

Transcriptional induction of DLC-1 gene through Sp1 sites by histone deacetylase inhibitors in gastric cancer cells

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Abbreviations: DLC-1, deleted in liver cancer-1; EMSA, electrophoretic mobility-shift assay; GAP, GTPase-activating protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; NaBu, sodium butyrate; TSA, trichostatin A

Abstract

We previously reported that trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, induced DLC-1 mRNA expression and accumulated acetylated histones H3 and H4 associated with the DLC-1 promoter in DLC-1 non-expressing gastric cancer cells. In this study, we demonstrated the molecular mechanisms by which TSA induced the DLC-1 gene expression. Treatment of the gastric cancer cells with TSA activates the DLC-1 promoter activity through Sp1 sites located at -219 and -174 relative to the transcription start site. Electrophoretic mobility-shift assay (EMSA) revealed that Sp1 and Sp3 specifically interact with these Sp1 sites and showed that TSA did not change their binding activities. The ectopic expression of Sp1, but not Sp3, enhances the DLC-1 promoter responsiveness by TSA. Furthermore, the TSA-induced DLC-1 promoter activity was increased by p300 expression and reduced by knockdown of p300. These results demonstrated the requirement of specific Sp1 sites and dependence of Sp1 and p300 for TSA-mediated activation

of DLC-1 promoter.

Keywords: DLC1 protein, human; histone deacetylases; p300-CBP transcription factors; promoter regions, genetic; Sp1 transcription factor; trichostatin A

Introduction

The human DLC-1 (deleted in liver cancer) has been known as tumor suppressor showing that DLC-1 inhibits cell growth, colony formation, and invasion capacity in hepatocellular, breast, and non-small cell lung carcinoma (Ng *et al.*, 2000; Yuan *et al.*, 2003a; Kim *et al.*, 2007; Healy *et al.*, 2008). DLC-1 and its rat homolog p122 RhoGAP contain three functional domains such as an amino terminal SAM (sterile α -motif), a central RhoGAP, and a carboxy terminal START (steroidogenic acute regulatory related lipid transfer) domain (Durkin *et al.*, 2007). The RhoGAP domain is well known as a catalyst molecule converting active GTP-bound Rho proteins into inactive GDP-bound protein (Mackay and Hall, 1998), on the other hand the precise functions of the SAM and START domains remain answered.

DLC-1 gene has first been identified to be deleted in primary human hepatocellular carcinoma (Yuan *et al.*, 1998), but further studies demonstrated that DLC-1 expression is down-regulated mainly by DNA hypermethylation in several types of cancer including liver, breast, colon, prostate, gastric, lung, nasopharyngeal, esophageal, cervical carcinomas, and lymphoma (Kim *et al.*, 2003; Yuan *et al.*, 2003b, 2004; Seng *et al.*, 2007; Ying *et al.*, 2007)

We have previously reported that TSA, a HDAC inhibitor, induced DLC-1 mRNA expression in association with an accumulation of acetylated histones H3 and H4 with the DLC-1 promoter in human gastric cancer cell lines (Kim *et al.*, 2003), suggesting the possibility that histone deacetylation of the DLC-1 gene is a new epigenetic mechanism to contribute to down regulation of DLC-1 gene expression. The histone modifying enzymes histone acetyltransferase (HAT) and histone deacetylase (HDAC) have been reported to be involved in modifying chromatin structure and regulating transcription (Eberharter *et al.*, 2002). HATs acetylate the ϵ -amino group of lysine resi-

dues at the N-terminal domain of core histone, resulting in gene activation through relaxation of chromatin, whereas HDACs remove the acetyl group of core histone, leading to gene repression through condensation of chromatin.

In this study, we examined the molecular mechanism of the induction of DLC-1 gene by TSA in human gastric cancer cell lines. We show that Sp1 binding sites located at DLC-1 promoter play important roles in the activation of DLC-1 promoter by TSA. In addition, we found that Sp1, rather than Sp3, and p300 HAT are required for the activation of DLC-1 promoter by TSA, leading to the induction of DLC-1 gene expression. These findings demonstrate the molecular mechanism by which TSA induced the DLC-1 gene expression in gastric cancer cells.

Materials and Methods

Cell lines

Gastric cancer cell lines were obtained from the Korean Cell Line Bank (Seoul). Cell lines were maintained in RPMI-1640 supplemented with 10% (vol/vol) FBS and gentamicin (10 µg/ml) at 37°C in a humidified 5% CO₂ atmosphere.

RT-PCR and Western blot analysis

SNU-5 cells were treated with DMSO (control), 330 nM TSA, 1 µM SK-7068, or 5 mM sodium butyrate (NaBu) for 12 h and RT-PCR was performed as described previously (Kim *et al.*, 2003). Western blot analysis was performed with DMSO or 330 nM TSA-treated SNU-5 and SNU-668 cells as described previously (Kim *et al.*, 2007). Primary antibodies that recognize Sp1 and Sp3 (Santa Cruz Biotechnology) were used.

Construction of the DLC-1 promoter linked to luciferase gene with deletions and site mutations

A fragment of the DLC-1 gene promoter region (-577/+117) was generated by PCR using fetal lung fibroblast genomic DNA as a template and the resulting PCR product was inserted into the pGL3-basic luciferase reporter vector (pGL3b) (Promega) as described previously (Kim *et al.*, 2003). Sequence numbering is relative to the transcription start site (+1) (Genbank Accession NM_006094). The serial deletion constructs of the DLC-1 promoter-luciferase constructs were generated from the -577/+117-pGL3b by PCR and inserted into the pGL3b vector. The site mutation constructs of the DLC-1 promoter were generated using a Quick-

Change site-directed mutagenesis kit (Stratagene), based on the DLC-1 promoter-luciferase construct (-219/+117-pGL3b) that contains Sp1 binding sites. The sequences and orientations of the inserts were determined by direct sequencing.

Transient transfection and luciferase assay

To assess basal DLC-1 promoter activity in gastric cancer cells, the DLC-1 promoter-luciferase construct (-577/+117-pGL3b) was transfected into gastric cancer cells using Lipofectamine 2000 (Invitrogen). To study the effects of TSA on DLC-1 promoter activity, SNU-5 and SNU-668 cells were transfected with the indicated DLC-1 promoter-luciferase construct or cotransfected with the DLC-1 promoter-luciferase constructs and expression vectors for Sp1, Sp3 (pEVR2/Sp1 and pRC/CMV/Sp3 respectively, provided by Dr. G. Suske) (Hagen *et al.*, 1994), or p300, or pSuper vector carrying the shRNA against p300 sequence (pSuper-p300) (Liu *et al.*, 2003) using Lipofectamine 2000. Twenty-four hours post transfection, cells were treated with 330 nM TSA, or DMSO as control, for additional 12 h. Cell lysates were collected for luciferase assays. Luciferase activities of cell lysates were measured using a TR717 Microplate Luminometer (Tropix, Inc., Bedford, MA) and a Bioluminescent Reporter Gene Assay System (Tropix, Inc.) according to the manufacturer's instructions. Luciferase activities were normalized for total protein concentration determined using BCA assay.

Nuclear extract preparation and electrophoretic mobility shift assay

Nuclear extracts from DMSO- or TSA-treated SNU-5 or SNU-668 cells were prepared as described previously (Hiramoto *et al.*, 1998). For EMSA, oligonucleotide probes from -213 to -190 (5'-GAG GCGGGCCTGGGCGGGGCGGGGC-3') were annealed and labeled with [γ -³²P]ATP and T4-polynucleotide kinase (NEB). Five µg of nuclear extract and 1 µg poly (dI-dC) were preincubated for 20 min at 4°C in an appropriate binding buffer (20 mM HEPES pH 7.5, 5 mM KCl, 1 mM MgCl₂, 0.5 mM DTT, 0.1 mM EDTA, 0.1% NP-40, 10% glycerol). The mixture was then incubated on ice for 30 min with 0.5 ng of the labeled oligonucleotide. For supershift experiments, 2 µg of anti-Sp1 and/or anti-Sp3 antibodies were added during preincubation. For competition experiments, 20-fold molar excess cold oligonucleotide was added to the binding mixture 20 min before addition of the labeled probe. Samples were loaded onto a 4%

polyacrylamide gel and run for 2 h at 200 V in $0.5\times$ TBE. Gels were dried, and protein-DNA binding was visualized by autoradiography.

Results

Histone deacetylase inhibitors activate the DLC-1 promoter and induce DLC-1 gene expression in SNU-5 and SNU-668

We first investigated basal DLC-1 promoter activity in gastric cancer cells by luciferase assay using DLC-1 promoter-luciferase construct (-577/+117-pGL3b). As shown in Figure 1A, the DLC-1 promoter was active in many gastric cancer cells regardless of their expression status except SNU-5

and SNU-668. The loss of DLC-1 gene expression in the gastric cancer cells whose promoter was active is due to methylation of the DLC-1 promoter region as demonstrated in our previous study (Kim *et al.*, 2003). We have also previously demonstrated that TSA, a specific histone deacetylase (HDAC) inhibitor, induced DLC-1 gene expression in the SNU-5 and SNU-668. Treatment of SNU-5 cells with another HDAC inhibitor, NaBu or SK-7068, also resulted in the induction of DLC-1 gene expression (Figure 1B). To evaluate whether the DLC-1 gene expression by HDAC inhibitors is due to DLC-1 promoter activation, SNU-5 and SNU-668 cells were transiently transfected with empty vector (pGL3b) or -577/+117-pGL3b and treated with DMSO or TSA for 12h before measuring the luciferase activity. As shown in Figure 1C,

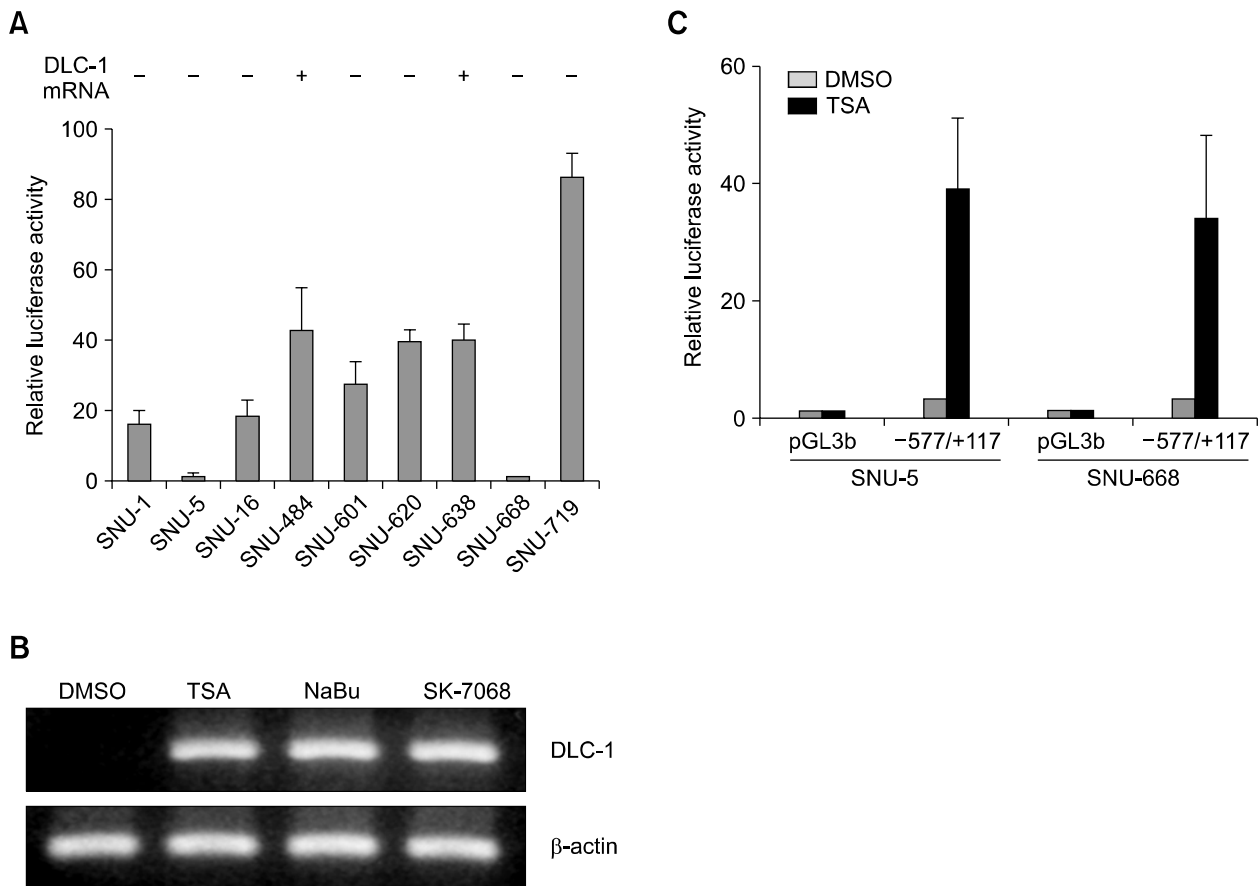


Figure 1. Induction of DLC-1 gene expression by HDAC inhibitors is via an activation of DLC-1 promoter in human gastric cancer cells. (A) The basal activity of DLC-1 promoter in gastric cancer cells. The DLC-1 promoter luciferase construct (-577/+117-pGL3b) was transiently transfected into gastric cancer cells for 24 h. Luciferase activity was measured and normalized by protein concentration. Data are representative of three independent experiments performed in triplicate. Expression of DLC-1 mRNA of each gastric cancer cells is shown in the top of the graph. (B) HDAC inhibitors induce DLC-1 gene expression. SNU-5 cells were treated with 330 nM TSA, 5 mM NaBu, or 1 μ M SK-7068 and cultured for 12 h. DLC-1 and β -actin mRNA expression was determined by RT-PCR as describe in "Materials and Methods" (C). TSA treatment increases the DLC-1 promoter activity. SNU-5 and SNU-668 cells were transiently transfected with empty reporter vector (pGL3b) or DLC-1 promoter luciferase construct (-577/+117-pGL3b) for 24 h followed by treatment with DMSO or 330 nM TSA for 12 h. Luciferase activity was measured and normalized by protein concentration. The data represent the combined results of three independent experiments performed in triplicate.

the DLC-1 promoter activity was dramatically increased after TSA treatment in both cells, but no significant induction was observed in cells transfected with empty vector. These results suggest that HDAC inhibitors induce the DLC-1 gene expression through the DLC-1 promoter activation in SNU-5 and SNU-668 cells.

Activation of the DLC-1 promoter by TSA requires Sp1 sites located between -219 and -174 of the DLC-1 promoter

To characterize the region responsible for activation of the DLC-1 promoter by TSA, serial deletion mutants of the DLC-1 promoter were constructed, transiently transfected into SNU-5 and

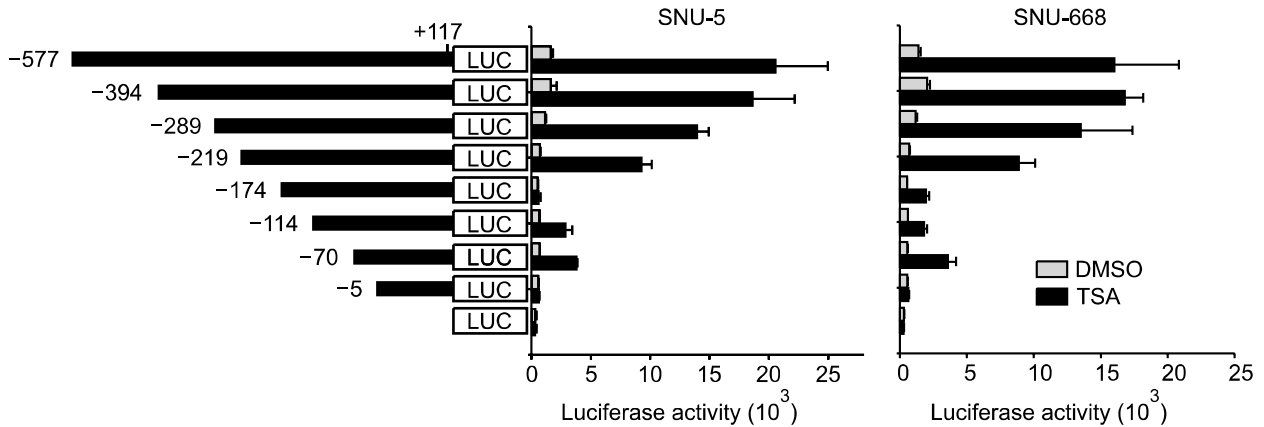


Figure 2. Activation of the deletion constructs of DLC-1 promoter in SNU-5 and SNU-668 cell. Serial deletion mutants of DLC-1 promoter were constructed as shown in the left panel. These constructs were transiently transfected into SNU-5 and SNU-668 cells followed by treatment with DMSO or 330 nM TSA for 12 h. Luciferase activity was measured and normalized to protein concentration in all luciferase reporter experiments. The data represent the combined results of three independent experiments performed in triplicate.

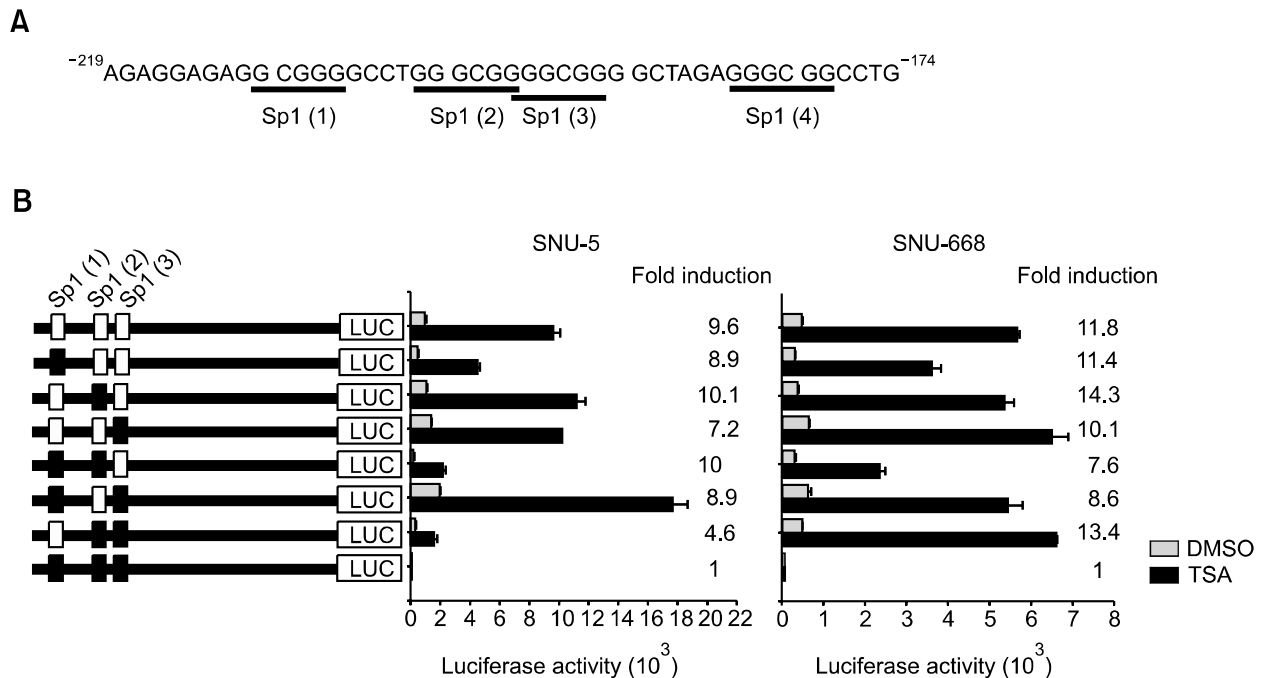


Figure 3. Identification of the Sp1 binding sites responsible for the activation of DLC-1 promoter by TSA. (A) Sequences of the DLC-1 promoter containing the region -219/-174 bp. The consensus sequences for Sp1 binding sites (GGCGGG or GGGCGG) are underlined. (B) Base substitution mutants using -219/+117 as a template were constructed as shown in the left panel. These constructs were transiently transfected into SNU-5 and SNU-668 cells followed by treatment with DMSO or 330 nM TSA for 12 h. Luciferase activity was measured and normalized to protein concentration in all luciferase reporter experiments. The data represent the combined results of three independent experiments performed in triplicate.

SNU-668, and luciferase activities were measured 12 h after treatment with DMSO or TSA. Deletion of the promoter to position -219 did not significantly affect the TSA-mediated *DLC-1* promoter activation, because the fold induction in response to TSA is similar to that of activation by -577/+117-pGL3b. On the other hand, further deletion to position -174 significantly decreased the activation of *DLC-1* promoter by TSA in both cells (Figure 2). These results suggest the TSA-responsive element is present within 45bp region between -219 and -174. Identification of potential transcription factor binding sites by the MatInspector program, using the TRANSFAC 3.5 matrices, revealed no TATA boxes could be identified. Notably, the *DLC-1* promoter region between -219 and -174 has four Sp1

binding sites (Figure 3A), suggesting that Sp1 might be involved in TSA-mediated *DLC-1* promoter activation. To evaluate the significance of these putative Sp1 sites, we focused on the first three Sp1 sites and constructed vectors containing various combinations of mutated Sp1 sites. Mutation of all three Sp1 sites abolished both basal and TSA-induced promoter activity in both cells. By contrast, other combinations of mutation showed a 4.6 to 10.1 and a 7.6 to 13.4-fold induction of *DLC-1* promoter activity in SNU-5 and SNU-668, respectively (Figure 3B). Taken together, these results suggest that Sp1 sites located between -219 and -174 are essential for basal *DLC-1* promoter activity and *DLC-1* promoter activation by TSA.

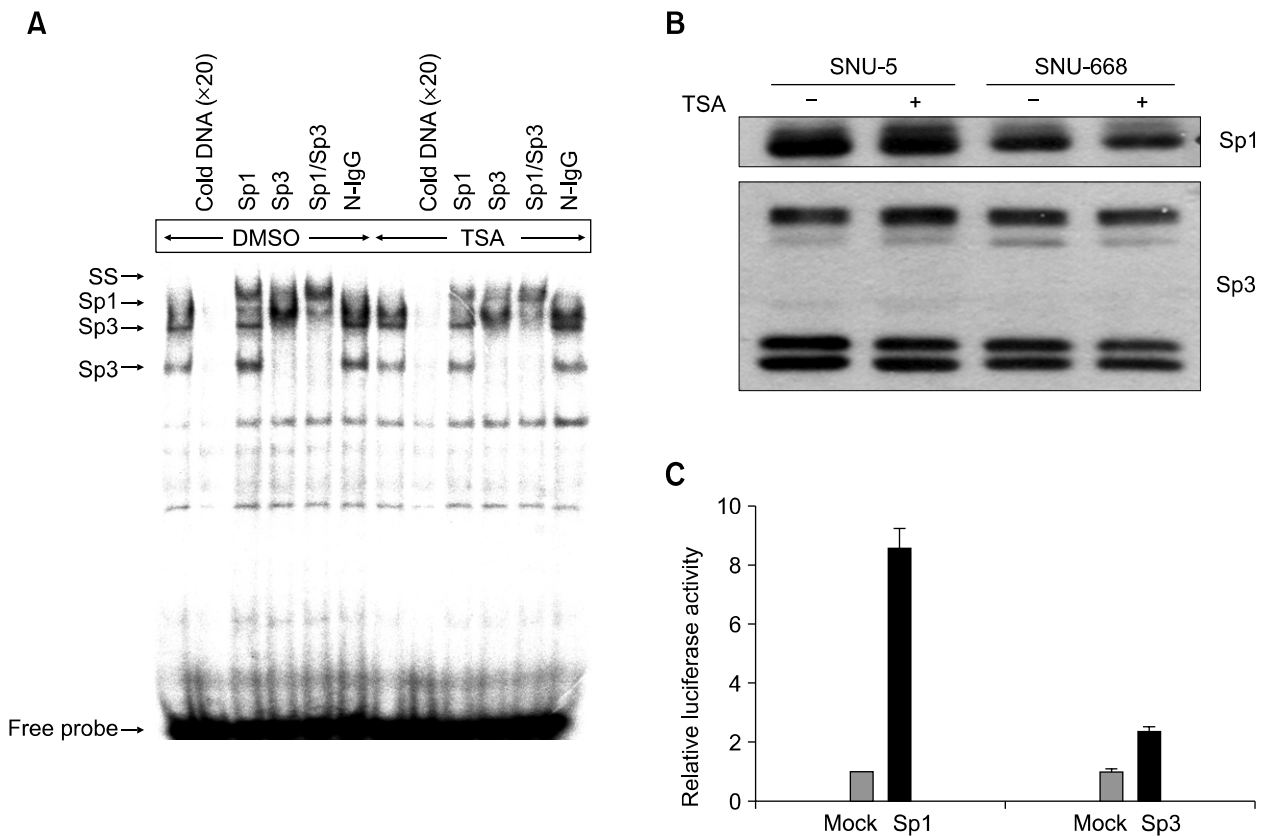


Figure 4. Sp1 and Sp3 bind to the *DLC-1* promoter and Sp1 is mainly involved in activation of the *DLC-1* promoter by TSA. (A) Sp1/Sp3 binds to the Sp1 sites located within *DLC-1* promoter. EMSA was carried out using [γ - 32 P] ATP-labeled oligonucleotide spanning Sp1 binding sites of *DLC-1* promoter and nuclear extracts prepared from SNU-5 cells treated with DMSO or 330 nM TSA for 12 h. The reaction was carried out in the absence (lanes 1 and 7) or presence of 20-fold molar excess of unlabeled probe (lanes 2 and 8) or in the presence of specific antibodies to Sp1 (lanes 3 and 9), Sp3 (lanes 4 and 10), both (lanes 5 and 11), or normal rabbit IgG (lanes 6 and 12). (B) TSA has no effect on the expression of Sp1 and Sp3 protein. Protein extracts from DMSO- or 330 nM TSA-treated SNU-5 and SNU-668 cells were analyzed by Western blotting using antibodies directed to Sp1 or Sp3. (C) Sp1 is the main transactivators of the *DLC-1* promoter in response to TSA. Empty vector (mock), Sp1, or Sp3 expression vectors were cotransfected with the *DLC-1* promoter luciferase construct (-219/+117-pGL3b) into SNU-668 cells and treated cells with 330 nM TSA for 12 h before measuring luciferase activity. Data are representative of two independent experiments performed in triplicate.

Sp1 and Sp3 bind to the -219 to -174 region of the DLC-1 promoter and Sp1 is the main transactivators of the DLC-1 promoter in response to TSA

To verify whether transcriptional factors of the Sp family (Sp1/Sp3) bind the Sp1 binding sites and the DLC-1 promoter activation by TSA could be attributed to a change of Sp family protein binding activities to this site, an EMSA was performed with nuclear extracts prepared from DMSO- or TSA-treated SNU-5 cells. Incubation of the 26mer oligonucleotide probe of the region representing the three Sp1 sites with nuclear extracts from SNU-5 cells resulted in the formation of three specific DNA protein complexes (Figure 4A). The addition of an excess of unlabeled oligonucleotide probe completely abolished the formation of all three complexes. To determine whether Sp1 and Sp3 were involved in the formation of the three complexes, antibody supershift assays were performed. The Sp1 and Sp3 antibodies were found to selectively supershift the complexes, whereas the normal rabbit antibody did not show any shifted bands. No apparent changes in complex formation were observed in DMSO- or TSA-treated SNU-5 cells. Similar results were obtained in DMSO- or TSA-treated SNU-668 cells (data not shown). In addition, TSA treatment did not show any significant effect on the expression of Sp1 and Sp3 protein in both cells (Figure 4B). Consequently, these results indicate that Sp1 and Sp3 proteins bind to the Sp1 sites of the DLC-1 promoter, and their expression level and binding affinity to the DLC-1 promoter are not affected by TSA treatment.

To determine whether both Sp1 and Sp3 are involved in the activation of DLC-1 promoter in response to TSA, we cotransfected Sp1 or Sp3 expression vectors with the DLC-1 promoter luciferase construct into SNU-668. Sp1 expression obviously increased the responsiveness of the DLC-1 promoter to TSA, but Sp3 expression had little effect on the induction of DLC-1 promoter activity by TSA (Figure 4C). This result suggests that Sp1 is the main transactivator of the DLC-1 promoter in response to TSA.

p300, a histone acetyltransferase, participates in the DLC-1 promoter activation by TSA

Because no changes in the expression level and binding affinity of Sp1 to DLC-1 promoter after TSA treatment were found, we considered that the activation of DLC-1 promoter by TSA results from interaction of Sp1 with other proteins. To examine whether p300 HAT is involved in the activation of DLC-1 promoter by TSA, a p300 expression vector or shRNA for p300 were cotransfected with the DLC-1 promoter luciferase construct into SNU-668. As shown in Figure 5, the expression of p300 increased stimulation of the DLC-1 promoter by TSA, whereas knock down of p300 inhibited DLC-1 promoter responsiveness to TSA in a dose-dependent manner. These results suggest that p300 acts as a co-activator and/or possibly the histone acetyltransferase activity of p300 is involved in the acetylation of histone protein around the DLC-1 promoter and the concomitant activation of DLC-1 promoter.

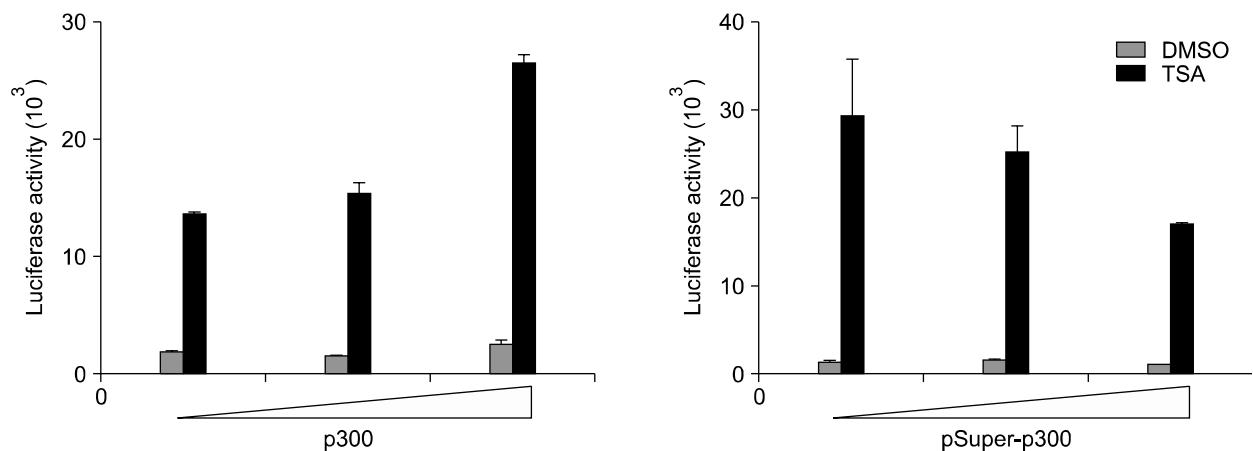


Figure 5. p300 is involved in the TSA-mediated DLC-1 promoter activation. p300 expression vector or pSuper vector carrying the shRNA against p300 (pSuper-p300) were cotransfected with the DLC-1 promoter luciferase construct (-577/+117-pGL3b) into SNU-668 cells and treated cells with DMSO or 330 nM TSA for 12 h before measuring luciferase activity. Data are representative of two independent experiments performed in triplicate.

Discussion

Several studies have shown that transcriptional silencing of DLC-1 gene is one of several mechanisms for tumor progression. Many human cancers express significantly reduced level of DLC-1 by genetic and epigenetic mechanism (Dunkin *et al.*, 2007). Reexpression of DLC-1 gene in DLC-1 null cells inhibits tumor cell growth and metastasis (Goodison *et al.*, 2005; Kim *et al.*, 2007). Inhibition of DLC-1 expression using RNA interference promoted cell proliferation and migration of in the colon cancer cell line (Jin *et al.*, 2008) and hepatocellular carcinoma in mice (Xue *et al.*, 2008). These results imply that DLC-1 acts as tumor suppressor gene and may be a candidate therapeutic target for cancer.

We have previously found that TSA induced DLC-1 gene expression in DLC-1 non-expressing gastric cancer cell lines, SNU-5 and SNU-668, which have the unmethylated alleles (Kim *et al.*, 2003). Since our report, several studies have demonstrated that DLC-1 gene expression was induced by treatment with either TSA alone or in combination with 5-aza-dC, a demethylating agent, in several cancers such as prostate cancer, multiple myeloma, and non-Hodgkin's lymphoma (Guan *et al.*, 2006; Song *et al.*, 2006; Shi *et al.*, 2007), suggesting that histone deacetylation of DLC-1 gene is not restricted to gastric cancer cells and is a new epigenetic mechanism for silencing of DLC-1 gene in human cancers. However, details of molecular mechanism underlying TSA-induced DLC-1 gene expression have not been elucidated. In this study, we have investigated the molecular mechanism of the induction of DLC-1 gene expression by TSA in SNU-5 and SNU-668 cells. We found that the DLC-1 induction by TSA requires Sp1 sites located between -219 and -174 of DLC-1 promoter and revealed that Sp1, rather than Sp3, and p300 HAT play an important role in the activation of DLC-1 promoter by TSA, resulting in DLC-1 gene expression.

Recently, the treatment of mammalian cells with HDAC inhibitors resulted in transcriptional activation of a variety of genes, particularly the genes which contain the Sp1 binding site such as p21 (WAF1), p19 (INK4D), TGF- β RII, and KLF4 (Sowa *et al.*, 1997; Zhao *et al.*, 2003; Chen *et al.*, 2004; Yokota *et al.*, 2004; Yi *et al.*, 2007). It has been also shown that p300 collaborates with Sp1 in the activation of genes by TSA such as p21 (WAF1) (Xiao *et al.*, 2000) and pyruvate dehydrogenase kinase 4 (PDK4) (Kwon *et al.*, 2006). Based on the observations that HDACs and p300 can directly interact with Sp1 (Owen *et al.*, 1998; de Ruijter *et*

al., 2003), we here suggest a possible mechanism for induction of DLC-1 gene expression by TSA. HDACs bound to DLC-1 promoter region via Sp1 renders histone hypoacetylation around DLC-1 promoter, leading to represses DLC-1 gene expression due to the compact chromatin structure. After TSA treatment, HDACs are released and p300 is recruited into the Sp1 to permit histone acetylation associated with the DLC-1 promoter region, resulting in the gene expression.

This is the first study showing the mechanism of induction of DLC-1 gene expression by a histone deacetylase inhibitor in human gastric cancer cell lines. Our present study suggests that the restoration of DLC-1 by HDAC inhibitors could be a major mechanism for suppressing tumor growth.

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