

EFFECT OF HIRA PROTEIN ON TRANSCRIPTIONAL REGULATIONS OF GENES IN VERTEBRATE CELLS

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ABSTRACT

For better understanding of DNA replicating-coupled chromatin assembly and transcription regulation in eukaryotes, we studied biochemical and genetic analysis of nuclear-related proteins from chicken DT40 cell lines. The genetic analysis of some nuclear proteins, such as HIRA and CAF-1, indicated that these proteins could play overlapping roles in chromatin dynamics and is consistent with the finding that HIRA protein exhibited binding ability to histones and ASF-1, as also ASF-1 bound directly with CAF-1p60. In this study, revealed not only that the N-terminal and C-terminal halves of HIRA mediate individually transcription repressions but also that even one of the seven WD dipeptide motifs and the LXXLL motif of HIRA are required for these mediations *in vivo*. Finally, we found that HIRA-mediated repression is sensitive to trichostatin TSA and it co-represses transcription together with HDAC-2. We believe our findings will contribute to a major break-through in future studies on the specific, individual roles of HIRA involved in numerous DNA-utilizing processes, through the formation and/or maintenance of the chromatin structure in vertebrate cells.

Keywords: histone regulator, transcriptional repression, trichostatin, chromatin

INTRODUCTION

Mammalian HIRA has been named for its homology to Hir1p and Hir2p (Hir is an acronym for histone regulator), two *Saccharomyces cerevisiae* transcriptional corepressors that strongly repress the transcription of one copy each of *H2A* and *H2B* histone genes located at the *HTA1-HTB1* locus throughout most of the cell cycle, and transiently recruit a SWI/SNF chromatin remodelling complex, thereby leading to transcription activation at this locus at the *G1/S* transition [1, 2]. HIRA proteins with seven WD repeat domains exhibited binding ability as to histones [3] and ASF-1 [4], and then ASF-1 interacted directly with CAF-1p60 [5, 6]. The HIRA protein family was predicted to form a β -propeller structure, which may be involved in protein-protein interactions, in its N-terminal half most similar to Hir1p and CAF-1p60 [1, 7-9]. The C-terminal half of HIRA similar to Hir2p is responsible for its binding ability as to homeodomains, Hir3p, Pax3 and core histones [3, 10]. Alterations in chromatin structure through the acetylation of core histones have been thought to be of fundamental importance in numerous DNA-utilizing processes [11-16]. The acetylation and deacetylation of core histones are catalyzed by HATs and HDACs, respectively. The members of the HAT and HDAC families play individual particular roles, and then participate in combination with one another in the processes of acetylation and/or deacetylation of core histones for the regulation of numerous cell functions. On the other hand, CAF-1p48 with seven WD repeat motifs, together with the p46 polypeptide, was found to

be associated with transcription repressor complexes of HDAC-1 and 2, and mSin3, Rb, or Mi2 plus MecP2.

Recently, we revealed not only that all seven WD dipeptide motifs of both HIRA and CAF-1p48 are necessary for their *in vitro* interaction but also that both the typical LXXLL motif of HIRA and a C-terminal region of HDAC-2 are necessary for their *in vitro* as well as *in vivo* interactions [17]. In this study, we revealed not only that the N-terminal and C-terminal halves of HIRA mediate individually transcription repressions but also that even one of the seven WD dipeptide motifs and the LXXLL motif of HIRA are required for these mediations *in vivo*. Finally, we also found that HIRA-mediated repression is sensitive to TSA and HIRA co-represses transcription together with HDAC-2.

MATERIALS AND METHODS

Material

The pM, pG5probasinLuc, pRL-CMV plasmids and Dual-Luciferase^R assay system were purchased from Promega. FuGENE 6 transfection reagent were purchased from Roche Molecular Biochemicals. DMEM medium and fetal bovine serum were purchased from GIBCO, Invitrogen Life Technologies.

Plasmids construction

We constructed GAL4 mammalian luciferase reporter plasmids as follows: pCiteHAHIRA and its derivatives [17] was digested with *NdeI* plus *XhoI*,

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followed by treatment with T4 polymerase. Resultant blunt-ended plasmids, the *NdeI/XhoI* blunt-ended fragments containing the full-length HIRA and its derivative cDNAs were excised and subcloned into the *SmaI* site of the pM vector to constructed pMHIRA, pMHIRA-(1-443), pMHIRA-(444-1019), pMHIRA-(WT7AA), pMHIRA-(L993A), pMHIRA-(WT7AA, L993A), pMHIRA-(1-443, WT7AA) and pMHIRA-(444-1019, L993A). Each end point of the deletions and point mutation plasmids was determined by sequence analysis (ABI 373A Sequencer DNA from Applied Biosystems Inc.) by the dye terminator method.

Cell culture

Adherent cell cultures of HeLa were maintained in DMEM supplemented with 10% fetal bovine serum (GIBCO, Invitrogen Life Technologies) and grown at 37°C in 5% CO₂ in air in a humidified incubator.

Luciferase assays

HeLa cells (2 X 10⁴) were cultured in 24-well dishes for 24 hr until 60-70% confluence. HeLa cells (60-70% confluence, 24-well plate) were cotransfected with 1.6 µg of pM or pMHIRA and its derivatives constructs, 0.4 µg of pG5probasinLuc, and 10 ng of pRL-CMV as internal control in 500 µl of DMEM containing 10% fetal bovine serum by lipofection with FuGENE 6 transfection reagent (Roche Molecular Biochemicals). After 24 hr, the medium was replaced, and cells were harvested and assayed for luciferase activity 36-48 hr after transfection. Luciferase assays were performed with the Promega Dual-Luciferase^R Assay System according to the manufacturers instructions. The reaction tube was discarded and proceeded to the next samples assay. Each transfection was performed in duplicate and repeated at least three times. The relative luciferase activity was normalized by dividing the measurement for the *firefly* luciferase activity with that for the *renilla* luciferase activity.

RESULT AND DISCUSSION

We previously reported that all the WD dipeptide motifs in the N-terminal half and the LXXLL motif in the C-terminal half of HIRA were essential for its *in vitro* and *in vivo* interactions with CAF-1p48 and HDAC-2, respectively [17]. On the other hand, HIRA has been shown to repress transcription of histones H1, H2A, H2B, H3 and H4 [18]. To determine whether or not HIRA possesses repressive potentiality, and if so, to determine whether or not the WD motifs and/or the LXXLL motif are responsible for the transcription repression *in vivo*, HIRA and its derivatives were fused to the DNA-binding domain of GAL4, and then repressor

activity of the fusion proteins was assayed in HeLa cells. A reporter plasmid, containing 5 copies of the GAL4 binding site (5X GAL4 probasinLuc), the cloned upstream of the SV40 promoter and the direction of expression of luciferase, was used to monitor the activity of GAL4-HIRA fusion proteins. Fold repression activity was determined as a luciferase activity relative to the basal transcription activity of the GAL4 (BD) reporter alone as a control.

HIRA, its N-terminal and C-terminal halves, and their single and/or double missense mutant derivats as to WD dipeptide and/or LXXLL motifs were fused to GAL4, and transiently expressed in HeLa cells, and then repressor activities were assayed as by luciferase activity. Figure 1 showed not only that GAL4-HIRA could repress the promoter activity to about 40% that of the control BD protein (lanes 2 and 1) but also that GAL4-HIRA-(1-443) and GAL4-HIRA-(444-1019) exhibited certainly repression activity, i.e. their repression activities were about 50% and 75%, respectively (lanes 3 and 4). To address whether or not the WD dipeptide motifs in N-terminal half and the LXXLL motif in C-terminal half of HIRA were responsible for these repressions, we co-transfected the high basal reporter plasmid 5X GAL4 probasinLuc along with GAL4-HIRA point mutations in the WD dipeptide and LXXLL motifs. The repression activity of the double point mutation (WT7AA) of the WD repeat motif was about 75% (lane 5), similar to that of GAL4-HIRA-(444-1019), indicating that this activity was due to the C-terminal half of HIRA.

Similarly, the repression activity of the single point mutation (L993A) of the LXXLL motif was about 50% (lane 6), similar to that of GAL4-HIRA-(1-443), demonstrating that this activity was due to the N-terminal half of HIRA. As expected, the simultaneous mutations in the WD dipeptide and LXXLL motifs, GAL4-HIRA-(WT7AA, L993A), exhibited no repression activity (lane 7). To confirm these findings, we introduced two mutations in the N-terminal and C-terminal halves of HIRA, and assayed their repression activity (lanes 8 and 9). Both of GAL4-HIRA-(1-443, WT7AA) and GAL4-HIRA-(444-1019, L993A) lost the transcription repression, indicating that the WD dipeptide and LXXLL motifs were individually important for the transcription repressing activity of HIRA *in vivo*. Thus, HIRA should participate at least in two distinguishable manners of transcription repressions through the corresponding interactions of its N-terminal and C-terminal halves with different proteins, i.e. CAF-1p48 and HDAC-2. For instance, the N-terminal half of HIRA may bind directly or indirectly with other transcription repression factor(s) through the interaction with CAF-1p48, without recruiting HDAC proteins [19], resulting in reducing of promoter activity, since CAF-1p48 was contained in transcription repression complexes with mSin3, Rb, or

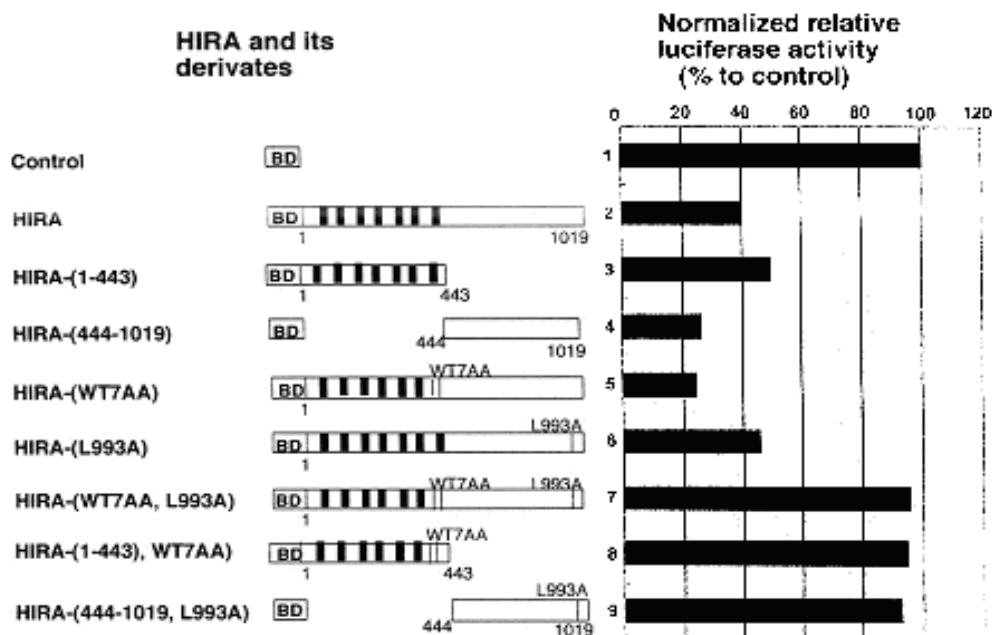


Figure 1. Necessity of WD dipeptide and LXXLL motifs in N-terminal and C-terminal halves of HIRA for the mediation of transcription repression *in vivo*.

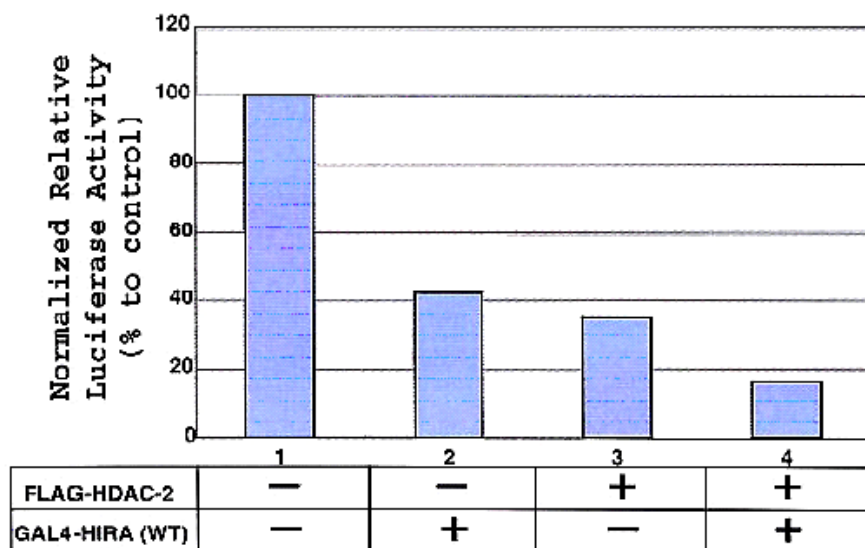


Figure 2. HIRA protein requires the HDAC-2 enzyme activity for transcriptional repression.

Mi2 plus MecP2 [20]. Further studies are necessary to resolve this repression mechanism.

Figure 1 showed not only that the N-terminal and C-terminal halves of HIRA mediate individually transcription repressions but also that even one of the seven WD dipeptide motifs and the LXXLL motif of HIRA are required for these mediations *in vivo*. On the other hand, HDAC activity has been shown to repress transcription [21]. Since HIRA associates with histone deacetylase activity and binds with HDAC-2 via its LXXLL motif *in vivo* [19], we evaluated whether HIRA

and HDAC-2 may act together to silence transcription. To this end, we performed transient transfections in HeLa cells using the reporter plasmid 5XGAL4-probassin-Luc reporter, with or without GAL4-HIRA and/or FLAG-HDAC-2. The basal activity of the reporter is normalized to a value of 100%. As illustrated in Figure 2, expression of a limiting amount of GAL4-HIRA (lane 2) or FLAG-HDAC-2 (lane 3) slightly repressed transcription. In contrast, co-transfection of GAL4-HIRA along with FLAG-HDAC-2 provided a

synergistic repressive effect on transcription (Figure 2, lane 4).

We next asked whether the repression mediated by GAL4-HIRA could be relieved by treatment with the specific HDAC inhibitor TSA. HeLa cells were transfected as in Figure 2. Cells were treated (lanes 2 and 4) or not (lanes 1 and 3) with the HDAC inhibitor TSA (0.4 μ M) harvested after 16 hr, and then assayed

for luciferase activity. The basal activity of the reporter is normalized to a value of 100%. As depicted in Figure 3, the repressive effect observed with GAL4-HIRA on reporter activity was relieved by the addition of TSA. Collectively, these results indicate that HIRA contains a transcriptional repressor domain, encompassing the LXXLL motif, and requires the HDAC-2 activity to exert this repressive effect.

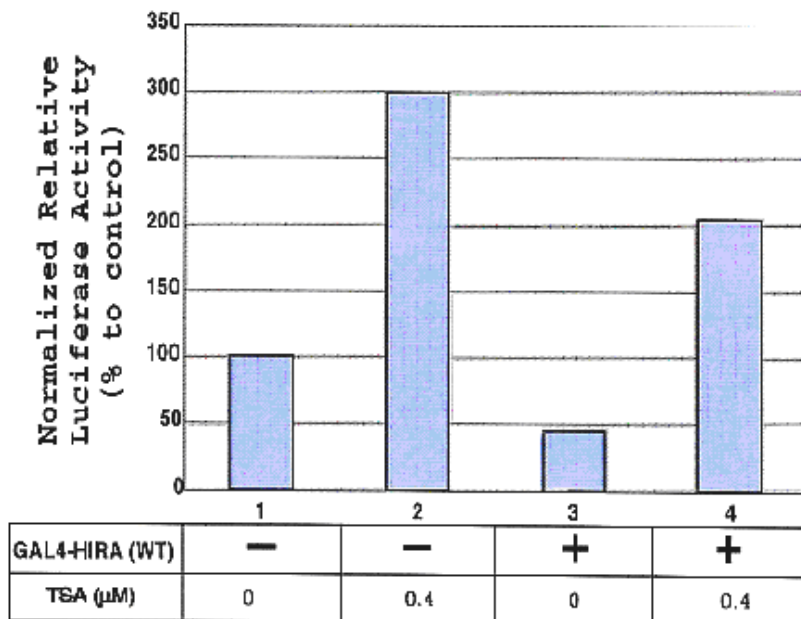


Figure 3. HIRA-mediated transcriptional repression is sensitive to histone deacetylases inhibitor TSA.

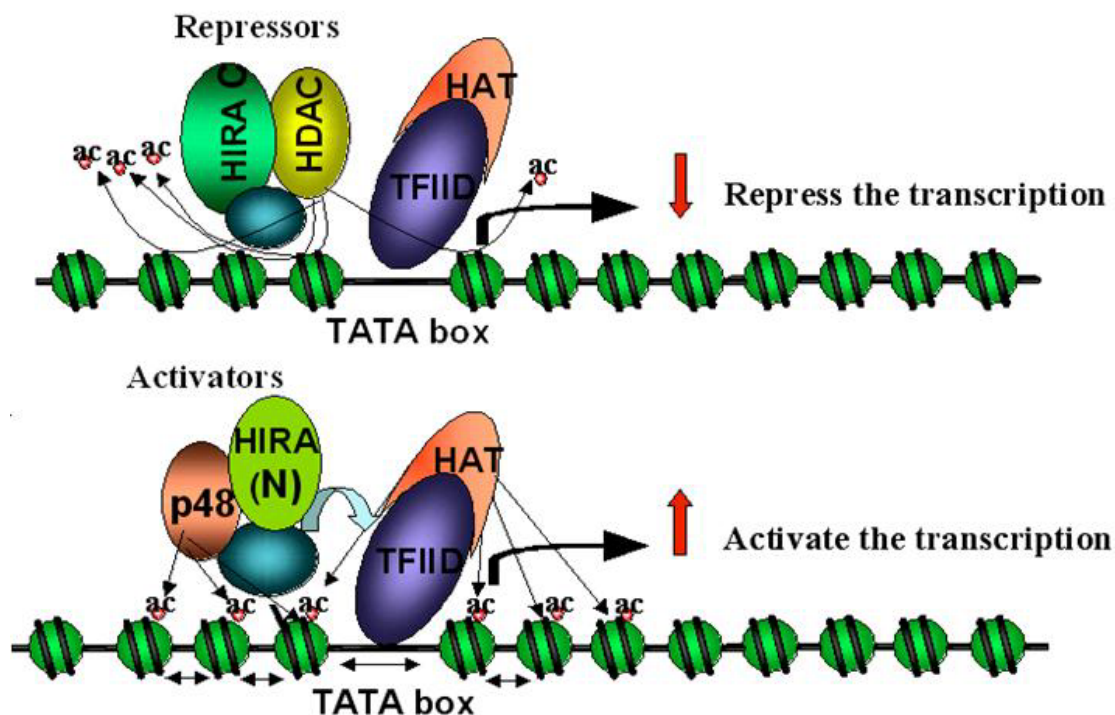


Figure 4. Model of a key role of HIRA protein on gene transcriptional regulations in vertebrate cells. The proteins used are denoted by appropriate abbreviations, i.e. HAT is the abbreviation for histone acetyltransferase.

As illustrated in Figure 4, if the C-terminal of HIRA bound to HDAC caused deacetylation of chromatin induces on close conformation that allows the down regulation of transcription and expression of gene activity. Oppositely, if N-terminal region of HIRA bound to p48 subunit caused acetylation of chromatin induces an open conformation that allows the transcription machinery access to promoter and up-regulation of transcription and expression of gene activity. Thus, these HIRA-participating interactions with CAF-1p48 and HDAC-2 (and probably other HIRA-carrying complexes) should be involved directly or indirectly in chromatin assembly or maintenance and alterations of chromatin structure, involving gene transcription. Our preliminary analysis of the HIRA-deficient DT40 mutant obtained showed not only that the HIRA-deficiency caused delayed cell growth, but also that the N-terminal and C-terminal halves of HIRA were oppositely involved in transcription regulation of various cell cycle-related genes [22]. Analysis of the HIRA-deficient and AFS1 conditional DT40 mutant [23], together with the CAF-1-deficient conditional mutant cells [24], will be of powerful tools for understanding the role of HIRA and the other histones chaperon in relation to DNA replication and transcription regulations in vertebrate cells.

CONCLUSION

In this study, revealed not only the WD repeat motif in N-terminal half and the LXXLL motif in C-terminal half of HIRA mediate transcription repression *in vivo*, but also that HIRA-mediated repression is sensitive to TSA and it co-represses transcription together with HDAC-2. We believe our findings will contribute to a major break-through in future studies on the specific, individual roles of HIRA, as well as CAF-1 involved in numerous DNA-utilizing processes, through the formation and/or maintenance of the chromatin structure in higher eukaryotes, including vertebrate cells.

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