Highly Acidic C-Terminal Domain of pp32 Is Required for the Interaction with Histone Chaperone, TAF-Ibeta

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We have previously reported that INHAT (inhibitor of acetyltransferases) complex subunits, TAF (template activating factor)-Ialpha, TAF-Ibeta and pp32 can inhibit histone acetylation and HAT (histone acetyltransferase)-dependent transcription by binding to histones. Evidences are accumulating that INHAT complex subunits have important regulatory roles in various cellular activities such as replication, transcription, and apoptosis *etc*. However, how these subunits interact each other remains largely unknown. Using immunoprecipitation (IP) and protein–protein interaction assays with TAF-Ibeta and pp32 deletion mutant proteins, we identify INHAT complex subunits, TAF-Ibeta and pp32 interaction requires highly acidic C-terminal domain of pp32. We also show that the interaction between the INHAT complex subunits is stronger in the presence of histones. In this study, we report that the synergistic inhibition of HAT-mediated transcription by TAF-Ibeta and pp32 is dependent on the highly acidic C-terminal domain of pp32.

Key words inhibitor of acetyltransferases (INHAT); template activating factor (TAF)-Ibeta; pp32; transcription; protein interaction

Chromatin structure plays central roles in the regulation of eukaryotic gene expression.[1)] It has been suggested that one of the chromatin remodeling mechanisms is the post-translational modifications of histone N-terminal residues.[2,3)] Among the modifications, the acetylation of nuclear core histones is thought to play important roles in various cellular functions.[4)] The biological importance of histone acetylation is correlated with the level of transcription. Histones H3 and H4 are hyperacetylated in active genes, whereas they are hypoacetylated in silent genes.[1—3)]

Previously we have reported the identification of a cellular complex termed INHAT (inhibitor of acetyltransferases) which binds to histones and inhibits p300/CBP- and PCAF-mediated histone acetylation through histone-masking.[3)] INHAT is a multiprotein complex with the oncoprotein template activating factor (TAF)-Ibeta, TAF-Ialpha, and a nuclear protein pp32 as the major subunits.[5)] These results suggest that INHAT have a role in transcription by binding to histones. We also have identified that TAF-Ibeta and pp32 have the properties to transduce the hypoacetylated chromatin into transcriptional repression in eukaryotes.

TAF-Ibeta has been purified from HeLa cells, which stimulates the replication from the Ad core and initially named TAF-I.[6,7)] There are two subtypes of TAF-I, designated as TAF-Ialpha and TAF-Ibeta, both of which have a common amino acid sequence except that N-terminal 30-amino acid sequences that are specific for each subtype. Both TAF-Is have long acidic tail in its C-terminal region that is required for the activation of the Ad core replication and transcription.[7)] TAF-Ibeta is the same as the product of the *set* gene, which is fused to the *can* gene by the translocation in an acute undifferentiated leukemia.[8)] TAF-I shows amino acid sequence homology to nucleosome assembly protein (NAP)-I, which was originally identified as the protein involved in chromatin assembly.[9)] The other INHAT subunit, pp32 belongs to a family of acidic leucine-rich nuclear proteins that includes April, LANP, and PHAP1.[10,11)] LANP was shown to associate with ataxin-1 and pp32 has also been reported to suppress cell transformation induced by oncogenes, including Ras and Myc.[12,13)] Like TAF-Ibeta and TAF-Ialpha, pp32 also has C-terminal acidic tail, indicating an evolutionary conserved role for this domain in the function of these proteins. We have shown that the INHAT complex and each of its subunits show overlapping but distinct specificities in their HAT inhibitory activity by targeting different histone subunits.[1)] It has been reported that TAF-Ibeta and pp32 play important roles in the regulation of apoptotic cell death. Granzyme A can activate apoptosis by inducing DNase, NM23-H1, and TAF-Ibeta can inhibit the process.[14)] Also, the induction of caspase-dependent apoptosis by pp32 and its homolog PHAPs was reported.[15)]

Besides the role of TAF-Ibeta and pp32 in regulation of histone acetylation and transcription, very little is known about the interaction between INHAT complex subunits. In this study, we have identified that the interaction between TAF-Ibeta and pp32 is facilitated by C-terminal acidic domain of pp32 both *in vitro* and *in vivo*. The interaction between INHAT complex subunits; especially pp32 interaction with TAF-I proteins is influenced by the presence of histones. Consistent with the results of interaction studies between TAF-Ibeta and pp32, HAT-dependent transcriptional repression activity of TAF-Ibeta and pp32 is dependent on the C-terminal acidic domain of pp32.

MATERIALS AND METHODS

Plasmids and Proteins For bacterial and eukaryotic expression constructs of INHAT subunits, the appropriate PCR-amplified fragments were cloned into pGEX 2TK (Amersham Pharmacia Biotech, NJ, U.S.A.) and CMX-PL1 vectors, respectively.[3)] Sequences of all constructs surrounding the cloning sites were verified by automated sequencing. Recombinant proteins were expressed in BL 21 (DE3) *E. coli* cells (Novagen, WI, U.S.A.), purified using glutathione beads (Amersham Pharmacia Biotech, NJ, U.S.A.), and cleaved by thrombin.

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Fig. 1. Interaction between INHAT Complex Subunits, TAF-Ibeta, TAF-Ialpha, and pp32

(A) [^{35}S]-labeled TAF-Ialpha and TAF-Ibeta were used for *in vitro* interaction assays with GST and GST-pp32 protein. Lane 1 of panel A indicated 10% of the input. Associated proteins were detected by SDS-PAGE and phosphorimaging device. (B) HeLa nuclear extract (NE) was incubated with GST and GST-pp32 protein. After washing, the complex proteins were separated by SDS-PAGE and analyzed by Western blot analysis with anti-TAF-Ibeta antibody.

Immunoblot Analysis and Pull Down Assay For pull down assay, HeLa cell extract was incubated with GST or purified GST-pp32 protein bound to beads. The GST beads were washed extensively, bound proteins separated by SDS-PAGE, transferred onto membrane, and incubated with anti-TAF-Ibeta antibodies. The blots were probed with alkaline phosphatase-conjugated anti-goat secondary antibodies (Bio-Rad, CA, U.S.A.).

***In Vitro* Immunoprecipitation and Interaction Assays** *In vitro*, [^{35}S]methionine-labeled TAF-Ialpha, TAF-Ibeta, and pp32 were synthesized using the CMX plasmids in a coupled transcription–translation system (TNT, Promega, WI, U.S.A.), and incubated with histones for 30 min at room temperature in buffer containing 150 mM KCl, 20 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT, and 10 μg/ml BSA. The complex was immunoprecipitated using anti-TAF-Ibeta and protein A agarose. For GST pull-down assays, *in vitro* labeled TAF-Ialpha, TAF-Ibeta and pp32 were incubated with glutathione sepharose bound GST or GST-TAF-Ibeta, GST-pp32. Beads were washed extensively and bound proteins separated by SDS-PAGE and analyzed by phosphorimager.

Transfection Assay HeLa cells were seeded in 48 well dishes and transfected by lipofectamine 2000 (Invitrogen, CA, U.S.A.) with internal control MH100-TK-LUC (100 ng) reporters, CMXGal4-CBP (100 ng) in the absence or presence of CMXPL1 derivatives of pp32 (50 ng), pp32-C2 (50 ng), and TAF-Ibeta (50 ng) where indicated.[1)] The amount of DNA in each transfection was kept constant by addition of pCDNA3. Forty eight hours after transfection, the cells were harvested and assayed for luciferase activity using the luciferase assay system (Promega, WI, U.S.A.) and normalized by the GFP level after the pEGFP-C1 (20 ng) co-transfection. Each value is the mean of six replicates from a single assay. The results shown are representative of at least three independent experiments.

RESULTS AND DISCUSSION

Interaction between INHAT Complex Subunits, TAF-Ibeta, TAF-Ialpha, and pp32 It has been reported that the most of the TAF-Ibeta and TAF-Ialpha exist as a dimer in a amino-terminal domain of TAF-I dependent manner.[16)] Also, in our previous reports we have shown that TAF-Ibeta, TAF-Ialpha, and pp32 forms INHAT complex both *in vivo* and *in vitro* and exhibited synergistic HAT inhibitory activity.[3)] However, little has been known about the detailed interaction mechanism among INHAT complex subunits. To further examine the mechanism of interaction between the TAF-Ibeta, TAF-Ialpha, and pp32, we performed GST pull down assay using purified GST-protein beads and immunoprecipitation assay using antibodies. Previously it has been reported that TAF-Ibeta, TAF-Ialpha can dimerize in N-terminal dependent manner in HeLa cells.[16)] *In vitro* GST pull down experiment utilizing radiolabeled TAF-Ialpha revealed that GST-TAF-Ibeta beads strongly interacted with radiolabeled TAF-Ialpha (Fig. 1A lane 3). GST beads alone did not retain both TAF-Ibeta and TAF-Ialpha (Fig. 1A lane 2). To further confirm the TAF-Ibeta and pp32 interaction *in vivo*, we incubated HeLa cell nuclear extracts with GST-pp32 beads. Immunoblotting with anti-TAF-Ibeta antibodies revealed that GST-pp32 interacted with endogenous TAF-Ibeta although it is a weak interaction compare to that of *in vitro* result (Fig. 1B lane 1). GST beads alone did not pull down TAF-Ibeta from HeLa cell extracts (Fig. 1B lane 2). These results suggest that TAF-Ibeta and TAF-Ialpha show interaction both *in vitro* and *in vivo*.

Stronger INHAT Complex Subunit Interaction in the Presence of Histones We and others have purified INHAT complex from cell extracts by applying various biochemical methodology and proved complex formation *in vivo*.[3,14,15)] We also have shown that both TAF-Ibeta and pp32 exhibited strong interaction with histones in a highly acidic C-terminal dependent manner.[1,3)] In this study, we further investigated the hypothesis that INHAT subunits might interact stronger in the presence of histones. Radiolabeled TAF-Ialpha, TAF-Ibeta, and pp32 were incubated in the absence and the presence of histones and complex formation was investigated using immunoprecipitation with TAF-Ibeta antibodies. Interestingly, in this assay conditions, INHAT subunit pp32 exhibited strong complex formation in the presence of histones when compared to the interaction without histones (Fig. 2 lanes 3, 4). Controls between anti-TAF-Ibeta antibodies and TAF-Ibeta or pp32 indicated the right assay conditions for both positive and negative controls (Fig. 2 lanes 2, 5). These results reveal that INHAT subunits might require histones for strong complex formation.

C-Terminal Acidic Domain of pp32 Dependent Interaction with TAF-Ibeta A series of pp32 N- and C-terminal deletion mutants were previously generated and purified to test the ability to inhibit histone acetylation and effects on transcription.[4)] The wild type pp32 showed histone binding, HAT inhibition, and transcriptional repression activities.[1)]

However, acidic C-terminal deletion mutant pp32-C2 lost all three of the above activities of the full length pp32. These strongly indicated the importance of the highly acidic C-terminal domain (amino acids 150—180) in HAT mediated transcriptional regulation. To further investigate the binding specificity between INHAT complex subunits, TAF-Ibeta and pp32, we performed GST pull down assay using GST beads of pp32 deletion mutants and GST cleaved full length TAF-Ibeta (Fig. 3A). Besides full length GST-pp32 protein, both N1 and N3 deletion mutants were retained after GST pull down assay though GST-pp32-N3 showed rather weak interaction compare to that of full length pp32 (Fig. 3B upper panel). Only pp32-C2 that is missing the highly acidic C-terminal domain did not interact with TAF-Ibeta (Fig. 3B upper panel GST-pp32-C2). These results strongly indicate that the interaction between INHAT complex subunits, TAF-Ibeta and pp32 is highly acidic pp32 C-terminal domain dependent. Coomassie staining of the deletion mutant proteins indicates the same amount of proteins were used for the pull down assay (Fig. 3B lower panel).

Acidic C-Terminal Domain of pp32 Is Required for the Synergistic Blocking of HAT-Dependent Transcription with TAF-Ibeta While each INHAT subunit has the inhibitory activity towards HAT-mediated transcriptional activation, the maximal inhibition was observed when TAF-Ibeta, TAF-Ialpha, and pp32 were combined.[1)] Also, we have identified C-terminal acidic domain of pp32 was responsible for the inhibition of HAT-mediated transcriptional activation by Gal4-CBP.[1)] To confirm the importance of this domain in combinatory transcriptional repression activity with TAF-

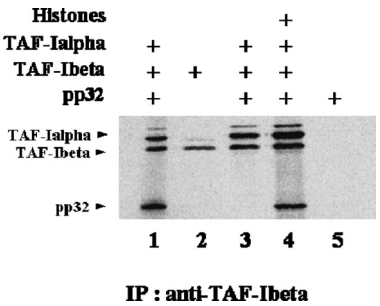

Fig. 2. INHAT Complex Subunit Interaction in the Presence of Histones

[^{35}S]-labeled INHAT subunits were incubated with or without histones. And proteins were immunoprecipitated with anti-TAF-Ibeta antibody. Lane 1 indicated 10% of the input. The reaction samples were separated by SDS-PAGE. The radioactivity of individual INHAT subunits was determined from the phosphorimager.

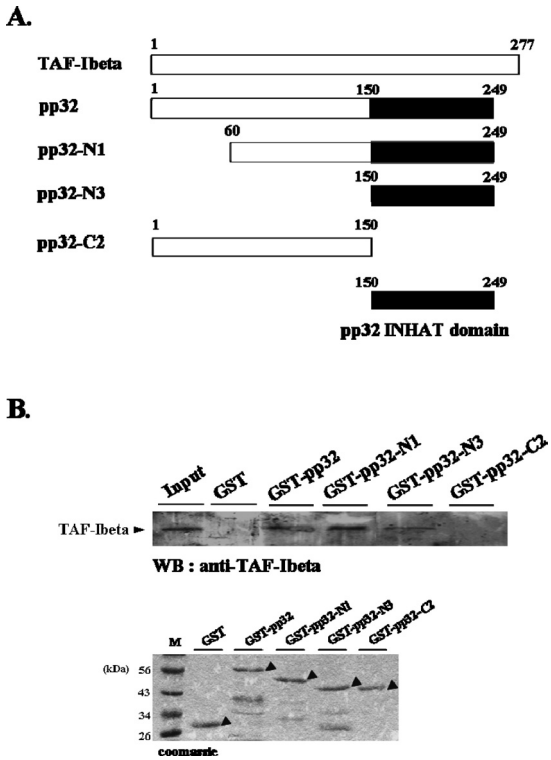

Fig. 3. C-Terminal Acidic Domain of pp32 Dependent Interaction with TAF-Ibeta

(A) Recombinant GST-pp32 and deletion proteins were assayed in pull down assay. The highly acidic domains are marked with dark boxes. (B) GST cleaved full length TAF-Ibeta was incubated with full length or deletion mutant GST-pp32 protein and pull down with GST-beads. Bound proteins were detected by immunoblot analysis using anti-TAF-Ibeta antibodies. The first lane indicates 15% of the input. Purified GST-pp32 and deletion proteins were shown in coomassie-stained gel.

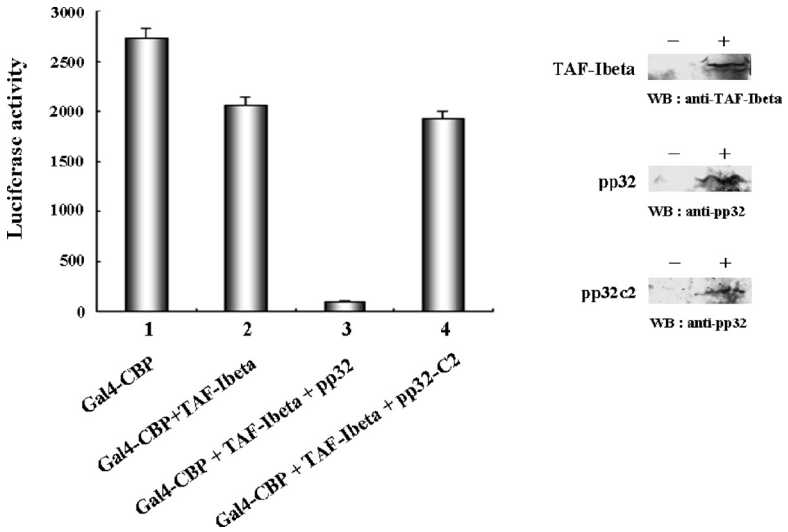

Fig. 4. Acidic C-Terminal Domain of pp32 is Required for the Synergistic Blocking of HAT-Dependent Transcription with TAF-Ibeta

Overexpression of pp32 inhibits CBP-HAT-dependent transcription. HeLa cells were transfected with the reporter pMH100-TK-Luc, CMXGal4-CBP, TAF-Ibeta, pp32 and pp32C2 derivatives as indicated. Following transfection, cells were grown for 48 h, and cell extracts were prepared and assayed for luciferase activity. Immunoblot controls are provided for the expression of TAF-Ibeta, pp32, and pp32-C2 proteins in co-transfected cells (right panel).

Ibeta, we co-transfected deletion mutants of pp32 with sub-optimal amount of TAF-Ibeta construct. As expected, Gal4-DBD fusion of CBP activated transcription (Fig. 4 lane 1). This Gal4-CBP HAT mediated transcription was inhibited marginally when TAF-Ibeta was transfected alone (Fig. 4 lane 2) but severely inhibited with the cotransfection of wild type pp32 (Fig. 4 lane 3). When pp32-C2 was cotransfected, which dose not contain the TAF-Ibeta interaction domain, the level of repression was substantially diminished and no additional transcriptional activity of pp32 was observed (Fig. 4 lane 4). The immunoblot analysis indicates that the expression of transfected proteins (Fig. 4 right panel). These results are consistent with the *in vitro* interaction analysis between TAF-Ibeta and pp32 that showed the importance of the acidic domain of pp32.

Despite of increasing evidences of regulatory roles of INHAT complex subunits, TAF-Ibeta and pp32 in various cellular pathways including transcription and apoptosis, little has been known about the interaction mechanism between the subunits. In this study, we have identified that the interaction between TAF-Ibeta and pp32 is C-terminal acidic domain of pp32 dependent. An understanding of the interaction mechanism between INHAT complex subunits will further elucidate that how INHAT complex subunits might interact and work together in various cellular activities.

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REFERENCES

1) Seo S. B., Macfarlan T., McNamara P., Hong R., Mukai Y., Heo S., Chakravarti D., *J. Biol. Chem.*, **277**, 14005—14010 (2002).
2) Strahl B. D., Allis C. D., *Nature* (London), **403**, 41—45 (2000).
3) Seo S. B., McNamara P., Heo S., Turner A., Lane W. S., Chakravarti D., *Cell*, **104**, 119—130 (2001).
4) Grunstein M., *Nature* (London), **389**, 349—352 (1997).
5) Macfarlan T., Kutney S., Altman B., Montross R., Yu J., Chakravarti D., *J. Biol. Chem.*, **280**, 7346—7358 (2005).
6) Matsumoto K., Nagata K., Ui M., Hanaoka F., *J. Biol. Chem.*, **268**, 10582—10587 (1993).
7) Okuwaki M., Nagata K., *J. Biol. Chem.*, **273**, 34511—34518 (1998).
8) von Lindern M., van Baal S., Wiegant J., Raap A., Hagemeijer A., Grosveld G., *Mol. Cell Biol.*, **12**, 3346—3355 (1991).
9) Ishimi Y., Hirosumi J., Sato W., Sugasawa K., Yokota S., Hanaoka F., Yamada M., *Eur. J. Biochem.*, **142**, 431—439 (1984).
10) Chen T. H., Brody J. R., Romantsev F. E., Yu J. G., Kayler A. E., Voneiff E., Kuhajda F. P., Pasternack G. R., *Mol. Biol. Cell*, **7**, 2045—2056 (1996).
11) Matsuoka K., Taoka M., Satozawa N., Nakayama H., Ichimura T., Takahashi N., Yamakuni T., Song S. Y., Isobe T., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 9670—9674 (1994).
12) Matilla A., Koshy B. T., Cummings C. J., Isobe T., Orr H. T., Joghbi H. Y., *Nature* (London), **389**, 974—978 (1997).
13) Brody J. R., Kadkol S. S., Mahmoud M. A., Rebel J. M., Pasternack G. R., *J. Biol. Chem.*, **274**, 20053—20055 (1999).
14) Fan Z., Beresford P. J., Oh D. Y., Zhang D., Lieberman J., *Cell*, **112**, 659—672 (2003).
15) Jiang X., Kim H. E., Shu H., Zhao Y., Zhang H., Kofron J., Donnelly J., Burns D., Ng S. C., Rosenberg S., Wang X., *Science*, **299**, 223—226 (2003).
16) Hiyaji-Yamaguchi M., Okuwaki M., Nagata K., *J. Mol. Biol.*, **290**, 547—557 (1999).