Histone Deacetylase-1 Represses Transcription by Interacting with Zinc-Fingers and Interfering with the DNA Binding Activity of Sp1

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Abstract
Sp1 activates the transcription of many cellular and viral genes, and histone deacetylase 1 (HDAC1) removes the acetyl group of nucleosomal core histones. Treatment of cells with the histone deacetylase 1 inhibitor, TSA, robustly activates the transcription of the Sp1-dependent promoters, suggesting the inhibition of Sp1 activity which is critical in the activation of transcription, by HDAC1. We assessed the protein-protein interactions occurring between Sp1 and HDAC1, and the transcriptional regulatory mechanism controlled by this interaction. In vitro GST fusion pull down assays, co-immunoprecipitation, and mammalian two-hybrid assays revealed that the HDAC1 noncatalytic domain (a.a. 237-482) interacts directly with the zinc-finger DNA binding domain of Sp1. DNase I footprinting revealed that this interaction prevents the binding of Sp1 zinc-fingers to the target GC-box. Gal4-HDAC1 fusion, targeted proximally to the GC-boxes, potently repressed the transcription of pG5-5x(GC)-Luc, in which Sp1 potently activates transcription. This repression of transcription does not involve the deacetylase activity of HDAC1, and is accomplished by the direct protein-protein interactions which occur between the Sp1 zinc-finger DNA binding domain and HDAC1, which interferes with the promoter GC-box binding of Sp1.

Introduction
Histone deacetylase (HDAC) catalyzes the removal of the acetyl group from lysine residues in the N-terminal tails of nucleosomal core histones. HDAC has been implicated in the repression of gene expression via the facilitation of chromatin condensation [1-4]. HDAC homologs have been discovered in a wide variety of eukaryotes, and each organism typically includes multiple members of this family. Eleven human HDACs have been identified thus far, and have been distinguished into 2 different classes [5]. The most clearly and thoroughly described class I histone deacetylases are members of a common family which includes the founding member in humans, HDAC1 [1], and yeast Rpd3 [6]. Histone deacetylases (HDACs) play a key role in the regulation
of gene transcription, and may be involved in cell-cycle regulation, differentiation, and development, as well as human cancer [7-9].

The most striking property exhibited by the HDACs is their ability to repress transcription via their deacetylase activity [1, 2, 6]. This process likely underlies many biological processes controlled by HDACs. However, the fact that HDACs can catalyze enzymatic reactions on histone substrates in vitro does not necessarily indicate that histones are physiologically relevant only to substrates in vivo. Recent studies suggest, in fact, that their substrates or interacting partners form a quite diverse group, a group which is growing rapidly. HDACs tend to be found in large multiprotein complexes, which include the mSin3A corepressor [10-12] and are also associated with transcriptional corepressors for nuclear receptors including SMRT [13], NCoR [2, 12, 14], and BCoR [15, 16]. Also, these HDAC complexes associate with DNA-binding repressors including Mad [10, 17], Ume6 [18], YY1 [19], PLZF [20], retinoblastoma protein (Rb) [21], TG-interacting factor (TGIF) [22], and Smad [23]. Chromatin compaction by the HDAC complex when recruited by corepressors or transcription factors has been suggested to repress transcription [10-20]. Interestingly, transcription activator Sp1 factor has also been shown to interact with HDAC1 [7].

Specificity protein 1 (Sp1) is a sequence-specific transcription factor which binds to the GC-box, and activates a host of viral and cellular genes [24, 25]. Sp1 belongs to the Krüppel-like C2H2-type zinc-finger superfamily [26]. This protein is composed of several modules, including an N-terminus inhibitory domain (a.a. 1-82), serine/threonine rich domains (a.a. 87-143; a.a. 243-350), glutamine rich domains (a.a. 351-500), a zinc–finger DNA binding domain (a.a. 622-720), and a C-terminus D-domain (a.a. 721-788) [24-26]. As almost all genes contain the Sp1 binding GC-box in their promoters, and because Sp1 often plays a critical role in the activation of many genes’ transcription, there must be a certain mechanism which regulates the activity of Sp1. Indeed, Murata et al. have suggested that Sp1 activity might be negatively regulated by cellular proteins which interact with the amino terminus of Sp1 [27]. The zinc-finger of Sp1 has been shown to interact with HDAC1 and protein kinase C ζ and Sp1 activity is regulated by this interaction [7, 28]. Also, p300 and FBL-I have been demonstrated to regulate Sp1 activity via interaction with the zinc-finger DNA binding domain [29, 30]. However, the molecular and biochemical nature of interaction between Sp1 and histone deacetylase 1 (HDAC1) or protein kinase C ζ remains largely unknown [7, 28].

Recently, we and others discovered that the treatment of mammalian cells with histone deacetylase inhibitor resulted in drastic increases in the transcription of a variety of genes, particularly the genes which contain the Sp1 binding site in their proximal promoters, as in human ADH5/FDH, p21waf/cip1, and the pG5-5x(GC)-Luc promoter (Fig.1) [31, 32]. However, the mutagenesis of Sp1 binding sites or deletion of these sites resulted not only in large overall decreases in transcription, but also insensitivity to histone deacetylase inhibitors, including TSA and apicidine, which suggested that the Sp1 activity critical to transcription might be regulated by HDACs [31, 32, 40].

In this paper, we investigated and discovered a novel molecular mechanism underlying the repression of Sp1 transcription by HDAC1 on the human ADH5/FDH promoter and pG5-5x(GC)-Luc. The HDAC1 noncatalytic domain (a.a. 287-482) interacts with the Sp1 zinc-finger DNA binding domain, and represses transcription by interfering with Sp1’s ability to recognize the GC-box.

**Materials and Methods**

*Plasmids, recombinant proteins, antibodies, and chemicals*

pCAT-ADH5/FDHWt and pCAT-ADH5/DFDHt reporter plasmids were reported elsewhere [33]. The pG5-Luc plasmid was purchased from Promega (WI). The pG5-5x(GC)-Luc plasmid was prepared by subcloning 5 copies of Sp1 consensus sequence (sense, 5’-CACTCTATCAGGCGGGCCGGCCGGCGG, antisense, 5’-CTAGCCCTCCCGCCCAGATCGAG) into the pG5-Luc plasmid/Nhel (Promega, WI). The GST-fusions of various proteins were constructed by cloning cDNAs into the pGEX4T series vectors (Pharmacia, NJ), and were tagged with FLAG at the C-terminal, in the case of various Sp1-derived polypeptides. The a.a. 1-621 polypeptide, serine/threonine rich region (a.a. 244-350), Q-rich region (a.a. 351-500), and zinc-fingers of Sp1 (a.a. 622-720, or a.a. 622-788) were expressed in the GST-fusions. The His-tagged HDAC1 (a.a. 237-482) was produced by cloning the cDNA sequence into pQE30 (Qiagen, Germany), and via induction with IPTG. For mammalian two hybrid-assays, pGal4-Sp1ZFDBD and pVP16-HDAC1 (a.a. 237-482; a.a. 326-482) plasmids were prepared by subcloning Sp1ZFDBD or HDAC1 cDNAs into pGal4 or pVP16 fusion protein expression vectors. All the plasmid constructs were verified by sequencing. Sp1 was purchased from Promega (WI). Antibodies against His tag, HDAC1 and Sp1 were purchased from Qiagen (Germany) and Upstate Biotechnology (VA). Rabbit IgG was purchased from LabFrontier (Seoul, Korea).
Protein-protein interaction assays

When conducting the Sp1-HDAC1 interaction studies, three GST-Sp1 fragment polypeptides (2 µg each of a.a. 622-788, 622-720, 695-788 polypeptides) were incubated with His-tagged HDAC1 polypeptide (a.a. 237-482) (10 µg) in binding buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.1% NP-40, 10% glycerol, 2 mM EDTA, 0.5% skim milk, 5 mM DTT, protease inhibitor cocktail) for 6 hrs at 4°C. After extensive washing, the bound proteins were resolved by 10% or 8% SDS-PAGE, and were visualized via Western blot analysis using anti-His antibody (Qiagen, Germany) and an ECL detection kit (Amersham, CA).

To demonstrate the interaction between Sp1 and HDAC1 in vivo, HeLa nuclear extract (100 µg) were incubated with rabbit polyclonal anti-Sp1 antibody or IgG for 12 hrs, and precipitated after incubating further with protein A agarose (Sigma, MO). The precipitants were separated with 10% SDS-PAGE and analyzed by Western blot analysis using rabbit polyclonal anti-HDAC1 antibody (Upstate Biotechnology, VA) and ECL detection kit (Amersham, CA).

For mammalian two-hybrid assays, a mixture of the reporter pG5-Luc (0.2 µg, Promega), the Sp1ZFDBD expression plasmid (0.2 µg, pGal4-Sp1ZFDBD) the HDAC1 (a.a. 237-482 or 326-482) expression plasmid (0.6 µg, pVP16-HDAC1), and the control pCMV-β-galactosidase plasmid (0.2 µg) were cotransfected into the CV-1 cells. The cells were then cultured for 40 hrs. The cell extracts were assayed for reporter luciferase activity and normalized with β-galactosidase activity of the cell extracts. Data are presented as relative luciferase activity as compared with the control, and represent the average of 4 independent assays.

Transient expression assays

African green monkey kidney cells (CV-1) were cultured on 6-well dishes in Dulbecco’s MEM supplemented with 5% FBS (GIBCO-BRL, MD). At 50-60% confluency, the cells were transfected with a mixture of 1.0 µg of pCAT-ADH5/FDH Wt or ADH5/FDH M1 (pG5-Luc or pG5-5x(GC)-Luc) and 0.2 µg of pCMV-β-galactosidase plasmid using Lipofectamine plus (GIBCO-BRL, MD), as was reported previously [33]. Mutations at the Sp1 binding sites (-22 to +2 bp and +1 to +22 bp) were introduced with a site-directed mutagenesis kit (Strategene, CA) [33]. The cells were then cultured for 40 hrs. When necessary, the cells were treated with Trichostatin A (3 µl, 1000x, 0.1 mg/ml in DMSO) for 24 hrs prior to harvesting. The cell extracts representing the same quantities of protein were then assayed for reporter CAT or luciferase activity. Data are presented as the relative luciferase activity compared to the control, and represent the average of 4 independent assays.

In order to determine the degree to which Sp1 activity was inhibited by the Gal4-HDAC1 fusion, a mixture of the reporter plasmids, pG5-Luc or pG5-5x(GC)-Luc (0.3 µg, Promega, WI), Gal4 HDAC1 fusion expression vector (0.3 µg, pGal4-HDAC1 (a.a. 237-482), and the pCMV-β-galactosidase control vector (0.1 µg) were cotransfected into the CV-1 cells, and were assayed as described above. Cell extracts were assayed for reporter luciferase activity and normalized with cotransfected β-galactosidase activity. Data are presented as the relative luciferase activity compared to the control, and represent the average of 3 independent assays.
DNAse I footprinting assays of ADH5/FDH promoter

For DNase I footprinting, Sp1 (0.25 fpu, Promega, WI) was preincubated for 15 min. at room temperature with bovine serum albumin (BSA), or with the recombinant partial or full length HDAC1 (300 ng) before the addition of the 32p-α-[dCTP] labeled ADH5/FDH promoter probe [33]. A preincubated mixture containing Sp1 and the probe were mixed together and incubated further for 15 min. at room temperature. The mixtures were digested with DNase I and then stored overnight at -70 °C with a MS image-enhancing screen (Kodak, CT).

Results

Transcription activation by Trichostatin A requires Sp1 binding GC-box

HDAC1 was suggested to repress the transcription of S-phase specific promoters via interaction with Sp1 [7]. Although the Sp1 activity which is critical to transcription activation was suspected to be modulated by HDAC1, the nature of this repression remained unknown. We attempted to ascertain whether Sp1 was directly involved in the HDAC-mediated transcriptional repression, and whether the HDAC-specific inhibitor TSA might reverse the repression. First, we searched for the promoter in which Sp1 plays a major role in transcription. We selected the ADH5/FDH promoter, and the pG5-5x(GC)-Luc construct. We then introduced mutations at the two GC boxes of the ADH5/FDH promoter, both of which were critical with regard to the activation of the Sp1-induced transcription of the gene [33]. We also chose pG5-Luc and pG5-5x(GC)-Luc, which differ only with respect to Sp1-binding GC-boxes (Fig. 1A). The natural or mutant forms of ADH5/FDH-CAT fusion gene plasmid, pG5-Luc, and pG5-5x(GC)-Luc, were introduced into CV-1 cells and cultured in either the presence or absence of the HDAC-specific inhibitor TSA, and then analyzed in regard to reporter gene expression. When the transfected cells were treated with TSA, we observed a 4 fold increase in reporter expression (Fig. 1B, D). TSA alleviated the strong HDAC-induced repression of the ADH5/FDH minimal promoter. Mutations in the two Sp1 binding sites lowered transcription levels to 25% of those of the wild type promoter [28], and abolished Trichostatin A responsiveness (Fig. 1B).

We observed similar results with the pG5-5x(GC)-Luc fusion gene plasmid. The absence of the Sp1-binding GC-box in pG5-Luc showed neither strong transcription nor derepression as a result of TSA treatment. However, the presence of the Sp1-binding GC-box in pG5-5x(GC)-Luc resulted not only in the strong activation of transcription, but also derepression upon TSA treatment, and a 4-fold increase in transcription activation. This indicates that HDAC-induced transcription repression requires an intact proximal promoter GC-box, and the repression of transcription probably involves the action of HDAC directly on Sp1, perhaps by the inhibition of GC-box binding (Fig. 1).

HDAC1 interacts with the Sp1 zinc-finger DNA binding domain

The above data suggest that Sp1 and HDAC1 may interact directly to repress the activation of Sp1 transcription. Therefore, we evaluated the protein-protein interactions between Sp1 and HDAC1 by GST-fusion protein pull down and Western blot assays. Four different Sp1 polypeptides (a.a.1-622, S/T rich region, Q-rich region, zinc-finger + D region, zinc-finger, D-domain) covering the entirety of Sp1 were tested, in order to identify which part of Sp1 was interacting with HDAC1. Neither the S/T rich domain, the Q-rich region, nor the D-domain of Sp1 were found to be involved in this interaction with the HDAC1 (data not shown). However, the Sp1ZFDBD (a.a. 622-788) interacted with the noncatalytic HDAC1 C-terminus domain (a.a. 237-482). The interacting portion of Sp1 was narrowed down more precisely, to the zinc-finger DNA binding domain (a.a. 622-720) (Fig. 2A, C).

Furthermore, we tested whether Sp1 and HDAC1 interact in vivo by co-immunoprecipitation of HeLa nuclear extract. Western blot analysis of the complex precipitated by Sp1-protein A agarose showed that the complex clearly contained HDAC1, suggesting that Sp1 and HDAC1 interact in vivo (Fig. 2B).

We also investigated the in vivo molecular interactions between HDAC1 and Sp1ZFDBD by mammalian two-hybrid assays. Once VP16-HDAC1 (a.a. 237-482 or 327-482) fusions were targeted to the proximal promoter by interacting with Gal4-Sp1ZFDBD, the transcription of the reporter gene was reduced by approximately 65% or 36% of the control (Fig 2C, lanes 2, 3). In many cases of mammalian two-hybrid assays, VP16 polypeptide brought close to proximal promoter by bait-prey interaction activates transcription, but in our case, the interaction resulted in the transcription repression. We were puzzled by this, but later determined that Sp1ZFDBD interacts vigorously with corepressor proteins, including mSin3A, SMRT/NCoR, and BCoR (Lee et al. in preparation). Also, HDAC1 interacts with corepressor proteins such as mSin3A and is an important component.
Fig. 2. HDAC1 interacts with the Sp1 zinc-finger DNA binding domain. (A) GST fusion protein pull-down assays of various GST-Sp1 fusion polypeptides with His-tagged HDAC1 (a.a. 326-481). POZ-domains interact with Sp1, in particular, via the C-terminus Sp1 polypeptide (a.a. 622-788). Three zinc-fingers of Sp1 (a.a. 622-720), but not the D-domain (a.a. 695-788) interact with His-HDAC1 (a.a. 326-481). (B) Co-immunoprecipitation of HeLa nuclear extract with antibody against Sp1. HeLa nuclear extract was incubated with anti-Sp1 antibody and subsequently with protein A agarose. The mixture was precipitated. The precipitants were washed, separated by SDS-PAGE, and analyzed by Western blot using anti-HDAC1 antibody. (C) Mammalian two-hybrid assays in CV-1 cells. CV-1 cells were transfected with expression plasmid bait, Gal4-Sp1ZFDBD, and effector plasmids VP16, VP16-HDAC1 (a.a. 237-482), and pCMV-β-Gal, were cultured for 40 hrs and analyzed for reporter activity. Luciferase activities were normalized with β-galactosidase activity, and represent the average of 4 independent assays. Bars represent standard deviations.

Fig. 3. HDAC1 polypeptide (a.a. 237-482) potently inhibits Sp1 binding to the GC-boxes. DNase I footprinting assays of the ADH5/FDH promoter. Sp1 (0.25 fpu) was preincubated for 15 min. at room temperature, with either BSA or the effectors HDAC1 polypeptides before mixing with the 32P labeled footprinting ADH5/FDH probe. The probe and Sp1 mixture were incubated for an additional 15 min. and subjected to DNase I digestion. Pre-incubation with BSA did not affect the Sp1 binding activity at the expected GC-box sites (lanes 3 vs. 4, 5). However, 15 min. of preincubation of Sp1 with the effectors (HDAC1 full length, HDAC1 a.a. 237-482) abolished Sp1 recognition and binding to its target GC-boxes (shaded ovals) (lanes 3-5 vs. 6, 7). The Sp1 recognition GC-boxes are marked with shaded ovals. An arrow indicates the transcription start site.

Sp1ZFDBD and HDAC1 represses transcription. The abilities of the Sp1ZFDBD and HDAC1 to interact with corepressors or HDAC complex might overcome, in this particular assay context, VP16’s potential to activate transcription and repress transcription. All these data, both in vitro and in vivo, point to the conclusion that the Sp1ZFDBD interacts with the C-terminus domain of HDAC1 (Fig. 2B).
HDAC1 binds to the zinc fingers DNA binding domain of Sp1, and interferes with Sp1 GC-box recognition on the ADH5/FDH promoter.

Our data suggest that HDAC1 represses transcription via direct action on Sp1 (Fig. 1), and that this repression of transcription may be accomplished by a mechanism involving direct molecular interactions between the Sp1 zinc-finger DNA binding domain and the C-terminal portion of HDAC1 (Fig. 2). We suspected that HDAC1 probably affects the Sp1 activity which is absolutely critical with regard to the activation of transcription, i.e. GC-box recognition. We attempted to characterize the molecular interactions occurring between the Sp1 zinc-finger and HDAC1 on the human ADH5/FDH promoter by DNase I footprinting assays. Sp1 binds to the bp -22 to +22 region flanking the transcription start point (Fig. 3). The addition of recombinant full length HDAC1 (a.a. 1-482) or partial HDAC1 polypeptide (a.a. 327-482) and a 32P-labeled footprinting probe to the Sp1 preincubated with BSA had no effect on the binding of Sp1 to the probe (Fig. 3, lanes 4, 5). In contrast, the preincubation of Sp1 with HDAC1 polypeptides for 15 min. interferes with Sp1 binding to the GC-boxes of the probes (Fig. 3, lanes 6, 7). These data regarding the protein-protein interaction indicated that HDAC1 represses transcription via interaction with the zinc-finger DNA binding domain of Sp1, and also by interfering with the recognition of the GC-boxes by Sp1.

HDAC1 represses the activation of transcription by Sp1 in vivo

We also attempted to determine whether the activation of transcription by Sp1 could be repressed by the HDAC1 polypeptide (a.a. 237-482) targeted...
proximally to the Sp1 binding GC-boxes in vivo by transient transfection assay in CV-1 cells. We constructed the pG5-5x(GC)-Luc reporter plasmid, which harbors 5 copies of the Sp1-binding GC-box, placed between the Gal4-binding UAS and the AdML minimal promoter (Fig. 4A). Presence of the GC-boxes potently activated transcription by endogenous Sp1 by more than 95-fold, suggesting the major role of Sp1 in the transcriptional activation of the reporter plasmid, as in the ADH5/FDH and p21Waf/Cip1 promoters (Fig. 4B, compare lanes 1 vs. 2). The Gal4-HDAC1 (a.a. 237-482) fusion protein, targeted to the proximal promoter of pG5-5x(GC)-Luc, represses transcription by more than 85%.

The three lines of data (Figs. 2-4) suggested that HDAC1 represses the transcription of human ADH5/FDH, p21Waf/Cip1, and the pG5-5x(GC)-Luc promoter, via interactions with the Sp1ZFDBD, as well as interference with the recognition of the GC-box by Sp1.

Discussion

The transcription of a eukaryotic gene is governed by the combined action of multiple sequence-specific transcription factors, histone modifiers (histone acetyltransferase, histone deacetylase), cofactors, and mediators which regulate the activities of the transcription factors, as well as the chromatin structure [34-36].

In particular, the histone modifying proteins (HAT or HDAC) which are either associated with or are integral parts of the cofactor complex, are brought close to the target promoter via interaction with the transcription factor acting on the proximal promoter. They alter the acetylation status of the nearby nucleosomes, or modulate the activities of the general or sequence-specific transcription factors they encounter [2, 10, 13, 18, 21, 23]. Recent studies have suggested that histones are not the only substances which HDAC uses as substrates or interaction partners. HDAC1 and Rpd3 are found in large multiprotein complexes, which include the Sin3 corepressor, and are also associated with transcriptional corepressors for nuclear receptors, including SMRT [13] and NCoR [12, 14]. These HDAC/Rpd3 complexes also associate with DNA-binding repressors, such as Mad [10], Ume6 [18], YY1 [19, 37], and the transcription activator Sp1 [7].

Our study of HDAC1 and Sp1 revealed that the transcriptional repression associated with the ADH5/FDH promoter involves direct molecular interactions between the Sp1 zinc-finger DNA binding domains and the noncatalytic domain of HDAC1 (a.a. 327-482). This interaction interferes with the recognition of GC-boxes by Sp1 in vitro. Also, transient transfection studies with pG5-5x(GC)-Luc plasmid indicated that HDAC1 represses transcription by interference with the activation of transcription by Sp1 in vivo. Once HDAC is brought close to the proximal promoter by the Gal4DBD portion, HDAC1 represses transcription via interaction with the zinc-finger of Sp1 incoming to the nearby GC-boxes.

Sp1 binds to GC-boxes and activates a variety of viral and cellular genes [26, 38, 39]. The molecular mechanism elucidated here shows one of the ways in which the activity of the constitutive transcription activator, Sp1, can be regulated. Although other functional domains of Sp1 may be important with regard to the regulation of Sp1 activity, the zinc-finger DNA binding domain appears to be the key target in the regulation of Sp1 activity, as is evidenced by observations of protein-protein interactions involving POZ-domains, HDAC1, and p300 [7, 29, 30]. The molecular mechanisms elucidated here indicate that the zinc-finger DNA binding domain of the Sp-family, a module previously known to function only in binding to the promoter regulatory GC-box element, has the ability to harness diverse cellular regulatory information via protein-protein interaction, and also regulates biological processes by controlling transcription.

We also found that HDAC1 interacts with other zinc fingers in Sp3, Sp4, and FBI-1 (members of the Krüppel-like C2H-type zinc finger family) (data not shown). Taken together, it would appear that HDAC1’s repression mechanism is likely widespread, given the large number of transcription factors with Krüppel-like C2H-type zinc-fingers.

Abbreviations

Sp1 (specificity protein 1); HDAC1 (histone deacetylase 1); TSA (Trichostatin A); ADH5/FDH (alcohol dehydrogenase 5/formaldehyde dehydrogenase); Sp1ZFDBD (Sp1 zinc-finger DNA binding domain).

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