Site-specific Acetylation of p53 Directs Selective Transcription Complex Assembly

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Histone deacetylase (HDAC) inhibitors are being investigated as possible adjuvant therapies for a number of diseases, including cancer. In addition to stabilization of acetylated histones, HDAC inhibitors stabilize the acetylation of a number of transcription factors, including p53. This study investigates the action of two HDAC inhibitors, CG-1521 and trichostatin A, which stabilize Ac-Lys-373 p53 and Ac-Lys-382 p53, respectively, in LNCaP prostate cancer cells. Real-time PCR demonstrates that CG-1521 induces \( p21 \) transcription whereas trichostatin A does not alter the steady state level of \( p21 \) mRNA. Co-immunoprecipitation demonstrates that the selective acetylation of p53 directs the recruitment of mutually exclusive co-activator complexes on the p53 response elements in the \( p21 \) promoter. Furthermore, the co-activator complexes initiate the recruitment of the components of the basal transcription apparatus to the \( p21 \) promoter with markedly different outcomes because only Ac-Lys-373 p53 promotes the assembly of the basal transcriptional apparatus on the \( p21 \) promoter. These data highlight the profound effects of post-translational modification, including acetylation, on the function of p53. The data also suggest a novel and critically important role for protein acetylation/deacetylation in the assembly of active transcription processes that may be as important as classical phosphorylation/dephosphorylation.

Histone deacetylases (HDACs) are a family of evolutionarily conserved enzymes that act in concert with histone acetyltransferases (HATs) to modulate the acetylation status of histones and a number of other regulatory and structural proteins. In humans at least 14 HDACs have been identified that can be divided into two or three classes based on phylogenetic analysis (1). These proteins have been localized in both the cytoplasm and nucleus, suggesting that they have diverse cytoplasmic and nuclear substrates. HDAC inhibitors (HDI) have shown considerable promise as therapeutic agents for the treatment of cancer and a number of other diseases. The most studied HDIs, such as sodium butyrate, trichostatin A (TSA), suberoylanilide hydroxamic acid, valproic acid, and trapoxins, have divergent structures, and to date there is little information regarding the structure-function relationship of HDIs, the specificity of the HDIs for individual HDACs, or the biological processes these compounds affect, even though some of these compounds are in phase I and II clinical trials (2). The efficacy of individual HDIs is likely to be dependent on which HDACs are expressed in the target tissue and the selectivity of the inhibitors for the specific HDACs present in the cell.

The \( p53 \) tumor suppressor exerts antiproliferative effects, including growth arrest and apoptosis, in response to various types of stress (3). Mutations within the \( p53 \) gene have been documented in more than half of all human tumors, and even in cells that retain wild-type \( p53 \) other defects in the \( p53 \) pathway play an important role in tumorigenesis (4). Most of the tumor-suppressive functions of \( p53 \) have been attributed to the ability of the protein to act as a transcriptional modulator of downstream target genes, particularly those involved in cell cycle arrest and apoptosis (5, 6), although the precise mechanism by which \( p53 \) is activated by cellular stress is not fully understood. It is generally accepted that post-translational modifications of \( p53 \), including phosphorylation and acetylation, play important roles in the stabilization and activation of \( p53 \) (7). Several lysine (Lys) residues in the C-terminal domain of \( p53 \) can be acetylated, including Lys-320, Lys-373, and Lys-382 (8, 9). Because these sites are also the target for mdm2-mediated ubiquitination, acetylation of these sites leads to an increase in the steady state level of \( p53 \). Thus HDIs that prevent the deacetylation of the protein effectively stabilize the acetylated isoforms of \( p53 \) by blocking mdm2-mediated ubiquitination (10). In addition to increasing the steady state level of \( p53 \), acetylation stimulates the sequence-specific binding of \( p53 \) and its transcriptional activation of downstream target genes such as \( p21 \) (11, 12); however, the identities of the specific HATs and HDAC enzymes that modulate the acetylation status of \( p53 \) protein and the functions of the acetylated isoforms in transcriptional regulation are still largely unknown. We have previously shown that two HDIs, CG-1521 (7-phenyl-2,4,6-hepta- trienoic hydroxamic acid) and TSA, stabilize acetylation of \( p53 \) at Lys-373 and Lys-382, respectively, in LNCaP prostate cancer cells. In these cells, acetylation of Lys-373 \( p53 \) correlates with both cell cycle arrest and cell death, whereas acetylation of Lys-382 \( p53 \) only correlates with cell cycle arrest (13). This suggests that the selective stabilization of the acetylated isoforms by individual HDIs modulates the expression of different subsets of genes.
In this report we show that site-specific stabilization of acetylated p53 results in the formation of mutually exclusive transcriptional complexes that are differentially recruited to the p53 response elements (p53REs) in the promoter of the p21 gene, leading to profoundly different effects on the assembly of the basal transcriptional apparatus. These data provide a mechanistic explanation for the selective effects of these two HDIs and suggest that the design of selective HDIs will provide exceptional opportunities for tailored therapies based on the knowledge of which HDACs are active in target cells.

EXPERIMENTAL PROCEDURES

Cell Culture—LNCaP human prostate cancer cells, obtained from American Type Culture Collection (Rockville, MD), were cultured in RPMI 1640 (Invitrogen) with 10% fetal bovine serum (Atlas, Fort Collins, CO). Cells were routinely passaged every 3–4 days.

Drug Treatments—LNCaP cells were treated with 7.5 \( \mu \text{M} \) CG-1521 (Errant Gene Therapeutics, LLC, Chicago, IL) or 5 \( \mu \text{M} \) TSA (T8552; Sigma) dissolved in MeSO.

Nuclear Fractionation—Cells were scraped, pelleted by centrifugation at 1500 rpm for 3 min at 4 °C, resuspended in ice-cold wash buffer (25 mM Tris, pH 7.5, 250 mM sucrose, 2.5 mM MgCl\(_2\), 10 mM benzamidine, 10 mM NaF, 1 mM sodium vanadate, 10 \( \mu \text{g/mL} \) leupeptin, 10 \( \mu \text{g/mL} \) aprotinin, 1 \( \mu \text{g/mL} \) pepstatin, and 1 mM phenylmethylsulfonyl fluoride), and pelleted at 1500 rpm for 3 min at 4 °C. Pellets were resuspended with three volumes of Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM sodium vanadate, 1 mM dithiothreitol, 25 \( \mu \text{g/mL} \) leupeptin, 25 \( \mu \text{g/mL} \) aprotinin, 25 \( \mu \text{g/mL} \) pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, and 20 mM NaF) and lysed with a Dounce homogenizer. Homogenates were centrifuged twice at 1500 rpm for 3 min at 4 °C, resuspended in ice-cold phosphate-buffered saline and once with ice-cold phosphate-buffered saline-Tween. Cross-linking was stopped by addition of glycine to 125 mM final concentration and formaldehyde solution for 10 min at 37 °C. Cross-linking was performed using the SYBR Green reagents (N8080234; Applied Biosystems, Foster City, CA). The reaction mixture was incubated for 10 min at 25 °C, 1 h at 37 °C, and 5 min at 95 °C and kept at 4 °C until further analysis. Each sample was replicated three times from three independent sets of RNA preparations.

Quantitative Real-time PCR—Real-time SYBR Green probes for p21 gene were designed using Primer Express\textsuperscript{TM} 1.5 (Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA). p21 forward 5’-CCT AAT CCG CCC ACA GGA A-3’ and p21 reverse 5’-ACC TCC GGG AGA GAG GAA AA-3’ SYBR Green reactions were performed using the SYBR\textsuperscript{®} Green PCR Master Mix (4309155; Applied Biosystems). SYBR Green reactions were performed using an ABI Prism\textsuperscript{™} 7700 Sequence Detector (Applied Biosystems) and incubated for 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C, and 1 min at 60 °C for 50 cycles. Relative expression levels of p21 in real time were analyzed using the 2\(^{-\Delta\Delta CT}\) method (14) and presented as ratio to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Each sample was replicated twice from three independent sets of RNA preparations. Results are tabulated as mean ± S.E. of three independent experiments. Means were considered statistically significant (*) at \( p < 0.05 \).

Chromatin Immunoprecipitation Assay—LNCaP cells were plated at a density of 1 \( \times 10^6 \) cells/dish in 20-mm dishes (08772E; Fisher Scientific), treated with 7.5 \( \mu \text{M} \) CG-1521 or 5 \( \mu \text{M} \) TSA, and harvested after 8 h of treatment. Cells were scraped from the dish and collected by centrifugation at 1200 rpm for 5 min at 4 °C. The cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in lysis buffer (10 mM Tris, pH 8.0, 1% NP-40, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 \( \mu \text{g/mL} \) aprotinin, 1 \( \mu \text{g/mL} \) pepstatin A). Cells were then pelleted at 12,000 rpm for 5 min at 4 °C. Chromatin immunoprecipitation was performed using the chromatin immunoprecipitation (ChIP) assay kit (17-295; Upstate Biotechnology). 1.5% of the diluted sample was set

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Aside as the input DNA. For immunoprecipitation, the sample was pre-cleared for 30 min at 4 °C with 75 μl of salmon sperm DNA/protein A-agarose-50% slurry (16-157C; Upstate Biotechnology) before addition of indicated antibodies. The antibodies used for the immunoprecipitation assay were: anti-total p53 mouse monoclonal (05-224; Upstate Biotechnology), anti-Ac-Lys-373 p53 rabbit polyclonal (06-916; Upstate Biotechnology), anti-Ac-Lys-382 p53 rabbit polyclonal (01-010-179M; American Proteomics, Carlsbad, CA), anti-p300 mouse monoclonal (554215; BD Biosciences), anti-CBP rabbit polyclonal (SC-369; Santa Cruz Biotechnology), anti-HDAC1 mouse monoclonal (05-614; Upstate Biotechnology), anti-HDAC2 rabbit polyclonal (07-222; Upstate Biotechnology), anti-Pol II (H-224) rabbit polyclonal (SC-9001; Santa Cruz Biotechnology), anti-TBP mouse monoclonal (AB818; Abcam Inc., Cambridge, MA), or anti-TFIH (Q-19) rabbit polyclonal (SC-292; Santa Cruz Biotechnology). 2 μg of each antibody were added to the reactions and incubated overnight at 4 °C. An isotype control was performed in the presence of the respective IgGs. Collection of the antibody-histone complex was performed by addition of 60 μl of salmon sperm DNA/protein A-agarose-50% slurry for 1 h at 4 °C. Agarose beads containing the antibody-histone complex were collected by gentle centrifugation (3000 × g for 1 min at 4 °C) and washed several times, and immunocomplexes were eluted twice for 15 min each at room temperature with 1% SDS and 0.1 M NaHCO₃. Cross-linking was reversed by addition of 20 μl of 5 M NaCl (20-159; Upstate Biotechnology) to 500 μl of eluates and incubating for 4 h at 65 °C. DNA was purified by phenol-chloroform extraction, and 1 μg of purified DNA was used as a template in PCR reactions using primers for −1.3 kb p53RE, −2.4 kb p53RE, and p21 basal promoter (15) and −5 kb control region in the p21 promoter (16) (supplemental data). All reactions were incubated initially for 5 min at 95 °C and then for 45 s at 95 °C, 1 min at 61.5 °C (65.5 °C for basal promoter), and 1 min at 72 °C for 35 cycles, with the final extension for 7 min at 72 °C. Amplification products were electrophoresed in 2% agarose gels and visualized by ethidium bromide. 1.5% of total input DNA was subjected to PCR (Input). PCR products for −1.3 and −2.4 kb p53REs, −5 kb control region, and p21 basal promoter were 197, 282, 500, and 214 bp, respectively, and were quantified by densitometry using Scion Image software (Scion Corp, Frederick, MD). Percentage of immunoprecipitated DNA was calculated by comparison to input signals and plotted as bar graphs with statistical significance (*) measured at p < 0.01. Data represent the average of three independent sets of experiments.

Statistical Analysis—All data are expressed as the mean ± S.E. One-way analysis of variance was used to assess statistical significance between means. Differences between means were considered significant when p < 0.05 using the Bonferroni post-test. All statistical analyses were performed with the GraphPad Instat software (Intuitive Software for Science, San Diego, CA).

RESULTS

Ac-Lys-373 p53 and Ac-Lys-382 Associate with Mutually Exclusive Transcription Factors—Treatment with CG-1521 induces the formation of Ac-Lys-373 p53, whereas treatment with TSA induces Ac-Lys-382. Analysis of the phosphorylation status of p53 after treatment shows that Ac-Lys-373 p53 is also phosphorylated at Ser-15, whereas Ac-Lys382 p53 is not (Fig. 1A). The stabilization of Ac-Lys-373 p53 in response to CG-1521 correlates with the substantial (20-fold) increase in the steady state levels of p21 mRNA, measured by real-time PCR (Fig. 1B), suggesting that CG-1521 up-regulates p53-mediated p21 transcription. In marked contrast TSA does not alter the steady state level of p21 mRNA at any time point, suggesting that the post-trans-
lational modifications induced by these HDIs have very different effects on the transcriptional activity of p53.

Co-immunoprecipitation using site-specific antibodies demonstrates that treatment with CG-1521 or TSA induces a striking segregation of transcription factors associated with Ac-Lys-373 p53 (after treatment with CG-1521) and Ac-Lys-382 p53 (after treatment with TSA) (Fig. 2A). In particular, p300 is found exclusively associated with Ac-Lys-373 p53, whereas CBP is only bound to Ac-Lys-382 p53. Brg-1 and Brm-1, components of the SWI/SNF complex, also segregate in mutually exclusive fashion. Baf60, a regulatory subunit of the Brg-1 complex, segregates with Ac-Lys-373 p53, whereas Brm-1 associates exclusively with Ac-Lys-382 p53. The androgen receptor (which is itself acetylated) and the forkhead transcription factors Foxa1 and Foxm1 also segregate only with complexes containing Ac-Lys-373 p53. Because Pol II and TFIIH, components of the basal machinery, are associated with both Ac-Lys-373 p53 and Ac-Lys-382 p53, both complexes appear to be capable of initiating transcription. Simultaneous treatment of LNCaP cells with CG-1521 and TSA shows that acetylation of the Lys-373 and Lys-382 sites is not mutually exclusive and p53 can be simultaneously acetylated at both sites (Fig. 2B). However, because the dual acetylated Ac-Lys-373/382 p53 associates preferentially with p300 and not CBP, modification of Lys-373 appears to dictate the assembly of transcriptional complexes.

Acetylated p53 Isoforms Assemble Different Cofactor Complexes on the p21 Promoter—We have used ChIP to investigate the assembly of transcription factors on the p21 promoter after treatment with CG-1521 or TSA. The p21 promoter contains two well-characterized p53REs at −2.4 and −1.3 kb upstream of the transcription start site (15) (Fig. 3A). The relative enrichment of specific transcription factors at each site after treatment with CG-1521 and TSA was determined after chromatin immunoprecipitation using PCR primers flanking these two sites. Prior to treatment, p53 is associated mainly with the −2.4 kb site. Treatment with CG-1521 significantly increases the binding of Ac-Lys-373 p53 at both p53REs, but most significantly at the −1.3 kb site. In contrast, TSA slightly increases the binding of Ac-Lys-382 p53 to the −2.4 kb site and to a lesser extent to the −1.3 kb site (Fig. 3B).
FIGURE 4. Association of transcription factors with p21 promoter after treatment with CG-1521 or TSA. A, schematic of the p21 promoter showing the TATA box, −1.3 and −2.4 kb p53 response elements, and control region at −5 kb. B, protein extracts from untreated (−) or CG-1521- and TSA-treated (+) LNCaP cells after 8 h of treatment were subjected to ChIP analysis of the proximal promoter using antibodies against p300, CBP, HDAC1, HDAC2. C, ChIP analysis of the basal promoter using antibodies against Pol II, TBP, and TFIIH. Control immunoprecipitations with normal IgGs were also performed (Mock).
As shown in Fig. 4B, the two acetylated p53 isoforms recruit different cofactor complexes to the p53REs. Ac-Lys-373 p53 selectively recruits p300 to −2.4 and −1.3 kb p53REs, whereas Ac-Lys-382 p53 recruits CBP to the two sites. This is entirely concordant with the co-immunoprecipitation data presented in Fig. 2A. Although both HDAC1 and HDAC2 are associated with −2.4 and −1.3 kb sites prior to treatment, Ac-Lys-373 p53 selectively stabilizes the binding of HDAC1 to both p53REs, whereas HDAC2 is not found in the complexes on either response element. This is consistent with our earlier observation that treatment with CG-1521 leads to rapid degradation of HDAC1 (13). TSA on the other hand stabilizes Ac-Lys-382 p53 and enhances the formation of transcription complexes containing CBP and HDAC2, but not HDAC1.

As shown in Fig. 4C, these complexes have markedly different abilities to recruit components of the basal transcription apparatus, namely TATA box-binding protein (TBP), RNA polymerase II, and TFIIH. Prior to treatment, RNA Pol II and TBP already associate with the basal promoter of the p21 gene but TFIIH is not associated with the complex. After treatment with CG-1521, there is a substantial increase in binding of TBP to the basal promoter and TFIIH is recruited to the basal promoter, coinciding with a decrease in RNA Pol II occupancy, which is consistent with the initiation of transcription. In contrast, after treatment with TSA, neither TBP nor TFIIH is associated with the basal promoter and binding of RNA Pol II to the basal promoter is significantly reduced. This most likely reflects the decreased occupancy of the TATA box by TBP.

These data indicate that a preassembled initiation complex containing a paused RNA Pol II is present at the p21 promoter. Binding of Ac-Lys-373 p53 and its associated cofactors (including p300 and HDAC1), to the p53REs leads to the recruitment of TFIIH to the basal transcriptional complex and activation of RNA Pol II. In contrast, even though Ac-Lys-382 p53 and its associated cofactors (CBP and HDAC2) bind to the p53REs, the complexes do not initiate the assembly of RNA Pol II on the basal promoter (Fig. 5).

**DISCUSSION**

The importance of histone acetylation in the epigenetic regulation of gene expression has been recognized for many years (17), and the role of HATs and HDACs in regulating chromatin conformation has been studied extensively (18, 19). It has also become apparent over the last five years that many nuclear and cytoplasmic substrates are also acetylated. This includes transcription factors such as p53 (20), the forkhead transcription factors (21), and nuclear steroid receptors including the androgen receptor (22), structural proteins such as tubulin (23, 24), and signaling molecules such as PTEN (25). This has led to the realization that acetylation is an important intracellular signaling pathway analogous to phosphorylation (20).

LNCaP cells express several HATs, including P/CAF, p300, CBP, and Tip60 (26, 27), as well as the zinc-dependent class I and II HDACs 1, 2, 3, and 4 (13, 28) and the NAD+-dependent class III enzyme Sirt1 (29). Thus, the effect of individual HDIs on these cells represents a composite effect on the biology of cells that have similar HATs and HDACs. Comparison of the effects of CG-1521 and TSA, two hydroxamic acid-based inhibitors, has shown that despite the similarity in structure these compounds have very different effects on LNCaP cells. CG-1521 induces G2/M arrest and apoptosis, whereas TSA induces G1/S arrest but does not induce apoptosis (13). These two compounds induce markedly different effects on the acetylation status of p53: CG-1521 stabilizes Ac-Lys-373 p53 whereas TSA stabilizes Ac-Lys-382 p53. Our previous studies have shown that Lys-320 is constitutively acetylated in LNCaP cells and the acetylation at Lys-320 is not affected by CG-1521 or TSA (13). Acetylation of p53 at either Lys-373 or Lys-382...
blocks the mdm2-mediated ubiquitinylation of p53, which increases the steady state level of the acetylated proteins. However, as the data presented here show, the differences in acetylation status are not due solely to the increased steady state levels of p53. The ability of the different acetylated isoforms to recruit distinct co-activator complexes to the p21 promoter is the major factor in determining the downstream events associated with these HDIs. Ac-Lys-373 p53 recruits p300, forkhead transcription factors, the androgen receptor, and Brg-1. When assembled on the p53REs in the p21 promoter, this complex induces the assembly of TBP, RNA Pol II, and TFIIH on the basal promoter and initiates p21 transcription. In contrast, Ac-Lys-382 p53 recruits CBP and Brm-1 to the p53REs in the proximal p21 promoter, but this complex does not recruit TBP, RNA Pol II, or TFIIH to the basal promoter, suggesting that Ac-Lys-382 p53 promotes the assembly of a non-functional complex on the p21 promoter. Thus, in addition to the general effects on steady state mRNA levels, these HDIs have specific effects on the assembly of transcription complexes, at least on the p21 promoter. These data lend support to the concept proposed by Knights et al. (30) that distinct p53 acetylation cassette influence gene expression. Genome-wide array suggests that, in addition to p21, CG-1521 modulates the expression of 233 genes in total, of which a highly circumscribed group of 40 genes are involved in cell cycle, cell proliferation, or cell death. In contrast, TSA modulates over 1700 genes, 1400 of which are down-regulated. Of these, 150 genes are associated with cell cycle, cell proliferation, or apoptosis as described by Gene Ontology (GeneSpring GX 7.2 software; Agilent Technologies). This extensive transrepression of gene expression has also been noted in the p53-mediated response to hypoxia (31). Although it is tempting to attribute the dramatic difference in the effects of CG-1521 and TSA solely to the site of acetylation in the C-terminal domain of p53, Ac-Lys-373 p53 is also selectively phosphorylated in the N-terminal domain at Ser-15. Based on the available evidence it is not possible to determine whether one or both post-translational modifications are necessary for the assembly of the active transcription complex on the p21 promoter. Nevertheless, these data suggest that the effects of CG-1521 and TSA are attributable, at least in part, to the selective effects of the HDIs on HDAC activities that lead to differences in the p53-mediated induction of p21 transcription. However, because HDACs have multiple target proteins and the specificity of the HDIs has not been precisely delineated, it is not possible to exclude the possibility that the overall biological effect of these compounds, particularly the global effects of TSA, is due to the acetylation of other transcription factors.

In summary, although the interactions between HATs, HDACs, and HDIs are clearly complex and not fully understood, the data presented here demonstrate that selective inhibition of HDACs is possible and can lead to significant changes in gene expression through the acetylation of transcription factors such as p53. This holds out promise that tailored therapies based on a more refined knowledge of the cellular HDAC composition will be possible.

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