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Regulation of Histone Acetylation and Transcription by Nuclear Protein pp32, a Subunit of the INHAT Complex*

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Histone acetylation by p300/CBP and PCAF coactivators is considered to be a key mechanism of chromatin modification and transcriptional regulation. A multiprotein cellular complex, INHAT (inhibitor of acetyltransferases), containing the Set/TAF-Iβ oncoprotein and pp32 strongly inhibits the HAT activity of p300/CBP and PCAF by histone masking. Here we report that the INHAT complex and its subunits have overlapping but distinct HAT inhibitory and histone binding characteristics. We provide evidence suggesting that the histone binding and INHAT activity of pp32 can be regulated by its physical association with other INHAT subunits. *In vivo* colocalization and transfection studies show that pp32 INHAT domains are responsible for histone binding, HAT inhibitory activity, and repression of transcription. We propose that INHAT and its subunits may function by modulating histone acetyltransferases through a histone-masking mechanism and may play important regulatory roles in the establishment and maintenance of the newly proposed "histone code" of chromatin.

Chromatin plays important structural and regulatory roles in the control of gene expression in eukaryotes (1–4). It has been suggested that the modulation of chromatin structure and transcription can be achieved through the interaction between histones and chromatin-remodeling factors and/or post-translational modifications of N-terminal histone tails (2, 4–8).

Evidence is accumulating for the role of post-translational modifications such as acetylation (1, 3, 9), phosphorylation (10–12), and more recently methylation of N-terminal histone tails in chromatin-linked processes, including transcription (13–16). One such modification, which has been intensively studied, is acetylation of lysine residues of histone tails. It is generally believed that hypo/unacetylated histones associate with basal/repressed chromatin, whereas hyperacetylation of histones has been linked to transcriptionally active chromatin domains. Transcriptional coactivators such as p300/CBP,[1] PCAF, and ACTR have intrinsic acetyltransferase activity, and it is proposed that these coactivators contribute to transcription at least in part by acetylating histones and nonhistone proteins (17–19). Given that histone acetylation plays an important role in modulating transcription, it is conceivable that the processes leading to histone acetylation are regulated and that their misregulation by cellular and viral proteins could have significant influences on chromatin modification and gene expression (7, 20–26). Regulation of histone acetylation has been shown to be mediated by activities that bind either to the acetylases or the substrate histones. For example, while E1A inhibits histone acetylation by directly binding to p300/CBP and PCAF, RbAp 46/48 stimulates histone acetylation and transcription by p300/CBP by directly binding to histones (22, 25, 27). Additionally, although acetylation of histones by p300/CBP, PCAF, and ACTR promotes transcription, acetylation of ACTR by p300/CBP destabilizes interactions between nuclear receptors and ACTR, resulting in attenuation of hormonal signaling (28). Therefore, protein acetylation may have both positive and negative regulatory roles in transcription.

While investigating the cellular regulation of HAT activity of coactivators, we reported the identification of a cellular complex termed INHAT (inhibitor of acetyltransferases). INHAT binds to histones and inhibits p300/CBP- and PCAF-mediated histone acetylation. We referred to this mechanism as histone-masking (7). INHAT is a multiprotein complex with the putative oncoprotein Set/TAF-Iβ, TAF-Iα, and a nuclear protein, pp32, as the major subunits. We had previously shown that Set inhibits HAT-mediated transcription when overexpressed in intact cells. These results suggest that INHAT may play a role in transcription by binding to histones. Although we have previously characterized the role of Set/TAF-Iβ in regulation of histone acetylation and transcription, very little is known about the role of pp32 in HAT regulation and transcription.

Among the subunits, pp32 belongs to a family of acidic leucine-rich nuclear proteins that includes April, LANP, PHAP1, and Mapmodulin (29–33). Along with other INHAT subunits, pp32 is also a subunit of the RNA transport pathway (34). Additionally, LANP was shown to associate with ataxin-1, although the significance of that interaction is unclear (35). pp32 has also been reported to suppress cell transformation induced by multiple oncogenes, including Ras and Myc (30, 36). Like Set/TAF-Iβ and TAF-Iα, pp32 also has a long C-terminal acidic tail, indicating an evolutionary conserved role for this domain in the function of these proteins. Besides their C-terminal acidic domains, little is known about any common or distinct characteristics of Set/TAF-Iβ and pp32 in HAT and transcriptional regulation. Additionally, it is not clear how the activity of the individual INHAT subunits contributes to the overall activity of the complex and whether the activity of the subunits can be dictated based on whether they are free or part of the complex.

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[1] The abbreviations used are: CBP, CREB-binding protein; CREB, cAMP response element-binding protein; RbAp 46/48, Rb-associated protein 46/48; HAT, histone acetyltransferase; INHAT, inhibitor of acetyltransferases; GST, glutathione *S*-transferase; GFP, green fluorescent protein; DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride; FITC, fluorescein isothiocyanate; PCAF, p300/CBP-associated factor; ACTR, activator of thyroid and retinoid receptors.

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In this report, we have analyzed the HAT inhibitory characteristics of INHAT subunits with emphasis on pp32. We show that the INHAT complex and each of its subunits show overlapping but distinct specificities in their HAT inhibitory activity by targeting different histone subunits. The colocalization studies show that although the full-length pp32 colocalizes with distinct histone domains in the nucleus, a mutant pp32 defective in HAT inhibition and histone binding fails to colocalize with histones, indicating that histone binding is a prerequisite for pp32 function in the regulation of histone acetylation and transcription.

EXPERIMENTAL PROCEDURES

Plasmids and Proteins—For bacterial and eukaryotic expression constructs of Set/TAF-Iβ, TAF-Iα, pp32, CBP, and their derivatives, the appropriate PCR-amplified fragments were cloned into pGEX4T2, CMX-PL1, RHNCMX-PL1, CMXGAL4, and pEGFP-C1 (CLONTECH) vectors. Sequences of all constructs surrounding the cloning sites were verified by automated sequencing. Recombinant GST proteins were purified using glutathione beads (Amersham Biosciences). Purification of baculovirus-expressed FLAG-p300 and FLAG-PCAF was carried out as described (22). The INHAT complex was purified from HeLa cells as described (7).

HAT Assay—Histone and nucleosome acetyltransferase assays were performed as described previously (7).

In Vitro Immunoprecipitation and Interaction Assays—[^{35}S]methionine-labeled INHAT subunits and pp32-deletion mutant proteins were generated by *in vitro* transcription/translation and used in histone binding and immunoprecipitation assays as described (7). For histone binding specificities of INHAT subunits, individual histones were used for each assay instead of total histones.

Transfection Assays—CV-1 cells were seeded at 20,000 cells/well of a 48-well dish as described (7) with internal control pRLSV40 (5 ng) and MH100-TK-LUC (100 ng) as reporter. CMXGal4·CBP (200 ng) and CMXRHN·pp32 derivatives (100 and 50 ng) used for Fig. 6 were added where indicated. The amount of DNA in each transfection was kept constant by addition of pCDNA3 vector. The results in Fig. 6 are representative of three independent experiments. The margin of error was less than 10% between the mean values of each assay.

Peptides—The pp32 peptide (residues 150–180) was synthesized commercially (Annovis, Aston, PA). Product purity was greater than 85%, and the molecular weight of synthesized peptide was confirmed by mass spectrometry analysis.

Immunostaining—For endogenous pp32 detection, NIH3T3 cells seeded in 35-mm^2 plate were fixed and incubated with anti-pp32 antibodies and immunocytostained with secondary antibody conjugated with FITC (Jackson ImmunoResearch Laboratories). NIH3T3 cells were seeded overnight and transiently transfected with pEGFP·C1-INHAT subunits and pEGFP-C1-pp32-C2 as indicated. After 24 h, cells were washed with phosphate-buffered saline and fixed in 50% acetone/methanol. For histone staining, cells were incubated with antihistone antibodies (Chemicon International), followed by incubation with Cy3-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories). For nucleus detection, cells were incubated with DAPI (Molecular Probes) stain and mounted with Gel/Mount. Slides were examined under oil immersion with a confocal microscope with Bio-Rad 1024-ES using the Confocal Assistant™ program and Nikon Eclipse E-600 with X60 objectives. Raw data images were processed further using the Confocal Assistant™ program.

RESULTS

Specificity of Inhibition of Histone Acetylation by pp32—Using baculovirus-expressed FLAG-tagged p300, PCAF, and pp32 purified as GST fusion proteins from *Escherichia coli*, we tested the ability of pp32 to inhibit histone and nucleosome acetylation. In agreement with previous results, the acetylation of core histones by p300 and PCAF was inhibited by addition of saturating concentrations of pp32 (Fig. 1, compare *lanes 1* and *3* to *lanes 2* and *4*, respectively). Additionally, nucleosomal histone acetylation by p300 and PCAF was also inhibited by pp32 (data not shown). No proteolytic degradation of histones in the presence of pp32 was evident (Fig. 1, *Coomassie* and data not shown). These observations demonstrate that pp32 inhibits histone acetylation and prompted us to finely map the HAT inhibitory domain(s) of pp32.

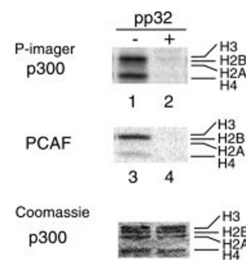

FIG. 1. **Recombinant pp32 blocks p300- and PCAF-mediated histone acetylation.** Histones were incubated with p300 and PCAF without (*lanes 1* and *3*) or with pp32 (*lanes 2* and *4*) as indicated. Samples were separated by SDS-PAGE and analyzed by phosphorimager. Positions of individual histones H3, H2B, H2A, and H4 are shown. Coomassie Blue staining of p300-HAT inhibition assay gel is shown.

Mapping of the pp32 INHAT Domain—pp32 contains previously characterized structural domains, including a highly acidic C-terminal domain homologous to that of TAF-I (amino acids 167–249), an α-helical N-terminal domain (amino acids 1–206), and a central domain containing the tumor suppressive activity that spans amino acids 150–174 (30, 36). We generated and purified a series of N- and C-terminal deletion mutants of pp32 as GST fusion proteins and examined their HAT inhibitory activity in order to identify the HAT inhibitory domain(s) of pp32 (Fig. 2*A*). Approximately the same amount of expressed mutant proteins was used for the HAT inhibitory activity assay, and purified GST protein alone had no effect on the assay system (data not shown). When compared with the full-length protein (*Construct 1*), deletion of large parts of the acidic C-terminal residues 181–249 of pp32 (*pp32-C1*, *Construct 2*) overall had no effect on the INHAT activity. However, further deletion of additional C-terminal sequences (residues 151–180) resulted in the loss of the entire inhibitory activity (*pp32-C2*, *Construct 3*). Interestingly, this domain overlaps with the tumor suppressor domain of pp32 (36). These results suggest that a major HAT inhibitory domain of pp32 resides between amino acids 151 and 180.

A series of pp32 N-terminal deletion mutant proteins was also generated and tested for the ability to inhibit histone acetylation. It is interesting that the deletion of the first 1–59 residues of pp32 (*pp32-N1*, *Construct 4*) showed a slight increase in INHAT activity when compared with that of the full-length pp32. This suggests the possible existence of a negative autoregulatory domain in the N terminus of pp32 (residues 1–59). The overall role of this domain in pp32 activity *in vivo* remains to be determined. Consistent with the results of C-terminal deletion analysis, both pp32-N2 (*Construct 5*), which lacks residues 1–119, and pp32-N3 (*Construct 6*), which lacks residues 1–149 of the N terminus, showed strong HAT inhibitory activity. Although deletion of residues 181–249 did not significantly affect the HAT inhibitory activity of pp32-C1 (*Construct 2*), pp32-N5, which only contains the C-terminal residues 190–249, still retained reasonably strong INHAT activity (*Construct 7*). Thus, the removal of amino acids 150–180 uncovered a direct role for the C-terminal acidic domain of pp32 that is similar to the INHAT domain of Set/TAF-Iβ and TAF-Iα in the inhibition of histone acetylation (7). Together, these results putatively map the HAT inhibitory domains of pp32 at the C terminus of the protein in two separable subdomains termed pp32 INHAT domain I (residues 151–180) and domain II (residues 190–249).

To directly test whether pp32 INHAT domain I functions independently in inhibiting histone acetylation, a peptide covering the INHAT domain I of pp32 (residues 150–180) was

FIG. 2. **Mapping of the pp32 INHAT domains.** *A*, recombinant wild type (*Construct 1*) and mutant pp32 proteins (*Constructs 2–7*) were utilized in p300-mediated acetylation assays of total histones. The pp32 INHAT domains I and II are shown as two different *striped boxes*. The C-terminal domain rich in acidic amino acids is indicated by a *two-headed arrow*. *B*, sequence of the synthesized pp32 peptide representing pp32 INHAT domain I is indicated as *peptide pp32: 150–180*. The full-length pp32 protein is shown in the *upper panel* with pp32 INHAT domains. *C*, different concentrations of peptide pp32 were assayed for their INHAT activities with individual histone subunits H2B and H2A (*left panel*) and H3 and H4 (*right panel*) as indicated.

synthesized and analyzed in HAT assays. A schematic diagram of pp32 illustrating the putative INHAT domain and the amino acid composition of peptide pp32 is shown in Fig. 2*B*. The pp32 INHAT peptide strongly inhibited p300-mediated acetylation of core histones (data not shown) and four individually purified histone subunits in a dose-dependent manner (Fig. 2*C*). The relative IC_{50} values of this peptide toward the individual histones H2B, H2A, H3, and H4 ranged between 0.6 and 1.5 μM (Fig. 2*C*). These results are consistent with those obtained with purified full-length pp32 protein and further directly implicate INHAT domain I, spanning amino acids 150–180, as a major determinant of the INHAT activity of pp32.

Histone Binding Specificity of pp32 and INHAT—Histone and chromatin binding activity of both TAF-I proteins has been previously reported (37). Additionally, we have shown that the INHAT complex associates with histones on chromatin *in vivo* and blocks them from serving as acetylase substrates, implying a critical role for histone binding in the INHAT-mediated inhibition of acetylation (7). To determine whether HAT inhibitory properties of pp32 mutants correlate with their histone binding characteristics, we compared the histone binding properties of pp32 mutants.

In vitro immunoprecipitation experiments utilizing radiolabeled full-length and mutant pp32 proteins with total histones were carried out to determine the histone binding properties of pp32. pp32-C1, which retained histone acetylation inhibitory activity, was efficiently immunoprecipitated with antihistone antibodies (Fig. 3*A*, *lane 3*) in a manner similar to the histone binding properties of full-length pp32 (Fig. 3*A*, *lane 8*). Importantly, however, pp32-C2, which lacks INHAT domains I and II and had lost its HAT inhibitory activity (Fig. 2), did not bind to histones (Fig. 3*A*, *lane 6*), providing a strong correlation between histone binding and the HAT inhibitory activity of pp32. The N-terminal mutant proteins were also tested for their histone binding ability, and as predicted from their HAT inhibitory activity, all N-terminal mutants tested bound to histones with comparable affinity (Fig. 3*B*, *lanes 3*, *6*, and *9*). Together these results suggest histone binding as a prerequisite for HAT inhibition by pp32.

FIG. 3. **Histone binding properties of pp32 correlate with its INHAT function.** *In vitro* radiolabeled full-length pp32 (*panel A*, *lane 7*), pp32-C1 (*panel A*, *lane 1*), and pp32-C2 (*panel A*, *lane 4*) were incubated with (*panel A*, *lanes 3*, *6*, and *8*) or without (*panel A*, *lanes 2*, *5*, and *9*) total histones and immunoprecipitated with antihistone antibodies. *Lanes 1*, *4*, and *7* of *panel A* indicated 10% of the input. *In vitro* radiolabeled pp32-N1, N2, and N3 were incubated with (*panel B*, *lanes 3*, *6*, and *9*) or without (*panel B*, *lanes 2*, *5*, and *8*) total histones and immunoprecipitated with antihistone antibodies. *Lanes 1*, *4*, and *7* of *panel B* indicated 10% of the input.

We also tested the hypothesis that pp32 and other INHAT subunits might have distinct binding affinities toward different histone subunits. To address this question of histone binding specificity, we analyzed the binding properties of pp32 and the other INHAT subunits to individual histones *in vitro*. For that purpose, radiolabeled full-length pp32 was incubated with each histone subunit separately and immunoprecipitated with antihistone antibodies. The antihistone antibodies bound specifically to an antigenic determinant that is present on all four histone subunits and pulled down approximately the same amount of isolated histone subunits (data not shown). Histones H2B and H3 showed stronger interaction with pp32 (Fig. 4, *panel I*). Analysis of histone subunit binding by Set/TAF-Iβ demonstrated that this subunit has the strongest affinity to histone H3 and histone H4 (Fig. 4, *panel II*). TAF-Iα bound with higher affinity to histones H4 and H2B (Fig. 4, *panel III*). It is interesting that the reconstituted INHAT complex, where all three INHAT subunits are combined, showed stronger interactions with histones H3 and H4 (Fig. 4, *panel IV*) and little interaction with histone H2B. Binding to histone H2A was very low with all three individual subunits of the INHAT complex. The above results indicate the INHAT complex displays a histone binding property that is overall different from that of its individual subunits. From these *in vitro* results we have concluded that INHAT and its subunits have distinct but overlapping histone binding properties, and we suggest that individual

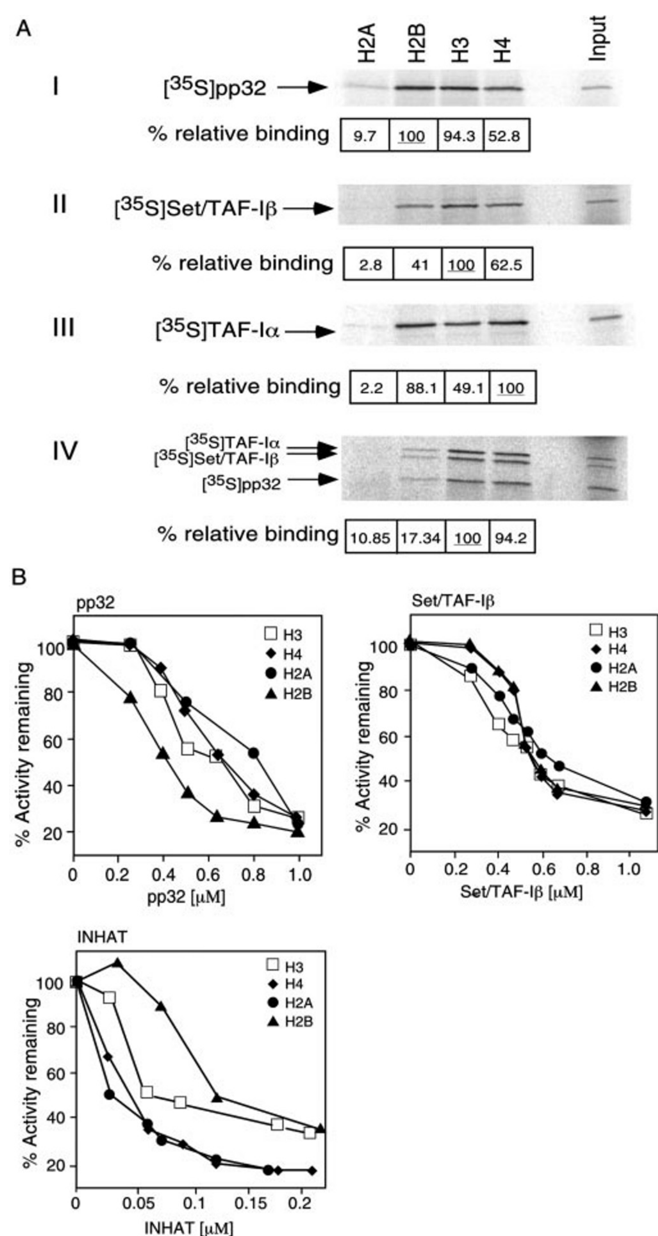

FIG. 4. **Determination of histone binding specificities of the INHAT subunits.** *A*, the INHAT subunits and complex have overlapping but distinct histone subunit binding specificity. *In vitro* radiolabeled pp32, Set/TAF-Iβ, and TAF-Iα were incubated individually or as a reconstituted complex with each individual histone subunit and immunoprecipitated with antihistone antibodies. The percentage of each binding relative to the strongest binding (arbitrarily set at 100%) in each set of experiments was quantitated by the phosphorimager ImageQuant program. The percent of binding numbers represent the mean values of two independent experiments. The input represents 10% of the materials used for immunoprecipitation. *B*, histone specificity of inhibition by INHAT and its subunits. Increasing amounts of purified pp32 (*upper left panel*) or Set/TAF-Iβ (*upper right panel*) and the INHAT complex (*lower left panel*) were incubated separately with 500 ng of individual histones H3, H4, H2A, and H2B prior to the addition of p300. Reaction samples were separated by SDS-PAGE. The radioactivity of individual histone bands was determined from the phosphorimager, and percent activity remaining was calculated using the value of acetylation of each histone in the absence of INHAT and its subunits as 100%.

INHAT activity of subunits may be regulated by their physical association with other subunits.

To determine whether histone binding properties of INHAT and its subunits correlate with the ability to inhibit individual histone acetylation, the inhibitory activity of INHAT and its subunits toward each histone subunit was titrated (Fig. 4*B*). In agreement with the histone subunit binding properties of pp32 (Fig. 4*A*), inhibition of histone H2B acetylation by pp32 was slightly higher than that of the other subunits (Fig. 4*B*, *panel pp32*). Inhibition by purified Set/TAF-Iβ protein was very similar for all histone subunits, although it displayed a lower binding affinity for histone H2B. However, the INHAT complex showed slightly lower inhibitory activity toward histone H2B, in agreement with its histone subunit binding properties. Together these results indicate that INHAT and its individual subunits display distinct but overlapping histone substrate preferences *in vitro* and that the inhibition of histone acetylation specificity of INHAT subunits may be regulated by their physical association with other subunits.

Colocalization of INHAT Subunits with Histones—The observation that pp32 and other INHAT subunits associate with histones *in vitro* led us to investigate whether they would individually colocalize with histones *in vivo*. We have previously shown, using ChIP (chromatin immunoprecipitation) assays, that INHAT forms a complex with histones *in vivo* (7). Localization of endogenous pp32 was examined using anti-pp32 antibodies and a secondary antibody conjugated with FITC. Next we generated C-terminal GFP fusions of pp32, TAF-Iα, and TAF-Iβ proteins and carried out colocalization studies to demonstrate the association between individual INHAT subunits and histones in intact cells. NIH3T3 cells transfected with expression constructs coding individual GFP-fused INHAT subunits were immunostained with antihistone antibodies and Cy3-conjugated secondary antibodies. They were DAPI stained and analyzed by confocal fluorescence imaging microscopy. Intact histones and nuclei were detected by anti-Cy3-histone antibodies and DAPI staining, respectively.

Endogenous pp32 was predominantly found in the nucleus, with some cytoplasmic distribution (Fig. 5*A*, *FITC-pp32*). The overlay and individual detection of endogenous pp32 and histones show both proteins are in the nucleus (Fig. 5*A*). In agreement with the localization of endogenous pp32, GFP·pp32 was also detected mainly in the nucleus (Fig. 5, *A* and *B*, *FITC-pp32* and *GFP·pp32*), whereas GFP·TAF-Iα and GFP·Set/TAF-Iβ exhibited both cytoplasmic and nuclear staining (Fig. 5, *D* and *E*, *GFP·TAF-Iα* and *GFP·Set/TAF-Iβ*) in our assay conditions. No specific immunofluorescence signal was detected in the nuclei of NIH3T3 cells when primary antibodies were substituted with anti-mouse control antibodies (data not shown). Based on the hypothesis that INHAT binds to histones on chromatin, prevents them from being acetylated, and serves as a component of the repressive/basal state of chromatin, one would expect that under normal circumstances INHAT and histones would localize on some but not all chromatin domains. The overlay of staining of endogenous as well as GFP·pp32 and histones showed colocalization of pp32 and histones inside the cell nucleus. As expected, it was also evident that a significant fraction of histones did not colocalize with pp32 under these assay conditions, suggesting that the histone-INHAT interaction is specific and implying that binding of histones to pp32 may be regulated at different chromatin loci in the nucleus. It remains to be determined whether pp32-histone colocalized domains represent repressed/basal chromatin and whether histone domains lacking pp32 represent the active state of chromatin. Although a significant amount of TAF-I proteins displayed cytoplasmic localization, it nonetheless showed nuclear colocalization with histones at varying levels (Fig. 5, *D* and *E*).

Our *in vitro* binding studies demonstrated that pp32-C2 failed to inhibit histone acetylation and did not bind to histones. We utilized GFP·pp32-C2 in colocalization analysis to further demonstrate the interaction specificity between pp32

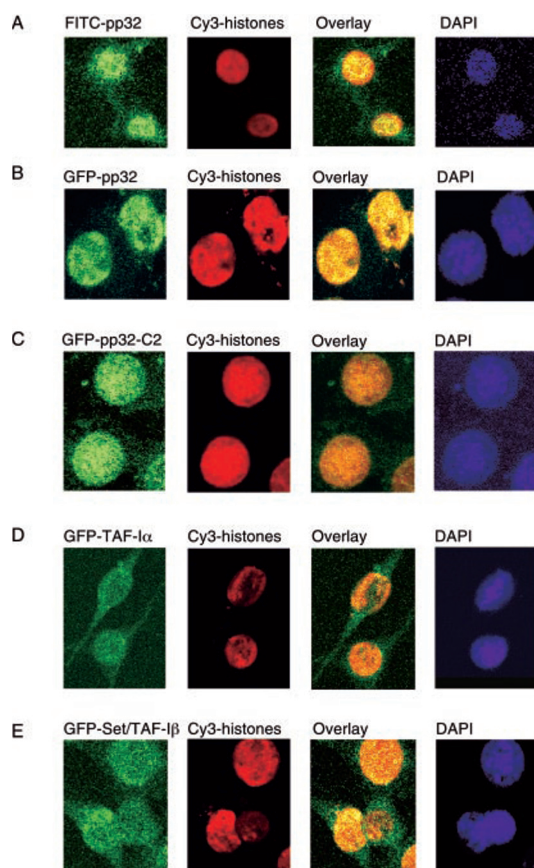

FIG. 5. **Localization of INHAT subunits with histones.** Endogenous pp32 in NIH3T3 cells was detected by immunocytostaining with anti-pp32 antibodies and FITC-conjugated secondary antibodies (*A*, *FITC-pp32*). NIH3T3 cells were transfected with constructs representing GFP·pp32 (*B*), GFP·pp32-C2 (*C*), GFP·TAF-Iα (*D*), and Set/TAF-Iβ (*E*) fusion proteins. Cells were immunocytostained with antihistone antibodies and Cy3-conjugated secondary antibodies (*Cy3-histones*). The overlays of GFP proteins and Cy3-stained histones are shown in all cases. DAPI staining which detected the nuclei of cells is also shown.

and histones *in vivo*. Like GFP·pp32, GFP·pp32-C2 localized in the nucleus (Fig. 5*C*). However, in contrast to the GFP·pp32, which showed extensive colocalization with histones (Fig. 5*B*, *overlay*), a dramatic reduction in colocalization of GFP·pp32-C2 and histones, as judged by the decreased number of bright yellow speckles, was observed (Fig. 5*C*, *overlay*). These results again demonstrate the specificity of the *in vivo* interactions between histones and pp32. Taken together, these *in vivo* colocalization studies are consistent with the hypothesis that the HAT inhibitory activity of the individual INHAT subunits is accomplished through mechanisms that at least in part involve histone binding.

pp32 Blocks HAT-dependent Transcription—The demonstration that HAT inhibition by pp32 occurs through histone binding mediated by its INHAT domain implies that this domain may play a regulatory role in HAT-mediated transcription. We and others have shown that a Gal4 DNA binding domain fusion of CBP (Gal4·CBP: 1092–2002) activates transcription in a HAT-dependent manner (7, 38). Furthermore, we have shown that Gal4·CBP-mediated transcriptional activation was severely inhibited by the overexpression of Set/TAF-Iβ in an INHAT domain-dependent manner *in vivo* (7). To determine the effect of pp32 and its INHAT domains on CBP-HAT-directed transcription, the Gal4·CBP (1092–2002) construct was cotransfected with various pp32 constructs, and the expression of the Gal4 responsive reporter gene was analyzed. The Gal4·DBD fusion of CBP activated transcription as expected

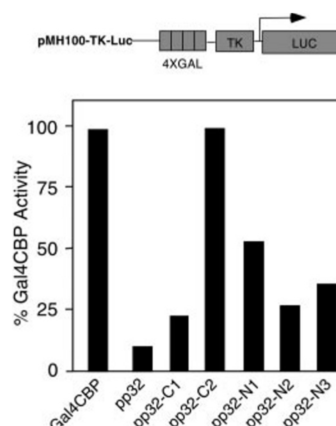

FIG. 6. **Overexpression of pp32 inhibits HAT-dependent transactivation.** Overexpression of pp32 inhibits CBP-HAT-dependent transcription. CV-1 cells were transfected with the reporter pMH100-TK-Luc, Gal4·CBP (1092–2002), and pp32 derivatives as indicated. Following transfection, cells were grown for 48 h, and cell extracts were prepared and assayed for luciferase activity. The percent reporter activity was determined utilizing the values of luciferase activity of Gal4·CBP as 100%.

(Fig. 6, *lane Gal4·CBP* and Ref. 7). This Gal4·CBP HAT-mediated transcription was severely inhibited at varying degrees by all pp32 constructs tested except for pp32-C2, which does not contain the INHAT domains (Fig. 6). These overexpression studies imply a regulatory role for pp32 in HAT-mediated transcription and suggest that pp32 may serve as a component of the basal/repressive state of genes.

DISCUSSION

In this study we have characterized differential HAT inhibitory activity of the INHAT subunits with special emphasis on pp32 and have proposed a role for INHAT in the maintenance of a basal/repressive chromatin state. We provide *in vitro* evidence demonstrating that the INHAT complex and its individual subunits show distinct but overlapping histone target and HAT inhibition specificities and that these properties may be regulated based on their physical existence in free or complexed form with other subunits. *In vivo* colocalization and transcription studies suggest a role for the INHAT domain of pp32 in histone binding, HAT inhibitory activity, and transcriptional repression.

Using N- and C-terminal deletion mutagenesis, we show that pp32 contains two independent HAT inhibitory domains. Although the pp32 INHAT domain I maps between amino acids 150 and 180, pp32 INHAT domain II, which is similar to the INHAT domain of TAF-I proteins, resides between amino acids 190 and 249. Because the INHAT domain I overlaps with the tumor suppressor domain, it will be important to determine in the future whether the INHAT activity plays any role in this latter property of pp32. In agreement with the HAT inhibitory properties, a pp32 mutant defective in HAT inhibition also fails to bind histones, suggesting that histone binding is an integral part of pp32-mediated HAT inhibition. Although isolated pp32 binds to and inhibits acetylation of histone H2B and H3 with high affinity, this H2B preference is lost when pp32 is incorporated into the INHAT complex, which predominantly binds to and inhibits acetylation of histones H3 and H4. These results are consistent with the distinct HAT inhibitory properties of INHAT and its subunits and are quite remarkable in that they suggest that the *in vivo* function of TAF-Iα, Set/TAF-Iβ, and pp32 may well be determined by their existence either as free proteins or as subunits of the INHAT complex. This prediction becomes more relevant because the INHAT subunits have also been purified in other biological contexts, either in free forms or

as part of a complex with other proteins (31, 33, 34, 39–41). The *in vivo* implication of these differential histone binding and HAT inhibitory properties of the INHAT subunits remains to be determined.

Consistent with previous reports, our colocalization studies demonstrate that pp32 is predominantly nuclear and show that it associates with certain subnuclear domains of histones, suggesting the pp32 and/or INHAT bind to selected sites on chromatin. Although the identities of these sites have not been determined, it is possible that pp32-targeted histones may represent repressed chromatin, and histone domains free of pp32 and INHAT may indicate the active state of chromatin. This *in vivo* binding of pp32 to histones is specific, because a pp32 mutant defective in HAT inhibition and *in vitro* histone binding demonstrated a significant reduction in colocalization with histones *in vivo*.

When overexpressed, pp32 inhibits Gal4·CBP-mediated transactivation, implicating a role for pp32 in regulating HAT-mediated transcriptional events and suggesting a role for pp32 in the maintenance of the basal/repressive state of genes. Based on the implication that INHAT subunits are components of the basal/repressive state of genes, we propose that the regulation of INHAT activity by cellular mechanisms, which prevent their binding to histones, will play an important role during activation of transcription.

In summary, our results describe overlapping but distinct HAT inhibitory and histone binding properties of INHAT and its subunits. The recently proposed histone code hypothesis envisions that the level and combination of epigenetic marking, including acetylation, methylation, and phosphorylation on histones, will play a fundamental role in chromatin-based processes, including transcription (2, 6). An extension of this hypothesis is that the epigenetic marking of histones (including acetylation) therefore should be regulated. Given that the histone code dictates the transition between transcriptionally active and silent chromatin states, we propose that INHAT and other yet to be identified parallel complexes will play a significant role in the establishment and maintenance of the histone code, reflecting the transcriptional off or on states of chromatin domains.

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