Restoration of Transforming Growth Factor-β Signaling through Receptor RI Induction by Histone Deacetylase Activity Inhibition in Breast Cancer Cells

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The loss of transforming growth factor-β (TGF-β) response due to the dysregulation of TGF-β receptors type I (RI) and type II (RII) is well known for its contribution to oncogenesis. Estrogen receptor-expressing breast cancer cells are refractory to TGF-β-mediated growth control because of the reduced expression of TGF-β receptors. Although RII is required for the binding of TGF-β to RI, RI is responsible for directly transducing TGF-β signals through the Smad protein family. Treatment of estrogen receptor-expressing MCF-7L and ZR75 breast cancer cells with the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) led to a dramatic induction of RI. Accumulation of acetylated histones H3 and H4 was observed in the SAHA-treated cells. Chromatin immunoprecipitation analysis followed by PCR with RI promoter-specific primers indicated an accumulation of acetylated histones in chromatin associated with the RI gene, suggesting that histone deacetylase inhibition was involved in the transcriptional inactivation of RI. SAHA treatment stimulated RI promoter activity through the inhibition of Sp1/Sp3-associated HDAC activity. Histone acetyltransferase p300 stimulated RI promoter activity, thus further confirming the involvement of HDAC activity in the transcriptional repression of RI. Significantly, SAHA-mediated RI regeneration restored the TGF-β response in breast cancer cells.

Transforming growth factor-β (TGF-β),1 a 25-kDa homodimeric polypeptide, plays an important role in the growth inhibition of most normal epithelial and some cancer cells (1). TGF-β functions through cell surface receptors referred to as type I (RI) and type II (RII). RI requires RII for the binding of TGF-β. However, RI is a direct player in the TGF-β signaling pathway as it conveys signals from TGF-β through the activation of Smad protein family members (2). The loss of TGF-β response is well known for its contribution to oncogenesis and tumor progression. Direct involvement of RI in TGF-β signal transduction would suggest that loss or reduced expression of RI could contribute to TGF-β resistance resulting in a growth advantage that contributes to tumor progression.

TGF-β resistance due to methylation of the RI promoter or RI promoter repression by Sp1 deficiency was reported to be a cause of TGF-β resistance in gastric and colon carcinomas (3, 4). The RI gene is frequently mutated in ovarian carcinomas (5). Decreased expression of RI is associated with poor prognosis in bladder transitional cell carcinomas (6). Reduced RI expression is also associated with unfavorable prognosis in esophageal squamous cell carcinoma (7). RI*6A, a polymorphic allele of RI, is emerging as a high frequency, low penetrance tumor susceptibility allele that predisposes to the development of breast, ovarian, and colorectal cancer, as well as hematologic malignancies (8). Polymorphisms in the microsatellite region of the RI gene were reported in head and neck cancers as well as non-small cell lung cancer (9, 10). Mutations in the kinase domain of the RI gene were associated with metastatic breast cancer (11). Consequently, loss of RI expression appears to play a significant role in determining the malignant phenotype of a broad variety of cancer cells. RI replacement restored TGF-β response in colon and pancreatic cancer cells (12, 13).

The promoter for the RI gene has been partially characterized (14). The RI promoter lacks a distinct TATA box, is GC-rich, and depends on the Sp1 transcription factor for the initiation of transcription. The RI promoter contains four consensus and several putative Sp1 sites. It also contains an inverted CCAAT box, a putative NF-Y binding site, and an AP-2 element. Although members of the Sp gene family of transcription factors, Sp1, Sp2, and Sp4, are known to be activators of gene transcription, Sp3 can either be an activator or a repressor (15). Sp1 and Sp3 transcription factors recognize the same DNA sequence and have similar DNA-binding affinities. We have reported previously that Sp1/Sp3 proteins control RI promoter activities (16).

There is growing evidence that changes in chromatin structure by histone modification appear to play an important role in the regulation of gene transcription. Acetylation of core nucleosomal histones is regulated by the opposing activities of histone deacetylases (HDACs) and histone acetyltransferases (HATs) such as CBP/p300 and P300/PCAF (17). Both HDACs and HATs are recruited to the target genes in complexes with sequence-specific factors and cofactors to regulate gene expression (18). A broad variety of human cancer cell lines, including estrogen receptor-expressing (ER+) breast cancer cell lines exhibit TGF-β resistance without detectable changes in the RI and RII genes (19, 20). These cells express low or reduced levels

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1 The abbreviations used are: TGF-β, transforming growth factor-β; RI and RII, TGF-β receptors type I and type II; SAHA, suberoylanilide hydroxamic acid; HDAC, histone deacetylase; ER+, estrogen receptor-expressing; CMV, cytomegalovirus; DBD, DNA-binding domain; CREB, cAMP-responsive element-binding protein; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor; HAT, histone acetyltransferase.
of RI and RII, suggesting that abnormalities in transcriptional regulation, alterations in mRNA processing, or mRNA instability might be contributing to reduced receptor expression and hence TGF-β resistance in these cells.

In this report, we present data indicating treatment of ER+ MCF-7L and ZR75 breast cancer cells with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) results in the accumulation of acetylated histones in the chromatin of the RI gene, and this increase is associated with an increased RI expression in breast cancer cells. RI promoter activity is regulated by Sp1/Sp3 transcription factors (16). SAHA treatment induced Sp1/Sp3-associated HDAC activity leading to enhanced RI promoter activity and the concomitant RI expression. The wild type p300 (but not the histone acetyltransferase activity mutant p300) stimulated RI promoter activity. These results demonstrate that histone acetylation/deacetylation plays a central role in the transcriptional regulation of the RI gene, and Sp1/Sp3-associated histone deacetylases contribute to the transcriptional repression of RI in breast cancer cells. Our data further suggest that TGF-β signaling could be rescued through HDAC inhibitor SAHA-mediated RI induction.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7L and ZR75 breast cancer cells were grown in McCoy’s 5A medium supplemented with 10% fetal bovine serum (Sigma), amino acids, antibiotics, pyruvate, and vitamins (Invitrogen). The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2. Wherever indicated, MCF-7L and ZR75 cells were treated for 24 h with 100 ng/ml SAHA.

Nuclear Extracts—Nuclear extracts from control and SAHA-treated MCF-7L cells were prepared using the following procedure. The cells were harvested by scraping, washed in cold phosphate-buffered saline, and incubated in 2 packed cell volumes of buffer 1 (10 mm HEPES, pH 7.9, 1 mM MgCl2, 10 mM KCl, 0.2 mM EDTA, 200 mM sucrose, 0.5 mM dithiothreitol, and 1.0 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 1 μg of leupeptin and aprotinin/ml, and 0.5% Nonidet P-40) for 5 min at 4 °C. The crude nuclei released by lysis were collected by microcentrifugation and resuspended in two-thirds of a packed cell volume of buffer 2 (20 mm HEPES, pH 7.9, 1.5 mM MgCl2, 420 mm NaCl, 0.2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 1.0 mM dithiothreitol, and 1.0 μg of leupeptin and aprotinin/ml). The nuclei were incubated on a rocking platform at 4 °C for 30 min and clarified by centrifugation for 5 min. The resulting supernatants were diluted 1:1 with buffer 3 (20 mm HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, and 1 μg of leupeptin and aprotinin/ml).

Western Blot Analysis—Nuclear extracts were obtained from control and SAHA-treated MCF-7L and ZR75 breast cancer cells. Equal amounts of nuclear extracts were resolved by 7.5% SDS-PAGE and then blotted with rabbit anti-human acetyl histone H3, histone H4, and HDAC1 antibodies (Upstate Biotechnology).

Reverse Transcriptase-PCR—Total RNA from control and SAHA-treated MCF-7L and ZR75 cells was reverse transcribed into cDNA. PCR analysis was then performed to determine the RI expression levels in control and SAHA-treated MCF-7L and ZR75 cells using the respective cDNAs as templates. Primers for actin were used for a control to determine the RI expression levels. Reverse transcriptase-PCR analysis allows a rough estimate of the changes in RNA levels to be obtained following SAHA treatment. A total of 30 cycles of amplification were performed. Primers for R1 generate a 865 bp fragment as follows: sense primers, 5'-TTG TGG CAC GGT GAG AGT GT3'- antisense primers, 5'-TGC TCC TGG GAT ATT GAA TCA-3'. Primers for actin generate a 621 bp fragment as follows: sense primers, 5'-AAG GGC CCA CCT CAT ACT CAT-3'; antisense primers, 5'-AGG GCC AGC CCT ACT CAT A-3'.

Chromatin Immunoprecipitation Assay—MCF-7L cells were plated at a density of 4 × 105 cells/15-cm dish and incubated overnight at 37 °C with 5% CO2. The next day, the cells were cultured with or without 100 ng/ml SAHA for 24 h. The chromatin immunoprecipitation assay was performed as described previously (21). RI and actin primers were used to determine RI expression levels in cDNA isolated from chromatin immunoprecipitation experiments. The optimal reaction conditions for PCR were determined for each primer pair. Parameters included denaturation at 95 °C for 1 min and annealing at 58 °C for 1 min following 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: RI promoter, sense: 5'-CTG GGG GCT GGG CAC AAA C-3'; antisense: 5'-GCC CTT TGG AAC TGC TCG GAG GAC-3'; β-actin, sense: 5'-CCA ACG CCA AAA CTC CC-3'; antisense: 5'-AGC CAT AAA AGG CAA CT-3'.

Transfections and Luciferase Assay—The RI (−618 bpRI-Luc) promoter-luciferase reporter construct was used to determine RI promoter activity (16). The RI-Luc construct and control null vector without the RI promoter insert (pGL3) were transiently transfected into MCF-7L breast cancer cells using the FuGENE 6 method (Roche Applied Science) with a β-galactosidase plasmid for normalization (16). The cells were treated with 100 ng/ml SAHA 4 h following transfection. The cells were harvested 24 h following SAHA treatment, and promoter activities were determined using a commercial luciferase assay (Luciferase Assay System, Promega). To determine the effects of histone acetyltransferase p300 on RI promoter activity, wild type p300 (CMV-p300) or HAT domain-deleted mutant p300 (CMV-p300ΔHAT) along with RI-Luc plasmid were transfected into MCF-7L cells. The cells were harvested 48 h following transfection, and luciferase activity was determined following normalization to β-galactosidase. To determine that p300-dependent RI promoter activity is also Sp1-dependent, wild type Sp1 (CMV-Sp1) or mutant Sp1 (CMV-Sp1 DBD) along with p300 (CMV-p300) and RI-Luc plasmid were transfected into MCF-7L cells. The cells were harvested 48 h following transfection, and luciferase activity was measured. The TGF-β-responsive plasminogen activator inhibitor promoter-luciferase reporter construct (p3TP-Lux) was used to determine TGF-β sensitivity as described previously (22). The TGF-β-responsive p3TP-Lux vector or pGL2-Lux null vector without the TGF-β response element was transiently transfected into MCF-7L cells. The cells were treated with 100 ng/ml SAHA 4 h following transfection. 24 h following SAHA treatment, the cells were treated with 5 ng/ml TGF-β for an additional 24 h, and the cells were lysed and luciferase activity determined following normalization to β-galactosidase.

Histone Deacetylase Assay—We immunoprecipitated endogenous Sp1/Sp3 from 500 μg of MCF-7L nuclear extracts using agarose-conjugated anti-rabbit Sp1/Sp3 polyclonal or control IgG antibodies. The beads were washed four times with 1 ml of phosphate-buffered saline and used for deacetylation activity using the HDAC fluorescent activity assay/drug discovery kit (AK-500; BIOMOL Research Laboratories). Briefly, the beads were incubated with 100 μM acetylated substrate in 100 μl of assay buffer containing or lacking 1 μM SAHA. Incubation of the reaction at 37 °C for 30 min allowed deacetylation of the substrate, which sensitized it to treatment with the developer and produced a fluorophore detectable on a fluorometric reader (excitation at 360 nm and emission at 450 nm).

RESULTS

Inhibition of Histone Deacetylation Activates RI Expression—Besides DNA methylation, another epigenetic mechanism by which gene expression can be repressed involves deacetylation of chromosomal histones. Hypoacetylated chromatin is transcriptionally silent (17). Inhibition of histone deacetylation can be accomplished by treatment with histone deacetylase inhibitors such as trichostatin A and SAHA (23). To determine whether SAHA induces acetylation of histones and the concomitant induction of RI expression, we have treated the ER+ MCF-7L and ZR75 breast cancer cells with 100 ng/ml SAHA for 24 h and performed immunoblot analysis on the nuclear extracts using antibodies to acetylated histones H3 and H4. Accumulation of acetylated histones was observed in SAHA-treated MCF-7L and ZR75 cells (Fig. 1a). HDAC1 expression levels were used to normalize protein. If histone deacetylation is associated with transcriptional repression, then histone acetylation following SAHA treatment should lead to RI expression. We isolated the total RNA from the control and the SAHA-treated MCF-7L and ZR75 cells and reverse transcribed the RNA into cDNA. PCR analysis using RI and actin primers was then performed. The RI message was induced in SAHA-treated MCF-7L and ZR75 cells and reverse transcribed the RNA into cDNA. PCR analysis using RI and actin primers was then performed. The RI message was induced in SAHA-treated MCF-7L and ZR75 cells (Fig. 1b). Reverse transcriptase-PCR analysis is a rough estimate of the changes in RNA levels following SAHA treatment. We have used MCF-7L cells in subsequent experiments to characterize the mechanism of RI repression in the breast cancer cells.
Effect on RI is selective because the involved in the transcriptional repression of RI. The SAHA tones H3 and H4 confirmed that histone deacetylation was untreated control cells. The accumulation of acetylated histones H3 and H4 in chromatin associated with the RI gene promoter. Chromatin fragments from control and SAHA-treated MCF-7L and ZR75 cells, and Western blot analysis was performed using anti-acetyl histone H3, anti-acetyl histone H4, and HDAC1 antibodies. Total RNA from control and SAHA-treated MCF-7L and ZR75 cells was reverse transcribed into cDNA, and PCR analysis was performed using primers for RI and actin as described under "Experimental Procedures."

SAHA Induces Accumulation of Acetylated Histones in Chromatin Associated with the RI Gene—Chromatin immunoprecipitation analysis was used to examine the effect of HDAC inhibition on the acetylation of histones H3 or H4 associated with the RI gene promoter. Chromatin fragments from MCF-7L cells cultured with or without SAHA for 24 h were immunoprecipitated with antibodies to acetylated histones H3 or H4. DNA from the immunoprecipitates was isolated, and PCR was performed using RI promoter primers (Fig. 2). Accumulation of RI with highly acetylated histones H3 and H4 was observed in SAHA-treated MCF-7L cells in comparison with untreated control cells. The accumulation of acetylated histones H3 and H4 confirmed that histone deacetylation was involved in the transcriptional repression of RI. The SAHA effect on RI is selective because the β-actin gene was not affected.

SAHA Treatment Affects RI Promoter Activity—SAHA-treated MCF-7L as well as ZR75 cells showed enhanced RI expression (Fig. 1b). To determine whether the enhanced RI expression levels following SAHA treatment were due to increased RI transcription, we analyzed RI promoter activities using an RI promoter-luciferase reporter construct in control and SAHA-treated MCF-7L cells. The RI promoter construct was described previously (14). The RI promoter lacks a distinct TATA box and contains two Sp1 binding sites. We reported previously that Sp1/Sp3 transcription factors control the activity of this RI promoter construct (16). The RI promoter exhibited enhanced activity in the presence of SAHA (Fig. 3), suggesting that stimulation of the RI promoter activity was contributing to enhanced RI expression.

Sp1/Sp3 Associates with Histone Deacetylase Activity—We performed co-immunoprecipitation experiments followed by Western analysis to determine whether Sp1/Sp3 transcription factors associate with HDAC1 in MCF-7L cells. Sp1 as well as Sp3 associated with HDAC1. However, this interaction was not disrupted in the presence of HDAC inhibitor, SAHA, thus ruling out alterations in the association of Sp1/Sp3 and HDAC1 as a cause for RI induction in MCF-7L cells (Fig. 4a). Consequently, we hypothesized that SAHA was acting by inhibiting HDAC enzymatic activity associated with Sp1 and Sp3. To determine whether SAHA stimulates RI promoter activity through the inhibition of Sp1/Sp3-associated histone deacetylase activity, we immunoprecipitated endogenous Sp1/Sp3 from MCF-7L nuclear extracts using anti-Sp1 and -Sp3 or control IgG antibodies. The precipitated complexes were tested for their ability to deacetylate an acetylated histone substrate (Fig. 4b). The results show that Sp1 as well as Sp3 associates with deacetylase activity, and this activity is abolished when the deacetylase inhibitor SAHA is included in the deacetylation reaction. These data suggest that the histone deacetylase activity associated with Sp1 and Sp3 is completely sensitive to SAHA.

Acetyltransferase p300 Stimulates RI Promoter Activity—Histone acetyltransferase p300 has been shown to up-regulate Sp1-dependent promoter activities (24). To ascertain whether the acetyltransferase activity of p300 was able to stimulate Sp1-dependent RI promoter activity, we co-transfected wild type CMV-p300 or HAT domain-deleted mutant p300 vector (CMV-p300ΔHAT) along with the RI promoter-luciferase construct in MCF-7L cells and analyzed the RI promoter activities. Western analysis indicated ectopic wild type p300 as well as mutant p300 are expressed in MCF-7L cells (Fig. 5a). Actin was...
used as a loading control. The wild type p300 stimulated Sp1-mediated RI promoter activity but not the acetyltransferase activity-null p300 mutant (Fig. 5b). This result suggests that p300 acts as a co-activator of Sp1-mediated RI promoter activity. To further determine that p300-dependent RI promoter activity is Sp1-dependent, we have analyzed the RI promoter activity using either wild type Sp1 or mutant Sp1 (Sp1 DBD) in which the DNA-binding domain is deleted. The p300 protein was able to up-regulate wild type Sp1-mediated RI promoter activity but not the DNA-binding domain-deleted mutant Sp1 (Fig. 5c), thus confirming that Sp1 and p300 collaborate in the trans-activation of RI promoter activity.

SAHA-mediated RI Induction Restores TGF-β Response—To evaluate whether SAHA-mediated RI expression in MCF-7L cells restored TGF-β sensitivity, TGF-β-dependent promoter activity was analyzed using the p3TP-Lux plasmid as a TGF-β-responsive element in tandem with a luciferase reporter. The TGF-β-responsive vector or null vector without the TGF-β-responsive element was transiently transfected into MCF-7L cells. Following transfection (4 h), the cells were treated with 100 ng/ml SAHA. 48 h after SAHA treatment, the cells were transfected with 5 ng/ml TGF-β for an additional 24 h, at which time the cells were lysed and luciferase activity determined following normalization to β-galactosidase (Fig. 6). RI expression restored TGF-β sensitivity, as indicated by the enhanced activity of the TGF-β response element in the SAHA-treated MCF-7L cells.

DISCUSSION

TGF-β plays a central role in the regulation of many cellular processes including the growth control of most normal epithelial cells and some cancer cells. TGF-β functions through the specific cell surface receptors RI and RII. RI plays a direct role
in the TGF-β signaling pathway through recruitment and activation of Smad protein family members. Consequently, loss or reduced expression of RI could generate TGF-β resistance resulting in a growth advantage that contributes to tumor progression. Losses of RI expression due to methylation, mutations, and polymorphisms in the RI promoter regions have been reported in a variety of cancers of epithelial origin (3, 5, 8–10). However, there are many cancer cell lines including ER+ breast cancer cells that show TGF-β resistance without changes in the RI gene structure, suggesting that abnormalities in the transcriptional or post-transcriptional regulation could be a contributing factor for reduced RI expression.

Besides DNA methylation, another epigenetic mechanism that frequently controls the transcriptional regulation of genes is the acetylation/deacetylation of chromosomal histones associated with target genes (23). In this study, we have shown that treatment of ER+ breast cancer cells with the HDAC inhibitor SAHA regenerates RI expression. The transcription of the RI gene may be repressed by a compact chromatin structure, which is maintained by increased HDAC activity in these cells. RII was induced to a lesser extent than RI. MS-275, another HDAC inhibitor, was shown to induce RI expression but not RI (25). This may be the result of the differential selectivity of the HDAC inhibitors used or the heterogeneity of the cells involved. Similar to many growth factors and their receptor gene promoters, the RI promoter also lacks a classical TATA box, is GC-rich, and depends on the Sp1 transcription factor for its activity. We reported previously that Sp1/Sp3 proteins control RI promoter activity in ER+ breast cancer cells (16). The HDAC inhibitor SAHA stimulated RI promoter activity in breast cancer cells. Further, our data indicated inhibition of Sp1/Sp3-associated HDAC activity is the contributing factor for the up-regulation of RI promoter activity. SAHA treatment suppresses the Sp1/Sp3-associated HDAC activity leading to a local disruption of the nucleosome structure of the RI promoter by acetylation of histones H3 and H4.

The finding that the p300 protein possesses intrinsic acetyltransferase activity and by chemically modifying histone tails affects the nucleosomal environment and transcription has greatly advanced our understanding of its function. Subsequent studies revealed that non-histone proteins are also acetylated by p300, e.g. p53, Sp1, and Sp3 proteins (18, 26). The present model of promoter recognition and nucleosomal remodeling involves the recruitment of acetyltransferases such as CBP/p300 and PCAF to the promoter by DNA-binding activators such as Sp1/Sp3, which results in a remodeling of the nucleosomal structure by acetylyating histones, possibly in concert with non-histone proteins (e.g. DNA-binding activators Sp1/Sp3) to allow for the initiation of transcription. Our data indicated that wild type p300 stimulates Sp1-dependent RI promoter activity but not the acetyltransferase activity-defective mutant p300. The p300 and Sp1 association was also shown to contribute to Sp1-dependent p21 promoter activity (24). PCAF and NF-Y association was reported to activate RII promoter activity (27). However, how the co-activator/acyetyltransferase interacts with DNA-binding activators to modulate their actions in the context of promoter access is not completely understood. DNA-binding activators bind in a sequence-specific manner to their cognate binding sites in enhancers and core promoter regions. DNA-binding activators with common DNA-binding domains often bind similar DNA sequences. However, additional regulatory steps must be present, as the complexity of these factors in undertaking specific functions cannot be readily explained by sequence-specific DNA-binding properties alone. Regulation through differential protein-protein interactions and/or chemical modifications, such as acetylation and phosphorylation, are likely to contribute to their different functions. Transcription factors Sp1/Sp3 have been reported to undergo post-translational modifications such as glycosylation, phosphorylation, and acetylation (15). Although Sp1 is known to be an activator of gene transcription, Sp3 is bifunctional and can either activate or repress target gene expression (15). For example, we reported previously that although unmodified Sp3 acts as a transcriptional repressor of TGF-β receptors, acetylated Sp3 acts as a transcriptional activator (16, 21).

HDAC inhibitor (SAHA)-mediated RI expression resulted in the restoration of TGF-β response as evidenced by the enhanced activity of a TGF-β-responsive plasminogen activator inhibitor promoter-luciferase reporter in the SAHA-treated breast cancer cells. Consequently, this result suggests that TGF-β tumor-suppressive function can be regained following regeneration of RI expression in ER+ breast cancer cells.
SAHA Induces RI Expression
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Induction by Histone Deacetylase Activity Inhibition in Breast Cancer Cells

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