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Requirement of a Specific Sp1 Site for Histone Deacetylase-mediated Repression of Transforming Growth Factor β Type II Receptor Expression in Human Pancreatic Cancer Cells[1]

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ABSTRACT

In this study, we demonstrate a novel mechanism by which down-regulation of transforming growth factor β type II receptor (TβRII) is mediated by a histone deacetylase (HDAC) in pancreatic ductal adenocarcinoma (PDAC) cells. Treatment of PDAC cell lines BxPC-3 and MIA PaCa-2 with a specific HDAC inhibitor, trichostatin A (TSA), strongly activates TβRII promoter activity and induces TβRII expression. The transcriptional activation of *TβRII* by TSA was correlated with a decrease in HDAC activity and an increase in acetylated histone H4 protein. Correspondingly, an increase in the association of TβRII promoter with acetylated histone H4 was detected in the TSA-treated cells as determined by a chromatin immunoprecipitation assay. We found that a specific Sp1 site (Sp1C, located at –102 bp relative to the transcription start site) adjacent to an inverted CCAAT box (−83 bp) is required for TSA-mediated activation of the TβRII promoter. Furthermore, we determined that HDAC1 complexed with Sp1 in PDAC cells and that TSA treatment interfered with this association. Diminished binding of HDAC1 to the –112 to –65 bp region of the TβRII promoter after TSA treatment was confirmed by a DNA affinity precipitation assay. This is the first study to demonstrate the requirement of a specific Sp1 site for TSA-mediated transcriptional activation of *TβRII*. This study further suggests that the specificity of this Sp1 site for HDAC-mediated repression of *TβRII* may involve the interaction of the Sp1-HDAC1 complex with components of the cognate transcriptional regulators that bind to the inverted CCAAT box.

INTRODUCTION

TGF-β[3] is a multifunctional cytokine that inhibits epithelial cell growth (1, 2). Epithelial-derived tumors often lose their normal growth-inhibitory response to TGF-β. Restoration of a functional TGF-β signaling pathway causes a decrease in the tumorigenic phenotype of colon, breast, and pancreatic cancer cells that were previously insensitive to TGF-β (3–7). TGF-β and its downstream signaling molecules are thought to act as tumor suppressors (2, 4–6, 8). About half of pancreatic cancers show either biallelic inactivation or intragenic mutations of *DPC4* (deleted in pancreatic cancer locus 4), which codes for Smad 4 (9–11). In addition to alterations of the *DPC4* gene, mutations of the *TβRII* gene account for the lack of sensitivity to TGF-β in many tumor types (12). Mutations have also been reported to occur for the *TβRI* gene, although they appear to be less common than those for *TβRII* (13). In previous studies, we found that most PDACs do not harbor mutations or deletions in the *TβRII* gene (14) and that many of PDAC specimens or cell lines express low or undetectable levels of TβRII (15). This suggests that the down-regulation of *TβRII* in PDACs may be caused by epigenetic alterations. Several studies have demonstrated that transcriptional repression of the *TβRII* gene plays an important role in modulating TGF-β responsiveness in human cancer cell lines (16, 17).

The promoter of the *TβRII* gene has been cloned and partially characterized (18). The TβRII promoter lacks TATA box, and four major regulatory elements have been identified. They are two positive regulatory elements [PRE-1 and PRE-2 (located at −219 to −172 and +1 to +50, respectively)], a negative regulatory element [NRE-2 (located at −100 to −67)], and a core promoter (located at −47 to −1). Similar to other promoters that lack TATA boxes, Sp1 is required for transcription of *TβRII*. There are two consensus Sp1 binding sites, one in the core promoter (located at −25), and one upstream of the NRE-2 (located at −147; Ref. 18). Recently, two novel regulatory sites at position –102 and –59 were identified that bind Sp family proteins (19). An AP-1/cAMP-responsive element-binding protein site is located in the PRE-1 of the TβRII promoter, and an ets binding site is located in the PRE-2 of the TβRII promoter (16).

Transcriptional repression can be mediated by alterations in the activity or DNA binding properties of *trans*-activators or repressors or by epigenetic mechanisms that alter DNA structure. A suboptimal level of the transcription factor Sp1 due to methylation is responsible for the lack of TβRII expression in the late passage of MCF-7 cells (20). Similarly, we recently showed that increasing Sp1 expression was sufficient to reverse transcriptional repression of *TβRII* in MIA PaCa-2 cells (21). More recently, two separate studies indicate that HDAC causes loss of TβRII expression in lung and breast cancer cells (22, 23). HDACs, together with the opposing enzymes, HATs, control acetylation of histones (24, 25). Histone acetylation by HATs mediates transcription by facilitating the binding of transcription factors to DNA. Histone deacetylation by HDACs opposes this effect by restricting the access of transcription factors (25). HDAC activity is reported to be increased in some tumors compared with normal tissue, and this increase in HDAC activity has been associated with transcriptional repression of tumor suppressor genes that cause growth inhibition and apoptosis (24, 25).

We demonstrate here that treatment of PDAC cells with a HDAC inhibitor, TSA, increases TβRII promoter activity and TβRII expression. The aim of the present study was to determine the mechanism of HDAC-mediated repression of *TβRII* gene expression. During the course of our study, reports on lung (22) and breast cancer cells (23) showed that an inverted CCAAT box was required for HDAC-mediated transcriptional repression of *TβRII*. We show here, for the first time, that a specific Sp1 site (Sp1C) adjacent to the inverted CCAAT box is required for HDAC-mediated silencing of *TβRII*. Furthermore, we found that HDAC1 is complexed with Sp1 in PDAC cells and that TSA treatment interferes with this association. These

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[3] The abbreviations used are: TGF-β, transforming growth factor β; PDAC, pancreatic ductal adenocarcinoma; HDAC, histone deacetylase; HAT, histone acetyltransferase; TβRII, transforming growth factor β type II receptor; TSA, trichostatin A; RT-PCR, reverse transcription-PCR; PMSF, phenylmethylsulfonyl fluoride; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; DAPA, DNA affinity precipitation assay; CMV, cytomegalovirus; AP-1, activator protein 1; NF-Y, nuclear factor-Y.

findings indicate a novel mechanism by which HDAC1 mediates transcriptional repression of *TβRII*.

MATERIALS AND METHODS

Cell Culture, Transfection, Luciferase Activity Assay, and RT-PCR. The PDAC cell lines BxPC-3 and MIA PaCa-2 were maintained in DMEM and supplemented with 10% fetal bovine serum. For transfection, 1×10^5 cells/well were plated in a 12-well plate. FuGENE 6 (Roche, Indianapolis, IN) was used according to the manufacturer's instructions. Different TβRII promoter-luciferase constructs (0.5 μg) were cotransfected with CMV-*Renilla* (4 ng) plasmid as an internal control for transfection efficiency. Twenty-four h after transfection, the cells were treated with 100 ng/ml TSA or vehicle alone for an additional 24 h. The TβRII promoter activities were measured using the Dual Luciferase Assay Kit (Promega, Madison, WI). RNA extraction and RT-PCR of TβRII were performed as described previously (15).

HDAC Activity Assay. Cellular HDAC enzymatic activity was determined using a HDAC assay kit (Upstate Biotechnology, Lake Placid, NY) per the manufacturer's instructions. Briefly, 10 μg of nuclear extracts from TSA-treated and untreated cells were incubated with [^{3}H]acetate-labeled histone H4 peptide in HDAC assay buffer for 24 h. The released [^{3}H]acetate was extracted with 600 μl of ethyl acetate and separated by centrifugation. Aliquots (200 μl) of ethyl acetate phase in duplicates were measured for radioactivity.

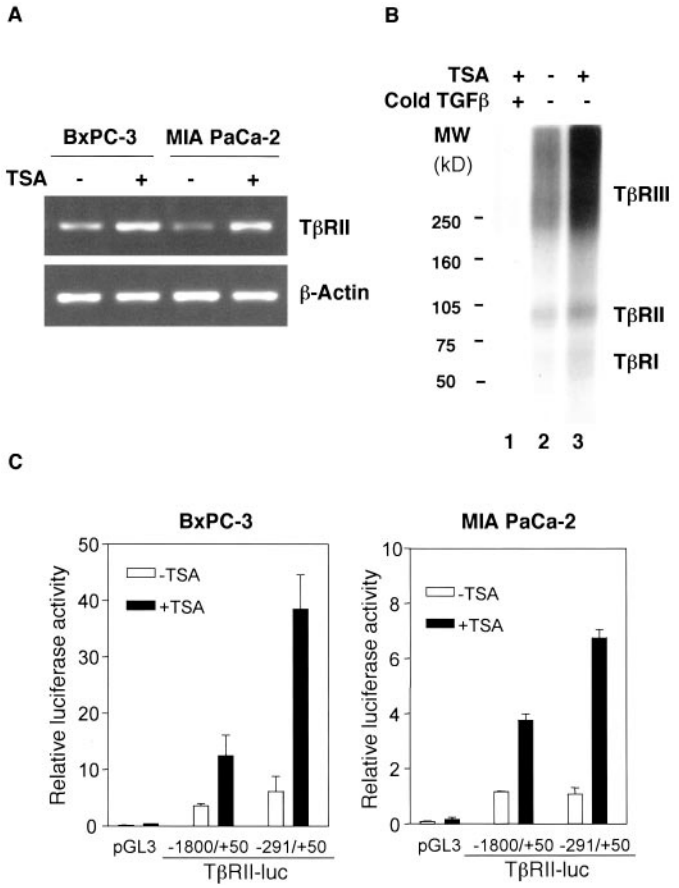

Fig. 1. TSA activates the TβRII promoter and increases TβRII expression. *A*, treatment of BxPC-3 or MIA PaCa-2 cells with 100 ng/ml TSA for 24 h increases TβRII mRNA expression as determined by RT-PCR. *B*, TGF-β1 cross-linking assay was performed as described in "Materials and Methods." Treatment of BxPC-3 cells with TSA increases cell surface expression of TβRII. Binding specificity was demonstrated by competing with 25-fold excess of cold TGF-β1 (*Lane 1*). *C*, treatment of PDAC cells with TSA increases the TβRII promoter activity. BxPC-3 and MIA PaCa-2 cells were transiently transfected with TβRII promoter-luciferase reporter constructs and treated with TSA or vehicle control for 24 h. Luciferase activity was measured at 48 h after transfection and normalized by CMV-*Renilla* luciferase activity. The data represent the mean ± SE from three independent experiments performed in duplicate.

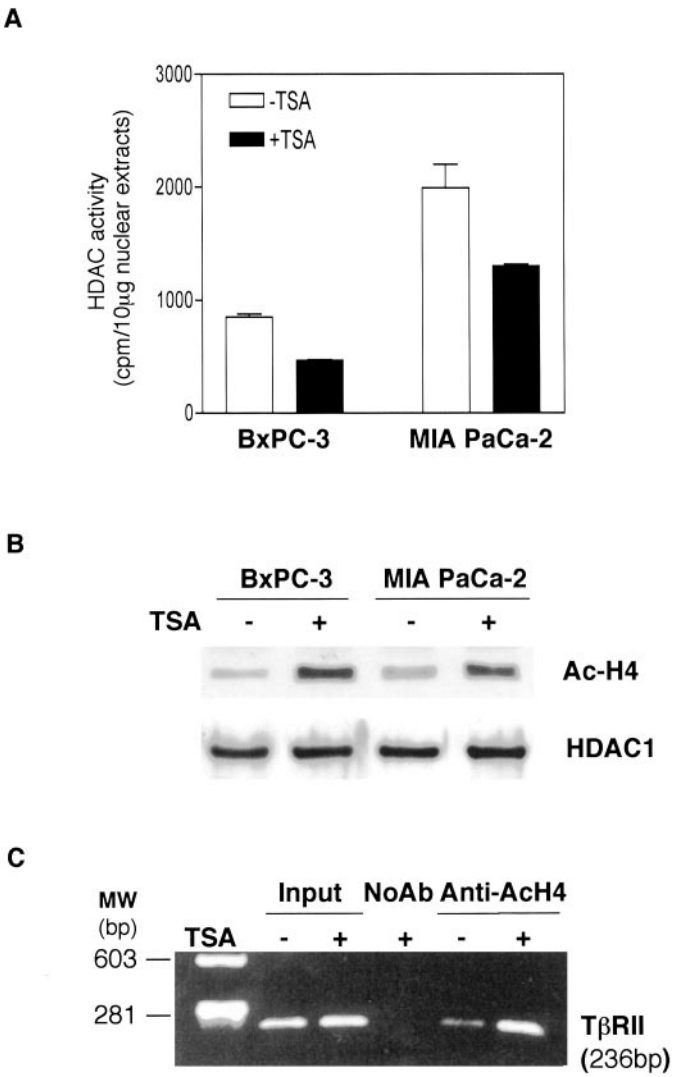

Fig. 2. Inhibition of HDAC activity increases the association of the TβRII promoter with acetylated histone H4. *A*, TSA treatment inhibits cellular HDAC enzymatic activity. Nuclear extracts (10 μg) from TSA-treated and untreated cells were incubated with [^{3}H]acetate-labeled histone H4 peptide at room temperature for 24 h. The released [^{3}H]acetate was extracted by ethyl acetate and counted for radioactivity as described in "Materials and Methods." *B*, TSA treatment increases the nuclear accumulation of acetylated histone H4. Fifteen μg of nuclear extracts from TSA-treated and untreated cells were separated by SDS-PAGE. Acetylated histone H4 and HDAC1 were detected by Western blotting analysis with respective antibodies. *C*, ChIP assay shows that TSA treatment increases the association of the TβRII promoter with acetylated histone H4. Untreated or TSA-treated MIA PaCa-2 cells were formaldehyde cross-linked. Chromatin was sonicated to yield 500–600-bp DNA fragments and immunoprecipitated with anti-acetylated histone H4 antibody. The TβRII promoter region was amplified by PCR using specific primers as described in "Materials and Methods."

TGF-β Cross-linking Assay. Exponentially growing cells were treated with 100 ng/ml TSA or vehicle control for 24 h. Cell monolayers were then incubated with 200 pM ^{125}I-TGF-β1 (Amersham Pharmacia) at 4°C for 3 h followed by cross-linking with disuccinimidyl suberate for 15 min. The labeled cells were solubilized in 200 μl of 1% Triton X-100 with 1 mM PMSF. Equal amounts of cell lysates were separated by a 4–10% gradient SDS-PAGE under the reducing condition and exposed for autoradiography.

ChIP Assay. ChIP assay was carried out as described by Boyd *et al.* (26), with some modifications. Briefly, 2×10^7 cells treated with and without 100 ng/ml TSA were cross-linked in 1% formaldehyde for 15 min at room temperature, and cells were collected in cold PBS. Cells were then lysed on ice in cell lysis buffer [5 mM PIPES (pH 8.0), 85 mM KCl. 0.5% NP40, 1 mM PMSF, and 10 μg/ml of both leupeptin and aprotinin] for 10 min. Nuclei were collected by centrifugation and resuspended in nuclear lysis buffer [50 mM Tris-HCl (pH 8.1), 1% SDS, 10 mM EDTA, and protease inhibitors as indi-

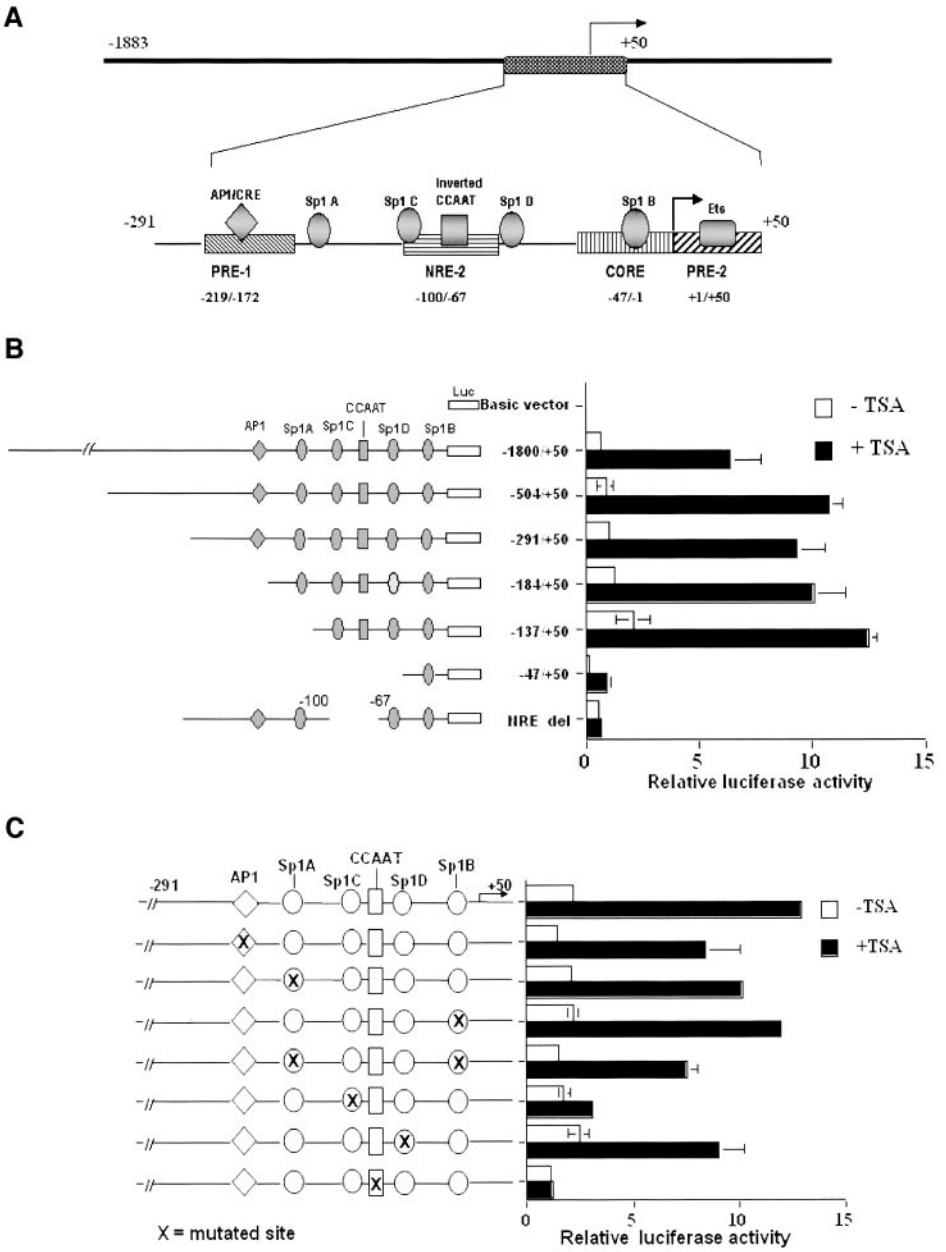

Fig. 3. Activation of the TβRII promoter requires a specific Sp1 site and an inverted CCAAT box of the TβRII promoter. *A*, a schematic diagram of the multiple regulatory elements within the TβRII promoter. *B*, deletion of the NRE-2 region (−100/−67) of the TβRII promoter diminishes the TSA-mediated induction of promoter activity. Serial 5′-flanking region deletion mutants of the TβRII promoter-luciferase constructs were transiently transfected into MIA PaCa-2 cells, and the cells were treated with 100 ng/ml TSA or vehicle control for 24 h. Luciferase activities were determined and normalized by CMV-*Renilla* luciferase activities. *C*, mutation of the Sp1C site or inverted CCAAT box attenuates the TSA-mediated induction of TβRII promoter activity. The TβRII promoter-luciferase reporter constructs with the indicated site mutations were transfected, and the cells were treated with TSA as described above. The data in *B* and *C* represent the mean ± SE from three independent experiments performed in duplicate.

cated above]. Samples were then sonicated on ice to break the chromatin DNA to an average length of about 500–600 bp and precleaned with protein A-agarose. The chromatin-protein complexes were immunoprecipitated with anti-acetylated histone H4 antibody (Upstate Biotechnology). The cross-links were reversed by heating at 65°C for 5 h. DNA was then extracted with phenol/chloroform/isoamyl alcohol and subjected to PCR. The primers used for TβRII promoter were as follows: forward primer, 5′-GTAAATACTTG-GAGCGAGGAAC-3′ (−182/−161); and reverse primer, 5′-ACTCACT-CAACTTCAACTCAGC-3′(+54/+33).

Construction of the TβRII Promoter-luciferase Reporter Plasmids with Deletions and Site Mutations. The 1.8-kb full-length TβRII promoter region from the TβRII promoter-CAT vector was subcloned into pGL3 basic luciferase vector. The serial deletion constructs of the TβRII promoter-luciferase reporter vectors were generated from the full-length construct by PCR using primers with an integrated *Xho*I site and *Hin*dIII site for the forward and reverse primers, respectively. The amplified TβRII promoter regions were then inserted into pGL3 basic vector. The site mutation constructs of the TβRII promoter were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) based on the TβRII promoter-luciferase construct (−291/+50) that contains the major regulatory elements. The mutations were confirmed by DNA sequencing.

Immunoprecipitation and Western Blotting Analysis. Nuclear extracts of the cells were prepared as described previously (21). Nuclear extracts (100 μg) from BxPC-3 and MIA PaCa-2 cells were incubated with 0.5 μg of the respective antibodies [Sp1 and c-Jun/Ap-1 from Santa Cruz Biotechnology (Santa Cruz, CA) and HDAC1 from Upstate Biotechnology] for 16 h at 4°C in 300 μl of radioimmunoprecipitation assay buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM PMSF, 1 μg of aprotinin, and 1 μg of leupeptin]. Protein A-agarose beads (25 μl) were used to precipitate the associated proteins. Samples were then separated by SDS-PAGE and detected by Western blot analysis using the indicated antibodies.

EMSA. EMSA was performed as described previously (21). Briefly, 5×10^4 cpm of ^{32}P-labeled probes (the sequences from −112 to −65 of the TβRII promoter) and 6 μg of nuclear extracts from MIA PaCa-2 cells were incubated at room temperature for 20 min in binding buffer [10 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 μg/μl BSA, and 1.5 μg of poly(dI-dC)·(dI-dC)]. For competition analysis, a 100-fold molar excess of unlabeled oligonucleotides was used. For the supershift assay, 2 μg

Table 1 *Primer sequences used to generate site mutation constructs of the TβRII promoter*

Sites	Positions	Sequences
AP-1	−195	5′-CTGTGTGCAC**TTAGTCA**TTCTTGAG-3′[a]
		5′-CTGTGTGCACTTAGT***GC***TTCTTGAG-3′[b]
Sp1A	−143	5′-GTGGTGTGGGA**GGGCGG**TGAGGGGCAG-3′[a]
		5′-GTGGTGTGGGA***TTT***CGGTGAGGGGCAG-3′[b]
Sp1B	−25	5′-GAAGGCTCTC**GGGCGG**AGAGAGGTCC-3′[a]
		5′-GAAGGCTCTCGGG***TCC***AGAGAGGTCC-3′[b]
Sp1C	−102	5′-GCTCTCGGA**GGGGCTGG**TCTAGGAAAC-3′[a]
		5′-GCTCTCGGAGG***AATTC***GTCTAGGAAAC-3′[b]
Sp1D	−59	5′-GAGAGCTA**GGGGCTGG**ACGTCGAGGAG-3′[a]
		5′-GAGAGCTAGG***AATTC***GACGTCGAGGAG-3′[b]
Inverted CCAAT	−83	5′-CTAGGAAACATG**ATTGG**ACAGCTACGAG-3′[a]
		5′-CTAGGAAACATG***GTGTA***ACAGCTACGAG-3′[b]

The consensus binding sites are shown as bold letters; the mutated sequences are shown as bold italics.

[a] Wild-type primer sequences.

[b] Mutated primer sequences.

of Sp1 antibody (Santa Cruz Biotechnology) or, as a control, mouse IgG were added and incubated for another 30 min. Samples were resolved on a 5% nondenaturing polyacrylamide gel and exposed for autoradiography.

DAPA. DAPA was performed as described by Walker *et al.* (27). Briefly, 1 μg of biotin end-labeled double-strand oligonucleotides corresponding to the sequence from –112 to –65 of the TβRII promoter was incubated with 100 μg of nuclear extracts from untreated or TSA-treated MIA PaCa-2 cells for 16 h in DAPA buffer [25 mM HEPES (pH 7.6), 60 mM KCl, 5 mM MgCl, 7.5% glycerol, 0.1 mM EDTA, 1 mM DTT, and 0.25% Triton X-100]. The DNA-protein complexes were then precipitated with 50 μl of Neutravidin-coated agarose beads (Pierce Chemical Co., Rockford, IL). Complexed proteins were resolved by 7.5% SDS-PAGE and detected by Western blot using antibodies to Sp1, HDAC1, and NF-YA.

RESULTS

TSA Activates the TβRII Promoter and Increases TβRII Expression. In this study, we investigated whether loss of TβRII in PDAC is due to histone deacetylation. Two PDAC cell lines, BxPC-3 and MIA PaCa-2, were treated with TSA and analyzed for an induction of TβRII expression. RT-PCR analysis showed that TSA treatment increased expression of TβRII mRNA (Fig. 1*A*). An increase in the cell surface expression of TβRII was also observed in TSA-treated cells as determined by a TGF-β cross-linking assay (Fig. 1*B*). To determine whether TSA-induced expression of TβRII was due to transcriptional activation, cells were transiently transfected with the TβRII promoter-luciferase reporter constructs and treated with and without 100 ng/ml TSA for 24 h. As shown in Fig. 1*C*, the TβRII promoter activity was dramatically increased after TSA treatment. No significant induction in basic vector and the insulin-like growth factor I receptor promoter activity was observed (data not shown), suggesting that transcriptional activation of *TβRII* by TSA is gene specific and that this activation leads to reconstitution of TβRII expression.

Inhibition of HDAC Activity Increases the Association of the TβRII Promoter with Acetylated Histone H4. We next tested the effect of TSA on inhibition of HDAC activity in PDAC cells. Treatment of BxPC-3 and MIA PaCa-2 cells with TSA inhibited cellular HDAC activity by about 40% (Fig. 2*A*). TSA treatment also caused accumulation of acetylated histone H4 in these cells, whereas HDAC1 protein levels were unchanged (Fig. 2*B*). Correspondingly, an increase in association of the TβRII promoter with acetylated histone H4 protein was determined by a ChIP assay (Fig. 2*C*). These results suggest that repression of *TβRII* in PDAC cells may be caused, at least in part, by HDAC-mediated condensation of chromatin.

Activation of the TβRII Promoter by TSA Requires a Specific Sp1 Site (−102 bp) and an Inverted CCAAT Box (−83 bp) of the TβRII Promoter. The known regulatory elements of the TβRII promoter are illustrated in Fig. 3*A*. To characterize the regions and *cis* elements that are required for TSA-mediated activation of the TβRII promoter, serial deletion mutants of the TβRII promoter-luciferase reporter constructs were transiently transfected into MIA PaCa-2 cells. Twenty-four h after transfection, cells were treated with either TSA or vehicle control for 24 h, and luciferase activities were measured. A strong induction of the TβRII promoter activity by TSA depends on the –137 to –47 bp region that contains the NRE-2 (–100 to –67) of the TβRII promoter. When the NRE-2 region was deleted, the TSA-mediated induction of the TβRII promoter activity was completely abolished (Fig. 3*B*). To further define the *cis* elements that regulate responsiveness of TSA, specific site mutations of TβRII promoter-luciferase constructs were generated by site-directed mutagenesis. Sequences of the primers used to generate these site mutations are shown in Table 1. As shown in Fig. 3*C*, mutations of either Sp1C (−102) or inverted CCAAT box (−83) attenuated the TSA-mediated activation of TβRII promoter. Mutation of other Sp1 sites (Sp1A, Sp1B, and Sp1D) or the AP-1 site caused only a minor reduction of TSA-induced promoter activity. These results suggest that the Sp1C site and the inverted CCAAT box play a key role for TSA-mediated activation of TβRII promoter.

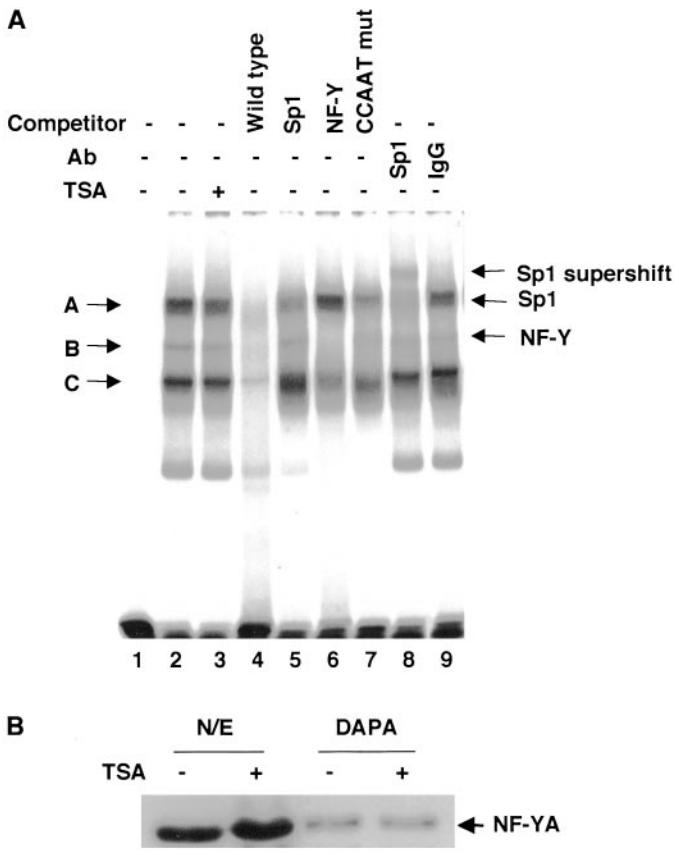

Fig. 4. Sp1 and NF-Y bind to the –112/–65 bp region of the TβRII promoter that contains both the Sp1C site and an inverted CCAAT box. *A*, an EMSA was performed using ^{32}P-labeled oligonucleotide probes spanning the region from –112 to –65 of the TβRII promoter. Five × 10^4 cpm of ^{32}P-labeled probes were incubated without nuclear extracts (*Lane 1*), with nuclear extracts from untreated or TSA-treated MIA PaCa-2 cells (*Lanes 2* and *3*), or with nuclear extracts from untreated cells in the presence of 100-fold molar excess of unlabeled competitive oligonucleotides (*Lanes 4–7*). To identify whether the bound proteins contain Sp1, supershift analysis was performed using a monoclonal antibody against Sp1 (*Lane 8*). Supershift with a mouse IgG was used as a negative control (*Lane 9*). The DNA-protein complexes were then separated on a 5% nondenaturing polyacrylamide gel. The Sp1 and NF-Y complexes and the Sp1 supershift as indicated. *B*, NF-YA bound to the −112/−65 bp region of the TβRII promoter. DAPA was performed as described in "Materials and Methods." One hundred μg of nuclear extracts from MIA PaCa-2 cells treated with TSA or vehicle control were analyzed. The complexed proteins were resolved on a 7.5% SDS-PAGE and detected by Western blotting using a monoclonal antibody against NF-YA. Fifteen μg of nuclear extracts were used as control. *N/E*, input nuclear extracts.

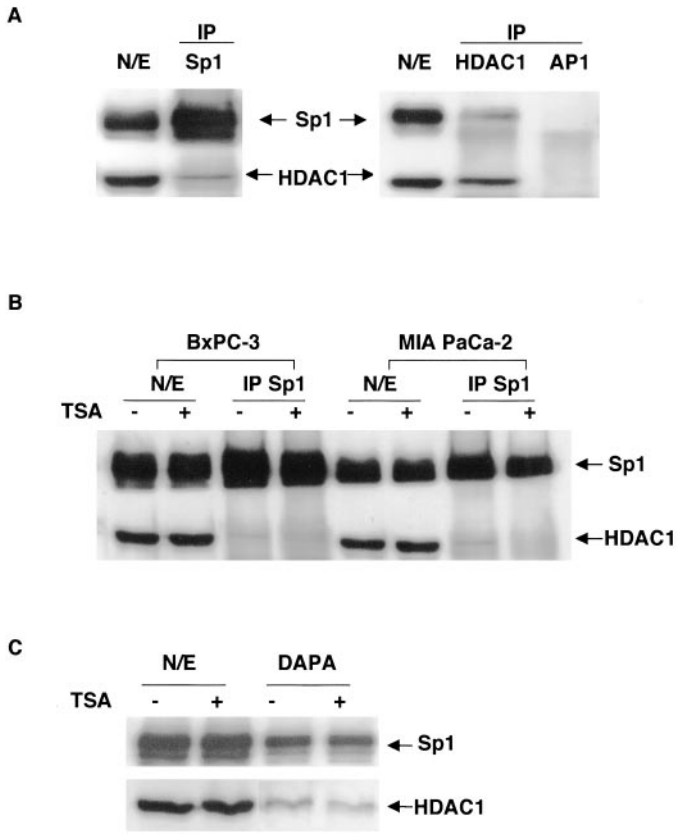

Fig. 5. TSA disrupted the association of HDAC1 with Sp1. *A*, HDAC1 is associated with Sp1 in PDAC cells. Nuclear extracts (100 μg) from MIA PaCa-2 cells were immunoprecipitated with antibodies to Sp1 or HDAC1. The protein complexes were resolved by SDS-PAGE and detected with the indicated antibodies. *N/E*, input nuclear extracts; *IP*, immunoprecipitation. *B*, treatment of cells with TSA diminishes the association of HDAC1 with Sp1. One hundred μg of nuclear extracts from untreated or TSA-treated BxPC-3 and MIA PaCa-2 cells were immunoprecipitated using an antibody to Sp1. The associated proteins were separated by SDS-PAGE and detected by the indicated antibodies. Fifteen μg of nuclear extracts were used as control. *N/E*, input nuclear extracts; *IP*, immunoprecipitation. *C*, TSA treatment decreases the binding of HDAC1 to the TβRII promoter. DAPA of MIA Pa Ca-2 cells was performed as described in "Materials and Methods." The DNA-protein complexes were resolved on a 7.5% SDS-PAGE, and bound proteins were detected by Western blot using antibodies to Sp1 or HDAC1, respectively. *N/E*, input nuclear extracts.

Sp1 and NF-Y Bind to the –112 to –65 Region of the TβRII Promoter That Contains Both the Sp1C Site and the Inverted CCAAT Box. We used EMSA and DAPA to identify whether Sp1 or NF-Y binds to the Sp1C site and the inverted CCAAT box, respectively. Nuclear extracts from MIA PaCa-2 cells were used for these assays. As shown in Fig. 4*A*, three specific complexes (complexes A, B, and C) bound to this region as determined by the competition with unlabeled wild-type competitor (*Lane 4*). We found that both Sp1 and NF-Y bind to this region. Complex A contains Sp1 because it was partially competed from binding by the unlabeled consensus Sp1 oligonucleotides (Fig. 4*A*, *Lane 5*). The identity of the Sp1 protein in this complex was further confirmed by a supershift assay using an antibody specific to Sp1 (Fig. 4*A*, *Lane 8*). Although it may also be possible that other Sp family proteins are present in some of these complexes, we did not analyze for their presence in this study. TSA treatment appears to cause a minor reduction of complex A (Fig. 4*A*, *Lane 3*). However, whether this minor reduction is due to a lack of binding of other Sp family proteins, such as Sp3, should be further verified. Complex B was diminished by competition with unlabeled consensus CCAAT oligonucleotides (Fig. 4*A*, *Lane 6*); whereas oligonucleotides with mutations in the CCAAT sequences failed to compete with the binding of this complex (Fig. 4*A*, *Lane 7*), suggesting that complex B contains CCAAT-binding protein. To determine whether NF-Y protein binds to this region, DAPA was performed using the same oligonucleotides that were end labeled with biotin. NF-YA binds to the –112 to −65 region of the TβRII promoter as detected by using a monoclonal antibody specific to NF-YA (Fig. 4*B*). However, no obvious altered binding was observed between untreated and TSA-treated cells.

TSA Interferes with the Association of Sp1 with HDAC1. We further investigated how Sp1 mediates the activation of the TβRII promoter by TSA. The association of HDAC1 with Sp1 was reported to repress $p21^{WAF1/CIP1}$ transcription in breast cancer cells (28). We tested whether Sp1 is complexed with HDAC1 in PDAC cells. BxPC-3 and MIA PaCa-2 nuclear extracts were immunoprecipitated using antibodies to Sp1 or HDAC1. We found that HDAC1 is present in Sp1 immunoprecipitates and, *vice versa*, that Sp1 is also detected in HDAC1 immunoprecipitates. However, a negative control immunoprecipitation with antibody to c-Jun/AP-1 did not reveal the presence of HDAC1 or Sp1 (Fig. 5*A*). Treatment of the cells with TSA diminished the association of HDAC1 with Sp1 (Fig. 5*B*). To confirm these results, we performed DAPA. The same oligonucleotides (−112 to −65) used in the EMSA were end-labeled with biotin and incubated with nuclear extracts from untreated and TSA-treated MIA PaCa-2 cells. As shown in Fig. 5*C*, both Sp1 and HDAC1 were present in these complexes. TSA treatment reduced the amount of HDAC1 present in the complexes, suggesting that inhibition of HDAC activity affects the binding of HDAC1 to this region of TβRII promoter.

DISCUSSION

In the present study, we demonstrate a novel mechanism that causes down-regulation of *TβRII*. This mechanism involves the binding of an Sp1-HDAC1 complex to a specific Sp1 site of the TβRII promoter in PDAC cells. Treatment of BxPC-3 and MIA PaCa-2 cells with a HDAC inhibitor, TSA, strongly activates *TβRII* promoter and induces TβRII expression. An increase in the association of TβRII promoter chromatin with acetylated histone H4 was observed after TSA treatment. These findings suggest that histone deacetylation plays a role in repression of the *TβRII* gene in PDAC. Luciferase reporter assays using serial deletion constructs revealed that a major TSA response region is located within the NRE-2 (−100 to −67) region of the TβRII promoter. Analysis using site-specific mutation constructs demonstrated that a specific Sp1 site (Sp1C) and an inverted CCAAT element located at −102 and −83 bp relative to the transcription start site of TβRII promoter are required for TSA-mediated reversal of *TβRII* gene silencing. These findings are in agreement with Osada *et al.* (22) and Park *et al.* (23), who recently showed that an intact CCAAT box is required for HDAC-mediated repression of *TβRII* transcription in lung and breast cancer cells. We report here for the first time that TSA-mediated induction of TβRII promoter activity requires not only the CCAAT box but also a specific Sp1 site. The region −47/+50 appears to retain some responsiveness to TSA, although this region has low basal promoter activity (Fig. 3*B*). Therefore, it is possible that a separate TSA-responsive element may reside in this region.

The TβRII promoter lacks a TATA box and has several Sp1 binding sites. Two typical Sp1 sites located at –147 and –25 bp upstream of transcription start site are critical in regulating *TβRII* transcription (18, 20). Another two sites, located at –102 and –59, have recently been reported to bind Sp family proteins and also to play an important role in *TβRII* transcription (19). We have shown previously that overexpression of Sp1 protein restored TβRII expression in MIA PaCa-2 cells (21). These results support the premise that Sp1 is a key transcriptional regulator of the TβRII promoter. In this regard,

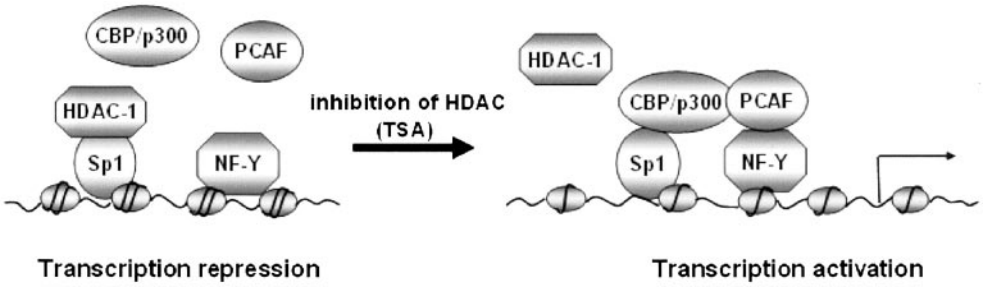

Fig. 6. A hypothetical model of transcriptional activation of the TβRII promoter by inhibition of HDACs. From this study, we propose a model in which Sp1 may serve as a scaffold to recruit HDAC1 to the TβRII promoter and causes chromatin deacetylation and gene silencing. Inhibition of HDAC activity by TSA disrupted the association of HDAC1 with Sp1, which in turn leads to the decondensation of local chromatin. This favors Sp1 and NF-Y interaction by recruiting factors that possess HAT activities, such as p300 and PCAF, to the promoter, thus activating the transcription of *TβRII*.

the BxPC-3 cell line is reported to have high levels of Sp1 expression and Sp1 activity (21, 29). This is consistent with our observation that BxPC-3 cells show a greater level of TβRII promoter activity compared with MIA PaCa-2 cells (Fig. 1*C*).

We found that both Sp1 and NF-Y bind to the –112/–65 region (which contains both Sp1C and NF-Y sites) of the TβRII promoter. The Sp1C site and the inverted CCAAT box are located close together (−102 and −83, respectively) and lie within a region originally described as a negative regulatory element termed NRE-2 (16). Thus, the NRE-2 possesses a Sp1 and inverted CCAAT sites that are generally considered to positively regulate promoter activity (30, 31). However, HDAC activity appears to selectively block the ability of these sites to activate the TβRII promoter. Studies indicate that HDAC1 can repress transcription by binding to Sp1 (32) and that Sp1 sites are involved in HDAC inhibitor-induced promoter activity (27, 28, 32). Sp1 and HDACs can directly interact *in vivo* and *in vitro* (32). In our study, immunoprecipitation assays of PDAC cells also suggest that Sp1 and HDAC1 are present in the same complex. Thus, Sp1 may serve as a scaffold to recruit HDAC to the promoter and causes chromatin condensation leading to transcriptional repression. NF-Y has also been reported to recruit HDACs (23). Sp1 and NF-Y have been reported to interact and lead to synergistic transcriptional activation of some genes (31, 33). It is therefore possible that these two factors may cooperatively regulate TSA-mediated *TβRII* transcription. Additional studies are required to determine whether HDAC1 recruited to the NRE-2 region is mediated independently or cooperatively through both of these transcription factors. Moreover, studies are necessary to assess whether a direct interaction of Sp1 with HDAC1 exists in these cells.

It is generally believed that the Sp family of transcription factors (Sp1, Sp2, Sp3, and Sp4) binds to the same consensus sequences. Sp3 is considered to be a transcriptional repressor in some instances (34). It is possible that Sp3 may also be involved in repression of TβRII or recruitment of HDAC to the TβRII chromatin. Whether an interaction of other Sp family proteins and HDACs occurs in PDAC cells also needs to be determined.

In this study, we provide the first evidence that HDAC1 mediates repression of *TβRII* transcription by associating with Sp1 in PDAC cells. Inhibition of HDAC activity by TSA interferes with the association of HDAC1 and Sp1. Based on these results, we propose a model in which Sp1 and NF-Y cooperatively regulate TSA-mediated *trans*-activation of the *TβRII* (Fig. 6). In this model, Sp1 may serve as a scaffold to recruit HDAC1 to the promoter and cause chromatin condensation and gene silencing. Inhibition of HDAC activity by TSA disrupts the association of HDAC1 with Sp1. This favors Sp1 and NF-Y interaction by recruitment of factors that possess HAT activity, such as p300 and PCAF, to the promoter, thus activating *TβRII* transcription. Because other transcription factors also interact with Sp1, competition between transcriptional regulators and HDACs might be a more general way to regulate gene expression via reversible chromatin modification.

In summary, we demonstrate here for the first time that activation of *TβRII* by TSA in PDAC cells is mediated by a specific Sp1 site and an inverted CCAAT box in the TβRII promoter. We found that HDAC1 is complexed with Sp1 in PDAC cells and that TSA treatment interferes with this association. This study further suggests that the specificity of this Sp1 site for HDAC-mediated repression of TβRII may involve the interaction of the Sp1-HDAC complex with components of the cognate *trans*-activators, such as NF-Y, that bind to the inverted CCAAT site.

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Requirement of a Specific Sp1 Site for Histone Deacetylase-mediated Repression of Transforming Growth Factor β Type II Receptor Expression in Human Pancreatic Cancer Cells

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