Acetylated Sp3 Is a Transcriptional Activator*

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Sp3 transcription factor can either activate or repress target gene expression. However, the molecular event that controls this dual function is unclear. We previously reported (Ammanamanchi, S., and Brattain, M. G. (2001) J. Biol. Chem. 276, 3348–3352) that unmodified Sp3 acts as a transcriptional repressor of transforming growth factor-β receptors in MCF-7L breast cancer cells. We now report that histone deacetylase inhibitor trichostatin A (TSA) induces acetylation of Sp3, which acts as a transcriptional activator of transforming growth factor-β receptor type II (RII) in MCF-7L cells. Mutation analysis indicated the TSA response is mediated through a GC box located on the RII promoter, which was previously identified as an Sp1/Sp3-binding site that was critical for RII promoter activity. Ectopic Sp3 expression in Sp3-deficient MCF-7E breast cancer cells repressed RII promoter activity in the absence of TSA. However, in the TSA-treated MCF-7E cells ectopic Sp3 activated RII promoter. Histone acetyltransferase p300 was shown to acetylate Sp3. Sp3-mediated RII promoter activity was stimulated by wild type p300 but not the histone acetyltransferase domain-deleted mutant p300 in MCF-7L cells, suggesting the positive effect of p300 acetylase activity on Sp3. Consequently, the results presented in this manuscript demonstrate that acetylation acts as a switch that controls the repressor and activator role of Sp3.

The Sp gene family consists of four members, which are referred to as Sp1–Sp4. Sp1, Sp2, and Sp4 are known to be activators of gene transcription, whereas Sp3 can be an activator or a repressor (1). Sp1 and Sp3 proteins contain two glutamine-rich activation domains at the N terminus and a highly conserved zinc finger region at the C terminus. Sp1 and Sp3 transcription factors bind to the same DNA sequence (consensus GC box) with similar affinities in many gene promoters (2). Unlike Sp1, Sp3 contains an inhibitory domain between the second glutamine-rich activation domain and zinc finger region (3). The repressor function of Sp3 has been attributed to a charged amino acid triplet (KEE) in the inhibitory domain (3). The lysine in this triplet has been reported to undergo acetylation with RII promoter DNA. Acetylation of transcription factors such as p303, E2F1, Myo D, and EKLF has been shown to enhance transcriptional potency and affect protein-protein in

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terature (4). TSA response is mediated through a GC box on the RII promoter, which was previously identified as an Sp1/Sp3-binding site that was critical for RII promoter activity in MCF-7L cells (15, 18). TSA did not affect RII expression by altering Sp1/Sp3 binding affinities. This was interesting because unmodified Sp3 acts as a repressor of RII in MCF-7L cells (18). So, we hypothesized that acetylated Sp3 acts as an activator of RII in TSA-treated MCF-7L cells. Ectopic Sp3 expression in Sp3-deficient RII-positive MCF-7E cells repressed RII promoter activity in the absence of TSA. However, in the TSA-treated MCF-7E cells ectopic Sp3 stimulated RII promoter activity. Sp3 undergoes acetylation by p300 (4). We observed that wild type p300 but not the histone acetyltransferase (HAT) domain deleted mutant p300 up-regulated Sp3-mediated RII promoter activity in MCF-7L cells, suggesting the positive influence of p300 acetylase activity on Sp3 transcriptional activity. Consequently, the results presented in this report demonstrate that acetylation status of Sp3 determines the activator or repressor function of Sp3.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7E, MCF-7L breast, and MIA PaCa-2 pancreatic cancer cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (Sigma), amino acids, antibiotics, pyruvate, and vitamins (Invitrogen). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Wherever indicated MCF-7E, MCF-7L, and MIA PaCa-2 cells were treated for 24 h with 100 ng/ml of TSA.

Chromatin Immunoprecipitation (ChIP) Assay—MCF-7L and MIA PaCa-2 cells were plated at a density of 4 × 10⁶ cells/15-cm dish and incubated overnight at 37°C with 5% CO₂. The next day, cells were cultured with 0 or 100 ng/ml TSA for 24 h. The ChIP assay was performed as described previously (20). RII and actin primers were used to carry out PCR from DNA isolated from ChIP experiments and input samples. The optimal reaction conditions for PCR were determined for each primer pair. Parameters were: denaturation at 95°C for 1 min and annealed at 58°C for 1 min, followed by elongation at 72°C for 1 min. PCR products were analyzed by 2.5% agarose/ethidium bromide gel electrophoresis. The following primers were used for PCR: RII promoter, sense, GAG AGA GCT AGG GGC TGG; antisense, CTC AAC TCA GCG CTG; moter, sense, GAG AGA GCT AGG GGC TGG; antisense, CTC AAC TCA GCG CTG; RII promoter activity. Sp3 undergoes acetylation by p300 (4). We observed that wild type p300 but not the histone acetyltransferase (HAT) domain deleted mutant p300 up-regulated Sp3-mediated RII promoter activity in MCF-7L cells, suggesting the positive influence of p300 acetylase activity on Sp3 transcriptional activity. Consequently, the results presented in this report demonstrate that acetylation status of Sp3 determines the activator or repressor function of Sp3.

Fig. 2. TSA induces RII promoter activities. MCF-7L cells were transiently transfected with the RII-Luc reporter or control vector without RII promoter (pGL2) along with β-galactosidase plasmid for normalization of transfection efficiency as described under “Experimental Procedures.” Cells were treated with TSA 4 h following transfection. Cells were harvested at 24 h following TSA treatment, and luciferase activity was determined and presented as relative units.

Fig. 1. TSA induces accumulation of acetylated histones H3 and H4 in chromatin associated with the RII gene. Chromatin fragments from cells cultured with and without TSA for 24 h were immunoprecipitated with antibody to acetylated histones H3 and H4 or control normal rabbit serum (NRS). PCR primers for the RII and actin gene promoters were used to amplify the DNA isolated from the immunoprecipitated chromatin as described under “Experimental Procedures.”
ously (15). The above constructs were transiently transfected into MCF-7L cells using the FuGENE 6 method (Roche Applied Science). For normalization of transfection efficiency, β-galactosidase plasmid was co-transfected into the cells. Cells were treated with TSA 4 h following transfection. Cells were harvested at 24 h following TSA treatment, and CAT assays were performed as described under “Experimental Procedures.” CAT assay results were analyzed by TLC, and the TLC plate was quantitated directly using an alpha imager system.

**RESULTS AND DISCUSSION**

**TSA Induces Accumulation of Acetylated Histones in Chromatin Associated with the RII Gene**—TGF-β receptors RI and RII are essential for TGF-β-mediated growth suppression of normal epithelial and some cancer cells. TGF-β resistance due to loss of expression of RI or RII has been linked to tumor formation and progression (7–9). Ectopic RII expression in receptor-deficient cancer cells reduced tumorigenicity in athymic nude mice, thus indicating the role of RII as a tumor suppressor (7, 8). MCF-7L breast and MIA PaCa-2 pancreatic cancer cells acquire resistance to growth inhibition by TGF-β due to reduced transcription of RII (15, 16). Recent studies (16, 19, 20, 23, 24) indicated DNA methylation and histone deacety-
lation as modes of inactivation of several genes. ChIP analysis was used to examine the effect of HDAC inhibition on the acetylation of histones H3 or H4 associated with the RII gene promoter. Chromatin fragments from cells cultured with or without TSA for 24 h were immunoprecipitated with antibodies to acetylated histones H3 or H4. DNA from the immunoprecipitate was isolated, and PCR using RII promoter primers was performed (Fig. 1). Accumulation of RII with highly acetylated histones H3 and H4 was observed in TSA-treated MCF-7L and MIA PaCa-2 cells in comparison to untreated control cells. The accumulation of acetylated histones H3 and H4 indicated histone deacetylation was involved in the transcriptional repression of RII. The TSA effect on RII is selective because the β-actin gene was not affected. The transcription of RII promoter may be repressed by a compact chromatin structure, which is maintained by increased HDAC activity in MCF-7L cells.

**TSA Effects Are Mediated by a GC Box on the RII Promoter**—TSA-treated MCF-7L cells showed enhanced RII mRNA expression (data not shown). To determine whether the enhanced RII expression levels following TSA treatment were due to increased RII transcription we analyzed RII promoter activities using an RII promoter-luciferase reporter construct in control and TSA-treated MCF-7L cells. The RII promoter exhibited enhanced activity in the presence of TSA (Fig. 2). The RII promoter lacks a distinct TATA box and is highly GC-rich. It contains two GC boxes at −25 bp and −143 bp relative to the transcription start site, which have been characterized as Sp1-binding sites (17). We have shown previously that the GC box at −25 bp is critical for RII promoter activity in MCF-7L cells (15). This site also mediates the transcriptional repression of RII by Sp3 (22). To determine whether the TSA effects are mediated through this GC box on the RII promoter, we analyzed the activities of wild type (−47 bp RII-CAT) and mutant GC box (−47 bp Spm RII-CAT) RII promoter constructs in control and TSA-treated MCF-7L cells. Although the activity of the wild type GC box RII promoter was up-regulated in the presence of TSA, the mutant GC box RII construct was not modulated, thus confirming that TSA effects are mediated through this GC box (Fig. 3).

**Effect of TSA on Sp1 and Sp3 Binding Affinities and Their Association with HDAC1 and p300**—We previously reported (15, 16) that MCF-7L and MIA PaCa-2 cells express reduced levels of Sp1 protein. In addition MCF-7L cells express high levels of Sp3 protein, which acts as a transcriptional repressor of RII (18). Inhibition of DNA methylation by 5-azacytidine induced RII expression through a combination of increased Sp1 and decreased Sp3 binding affinities (19). To determine whether the TSA-mediated RII expression is through modulation of Sp1 and Sp3 binding affinities, we carried out electrophoretic mobility shift assays on control and TSA-treated MCF-7L nuclear extracts using 32P-labeled consensus Sp1 oligonucleotide. Both the control and TSA-treated MCF-7L nuclear extracts showed the high Sp3 binding and low Sp1 binding pattern we had previously observed in these cells (18). This indicated TSA treatment did not enhance transcription through modulation of Sp1 and Sp3 binding affinities (data not shown). ChIP analysis using Sp1/Sp3 antibodies also did not show any change in the Sp1/Sp3-associated RII promoter DNA in TSA-treated MCF-7L cells (data not shown). Consequently, TSA mediates RII promoter activities by a mechanism other than alteration of the DNA binding activities of Sp1 and Sp3. This data is consistent with several other reports indicating

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**Fig. 5. TSA induces acetylation of Sp3.** Nuclear extracts from control and TSA-treated MCF-7L cells were immunoprecipitated with rabbit anti-human Sp3 polyclonal antibody, and the immunoprecipitates were resolved by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with rabbit anti-human pan-acetyl lysine antibody or goat anti-human Sp3 polyclonal antibody. Ac Sp3, acetylated Sp3.

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**Fig. 6. Ectopic Sp3 affects RII promoter activity.** The RII promoter-Luc reporter or control PGL2 vector without RII promoter and CMV-Sp3 vector along with a β-galactosidase plasmid was transiently transfected into Sp3-deficient MCF-7E cells. Cells were treated with TSA 4 h following transfection. Cells were harvested 24 h following TSA treatment, and luciferase activity was determined following normalization to β-galactosidase.

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**Table 1.** Summary of TSA effects on Sp1 and Sp3 binding affinities and their association with HDAC1 and p300.
Acetylation Controls Sp3 Function

Histone deacetylase inhibitors induce the expression of target genes without altering the Sp1/Sp3 binding affinities (23–25). The mechanism of RII induction by TSA may involve modifications of Sp1 and/or Sp3 proteins, alterations in their interaction with other proteins, or modulation of proteins directly or indirectly interacting with Sp1 and/or Sp3. Co-immunoprecipitation experiments using Sp1/Sp3 and HDAC1/p300 antibodies indicated that Sp1 as well as Sp3 interacts with HDAC1 and p300. However, these interactions were not affected by TSA, thus ruling out alterations in the association of Sp1/Sp3 and HDAC activity in MCF-7L cells. Thus, we hypothesized that TSA was acting by inhibiting HDAC enzymatic activity associated with Sp1 and Sp3.

Sp1/Sp3 Associates with Histone Deacetylase Activity—To test whether Sp1/Sp3 associates with an active histone deacetylase, we immunoprecipitated endogenous Sp1/Sp3 from MCF-7L nuclear extracts using anti-Sp1 and anti-Sp3 or control IgG antibodies. The precipitated complexes were tested for their ability to deacetylate an acetylated histone substrate (Fig. 4). We showed that Sp1 as well as Sp3 associate with deacetylase activity, and this activity is abolished when the deacetylase inhibitor TSA is included in the deacetylation reaction, suggesting that the histone deacetylase activity associated with Sp1 and Sp3 is completely sensitive to TSA. TSA treatment suppresses the Sp1/Sp3-associated HDAC activity leading to a local disruption of the nucleosome structure of the RII promoter by acetylation of histones H3 and H4. It is interesting to note that TSA induced RII expression in MCF-7L cells without decreasing Sp3 binding, because we have previously reported that Sp3 acts as a transcriptional repressor of RII in these cells (18). One plausible reason may be that unmodified Sp3 acts as a transcriptional repressor, and TSA-mediated Sp3 modification may convert Sp3 into transcriptional activator.

Sp3 Acetylation and RII Promoter Activity—The lysine residue in the inhibitory domain of Sp3 was shown to be susceptible to acetylation, and it was hypothesized that acetylation silences Sp3 activity (4). However, it was later reported that sumo modification of the same lysine residue of Sp3 silences Sp3 activity (5). Consequently, the functional role of Sp3 acetylation was unclear. We previously reported that unmodified Sp3 acts as a transcriptional repressor of RII in MCF-7L cells (18). To determine whether TSA-mediated Sp3 acetylation is involved in the transcriptional activation of RII, we analyzed the acetylation status of Sp3 using a pan-acetyl lysine antibody in control and TSA-treated MCF-7L cells. TSA induced acetylation of Sp3 in MCF-7L cells (Fig. 5). Sp3 expression levels were used to normalize protein. Acetylation of transcription factors such as p53, E2F1, Myo D, and EKLF has been shown to enhance transcriptional potency and affect protein-protein interactions (4). We have previously shown (18) that RII-positive MCF-7E breast cancer cells express Sp1 protein but were Sp3-deficient. To confirm that the TSA-mediated Sp3 modification affects RII promoter activity, we have analyzed effects of ectopic expression of Sp3 on the RII promoter activity in control and TSA-treated, Sp3-deficient MCF-7E breast cancer cells. Although ectopic Sp3 repressed RII promoter in the absence of TSA, Sp3 stimulated RII promoter activity in the TSA-treated cells (Fig. 6). Histone acetyltransferase p300 has been reported to acetylate Sp3 protein (4). Because MCF-7L cells express high levels of Sp3 and the protein was shown to repress RII promoter activity, we wanted to ascertain if histone acetyltransferase p300 was able to stimulate Sp3 transactivation of the RII promoter. We co-transfected wild type CMV-p300 or HAT domain deleted mutant p300 vector (CMV-p300ΔHAT) along with the RII promoter-luciferase construct in MCF-7L cells and analyzed the RII promoter activities (Fig. 7). The wild type p300 stimulated Sp3-mediated RII promoter activity but not the acetyltransferase activity null p300 mutant. This result suggests that p300 acts as a co-activator of Sp3 and/or possibly the acetylation activity of p300 is involved in the acetylation of Sp3 and the concomitant activation of RII promoter. Histone acetyltransferase p300 but not PCAF has been shown to acetylate Sp3 protein (4). PCAF has been shown to associate with NF-Y in the transcriptional activation of RII (27). It was also shown that binding to the GC box by Sp1/Sp3 was influenced by the presence of an intact NF-Y-binding site on the RII promoter (22). Consequently, it is plausible that p300-mediated Sp3 acetylation as well as PCAF and NF-Y association contributes to RII expression in TSA-treated MCF-7L cells. This is the first report indicating that acetylation turns Sp3 from a transcriptional repressor to transcriptional activator.
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REFERENCES
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