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Tip60 Acetyltransferase Activity Is Controlled by Phosphorylation*

Claudie Lemerrier^{‡§}, Gaëlle Legube[¶], Cécile Caron^{‡**}, Mathilde Louwagie^{‡‡}, Jérôme Garin^{‡‡}, Didier Trouche[¶], and Saadi Khochbin^{‡§§}

From the [‡]Laboratoire de Biologie Moléculaire et Cellulaire de la Différenciation, INSERM U309, Institut Albert Bonniot, Faculté de Médecine, Domaine de la Merci, 38706 La Tronche Cedex, France, ^{‡‡}Laboratoire de Chimie des Proteines, ERIT-M0201, 38054 CEA-Grenoble, France, and [¶]Laboratoire de Biologie Moléculaire Eucaryote, UPR 9006 CNRS, 118, route de Narbonne, 31062 Toulouse, France

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Abstract

Here we show that the phosphorylation of histone acetyltransferase Tip60, a target of human immunodeficiency virus, type 1-encoded transactivator Tat, plays a crucial role in the control of its catalytic activity. Baculovirus-based expression and purification of Tip60 combined with mass spectrometry allowed the identification of serines 86 and 90 as two major sites of phosphorylation *in vivo*. The phosphorylation of Tip60 was found to modulate its histone acetyltransferase activity. One of the identified phosphorylated serines, Ser-90, was within a consensus cyclin B/Cdc2 site. Ser-90 was specifically phosphorylated *in vitro* by the cyclin B/Cdc2 complex. Accordingly, the phosphorylation of Tip60 was enhanced after drug-induced arrest of cells in G₂/M. This G₂/M-dependent phosphorylation of Tip60 was abolished by treating cells with a specific inhibitor of the cyclin-dependent kinase, roscovitin. All together, these results strongly suggest a G₂/M-dependent control of Tip60 activity.

Introduction

Tip60 was first identified as a partner of human immunodeficiency virus, type 1-encoded transactivator protein Tat (1). Later, investigations clearly showed it to be a specific member of the MYST family of nuclear histone acetyltransferases (HATs)¹ (2). Although the targeting of Tip60 by Tat was found to interfere with its HAT activity and to disturb the expression of at least one cellular gene (3), the function of Tip60 remained elusive until recently. Indeed, the identification of proteins associated with ectopically expressed Tip60 showed the presence of associated ATPase and DNA helicase activities. Functional tests showed that Tip60 and associated proteins may play an essential role in DNA repair and apoptosis (4). Moreover, considering other recent reports, it appears that Tip60 is involved in a wide variety of cellular functions. For instance, Tip60 was found to interact with the androgen receptor and to

enhance its transactivation in a ligand-dependent manner (5). Moreover, androgen receptor was found to be a substrate for Tip60 (6). Other steroid receptors such as estrogen and progesterone receptors have also shown an enhanced activity in the presence of Tip60 and their ligands (5). Tip60 has also been involved in the NF κ B response system, because it was found to interact directly with BCL-3, a member of I κ B family. BCL-3 is thought to serve as an adaptor-bridging Tip60 to the NF κ B p50/p52 and to participate in the gene activation function of this transcription factor (7). Moreover, it has recently been shown that interleukin-1 β induces the activation of a specific group of NF κ B-responsive genes including *KAI1* in relation to the selective recruitment of Tip60 to the promoter of this gene (8). Other studies (9) showed that Tip60 can interact with interleukin-9 (IL-9) receptor, suggesting its possible role in IL-9 signaling. Tip60 was also found to interact directly with cAMP response element-binding protein (CREB) and interfere with its activity, implicating this HAT in the cAMP-dependent signaling process (10). Moreover, Tip60 was also shown to interact with one of the endothelin receptors, ETA (11). All of these examples point to an important involvement of Tip60 in various receptor-mediated signaling processes. In agreement with this hypothesis, it has recently been shown that Tip60 is an essential component linking the proteolytic cleavage of Amyloid- β precursor protein to transcriptional activation (8, 12).

The important function of Tip60 in cell signaling is probably the reason for its targeting by the HIV-1-encoded transactivator Tat. Indeed, it appears that Tat uses Tip60 to control cellular events for the benefit of the virus (13). Here we evidenced a new property of Tip60. First, Tip60 has been shown to be phosphorylated *in vivo*, and second, two major sites of Tip60 phosphorylation have been discovered. The sequence of one of these sites encompassing serine 90 perfectly matches a Cdc2 phosphorylation site. We showed that cyclin B/Cdc2 complex can specifically phosphorylate Ser-90 *in vitro* and *in vivo* and that the phosphorylation of Tip60 was enhanced in G₂/M phase of the cell cycle. This specific G₂/M phosphorylation of Tip60 was inhibited when cells were treated with a specific inhibitor of cyclin-dependent kinase, roscovitin. Our data clearly show that the phosphorylation of Tip60 controls its HAT activity and strongly suggest a role for Tip60 HAT activity in the control of G₂/M-related events.

Materials and Methods

Production and Purification of Proteins in Baculovirus-- Wild type or mutant Tip60 cDNA were cloned into pBacPAK9 transfer vector (Clontech) in-frame with a histidine tag at the C terminus or at the N terminus of the coding sequence. Viral particles were generated using the BacPAK baculovirus expression system (Clontech) and Sf21 insect cells. 2-3 days after infection, Tip60 proteins were purified from Sf21 cells by nickel affinity column (NiTA-agarose, Qiagen), eluted with 250 mM imidazole, and finally dialyzed against 20 mM Tris, pH 7.5, 10% glycerol, and 1 mM dithiothreitol. The purified proteins were kept at -20°C and used in phosphatase treatment experiment, mass spectrometry analysis, and HAT assay (see below).

Plasmids-- The plasmid pcDNA-HA-Tip60 has been described previously (14). The Tip60-(1-211) mutant was generated by PCR and cloned in-frame with the HA tag in the pcDNA-HA vector. The point mutations were generated by PCR, and the incorporation of all of the mutations was confirmed by DNA sequencing. In the Gly-380 mutant, glycine 380 was replaced by an alanine. In the Ser-86, Ser-90, and Ser-86/Ser-90 mutants, the serines were replaced by alanines. In the Leu-254/Leu-257 mutant, the two indicated leucines were replaced by alanines.

Phosphatase Treatment-- Five hundred nanograms of His-Tip60 proteins produced in baculovirus were incubated with 10 units of calf intestine phosphatase (CIP) (New England BioLabs) in the presence or absence of phosphatase inhibitor (5 mM NaF) for 30 min at 37 °C (or 1 h on ice when the dephosphorylation was followed by a HAT assay). Tip60 was then removed by incubation of the reaction mixture with NiTA-agarose beads and eluted with Laemmli sample buffer and subsequently analyzed by SDS-PAGE and Western blotting. Phosphatase treatment of endogenous Tip60 was performed as follows. HeLa cell nuclei isolated from 10⁷ cells were incubated with 50,000 units of CIP for 30 min at 37 °C. Nuclei were washed three times in the lysis buffer (15 mM NaCl, 60 mM KCl, 12% sucrose, 2 mM EDTA, 0.5 mM EGTA, 0.65 mM spermidine, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100) and directly lysed in protein loading buffer. Tip60 was detected using an anti-Tip60 antibody described by Legube *et al.* (14).

Histone Acetyltransferase Assays-- HAT assays were performed using 4 µg of free core histones or 5 µg of oligonucleosomes, wild type, or mutant Tip60 proteins (100-200 ng) and 0.15 µCi of [¹⁴C]acetyl-CoA (65 mCi/mmol) (ICN) in HAT buffer (25 mM Tris, pH 8.0, 10% glycerol, 100 mM NaCl, 1 mM dithiothreitol, 0.2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 5 mM sodium butyrate) for 30 min at 30 °C. 50% of the reactions was loaded on a 18% SDS-polyacrylamide gel followed by Coomassie Blue staining to ensure equivalent loading of histones in each lane, and fluorography was performed after incubation of the gel in the Amplify solution (Amersham Biosciences). In some assays, the HAT activity was analyzed with Tip60 immobilized on NiTA-agarose beads after alkaline phosphatase treatment.

MALDI-MS Analysis-- Mass spectra of the tryptic digests were acquired on a Biflex (Bruker-Franzen Analytik, Bremen, Germany) MALDI-TOF mass spectrometer equipped with a gridless delayed extraction. The instrument was operated in linear mode. 0.5 µl of the digest solution (in 25 mM NH₄HCO₃) was deposited directly onto the sample probe on a dry thin layer of matrix made of α-cyano-4-hydroxy-*trans*-cinnamic acid mixed with nitrocellulose (mixture 4:3, v/v) of a saturated solution of α-cyano-4-hydroxy-*trans*-cinnamic acid in acetone and a solution consisting of 10 mg of nitrocellulose dissolved in 1 ml of isopropyl alcohol/acetone (1:1, v/v). The deposits were washed with 5 µl of 0.1% trifluoroacetic acid before the analysis. A mass list of peptides was obtained for each protein digest.

Peptide Dephosphorylation-- Phosphorylated tryptic peptides were identified on the peptide mass fingerprint by a 80-Da (or multiples of 80 Da) mass shifts after the dephosphorylation step. The dephosphorylation reaction was done by treating the tryptic peptide mixture with bovine alkaline phosphatase in which the peptide digest (5 µl) was mixed with 5 µl of calf intestinal alkaline phosphatase immobilized on agarose beads (P0762, Sigma) in 25 mM NH₄HCO₃ buffer. After 30 min (37 °C), the peptide digest was directly deposited on the MALDI sample probe for analysis.

Phosphopeptide Microcropurification-- Phosphopeptides were purified from the crude tryptic digest using immobilized metal ion affinity chromatography essentially as previously described (15). The tryptic digest (2 µl in 10 mM NH₄HCO₃, 5% acetic acid, 30% acetonitrile) was loaded on a ZipTip_{MC} (ZT0MCS, Millipore,) that had been previously equilibrated following the recommendations of the manufacturer. The ZipTip_{MC} column was then washed with 0.1% acetic acid followed by 0.1% acetic acid, 30% acetonitrile, and 0.1% acetic acid and finally eluted in 2% ammonium hydroxide (2 µl). Eluted peptides were then mixed with 50 mM NH₄HCO₃ (v/v) and analyzed by MALDI-MS.

Nanoelectrospray-MS/MS Analysis-- The digest solution was dried in a vacuum centrifuge and desalted with ZipTip C18 (Millipore, Bedford, MA) before the nanospray MS/MS analysis. A Q-TOF instrument (Micromass, Manchester, United Kingdom) was used with a Z-spray ion-source working in the nanospray mode. Approximately 3-5 μ l of the desalted sample was introduced into a needle (medium sample needle, PROTANA Inc. Odense, Denmark) to run MS and MS/MS experiments. The capillary voltage was set to an average voltage of 1000 V, and the sample cone was set to 50 V. Glufibrinopeptide was used to calibrate the instrument in the MS/MS mode. MS/MS spectra were transformed using MaxEnt3 (MassLynx), and amino acid sequences were analyzed using PepSeq (BioLynx).

Kinase Assays-- Wild type or mutant Tip60 peptides (mutant Ser-90 and mutant Ser-86/Ser-90) encompassing serines at positions 86 and 90 were synthesized, purified (Sigma), and reconstituted in distilled water at 5 mM. For the phosphorylation assays, 15 nmol of wild type or mutant peptides at a final concentration of 500 μ M were incubated in Cdc2 kinase buffer supplemented with 100 μ M cold ATP, 100 μ M sodium vanadate, 10 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol, ICN), and 5 units of Cdc2 kinase/cyclin B (New England BioLabs) for 30 min at 30 $^{\circ}$ C (final volume of 30 μ l). In control reactions, the kinase or the peptide was omitted. Two volumes of 10% trichloroacetic acid and 20 μ g of carrier bovine serum albumin were added at the end of the reaction, and the samples were spotted on P81 cellulose phosphate paper. Filters were then washed in 0.5% phosphoric acid (4 times, 10 min), rinsed in acetone, and finally used to measure 32 P incorporation in a scintillation counter.

Cell Culture, Transfection, Drug Treatments, and Western Blot-- HeLa cells were grown as described previously (14) and transfected using Exgen (Euromedex) or FuGENE 6 (Roche Molecular Biochemicals) as indicated by the suppliers. 24-48 h later, cells extracts were prepared by lysing cells directly in Laemmli sample buffer and sonicated. Stably Tip60 expressing HeLa cells (a gift of Dr. V. Ogrysko) were grown in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%) and antibiotics.

Results

Phosphorylation of Tip60 in Vivo-- To investigate the biochemical property of Tip60, we have expressed a histidine-tagged human Tip60 in insect cells using a baculovirus-based expression system. The recombinant protein was found to be an active HAT on purified histones as well as on nucleosomes (Fig. 1A). Interestingly, whereas Tip60 efficiently acetylated free histone H4 and H3, in nucleosomes, it essentially acetylated histone H4. However, the acetylation of nucleosomal H4 was much less efficient than that of free histones. Indeed, the gel containing nucleosomal histones had to be exposed for a longer period of time to obtain a comparable signal for the acetylation of free and nucleosomal H4. We have also noticed that the purified recombinant Tip60 migrated as a doublet on a 8% SDS-PAGE, and the treatment with the protein calf intestinal alkaline phosphatase prior to electrophoresis caused a shift of Tip60 to a faster migrating band (data not shown). This observation showed that protein phosphorylation was responsible for the observed shift in the electrophoretic mobility of Tip60. To determine the region of Tip60 concerned by phosphorylation, two fragments of Tip60 were expressed in insect cells, purified, and analyzed on a 15% SDS-PAGE, one containing the N-terminal chromodomain of Tip60 encompassing the first 211 amino acids and the other corresponding to the rest of the protein (Fig. 1B, *schemes*). Fig. 1B shows that the N-terminal fragment migrated as a doublet and that the CIP treatment caused a shift of the majority of the protein into a single fast migrating band (compare *lane 1* with 2). The 212-513 region of Tip60

migrated as a single band, and the CIP treatment did not affect its electrophoretic mobility (Fig 1B, lanes 3 and 4).

Two Major Sites of Tip60 Phosphorylation in Vivo-- Data presented above showed that the major sites of Tip60 phosphorylation are located in the first 211 amino acids of the protein. To determine precisely the sites of Tip60 phosphorylation, we chose a mass spectrometry-based approach. First, the mass spectra of the tryptic fragments of the 1-211 region of Tip60 were compared with that of the tryptic fragments of the CIP-treated protein. The MALDI-TOF analysis showed that peptide-(81-93) bears two phosphate groups. The Q-TOF sequencing of peptide-(81-93) from phosphorylated and CIP-treated proteins confirmed the above conclusion and showed that serines 86 and 90 are phosphorylated. Finally, tryptic fragments of Tip60-(1-211) were loaded on a ZipTip-MC column capable of retaining phospho-peptides, and the MALDI-TOF analysis of these peptides confirmed again the presence of two phosphate groups on peptide-(81-93). This analysis also showed the possible presence of phosphate groups on 190-211 and 204-211 tryptic fragments (data not shown). To evaluate the respective participation of serines 86 and 90 in Tip60 phosphorylation, we mutated either serine 86 or serine 90 or both to alanine and monitored their migration on 8% SDS-PAGE after an ectopic expression in HeLa cells. As controls, we also expressed either a Tip60 mutant in which glycine 380 was replaced by an alanine or Tip60 Leu-254/Leu-257 mutant in which the two indicated leucines potentially involved in a nuclear export signal were also changed to alanines. Fig. 2B shows that only mutations affecting serines 86 and 90 affect the mobility of Tip60, which strongly suggests that these sites are major sites of Tip60 phosphorylation in mammalian cells. This hypothesis was further confirmed by showing that CIP-treated Tip60-(1-211) fragment expressed in HeLa cells migrated at the same position as a Tip60-(1-211) fragment containing the Ser-86/Ser-90 mutation (Fig. 2C). All of these results showed that Tip60 can be phosphorylated both in insect and mammalian cells, and serines 86 and 90 represent two major sites of phosphorylation *in vivo*. Finally, we showed that endogenous Tip60 detected in HeLa cell nuclear extracts migrated as a doublet and that the CIP treatment of the nuclei before the extract preparation led to the migration of Tip60 as a single band (Fig. 2D).

Phosphorylation of Tip60 Modulates Its HAT Activity-- To evaluate the role of phosphorylation in the activity of Tip60, the HAT activity of purified phosphorylated Tip60 was compared with that of the CIP-treated protein. Fig. 3A shows that the pretreatment of Tip60 with increasing amounts of CIP considerably reduced the HAT activity of the protein (compare lanes 2 and 3 with 1). In the same experiment when CIP was added in the presence of its inhibitor NaF (Fig. 3A, lanes 4-6) or heat-inactivated prior to its addition (Fig. 3B, lane 9), no detectable modification of Tip60 HAT activity was observed compared with the control. These experiments show that the inhibition of Tip60 HAT activity is not because of the presence of CIP itself in the reaction medium but because of its ability to dephosphorylate Tip60. Moreover, the activity of the CIP-treated Tip60 was comparable with that of a mutated Tip60, Tip60^{Gly-380}, containing a mutation severely affecting its HAT activity (Fig. 3B, lane 11). In this mutant, a glycine critical for acetyl-CoA binding in all MYST members of HATs

was replaced by an alanine. This mutation had been previously shown to inactivate the *Drosophila* Tip60 homologue MOF (16) and was also one of the two mutations introduced by Ikura *et al.* (4) to inactivate Tip60. The above experiment clearly showed that dephosphorylated Tip60 has a poor catalytic activity. To show the specific involvement of the serines 86 and 90 in the control of the HAT activity of Tip60, baculovirus-based expression was used to express and purify a mutated form of Tip60 (Tip60^{Ser-86/Ser-90}) in which these two serines were replaced by alanines. A HAT assay was set up to compare the HAT activity of the wild type, phosphorylated Tip60 with that of Tip60^{Ser-86/Ser-90}. Fig. 3C shows that, as in mammalian cells, the double mutant Tip60 migrated faster than the wild type protein (*upper panel*). Moreover, this experiment also showed that Tip60^{Ser-86/Ser-90} was not as efficient as wild-type Tip60 at acetylating histones.

Kinases Involved in the Phosphorylation of Tip60-- The data presented thus far showed the critical role of Tip60 phosphorylation in the control of its catalytic activity. Therefore, it appeared very important to identify the kinases involved to better understand the functional significance of this regulation of Tip60 activity by its phosphorylation. The analysis of the sequence encompassing Ser-86 and Ser-90 showed that Ser-90 corresponded to a potential p34 Cdc2 phosphorylation site XSPX(R/K) (17). To test the ability of cyclin B/Cdc2 to phosphorylate Ser-90, we have synthesized three peptides encompassing the amino acid 82-96 region of Tip60. One peptide corresponded to the wild type sequence, and the two others contained serine replacement (Ser to Ala) of either Ser-90 or both Ser-86 and Ser-90 (Fig. 4A). The peptides were incubated with purified cyclin B/Cdc2 complex in the presence of ³²P-labeled ATP, and peptide phosphorylation was monitored. Fig. 4B shows that only the wild type peptide was phosphorylated by the purified cyclin B/Cdc2 complex. The phosphorylation of a peptide containing the "Ser-90 to Ala" mutation was comparable with that of a peptide containing replacements of both serines, Ser-86 and Ser-90, to alanines or that of the wild type peptide incubated with [³²P]ATP in the absence of the enzyme. This experiment shows that cyclin B/Cdc2 specifically phosphorylates Ser-90 of Tip60. To show the participation of cyclin-dependent kinases (CDKs) in the phosphorylation of Tip60, HeLa cells were treated with nocodazole, inducing an arrest at G₂/M phase of the cell cycle because of its ability to depolymerize microtubules. Nocodazole-treated cells were lysed, and Tip60 was detected using an anti-Tip60 antibody (14) when ~90% of cells were in the G₂/M phase of the cell cycle (data not shown). Fig. 5A shows that, as expected, in cycling cells, Tip60 appeared as a doublet. Interestingly, after the treatment of cells with nocodazole, almost only the phosphorylated form of Tip60 was visible. However, in our hands for unknown reasons, the endogenous Tip60 could not be detected with a reasonable sensitivity in a reproducible manner. Therefore, to better investigate the involvement of the CDKs in the phosphorylation of Tip60, we used a characterized HeLa cell line stably expressing HA-tagged Tip60 (4) to evaluate Tip60 phosphorylation in response to specific drugs. In cycling HeLa cells, the HA-Tip60 appeared also as a doublet. After the treatment of cells with nocodazole, the HA-Tip60 behaved like the endogenous protein because the amount of the phosphorylated form of the protein increased (Fig. 5B, lane 3). This nocodazole-induced accumulation of phosphorylated HA-Tip60 was severely reduced when the nocodazole-treated cells were treated with

roscovitin, a specific inhibitor of CDKs (18), for 7 h before the cell harvest (Fig. 5B, lane 4). All together, these data show the participation of CDKs in the phosphorylation of Tip60 and strongly suggest a role for Tip60 in the control of cell cycle-related events.

Discussion

The MYST family of histone acetyltransferases are evolutionary conserved enzymes from yeast to humans. The specific function of several members of this family is now emerging in different species (19). For instance, in yeast, two HATs of the MYST family, SAS2 and SAS3, play a role in silencing. The HAT activity of SAS3 has been shown *in vitro* (20), and moreover, it has been found within the NuA3 complex, which specifically acetylates histone H3 in nucleosomes (21). The third member of MYST HATs in yeast, Esa1, appears to be responsible for cell cycle progression (22). This HAT is also present in a complex known as NuA4 (23) and is required for DNA double-stranded break repair (24). In *Drosophila*, MOF, a MYST HAT, has been clearly shown to be involved in the male X chromosome hyperactivation by specifically acetylating histone H4 lysine 16 (16). Interestingly, as Tip60, MOF has a canonic chromodomain, which possesses the ability to bind to RNA *in vitro* and to the X chromosome-associated RNA *in vivo* (25). The role of other vertebrate MYST members, HBO1, MORF, and MOZ, has not yet been defined (19). Therefore, it appears that the HATs of the MYST family are involved in the control of a variety of critical cellular events. This conclusion implies that the activity and the expression of these enzymes should be tightly regulated. In the literature, there is almost no hint regarding this issue. Our data provide the first indication of the regulated expression and activity of a member of the MYST acetyltransferases, Tip60. Indeed, we have previously shown that the cellular concentration of Tip60 is controlled by Mdm2-mediated ubiquitination and proteasome-dependent degradation of the protein (14). Here we show that the phosphorylation of Tip60 is a controlled phenomenon and modulates the activity of the protein. Tip60 is not the only HAT whose activity is controlled by phosphorylation. Indeed, it has been shown that the transcription factor ATF-2 possesses an intrinsic HAT activity and that its phosphorylation considerably stimulates its HAT activity (26). Another example is CREB-binding protein, which is phosphorylated at the G₁/S boundary, and this phosphorylation was also shown to stimulate its HAT activity (27). This phosphorylation-dependent control of CREB-binding protein activity allows this HAT to be responsive to distinct signaling pathways. Indeed, kinases as different as p44 MAPK/ERK1 (28), cell cycle-dependent kinases such as cyclin E/Cdk2 (27), as well as MEKK1 (29) are capable of phosphorylating and, hence, activating the CREB-binding protein catalytic activity. In the case of Tip60, Ser-90 is located within a canonic cyclin B/Cdc2 site and the enzyme was found here to specifically phosphorylate this specific serine. Interestingly, Ser-90 is highly conserved among Tip60 homologues in different species (data not shown), suggesting that this mode of regulation is also conserved during evolution. Furthermore, our data provide a new basis to reconsider the many functions of Tip60. Indeed, Tip60 appears as an important intermediate in several unrelated receptor-mediated signaling processes (5, 6, 8, 9, 11). Therefore, our data suggest that an additional control level, that of Tip60 phosphorylation, needs to be coordinated with signals induced by steroids (5, 6), IL-1 β (8), IL-9 (9), or endothelin (11). Besides, the HAT activity of Tip60 has been shown to be essential in the cellular response to DNA damage (4). Therefore, the phosphorylation of Tip60 regulating its HAT activity could well play an important role in this process and somehow link Tip60 function to the replication checkpoint. Finally, the HAT activity of Tip60 was found to mediate gene activation following the cleavage of amyloid- β precursor (12). These data

should also be reconsidered in the light of a linkage between Tip60 catalytic activity and its phosphorylation.

In summary, this work has allowed to establish a link between CDKs and the activity of Tip60. However, other kinases may also phosphorylate Tip60, establishing a specific linkage between Tip60 activity and one or several of the signaling pathways discussed above.

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Footnotes

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§§ To whom correspondence should be addressed. Tel.: 334-7654-9583; Fax: 334-7654-9595.

Abbreviations

The abbreviations used are: HAT, histone acetyltransferase; IL, interleukin; CIP, calf intestine phosphatase; CREB, cAMP response element-binding protein; CDK, cyclin-dependent kinase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

References

1. Kamine, J., Elangovan, B., Subramanian, T., Coleman, D., and Chinnadurai, G. (1996) *Virology* **216**, 357-366
2. Yamamoto, T., and Horikoshi, M. (1997) *J. Biol. Chem.* **272**, 30595-30598
3. Creaven, M., Hans, F., Mutskov, V., Col, E., Caron, C., Dimitrov, S., and Khochbin, S. (1999) *Biochemistry* **38**, 8826-8830
4. Ikura, T., Ogryzko, V. V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000) *Cell* **102**, 463-473

5. Brady, M. E., Ozanne, D. M., Gaughan, L., Waite, I., Cook, S., Neal, D. E., and Robson, C. N. (1999) *J. Biol. Chem.* **274**, 17599-17604
6. Gaughan, L., Logan, I. R., Cook, S., Neal, D. E., and Robson, C. N. (2002) *J. Biol. Chem.* **277**, 25904-25913
7. Dechend, R., Hirano, F., Lehmann, K., Heissmeyer, V., Ansieau, S., Wulczyn, F. G., Scheidereit, C., and Leutz, A. (1999) *Oncogene* **18**, 3316-3323
8. Baek, S. H., Ohgi, K. A., Rose, D. W., Koo, E. H., Glass, C. K., and Rosenfeld, M. G. (2002) *Cell* **110**, 55-67
9. Sliva, D., Zhu, Y. X., Tsai, S., Kamine, J., and Yang, Y. C. (1999) *Biochem. Biophys. Res. Commun.* **263**, 149-155
10. Gavaravarapu, S., and Kamine, J. (2000) *Biochem. Biophys. Res. Commun.* **269**, 758-766
11. Lee, H. J., Chun, M., and Kandror, K. V. (2001) *J. Biol. Chem.* **276**, 16597-16600
12. Cao, X., and Sudhof, T. C. (2001) *Science* **293**, 115-120
13. Caron, C., Col, E., and Khochbin, S. (2003) *Bioessays* **25**, 58-65
14. Legube, G., Linares, L. K., Lemercier, C., Scheffner, M., Khochbin, S., and Trouche, D. (2002) *EMBO J.* **21**, 1704-1712
15. Posewitz, M. C., and Tempst, P. (1999) *Anal. Chem.* **71**, 2883-2892
16. Akhtar, A., and Becker, P. B. (2000) *Mol. Cell* **5**, 367-375
17. Kennelly, P. J., and Krebs, E. G. (1991) *J. Biol. Chem.* **266**, 15555-15558
18. Meijer, L., Borgne, A., Mulner, O., Chong, J., Blow, J., Inagaki, N., Inagaki, M., Delcros, J., and Moulinoux, J. (1997) *Eur. J. Biochem.* **243**, 527-536
19. Sterner, D. E., and Berger, S. L. (2000) *Microbiol. Mol. Biol. Rev.* **64**, 435-459
20. Takechi, S., and Nakayama, T. (1999) *Biochem. Biophys. Res. Commun.* **266**, 405-410
21. John, S., Howe, L., Tafrov, S. T., Grant, P. A., Sternglanz, R., and Workman, J. L. (2000) *Genes Dev.* **14**, 1196-1208
22. Clarke, A. S., Lowell, J. E., Jacobson, S. J., and Pillus, L. (1999) *Mol. Cell. Biol.* **19**, 2515-2526
23. Allard, S., Utleay, R. T., Savard, J., Clarke, A., Grant, P., Brandl, C. J., Pillus, L., Workman, J. L., and Cote, J. (1999) *EMBO J.* **18**, 5108-5119
24. Bird, A. W., Yu, D. Y., Pray-Grant, M. G., Qiu, Q., Harmon, K. E., Megee, P. C., Grant, P. A., Smith, M. M., and Christman, M. F. (2002) *Nature* **419**, 411-415
25. Akhtar, A., Zink, D., and Becker, P. B. (2000) *Nature* **407**, 405-40
26. Kawasaki, H., Schiltz, L., Chiu, R., Itakura, K., Taira, K., Nakatani, Y., and Yokoyama, K. K. (2000) *Nature* **405**, 195-200
27. Ait-Si-Ali, S., Ramirez, S., Barre, F. X., Dkhissi, F., Magnaghi-Jaulin, L., Girault, J. A., Robin, P., Knibiehler, M., Pritchard, L. L., Ducommun, B., Trouche, D., and Harel-Bellan, A. (1998) *Nature* **396**, 184-186
28. Ait-Si-Ali, S., Carlisi, D., Ramirez, S., Upegui-Gonzalez, L. C., Duquet, A., Robin, P., Rudkin, B., Harel-Bellan, A., and Trouche, D. (1999) *Biochem. Biophys. Res. Commun.* **262**, 157-162
29. See, R. H., Calvo, D., Shi, Y., Kawa, H., Luke, M. P., and Yuan, Z. (2001) *J. Biol. Chem.* **276**, 16310-16317

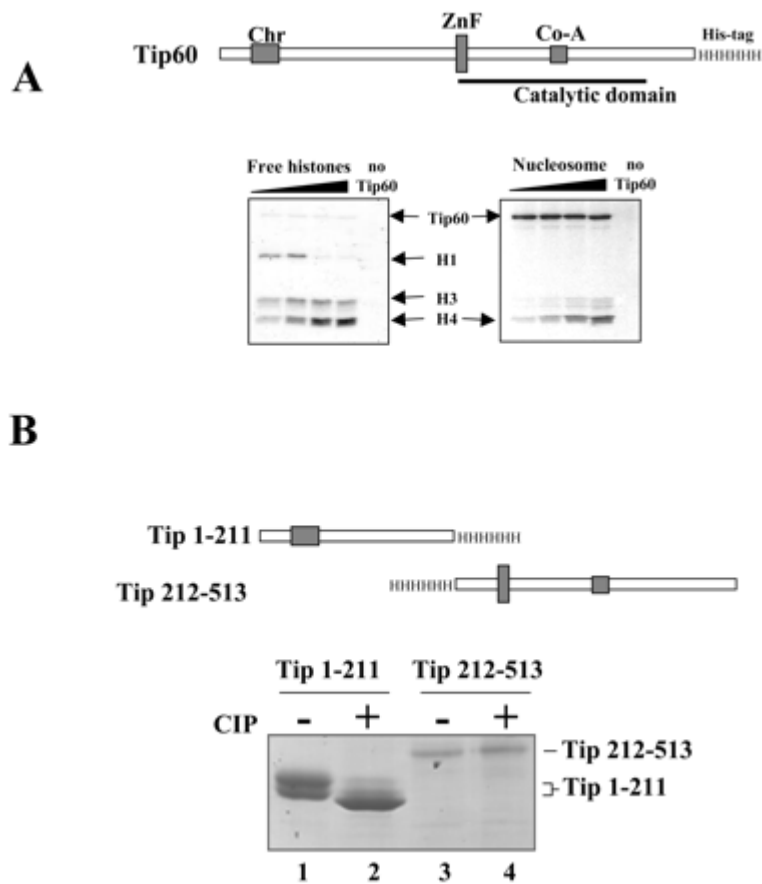


Fig. 1. The enzymatically active Tip60 expressed in insect cells is phosphorylated. Baculovirus-based expression system was used to produce His-tagged human Tip60 (*scheme*) in insect cells. *A*, the purified enzyme is capable of efficiently acetylating free histones (*left panel*) and nucleosomal histone H4 (*right panel*). *B*, the two indicated His-tagged fragments of Tip60 (*scheme*) were produced in insect cells, treated or not with CIP (+ and -, respectively) and analyzed on 15% SDS-PAGE stained with Coomassie Blue.

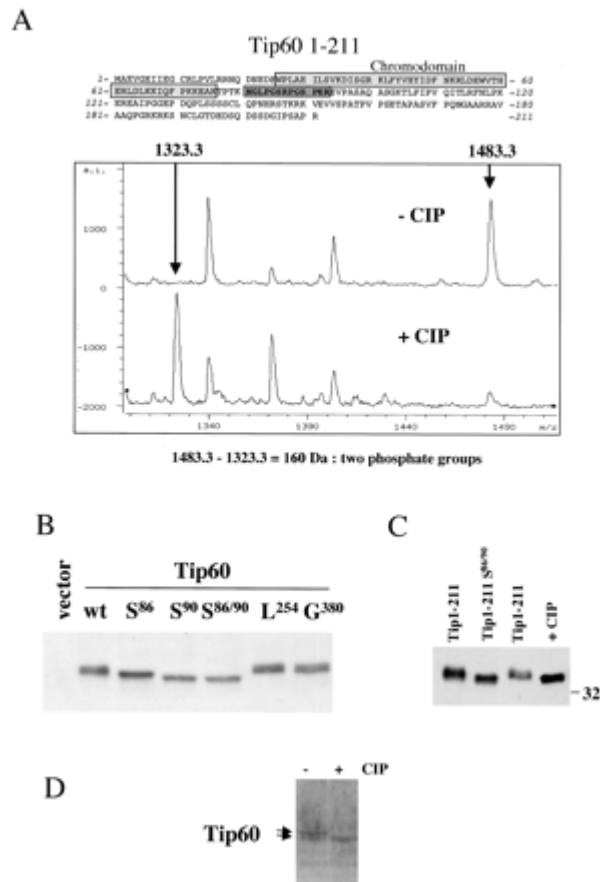


Fig. 2. Serines 86 and 90 are two major sites of Tip60 phosphorylation. *A*, amino acid 1-211 region of Tip60 expressed and purified from insect cells was digested with trypsin, and the CIP-treated and untreated tryptic fragments were analyzed by mass spectrometry. The CIP treatment led to the complete displacement of a peak at 1483.30 kDa and the appearance of a new one at 1323.30. The loss of mass attributed to the CIP treatment (160 Da) shows the removal of two phosphate groups by CIP. The Q-TOF sequencing of this peptide shows that it corresponds to the amino acid 81-93 region of Tip60 containing only two phosphorylatable residues, serine 86 and serine 90 (*gray box*). *B*, serines 86 (*S86*) and 90 (*S90*) are sites of phosphorylation in mammalian cells. Site-directed mutagenesis was used to replace Ser-86, Ser-90, or both by alanines. As a control, other irrelevant sites of Tip60 have been also mutated to alanines. These sites are glycine 380 (*G380*) and leucines 254 (*L254*) and 257. These proteins were expressed in HeLa cells, and their mobility was monitored after a Western blot and immunodetection with an anti-HA antibody. *C*, Tip60-(1-211) fragment containing the wild type sequence or mutated on Ser-86 and Ser-90 (*Tip1-211 S^{86/90}*) was also expressed in HeLa cells, and the mobility of the CIP-treated wild type peptide was compared with that of the Ser-86/Ser-90 mutant. *D*, purified HeLa cell nuclei were isolated, and a fraction was incubated with CIP before extract preparation (+). The extracts were then analyzed by Western blotting using an anti -Tip60 antibody (14).

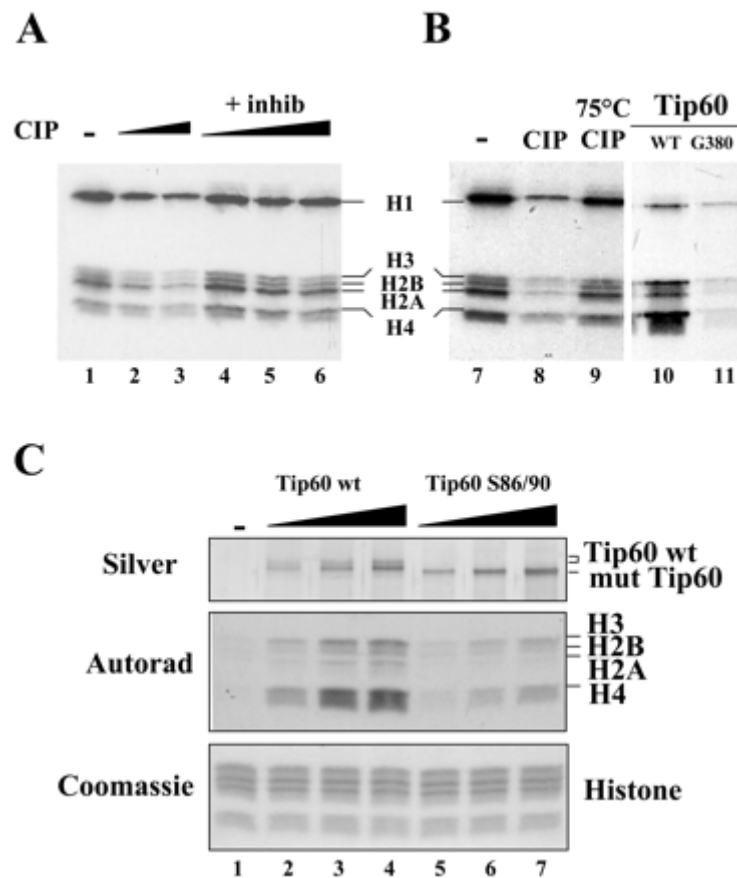


Fig. 3. Tip60 HAT activity is controlled by phosphorylation. *A*, purified Tip60 expressed in insect cells was treated with increasing amounts of CIP in the absence (*lanes 2 and 3*) or presence of CIP inhibitor NaF (*lanes 4-6*). *Lanes 1 and 4* show the activity of untreated Tip60. Treated and untreated Tip60 proteins were incubated with purified histone and [14 C]acetyl-CoA. The reaction was stopped, loaded on a 15% SDS-PAGE, and analyzed by autoradiography. *B*, purified Tip60 was left untreated (*lane 7*) or treated with CIP or heat-inactivated CIP (*lanes 8 and 9*, respectively). The activity of wild type Tip60 was also compared with a protein harboring a G to A mutation at position 380 (*lanes 10 and 11*, respectively). *C*, the Ser-86/Ser-90 Tip60 mutant shows a reduced HAT activity. Wild type Tip60 or a mutated version of the protein harboring double S to A mutations at positions 86 and 90 was produced in insect cells and purified, and their HAT activity was tested as noted above. After the reaction, a fraction was analyzed on a silver-stained 8% SDS-PAGE to compare the amounts of enzyme used (*upper panel*). The *middle panel (Autorad)* shows the labeling of histone after the reaction by wild type or mutated Tip60. The corresponding Coomassie Blue-stained gel before autoradiography shows histones used in each reaction (*lower panel*).

A

	86	90
wt	GLPGSRPG	SPEREVVP
mutS ⁹⁰	GLPGSRPG	APEREVVP
mutS ^{86/90}	GLPGARPG	APEREVVP

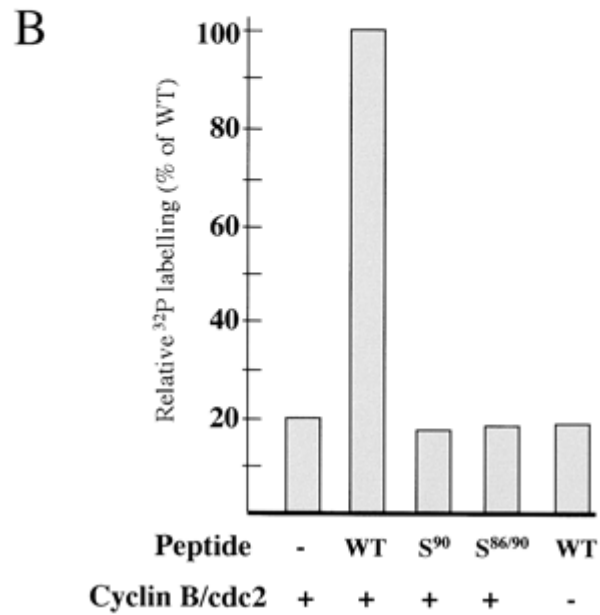


Fig. 4. Tip60 Serine 90 is phosphorylated by cyclin B/Cdc2 *in vitro*. A, peptides corresponding to the Tip60 amino acids 82-96 region, either wild type (*wt*) or containing the indicated mutations, were synthesized and used in a kinase reaction. B, the histogram shows that only the wild type peptide is a substrate for Cdc2 kinase.

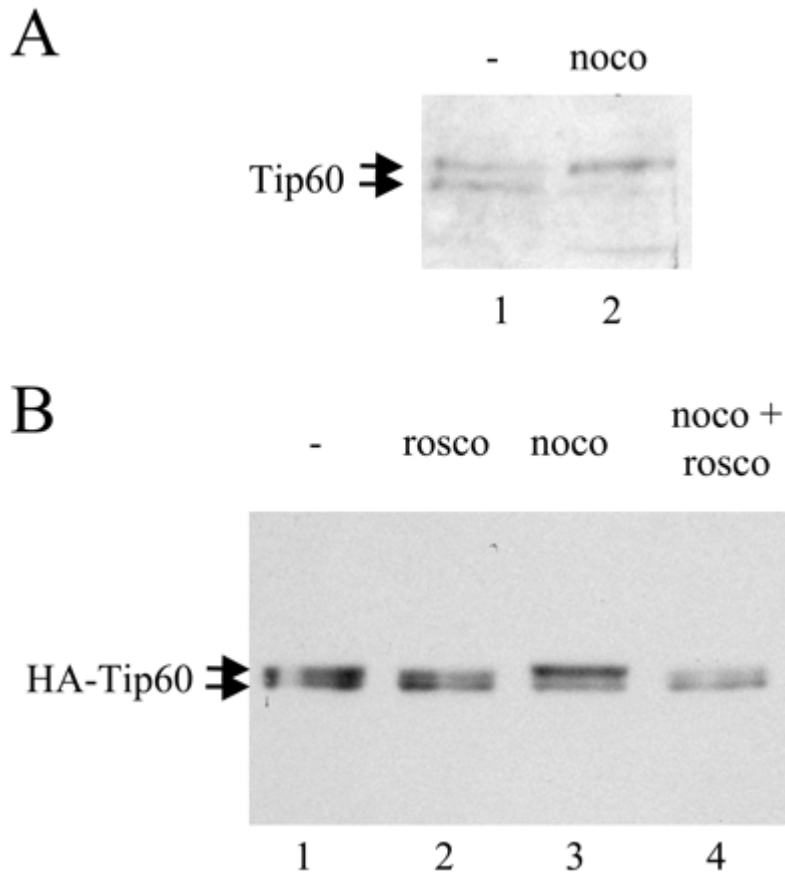


Fig. 5. The arrest of cells at the G₂/M phase of the cell cycle is associated with an enhancement of Tip60 phosphorylation. *A*, HeLa cells were treated with nocodazole (100 ng/ml) for 16 h and lysed, and the migration of the endogenous Tip60 was monitored by Western blotting using an anti-Tip60 antibody. *B*, HeLa cells stably expressing HA-Tip60 were treated with nocodazole for 23 h, and 7 h before harvest, roscovitin (50 μ M) was added where indicated. Cells were lysed and analyzed as above using an anti-HA antibody. -, non-treated cells.