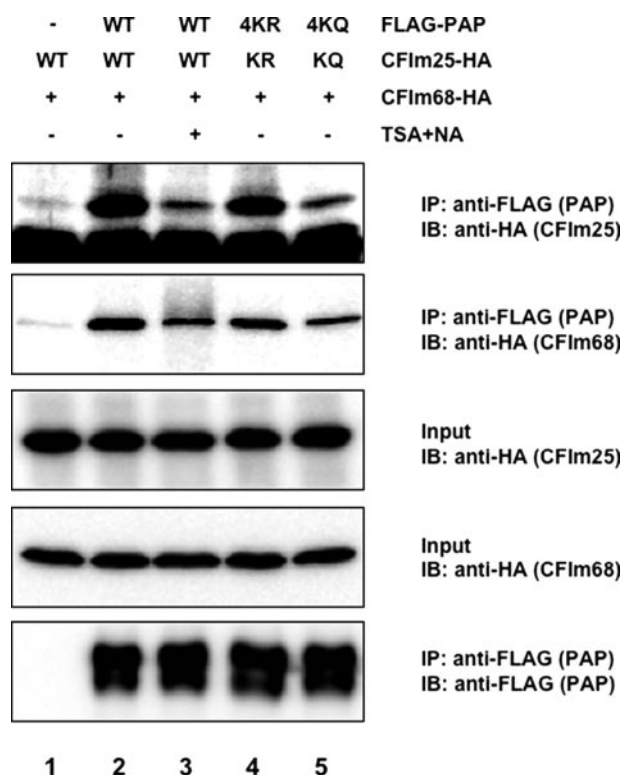


FIGURE 6. Acetylation dissociates PAP from pre-mRNA cleavage complex. Acetylation represses the interaction between PAP and the CF Im complex. COS-7 cells were transfected with 4 μ g of indicated HFF-PAP mutants, 6 μ g of CFIm25-HA mutants and 2 μ g of CFIm68-HA, and after 24 h, cells were treated with or without 3 μ M TSA and 5 mM NA for 12 h. The immunoprecipitates with anti-FLAG antibody were detected with the anti-HA antibody (top). 5% input of CF Im and immunoprecipitated PAP are shown in the lower panels.

and 4), when deacetylases were inhibited (lane 3; TSA plus NA treatment) or the acetylation mimic forms were used (lane 5; CFIm25-K23Q and PAP-4KQ). These results imply that acetylation regulates the interaction between PAP and the CF Im complex.

Acetylation Did Not Affect Polyadenylating Activity of PAP—Because phosphorylation of PAP is known to inhibit polyadenylating activity (7), we next checked whether the PAP activity was affected by acetylation. The plasmids for PAP mutants were transfected in COS-7 cells and the cells were treated with or without TSA+NA for 12 h. These PAP mutants were purified by the affinity to the anti-FLAG antibody. The amounts of purified PAP proteins used in this experiment were determined (Fig. 7A). We carried out the *in vitro* nonspecific polyadenylation assay under conditions identical to those previously reported (22), except that we used DIG-labeled adenovirus L3 pre- Δ 1 RNA as a substrate. As shown in Fig. 7B, the nonspecific polyadenylating activity was not affected by the acetylation or the KQ mutations. This observation was consistent with the previous report showing that the C-terminal deletion mutant of PAP (Δ 644–739 amino acids) did not lose the catalytic activity (19).

Acetylation Promotes Cytoplasmic Localization of PAP—Because lysine 644, one of the four acetylation sites in PAP, is located in the NLS2 (⁶⁴⁴KRTSSPHKEESP⁶⁵⁹), it is possible that acetylation affects the interaction with the importin α/β complex and the nuclear import of PAP. To test this possibility, we expressed the FLAG-tagged PAP mutants and

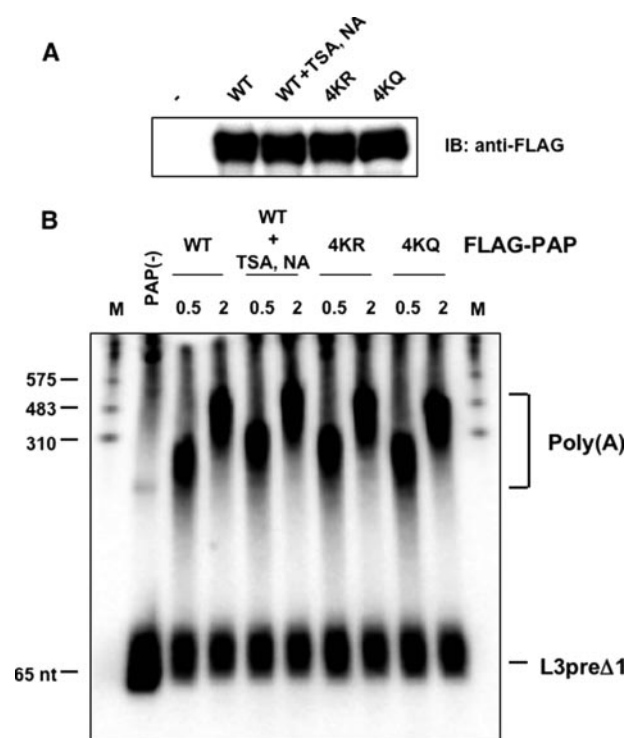


FIGURE 7. Acetylation does not affect nonspecific polyadenylation activity. A, expression and purification of PAP and its mutants. Purified PAPs were separated by SDS-PAGE, and the amounts were determined by Western blotting using the anti-FLAG antibody. B, *in vitro* nonspecific polyadenylation experiment. Purified FLAG-PAPs were incubated at 37 °C with DIG-labeled L3pre Δ 1. 0.5 h or 2 h after the reaction, RNA was separated by 10% TBE-urea gel, electroblotted to a nylon membrane, and detected with an anti-DIG antibody (Roche Applied Science).

V5-tagged importin β in COS-7 cells, and analyzed the effect of acetylation on the interaction between PAP and importin β by co-immunoprecipitation assay. As shown in Fig. 8A, wild type with the TSA+NA treatment or the 4KQ mutant were more weakly bound to importin β than wild type or 4KR. Indeed, the increased cytoplasmic localization of PAP was observed with TSA+NA treatment or by the 4KQ mutation, whereas wild type and the 4KR mutant localized exclusively in the nucleus (Fig. 8, C and D). These results show that acetylation of NLS2 in the PAP inhibits the binding to the importin α/β complex by neutralizing the basic lysine residue, thereby enhancing the cytoplasmic distribution of PAP.

DISCUSSION

Recent research has revealed that acetylation regulates the functions of many proteins other than histones. We have screened for acetylated proteins using an anti-acetylated lysine specific antibody and LC-MS/MS analysis. In this report, we focused on the acetylation of CFIm25, a subunit of the mammalian cleavage complex, and PAP. CFIm25 forms a complex named CF Im with a 68-kDa subunit and binds to pre-mRNA in the first step in pre-mRNA 3'-end processing. It is also shown to interact with PAP, and this interaction may assist polyadenylation by recruiting PAP to pre-mRNA. Although regulation of PAP by Ser phosphorylation has been described, we demonstrated that acetylation also regulates PAP. The present study shows that the interaction between CFIm25 and PAP decreases when

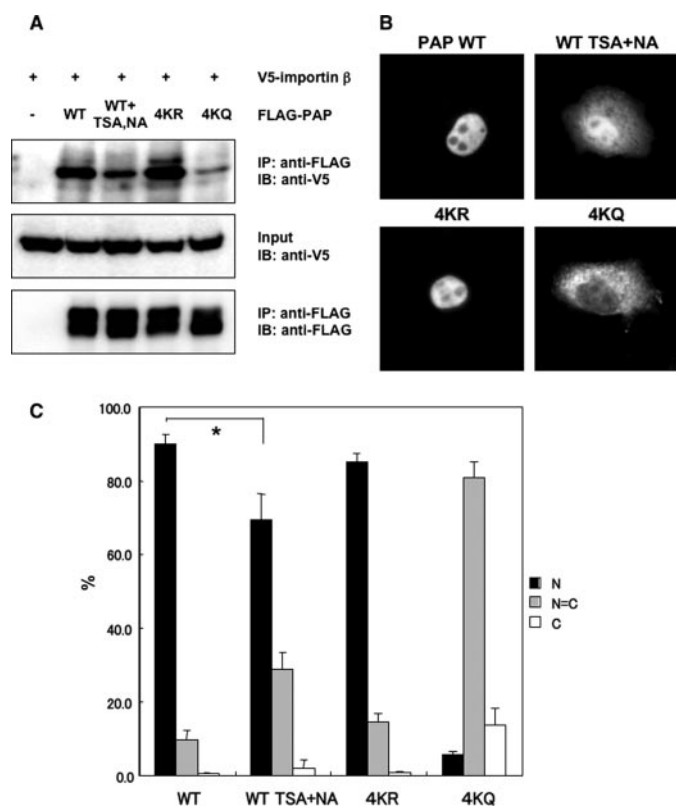


FIGURE 8. Acetylation of PAP promotes its cytoplasmic distribution. *A*, acetylation inhibits the interaction between PAP and importin β . COS-7 cells were transfected with the plasmids for V5-importin β (9 μ g) and of indicated HFF-PAP mutants (3 μ g). Twenty-four hours after transfection, cells were treated with or without TSA+NA for an additional 12 h. Then the cell lysates were immunoprecipitated with the anti-FLAG antibody and immunoblotted with the anti-V5 (top) and the anti-FLAG (bottom) antibody. 5% input of importin β is shown in the middle panel. *B*, acetylation promotes cytoplasmic localization of PAP. COS-7 cells were transfected with indicated HFF-PAP plasmids. Twenty-four hours after transfection, cells were fixed with 3.5% formaldehyde and stained with the anti-FLAG antibody, and the Alexa-fluor 488 anti-mouse secondary antibody. *C*, statistical analysis of subcellular localization of PAP. At least 200 cells expressing each mutant were counted and classified into three groups: nucleus (N), nucleus/cytoplasm (N = C) and cytoplasm (C) according to their subcellular localization. The average percentages of three independent experiments and respective S.D. are shown (*, $p < 0.01$).

lysine 23 on CFIm25 and lysine 635/644/730/734 on PAP are acetylated. The mechanism by which the acetylation decreases the interaction is still obscure but local conformational changes or loss of positive charges caused by acetylation might affect the interaction, because acetylation occurs in each interaction surface of these proteins.

Another effect of acetylation on the PAP function is changes in the localization. PAP re-localized to the cytoplasm when acetylated. This is probably because acetylation at the lysine residue in the NLS2 impairs the NLS function. Basic residues such as Arg and Lys are thought to be important for NLS, and acetylation of lysine residues results in the loss of their positive charges. Indeed, PAP interaction with the importin complex was greatly reduced by acetylation. However, it is still possible that acetylation of lysine residues other than NLS2 are responsible for the decrease in the importin binding. On the other hand, we could not observe any significant change in the CFIm25 localization by TSA plus NA treatment nor KR mutation (data not shown).

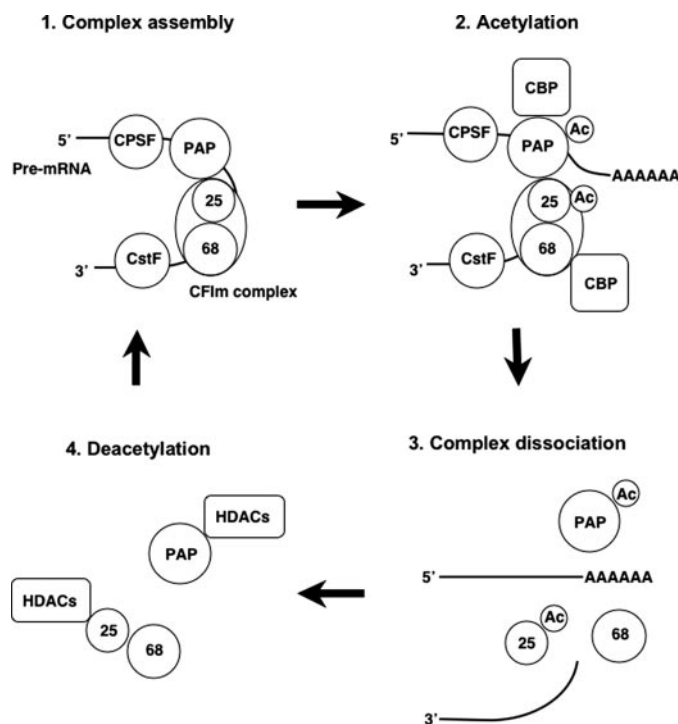


FIGURE 9. A proposed model of the acetylation and deacetylation cycle of pre-mRNA processing complex. In the early stages of pre-mRNA processing, CFIm binds to 3'-terminal of pre-mRNA. PAP is subsequently recruited to pre-mRNA by CFIm and other processing factors such as CPSF and CstF (Step 1). After the cleavage complex is formed, CBP will be recruited to this complex via CFIm68 and acetylate lysine-23 on CFIm25. At this stage, CBP may also interact with PAP and acetylate lysine 635/644/730/734 on PAP (Step 2). Acetylation of PAP and CFIm25 may promote dissociation of CFIm complex from PAP (Step 3). After the complex dissociation, HDACs interact with and deacetylate PAP and CFIm25 (Step 4).

Although nonspecific polyadenylation activity of PAP was not affected by acetylation (Fig. 7), it is possible that acetylation indirectly affects poly(A) addition and transcriptional termination through regulating the protein interactions. Although it is still unclear whether acetylation of PAP and CFIm25 activate or repress the mRNA processing, our data suggest that acetylation may have a negative role in the first assembly step by enhanced cytoplasmic localization of PAP and the weaker interaction between CFIm and PAP, while it may promote the following cleavage and polyadenylation steps by facilitating the dissociation of the CFIm complex from PAP. The fact that CFIm25 could directly interact with HDACs but not with CBP suggests that acetylation of CFIm25 occurs after the cleavage complex formation and deacetylation occurs when the complex is dissociated. Thus, it seems likely that deacetylation of CFIm and PAP promotes the assembly of the cleavage complex, whereas acetylation is coupled with the following dissociation steps (Fig. 9). As a consequence, reversible acetylation may facilitate the poly(A) addition of pre-mRNA, while permanent acetylation will lead to the dissociation of PAP and CFIm25 and the cytoplasmic localization of PAP, which may repress polyadenylation reactions. However, because general transcription and protein synthesis are not affected by treatment with both TSA and NA (data not shown), the role of reversible acetylation of CFIm25 and PAP may be controlling the rate of polyadenylation in response to the environmental conditions. Because

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protein acetylation is regulated by the synthesis of acetyl-CoA (23) and the deacetylating activity of class III HDACs depends on the cellular NAD levels (24), it seems possible that acetylation of CFIm25 and PAP is controlled by the metabolic activity.

It has been well established that HATs are involved in the initiation and elongation of transcription through acetylation (25). In this report, we provided evidence for the contribution of HATs and HDACs to the transcriptional termination through the acetylation of processing factors of pre-mRNA. Thus, the reversible acetylation of non-histone proteins appears to play a more profound role than previously thought, which should be carefully investigated.

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