Sp3, but not Sp1, Mediates the Transcriptional Activation of the p21/WAF1/Cip1 Gene Promoter by Histone Deacetylase Inhibitor

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Abstract

We previously reported that both sodium butyrate and trichostatin A (TSA), both of which are known as inhibitors of histone deacetylation, arrest human tumor cells at G1 and G2-M and activate the cyclin-dependent kinase inhibitor, the p21/WAF1/Cip1 gene promoter, through the Sp1 sites. In this study, we identified Sp1 and Sp3 as major factors binding to the Sp1 sites of the p21/WAF1/Cip1 promoter in MG63 cells through electrophoretic mobility shift assays and showed that TSA treatment did not change their binding activities. However, GAL4-Sp3 but not GAL4-Sp1 fusion protein supported the TSA-mediated gene induction from a luciferase reporter plasmid driven by five GAL4 DNA-binding sites. Moreover, the ectopic expression of dominant negative Sp3 repressed the enhancement by TSA of the p21/WAF1/Cip1 promoter and Sp1 site-driven promoter. Taken together, these results suggest that histone deacetylase inhibitor up-regulates p21/WAF1/Cip1 transcription by Sp3 but not by Sp1.

Introduction

Cancer results from a sequence of genetic changes that subvert the normal mechanisms of control over cell cycle, differentiation, and morphology. Many natural products have been isolated on the basis of their ability to arrest the deregulated cell cycle, induce differentiation, or revert the abnormal morphology back to normal morphology in various cancer cell lines and transformed cell lines. TSA (1) and trapoxin (2) have been also isolated as the potential products having detransforming activity. Furthermore, these compounds also induce differentiation and cell cycle arrest. It has been unclear how these compounds are able to show such antitumor activities. Recently, HDACs have been considered as likely molecular targets of these agents. Actually, both TSA and trapoxin inhibit the activity of HDAC at similar respective concentrations, for which antitumor activities are observed (3, 4). Accumulating evidence suggests that the acetylation and deacetylation of histones and/or nonhistone protein play significant roles in the regulation of transcription in eukaryotic cells (5, 6). With recent cloning of many histone acetyltransferases and the recognition that many transcription factors, coactivators, and basal transcription initiation complex proteins contain acetyltransferase activity, it has become apparent that histone acetyltransferases play an important role in transcriptional initiation and activation (7, 8). Also, with recent cloning of some HDACs and the recognition that some transcriptional repressors and corepressors form complexes with HDACs (9, 10), it has gradually become apparent that HDACs play an important role in transcriptional repression. Because HDAC inhibitors show antitumor activity, HDACs may repress the transcription of antitumor genes, the products of which induce arrest of cell proliferation or differentiation (11).

Previously, we demonstrated that sodium butyrate, which is a well-known differentiation-inducing reagent that also acts as a HDAC inhibitor at millimolar concentration, inhibits cell proliferation, and induces the expression of p21/WAF1/Cip1, cyclin-cyclin-dependent kinase inhibitor (negative cell cycle regulator), in a p53-independent manner (12). Furthermore, we also reported that both sodium butyrate and TSA activate the p21/WAF1/Cip1 gene promoter through the Sp1 sites (13). Interestingly, the region containing the Sp1 sites of the p21/WAF1/Cip1 promoter was also shown to be the minimal region for up-regulation of the p21/WAF1/Cip1 promoter mediated by TGF-β (14), phorbol ester (15), okadaic acid (15), and geranylogeraanyltransferase I inhibitor GGTI-298 (16). It is not known whether specific transcription factors that are capable of binding to the Sp1 sites mediate the HDAC inhibitor-transactivating signal.

Here, we show that Sp1 and Sp3 predominantly bind to the Sp1 sites of p21/WAF1/Cip1 promoter in MG63 cells and that TSA treatment does not change their binding activities. However, GAL4-Sp3 but not GAL4-Sp1 fusion protein can support TSA induction from a minimal promoter driven by five GAL4 DNA-binding sites. Moreover, we show that the ectopic expression of dominant negative Sp3 represses the enhancement by TSA of the p21/WAF1/Cip1 promoter. Thus, our results suggest that HDAC inhibitor up-regulates the p21/WAF1/Cip1 transcription by Sp3 but not Sp1.

Materials and Methods

Cell Culture and Preparation of Nuclear Extracts. MG63 cell line was maintained in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (Life Technologies, Inc.) and incubated at 37°C in a humidified atmosphere of 5% CO2 in air. Nuclear extracts were prepared from both TSA-treated and untreated MG63 cells, according to the method of Dignam et al. (17). After 24 h of treatment with 500 ng/ml TSA (Wako, Osaka, Japan), cells in a 100-mm-diameter plate were washed twice with ice-cold PBS containing 0.5 mM -ABSF (Wako), scraped, and incubated on ice for 10 min in 10 mM HEPES-KOH buffer (pH 7.9) containing 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, 5 mM sodium fluoride, 5 mM sodium orthovanadate, and 0.5 mM -ABSF. Cells were disrupted with a Dounce homogenizer. After centrifugation, nuclei were resuspended in 20 mM HEPES-KOH buffer (pH 7.9) containing 400 mM KCl, 20% glycerol, 0.1 mM EDTA, 1 mM DTT, 5 mM sodium fluoride, 5 mM sodium orthovanadate, and 0.5 mM -ABSF and incubated at 4°C for 60 min. The mixture was centrifuged at 35,000 rpm for 30 min at 4°C, and the supernatant was recovered as nuclear extracts.

Nuclear extracts were dialyzed against 20 mM HEPES-KOH buffer (pH 7.9) containing 400 mM NaCl, 1.5 mM MgCl2, 25% glycerol, 0.1 mM EDTA, 1 mM DTT, 5 mM sodium fluoride, 5 mM sodium orthovanadate, and 0.5 mM -ABSF. The dialyzed nuclear extract was used for protein concentration determination and was stored at −80°C.
EMSA. Annealed oligonucleotides containing the sequences between positions −87 and −72 from the transcription start site of the p21/WAF1/Cip1 promoter (AGTCGGGTCCCGCCCTCCCTT and TCGAAAGGAGCAGG-GACCGG) were labeled with [α-32P]dCTP using the Klenow fragment of *Escherichia coli* DNA polymerase and were used as a probe. The reaction mixture for the EMSA contained 8 mM Tris-HCl (pH 7.9), 24 mM HEPES-KCl (pH 7.9), 120 mM KCl, 24% glycerol, 2 mM EDTA, 2 mM DTT, 1 μg of poly(dI·dC) (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), and 8 μg of nuclear extract. After preincubation for 5 min, 32P-labeled probe DNA was added to the mixture, and the binding reaction was allowed to proceed at room temperature for 20 min. The reaction mixture was further incubated for 20 min in the presence or absence of anti-Sp1 or anti-Sp3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The product was resolved by electrophoresis on a 6% native polyacrylamide gel and analyzed by Fuji Image Analyzer Bas 2000 (Fujix, Tokyo, Japan).

**Plasmid Constructs.** Each of the plasmid constructs pM-Sp1, pM-Sp3, pM-DNSp1, and pM-DNSp3 expresses the GAL4 binding domain fused to active forms of Sp1 (amino acids 83–778) and Sp3 (amino acids 1–654), respectively, pM, expressing only the GAL4 binding domain, used as a negative control. Furthermore, pM-Sp3 (1–398), pM-Sp3 (81–160), pM-Sp3 (81–398), pM-Sp3 (161–398), pM-Sp3 (241–398), pM-Sp3 (1–80), pM-Sp3 (1–160), pM-Sp3 (1–240), and pM-Sp3 (1–320) express GAL4 binding domains fused to the Sp3 regions corresponding to amino acids 1–398, 81–160, 81–398, 161–398, 241–398, 1–80, 1–160, 1–240, and 1–320, respectively. The regions derived from Sp1 or Sp3 of all above plasmids were created by PCR amplification. The PCR products were subsequently cloned into a pM vector. The region and the junctions of all fusion proteins were sequenced to verify the accuracy of the PCR amplification. The expression plasmids were prepared from E. coli (Promega, Madison, WI). Luciferase activities of the cell lysates were measured according to the manufacturer’s recommendations (Promega, Madison, WI). Luciferase activities were normalized for the amount of the protein in cell lysates. All luciferase assays were carried out in triplicate. The level of induction was calculated by dividing the mean luciferase activity of samples treated with TSA by the mean activity of untreated control samples. Each experiment was repeated at least three times.

**Results**

**The Sp1 Sites, Which Are Required for the Transactivation of p21/WAF1/Cip1 Promoter by TSA, Interact with Sp1 and Sp3.** We have previously shown that TSA activates the p21/WAF1/Cip1 gene promoter through the Sp1 sites in a p53-independent manner and that the Sp1 sites were required for the up-regulation mediated by TSA (13). To determine the mechanism through which the p21/WAF1/Cip1 induction occurs, we performed EMSA using as a probe the sequence between positions −87 and −72 from the transcription start site of the p21/WAF1/Cip1 promoter. The EMSA, performed with nuclear extracts from both TSA-treated MG63 cells and untreated MG63 cells and 32P-end-labeled Sp1 probe (−87 to −72), revealed three specific bands (Fig. 1, Lanes 1 and 5). Supershift experiments in the presence of specific antibodies for Sp1 showed that the slowest-migrating complex was shifted (Fig. 1, Lanes 2 and 6). Both the fast complex and slow-migrating complex, which ran slightly faster than the slowest-migrating complex of Sp1, were shifted in the presence of specific antibodies for Sp3 (Fig. 1, Lanes 3 and 7). In the presence of antibodies for Sp1 and Sp3, used in combination, all three bands disappeared (Fig. 1, Lanes 4 and 8). These results are consistent with our previous report (12) and other reports (14, 16). They indicate that both Sp1 and Sp3 interact with Sp1 sites, which are required for the transactivation of the p21/WAF1/Cip1 promoter in MG63 cells. However, TSA treatment did not change DNA-binding activities of either Sp1 or Sp3 (Fig. 1, Lanes 1 versus 5, 2 versus 6, 3 versus 7, and 4 versus 8). These results suggest that a mechanism other than alteration of the DNA-binding activities of Sp1/Sp3 transcription factors is responsible for TSA-mediated transcriptional activation. This result is also consistent with our previous report using sodium butyrate (12) and another report using TGF-β (14) but is inconsistent with yet another report using geranylgeranyltransferase I inhibitor (16).

**Sp3 but not Sp1 Can Mediate Responsiveness to TSA.** As we have demonstrated previously, TSA activates the p21/WAF1/Cip1 promoter through the Sp1 sites and also activates three consensus Sp1 binding site-driven promoter (13). To determine whether Sp1 and/or Sp3 acts as a mediator of the TSA-transactivating signal, we used a system in which either Sp1 or Sp3 was fused to the DNA-binding domain of the bacterial transcriptional factor GAL4. Consequently, the ability of these fusion proteins to activate transcription from the consensus GAL4 DNA-binding site in both the presence and absence of TSA could be measured. In this way, an enhancement of the transcriptional activity in the presence of TSA over the transcriptional activity in the absence of TSA is a measure of the ability of each fusion protein to respond to TSA.

**Fig. 1.** The Sp1 sites that are required for the transactivation of the p21/WAF1/Cip1 promoter by TSA interact with Sp1 and Sp3. EMSA was carried out with nuclear extracts prepared from TSA-treated (Lanes 5–8) or untreated (Lanes 1–4) MG63 cells. Labeled oligonucleotide containing the sequence of the Sp1 sites required for the transactivation of the p21/WAF1/Cip1 promoter by TSA from position −87 to −72 from the transcriptional start site was used as a probe. Anti-Sp1 antibody (Lanes 2, 4, 6, and 8) or anti-Sp3 antibody (Lanes 3, 4, 7, and 8) was used as indicated. Left, positions of Sp1 and Sp3.
respectively. GAL4 dominant negative Sp3 fusion protein could not be activated by TSA treatment (Fig. 2, GAL4-DNSp3). These results suggest that the transactivation domain of Sp3 mediate the transcriptional activation through the Sp1 sites by TSA.

Analysis of the Necessity of the Sp3 Domain for the Mediation of Transcriptional Activation by TSA. To determine the TSA response region of Sp3, we also constructed various deletion mutants of transactivation domain of Sp3, which were fused to the GAL4 DNA-binding domain. Similar to the above experiments, we measured the ability of these GAL4 mutant Sp3 fusion proteins to activate transcription from consensus GAL4 DNA-binding site in both the presence and absence of TSA. As shown in Fig. 3, the results using gradual deletions from both NH2 and COOH termini of the transactivation domain of Sp3 suggest that the region between amino acids 81 and 160 of Sp3 is important for the mediation of the transcriptional activation by TSA. However, this domain alone, which corresponds to GAL4-Sp3 (81–160), could not mediate the transcriptional activation by TSA. Neither GAL4-Sp3 (241–398) nor GAL4-Sp3 (1–80) could be activated by TSA treatment. In contrast, mutant Sp1 site-driven promoter was not affected by the expression of dominant negative Sp3 expression plasmid to cotransfect with the promoter activity of the p21/WAF1 promoters (Fig. 4, A and B) and consensus Sp1 site-driven promoter (Fig. 4C), which would otherwise be activated by TSA treatment. In contrast, mutant Sp1 site-driven promoter was not affected by the expression of dominant negative Sp3 (Fig. 4D). Interestingly, the ectopic expression of dominant negative Sp3 did not repress the promoter activity of the p21/WAF1/Cip1 promoters (Fig. 4A and B) and consensus Sp1 site-driven promoter (Fig. 4C), which would otherwise be activated by TSA treatment. In contrast, mutant Sp1 site-driven promoter was not affected by the expression of dominant negative Sp3 (Fig. 4D). Interestingly, the ectopic expression of dominant negative Sp3 did not repress the promoter activity of the p21/WAF1/Cip1 promoter and Sp1 site-driven promoter in the absence of TSA (data not shown). These results suggest that, in the absence of TSA, Sp3 acts as an inactive transcription factor, but in the presence of TSA, Sp3 changes into a strong transcription factor.
through the Sp1 sites (12, 13). Here, we show that the Sp1 sites are capable of binding at least two transcription factors in vitro. Sp1 and the related protein, Sp3 (18), which recognizes the same sequence of Sp1. In this report, we determined whether either of these factors could act as a mediator for the TSA. Using chimeric GAL4 DNA-binding domain-Sp1 or Sp3 fusion protein, we have shown that TSA can activate the transcription from Sp3. This activation is specific to the transcription-domain of Sp3 because neither Sp1 nor the DNA-binding domain of Sp3 could activate transcription in a TSA-dependent manner. Furthermore, the deletion mutants of the Sp3 transcription domain indicate that at least one glutamine-rich domain of Sp3 is necessary for response to TSA treatment. Moreover, because the ectopic expression of dominant negative Sp3 could repress the enhancement by TSA of the p21/WAF1/Cip1 promoter and Sp1 site-driven promoter, it was also confirmed that Sp3 is involved in the Sp1 site-mediated transcription by TSA.

Interestingly, the region containing the Sp1 sites of the p21/WAF1/Cip1 promoter was also shown to be the minimal region for mediating up-regulation of p21/WAF1/Cip1 promoter by TGF-β, phorbol ester, okadaic acid, progesterone, and geranylgeranyltransferase I inhibitor (14–16, 19). In contrast to this report, several recent reports showed that Sp1 but not Sp3 is the mediator of the TGF-β signal through the Sp1 sites (20); geranylgeranyltransferase I inhibitor GGTI-298 up-regulates Sp1 transcriptional activity (16), and progesterone regulates transcription of the p21/WAF1/Cip1 gene through Sp1 and CBP/p300 (19). Probably, these agents might enhance the transcriptional activity of Sp1. However, it is considered that the function of TSA as a HDAC inhibitor might be to Sp3 to alter the its transcriptional activity from weak activator or repressor to strong activator. Histone acetylation appears to be critically involved in the activation of an increasingly recognized number of genes (7). Conversely, histone deacetylation has been linked to some genes’ repression. Several years ago, two transcriptional corepressors, called N-CoR and SMRT, were identified to bind the nuclear receptors and repress the transcription mediated by the receptors (21–23). These complexes then recruit a factor with HDAC activity and suppress gene activation (9, 24, 25). Actually, the recruitment of HDAC is critical to the transforming potential of the fusion proteins of acute promyelocytic leukemia, which are the fusions of the PML (promyelocytic leukemia) protein or the PLZF (promyelocytic leukemia zinc-finger) protein with retinoic acid receptor and a p53-negative human colon cancer cell line. J. Biol. Chem., 272: 22199–22206, 1997.

Recently, we proposed a novel approach for chemotherapy or chemoprevention against cancer, which we termed “gene-regulating chemotherapy or chemoprevention” (36). Our strategy is to activate the potent function of growth-inhibitory genes, which are activating targets of p53. p21/WAF1/Cip1 gene is one of the better candidates because the p21/WAF1/Cip1 gene appears to be rarely mutated in human common cancers (37, 38), whereas the p53 gene is frequently mutated (39, 40). Therefore, the elucidation of p53-independent activating mechanism by the HDAC of the p21/WAF1/Cip1 gene might contribute to the therapy or the prevention of cancer when p53 gene is mutated. Thus, HDAC inhibitors are regarded as possible drugs in the treatment of certain cancers. In fact, FR901228, which is a novel HDAC inhibitor (41), shows remarkable activity in vivo against experimental tumors and is now under clinical investigation (Phase I) at the National Cancer Institute (Bethesda, MD). Furthermore, clinical treatment with sodium phenylbutyrate, which is also a HDAC inhibitor and a drug previously tested for the treatment of thalassemia and certain hyperammonemias, is in combination with all-trans-retinoic acid to a patient with highly resistant acute promyelocytic leukemia induced complete clinical and molecular remission (42). These findings implicate HDAC inhibitors as possible molecular targets for the cancer therapy.

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References


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