Overexpression of the Oncoprotein Prothymosin α Triggers a p53 Response that Involves p53 Acetylation

Takahiko Kobayashi,[1] Ting Wang,[5] Masaji Maezawa,[1] Masanobu Kobayashi,[2] Shunsuke Ohnishi,[3] Kazuteru Hatanaka,[3] Shuhei Hige,[3] Yuichi Shimizu,[3] Mototsugu Kato,[3] Masahiro Asaka,[3] Junji Tanaka,[4] Masahiro Imamura,[4] Kiminori Hasegawa,[7] Yoshiyuki Tanaka,[6] and Rainer K. Brachmann[8]

[1]Hokkaido University Medical Hospital, Primary Care Medicine; [2]Division of Cancer Pathobiology, Institute for Genetic Medicine, Hokkaido University; Departments of [3]Gastroenterology and [4]Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; Departments of [5]Genetics and [6]Surgery, Washington University School of Medicine, St. Louis, Missouri; [7]Akita University School of Medicine, Akita, Japan; and [8]Division of Hematology and Oncology, Departments of Medicine and Biological Chemistry, University of California at Irvine, Irvine, California

Abstract

Activation of the tumor suppressor protein p53 is a critical cellular response to various stress stimuli and to inappropriate activity of growth-promoting proteins, such as Myc, Ras, E2F, and β-catenin. Protein stability and transcriptional activity of p53 are modulated by protein-protein interactions and post-translational modifications, including acetylation. Here, we show that inappropriate activity of prothymosin α (PTMA), an oncoprotein overexpressed in human cancers, triggers a p53 response. Overexpression of PTMA enhanced p53 transcriptional activity in reporter gene assays for p53 target gene promoters *hdm2, p21*, and *cyclin G*. Overexpressed PTMA resulted in increased mRNA and protein levels for endogenous p53 target genes, *hdm2* and *p21*, and in growth suppression. In contrast, reduction of endogenous PTMA through RNA interference decreased p53 transcriptional activity. Histone acetyltransferases (HATs) act as p53 coactivators and acetylate p53. PTMA, known to interact with HATs, led to increased levels of acetylated p53. PTMA did not increase the transcriptional activity of an acetylation-deficient p53 mutant, suggesting that p53 acetylation is an indispensable part of the p53 response to PTMA. Chromatin immunoprecipitation assays showed that excess PTMA associates with the *p21* promoter and results in increased levels of acetylated p53 at the *p21* promoter. Our findings indicate that overexpressed PTMA elicits a p53 response that involves p53 acetylation. (Cancer Res 2006; 66(6): 3137-44)

Introduction

Prothymosin α (PTMA) is a highly acidic protein considered to be a growth-promoting protein or oncoprotein because it can lead to cell proliferation in *in vitro* assays and is overexpressed in human cancers. Its high conservation in mammals and broad tissue distribution suggest that PTMA serves essential biological functions, and its exact physiologic roles continue to be elucidated (1, 2). PTMA acts both in the nucleus (3) and in the cytoplasm, where it was shown to negatively regulate proapoptotic pathways (4–6).

In the nucleus, PTMA affects the activity of specific transcription factors, including the estrogen receptor (7) and signal transducers and activators of transcription 3 (8). In the case of Nrf2, PTMA liberates Nrf2 from a Keap1-Nrf2 inhibitory complex under oxidative stress and contributes to Nrf2-dependent gene expression (9). Furthermore, PTMA is likely to have a broader effect on transcription, because it interacts with histones and affects chromatin remodeling processes (10–12). The effect of PTMA on chromatin remodeling is mediated, at least partially, through histone acetyltransferases (HATs). HATs function as transcriptional coactivators by acetylating histone tails and making promoters accessible to transcription factors and the transcriptional machinery (13). PTMA interacts with two HATs of high homology, CBP and p300 (14, 15), and CBP was recently shown to promote and stabilize the interaction of PTMA with the oncoprotein SET (16).

Similar to PTMA, p53, a central tumor suppressor protein and transcription factor with cellular effects quite opposite to PTMA, interacts with CBP and p300. p53 is activated in response to cellular stress signals and causes cell cycle arrest, apoptosis, DNA repair, and/or senescence by activating a number of target genes. p53 also represses genes (17) and is directly involved in cytochrome *c* release and apoptosis through interactions with Bcl-2 family members (18, 19). p53 stability and transcriptional activity are modulated by protein-protein interactions and post-translational modifications, such as phosphorylation and acetylation. p53 relies on several coactivators for full activity, including the HATs CBP, p300, and PCAF that also directly acetylate p53 (20–23).

We show here that inappropriate activity of the oncoprotein PTMA elicits a p53 response that is characterized by increased p53 transcriptional activity and involves acetylation of p53 at residues known to be acetylated by CBP, p300, and PCAF.

Materials and Methods

Cell culture and transfection. Human 293, A549, H1299, HCT116 *p53*$^{+/+}$, HCT116 *p53*$^{-/-}$, HepG2, Saos-2, and U2OS cells were grown in DMEM with 10% fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 μg/mL). Transient transfections were done with LipofectAMINE (Invitrogen, San Diego, CA) or Effecten (Qiagen, Chatsworth, CA).

Plasmids and plasmid constructions. The human *PTMA* cDNA was PCR amplified from EST clone AI289803 (Washington University Genome Sequencing Center, St. Louis, MO) with primers RB216 (GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGTCAGACGCAGCCGTAG) and RB217 (GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTCATCCTCGTCGGTC). The PCR product was subcloned into pDONR201 using the Gateway cloning

Requests for reprints: Takahito Kobayashi, Hokkaido University Hospital, Community Service Network, Kita 14 Nishi 5 Kita-ku, Sapporo 060-8648, Japan. Phone: 81-11-716-1161, ext. 5920; Fax: 81-11-706-7867; E-mail: 1961koba@med.hokudai.ac.jp.
©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-05-2112

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/6/3137/2558822/3137.pdf by guest on 12 April 2024

system (Invitrogen) to give PTMA-pCMVneo. The sequence of the entire cDNA was verified.

To generate *PTMA* short hairpin RNA (shRNA) plasmids, double-stranded oligonucleotides coding for siRNA that specifically target *PTMA* transcript (siPTMA1, 5′-ATCTAAACGTGGTCACCTT-3′ and siPTMA2, 5′-ATGACGATGTCGATACCAA-3′) were cloned into pcPURU6 (Takara, Tokyo, Japan) according to the manufacturer's protocol. Plasmids containing the inserts were confirmed by DNA sequencing.

Other plasmids used were pIC400 (24), pGL2hmdm2-HX-luc, pGL3-cyclin G-luc (25), pFLAG-PCAF (26), pBpuro (27), pTA-Luc, pp53-TA-Luc, pE2F-TA-Luc, pMyc-TA-Luc (Clontech, Palo Alto, CA), MG15-Luc, PG13-Luc, WWP-Luc, pC53-SN3 (28, 29), and p53-4KR (30).

Reporter gene assays. A luciferase reporter assay system (Promega, Madison, WI) was used as described (24). Cells with endogenous wild-type p53 were cotransfected with *PTMA* (2 μg) and *lacZ* control plasmid pIC400 (250 ng) in six-well dishes. Wild-type *p53* (pC53-SN3, 50 ng) was additionally transfected for *p53*-negative cells. p53 activity was assessed 24 hours later with *luciferase* reporter plasmids (250 ng) containing 13 copies of the ribosomal gene cluster p53 DNA binding site (DBS; PG13-Luc), the *p21* promoter (WWP-Luc), the *hdm2* promoter (pGLhmdm2-HX-luc), or the *cyclin G* promoter (pGL3-cyclin G-luc). pTA-Luc without DBS was compared with pp53-TA-Luc, pE2F-TA-Luc, and pMyc-TA-Luc with a DBS for p53, E2F, and Myc transcription factors, respectively. Transfection efficiency was standardized against β-galactosidase activity (pIC400). For the PCAF experiment, 1 μg each of *PTMA* and *PCAF* plasmid was used.

Puromycin selection. To pool transfected cells, cells were maintained in selection medium for 4 days containing 0.6 and 1.0 μg/mL puromycin for 293 and H1299 cells, respectively, starting 24 hours after transfection with pBpuro.

Immunoblotting. Cells were seeded at 3×10^6 per 10-cm plate and transfected with 1.75 μg *PTMA* and 250 ng pBpuro plasmid for puromycin selection. After puromycin selection for 4 days, the transfected cells were harvested and resuspended in lysis buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP40] supplemented with protease and phosphatase inhibitors. Extracts were sonicated and centrifuged at 15,000 rpm for 10 minutes. All immunoblots for H1299 and 293 cells used 60 μg of loaded protein. For PTMA analysis, samples were boiled in SDS sample buffer, separated with 15% SDS-PAGE gels, and electrotransferred to a positively charged nylon membrane (31). PTMA was detected with a mouse monoclonal anti-PTMA antibody (32). Other proteins were analyzed with standard methods and the following specific antibodies: mouse monoclonal anti-p53 (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-p21 (F-5, Santa Cruz Biotechnology), mouse monoclonal anti-MDM2 (OP-46, Oncogene Research Products, Uniondale, NY), mouse monoclonal anti-α-tubulin (Sigma, St. Louis, MO), and rabbit polyclonal anti-acetyl p53 antibodies that recognize p53 acetylated at Lys^{373} and Lys^{382}, at Lys^{373} only, or at Lys^{320} only (Upstate Biotechnology, Lake Placid, NY).

***In vitro* growth assay.** After transfection and puromycin selection, cells were seeded in six-well plates at a concentration of 1×10^5 per well. They were grown for 3 days and trypsinized, and viable cells were counted in a hematocytometer as assessed by trypan blue exclusion.

Cell proliferation assay. After transfection and puromycin selection, U2OS cells were plated in a chamber slide at a concentration of 1×10^5 per well and maintained in puromycin containing medium overnight. Proliferating cells were analyzed by bromodeoxyuridine (BrdUrd) incorporation with BrdUrd Labeling and Detection kit I (Roche Molecular Biochemicals, Indianapolis, IN).

Reverse transcription-PCR and quantitative real-time PCR. Total RNA was isolated from 293 cells transfected with *PTMA* or empty control plasmid and pooled in puromycin containing medium for 4 days. The first strand of cDNA was reverse transcribed from 1 μg of extracted RNA by MultiScribe Reverse Transcriptase (Perkin-Elmer, Norwalk, CT) with random hexamers or oligo dT primers at 42°C for 12 minutes. For standard reverse transcription-PCR, PCR amplification was done using specific primer sets for *PTMA* (upstream primer, 5′-TAATCCCCTTCATCGGATCA; downstream primer, 5′-GATTCTGAGACGGGAAGTGG-3′) and for *GAPDH* (upstream primer, 5′-ACCACAGTCCATGCCATCAC-3′; downstream primer, 5′-TCCACCACCCTGTTGCTGTA-3′). The conditions for amplification were denaturation at 95°C for 9 minutes followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, done on a GeneAmp PCR System 9700 (Perkin-Elmer). PCR products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining.

Quantitative PCR was done with specific primers and Taqman probes for *hdm2, p21*, and *GAPDH* with Pre-Developed Taqman Assay reagents according to the protocol of the manufacturer. Gene expression was measured using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were done using the chromatin immunoprecipitation assay kit (Upstate Biotechnology) according to the manufacturer's instructions. In brief, 2×10^6 293 cells were seeded into 10-cm dishes and transfected with pBpuro and *PTMA* or empty control plasmid. After puromycin selection for 4 days, genomic DNA and protein were cross-linked by adding 1% formaldehyde into the medium and incubated for 10 minutes at 37°C. Cells were lysed in 200 μL of SDS lysis buffer with a protease inhibitor mixture and sonicated to generate DNA fragments with lengths between 100 and 800 bp. The sonicated cell supernatant was diluted and precleared with protein A/G-Sepharose beads. The precleared supernatant was immunoprecipitated using mouse monoclonal anti-PTMA antibody, anti-p53 antibody (DO-1), and anti-acetyl p53 antibody that recognizes p53 acetylated at Lys^{320} (Upstate Biotechnology) or normal mouse IgG as a control antibody at 4°C for 16 hours. The immune complexes were precipitated, washed, and eluted. Cross-linking was reversed at 65°C for 4 hours. DNA was recovered by phenol-chloroform extraction and ethanol precipitation and resuspended in 30 μL of TE buffer; 1 μL of each sample was used as a template for PCR (40 cycles) with oligonucleotides that amplify a 233-bp fragment of the human *p21* promoter, including a p53 DNA binding site: 5′-CCTGCTTCCCAGGAACATGCTT-3′ (upstream primer) and 5′-CTGCTGGCAGATCACATACCCTGT-3′ (downstream primer).

Results

We identified PTMA in a screen of a HeLa cDNA expression library using a functional p53 yeast assay (33, 34). We did reporter gene assays to test whether PTMA influences p53 transcriptional activity in mammalian cells. *p53*-negative H1299 and Saos-2 cells were transfected with *p53* and *PTMA* plasmids and PG13-Luc containing 13 p53 DBS upstream of *luciferase*. In both cell lines, *PTMA* coexpression led to a 2- to 5-fold increase in transcriptional activity of exogenous p53 (Fig. 1*A-C*). PTMA alone had no effect in the reporter gene assays (Fig. 1*A* and *B*), and the p53-dependent increase was not seen with MG15-Luc containing 15 mutated p53 DBS (Fig. 1*A*). The effect of PTMA was dose (Fig. 1*B*) and time dependent (Fig. 1*C*). Increased p53 transcriptional activity corresponded to increased PTMA levels in H1299 cells cotransfected with *p53* and *PTMA* plasmids. Protein levels of overexpressed p53 did not change in the presence of overexpressed PTMA (Fig. 1*D*; data not shown).

PTMA also increased p53 activity in cells with endogenous wild-type p53. In HepG2, A549, and U2OS cells, ectopically expressed PTMA resulted in a 2-fold increase in p53 activity (Fig. 2*A*). We evaluated HCT116 colon cancer cells, because isogenic $p53^{+/+}$ and $p53^{-/-}$ cell lines exist (35). Overexpressed PTMA did not increase the transcriptional activity of endogenous wild-type p53 in HCT116 $p53^{+/+}$ cells or of overexpressed wild-type p53 in HCT116 $p53^{-/-}$ cells (data not shown). The effect of PTMA was especially pronounced in 293 cells with a 6- to 7-fold increase in p53 activity (Fig. 2*B*). Using a set of reporter gene plasmids that are identical except for the DBS, we established that PTMA does not have a general effect on transcription factors. All three plasmids,

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/6/3137/2558822/3137.pdf by guest on 12 April 2024

dependent either on p53, E2F, or Myc activity, showed significantly higher activity than the control plasmid pTA-Luc, indicating that all three transcription factors are active in 293 cells. However, p53 activity was the only one that increased in response to overexpressed PTMA (Fig. 2*C*). We evaluated whether PTMA enhances p53 activity with promoters of p53 target genes. H1299 cells (with or without cotransfected *p53*) and 293 cells (with endogenous p53) were transfected with *luciferase* reporter plasmids for the p53-responsive *hdm2, p21*, and *cyclin G* promoters. In all cases, PTMA resulted in increased p53 activity (Fig. 3*A-C*).

We next investigated whether endogenous PTMA affects p53 transcriptional activity. Two different plasmids for shRNA specifically targeting the *PTMA* transcript led to a significant reduction of *PTMA* mRNA and PTMA protein levels (Fig. 4*A* and *B*). The reduction of PTMA protein resulted in a corresponding decrease in p53 transcriptional activity in 293 cells in reporter gene assays for p53 DBS or p53-dependent promoters (Fig. 4*C*).

To investigate the effect of PTMA on transcription of endogenous p53-responsive genes, we did quantitative real-time PCR. PTMA caused increases of mRNA levels for *hdm2* and *p21* in 293 cells that were ~3- and 9-fold, respectively, whereas levels for the control *GAPDH* were not affected (Fig. 5*A*). A corresponding increase in protein levels for Hdm2 and p21 was observed upon overexpression of PTMA (Fig. 5*B*).

The increased transcriptional activity of p53 in response to overexpressed PTMA correlated with expected cellular outcomes of activated p53. Both H1299 (cotransfected with p53) and 293 cells showed a significant reduction in cell growth when transfected with *PTMA* compared with empty plasmid control (Fig. 5*C*). In *p53*-negative H1299 cells, PTMA alone resulted in some growth suppression as well, consistent with p53-independent functions of PTMA. This was not entirely surprising because overexpression of PTMA alone resulted to some extent in increased activity in reporter gene assays for the *p21* and *cyclin G* promoters in H1299 cells (Fig. 3*B* and *C*). Furthermore, we evaluated an additional cell line with endogenous wild-type p53, U2OS, that showed a PTMA-dependent increase in p53 activity in reporter gene assays (Fig. 2*A*). Overexpressed PTMA markedly reduced the percentage of proliferating BrdUrd-positive U2OS cells (Fig. 5*D*).

PTMA may modulate p53 activity, at least in part, through two HATs, CBP and p300, that are known to interact both with PTMA and p53 (14, 15, 21, 22, 36). We therefore determined whether

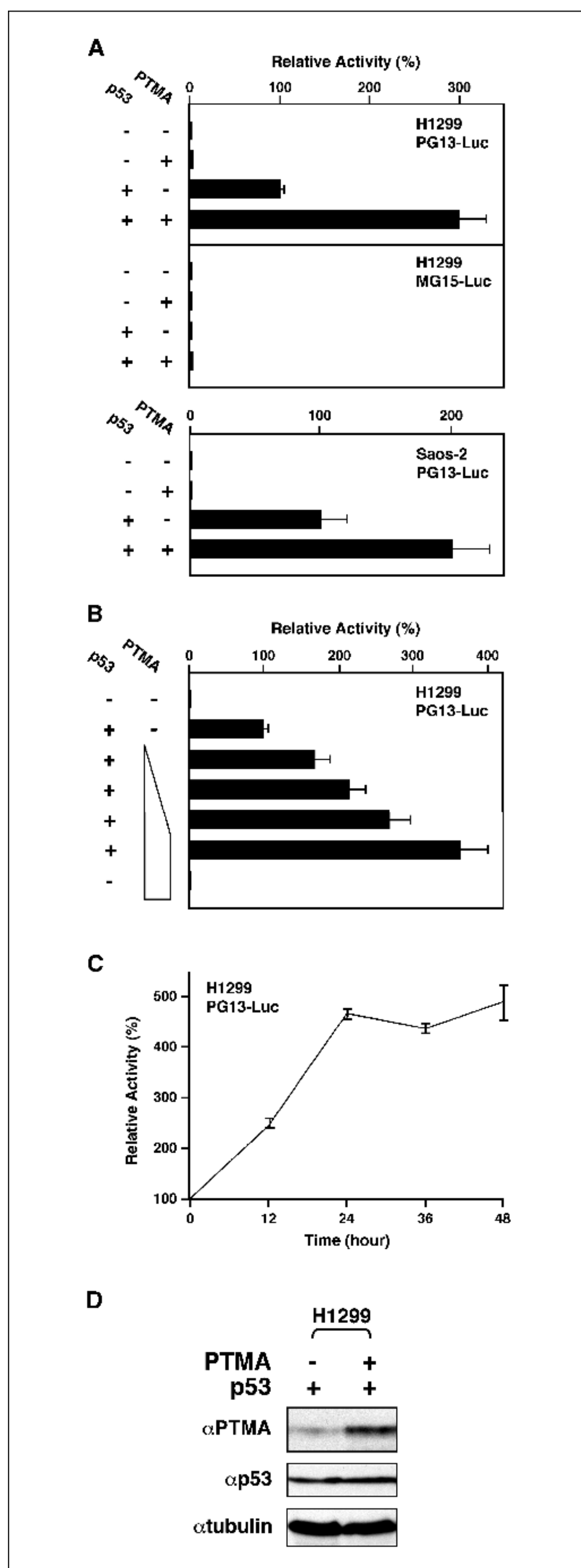

Figure 1. PTMA increases transcriptional activity of exogenous p53 in mammalian reporter gene assays with PG13-Luc. *p53*-negative cell lines H1299 and Saos-2 (*A*) were transfected with PG13-Luc (13 p53 DBS, upstream of *luciferase*), pIC400 expressing β-galactosidase and combinations of *p53* (pC53-SN3), *PTMA* (PTMA-pCMVneo), and/or empty control plasmids to ensure equal amounts of transfected plasmid. Luciferase activity, adjusted for transfection efficiency using β-galactosidase, was determined 24 hours after transfection. The adjusted luciferase activity of cells transfected with reporter plasmids and *p53* expression plasmid alone was set as 100%. *Columns,* mean for three independent experiments; *bars,* SD. *B,* PTMA increases p53 activity in a dose-dependent manner. H1299 cells were transfected with PG13-Luc and increasing amounts of *PTMA* plasmid (0, 0.5, 1, 1.5, and 2.0 μg). *C,* PTMA increases p53 activity in a time-dependent manner. H1299 cells were cotransfected with *p53* plasmid, reporter plasmids, and either *PTMA* or empty control plasmid and analyzed at several time points. Luciferase activity in the absence of PTMA at the various time points was arbitrarily set as 100%, and luciferase activity with PTMA was plotted as percentage of the negative control. *D, PTMA* expression plasmid results in increased PTMA protein levels. After puromycin selection of H1299 cells transfected with or without *PTMA*, as well as *p53*, immunoblotting was done to evaluate the protein levels of PTMA, p53, and control protein α-tubulin.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/6/3137/2558822/3137.pdf by guest on 12 April 2024

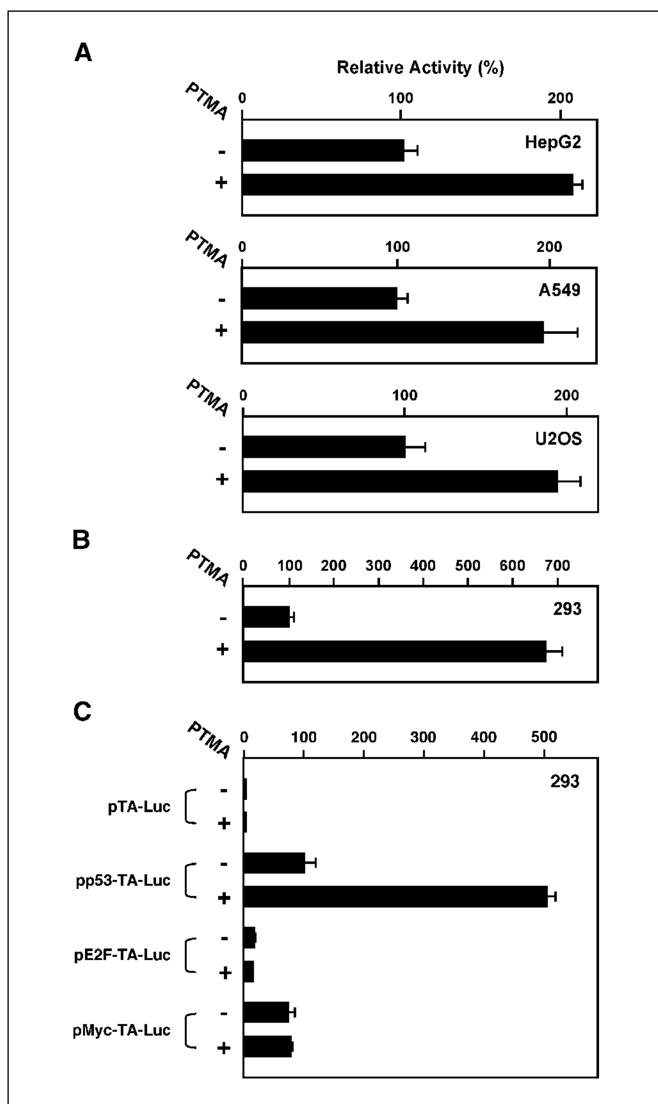

Figure 2. PTMA increases transcriptional activity of endogenous p53 in mammalian reporter gene assays. Experiments in HepG2, A549, U2OS (*A*), and 293 cells (*B*) with endogenous p53 were done as in Fig. 1, except that p53 was not cotransfected. *C,* PTMA does not enhance transcriptional activity of E2F and Myc transcription factors. Luciferase activity for reporter plasmids pTA-Luc lacking a specific DBS or containing a DBS for either p53, E2F, or Myc was assessed in 293 cells. All luciferase activities were compared with luciferase activity for pp53-TA-Luc in the absence of PTMA, arbitrarily set as 100%.

PTMA affects CBP-mediated and p300-mediated p53 acetylation (21, 22, 36). Using two different antibodies that recognize p53 acetylated at either Lys^{373} only or both Lys^{373} and Lys^{382}, immunoblotting of p53 from 293 cells overexpressing PTMA showed a significant increase in p53 acetylation (Fig. 6*A*). In the same experiment, increased levels of acetylated p53 coincided with increased p53 activity, as shown by increased protein levels for two p53 target genes, *hdm2* and *p21* (see Fig. 5*B*).

PCAF is a third HAT that functions as p53 coactivator and acetylates p53 (21, 22, 36–38). Immunoblots showed that PTMA also induces p53 acetylation on Lys^{320}, the target residue for PCAF (Fig. 6*A*). We investigated whether coexpression of PCAF with PTMA results in a further increase of p53 transcriptional activity. In reporter gene assays with PG13-Luc in 293 cells, expression of PCAF alone did not result in increased p53 activity, but coexpression of PCAF with PTMA almost doubled the effect of PTMA on p53 activity (Fig. 6*B*).

These results suggested that p53 acetylation is critical for activation of p53 in response to overexpressed PTMA. We investigated this hypothesis using transient reporter gene assays in H1299 cells that compared wild-type p53 to an acetylation-deficient p53 mutant that has Lys^{320}, Lys^{373}, Lys^{381}, and Lys^{382} replaced by arginines (4KR mutant; ref. 30). Whereas wild-type p53 responded to overexpressed PTMA with a 3-fold increase in

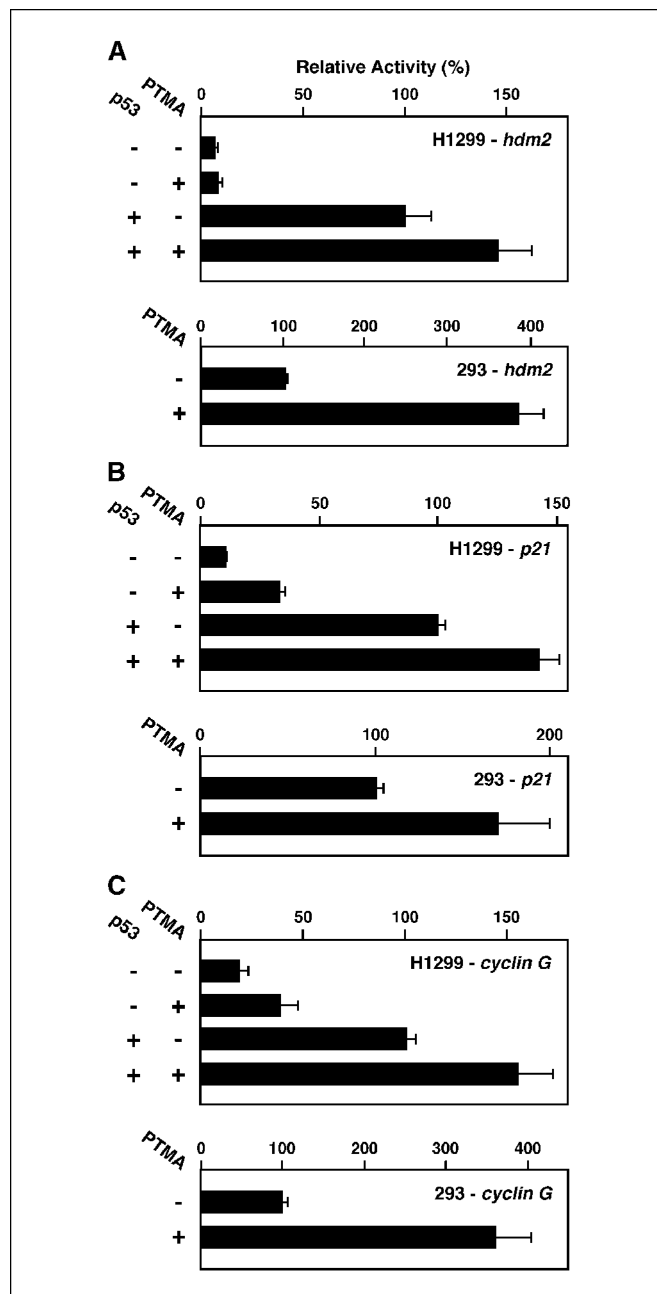

Figure 3. PTMA increases transcriptional activity of exogenous and endogenous p53 in reporter gene assays for p53-dependent promoters. Activity of exogenous p53 in H1299 cells (*top*) and endogenous p53 in 293 cells (*bottom*) was assessed in *luciferase* reporter assays for (*A*) the human *hdm2* promoter, (*B*) the human *p21* promoter, and (*C*) the murine *cyclin G* promoter.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/6/3137/2558822/3137.pdf by guest on 12 April 2024

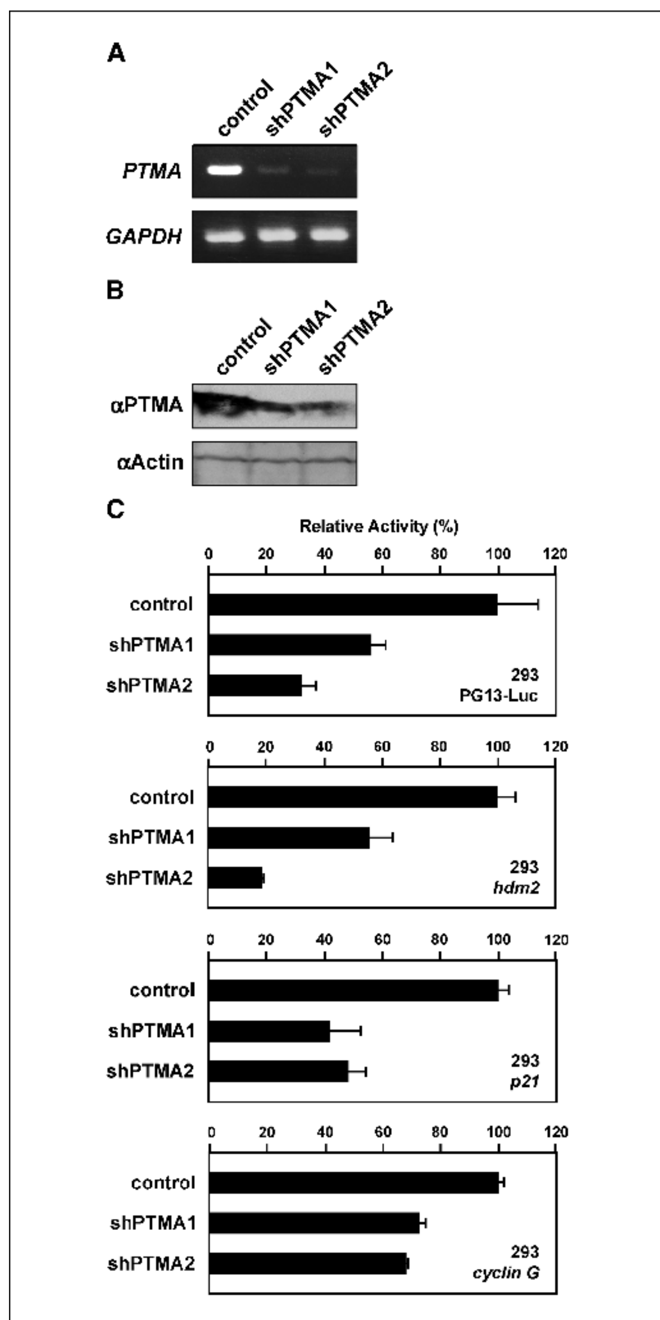

Figure 4. Reduction of endogenous PTMA results in reduced p53 transcriptional activity. 293 cells were transfected with either *PTMA* shRNA-expressing plasmids or with empty vector. Reduction of *PTMA* mRNA level (*A*) and PTMA protein level (*B*) through RNA interference was analyzed 2 days after transfection by RT-PCR and immunoblotting, respectively. *C,* p53-mediated transactivation in the presence of shRNA plasmids for *PTMA* was assessed in 293 cells 2 days after transfection as described in Fig. 2 using PG13-Luc or *luciferase* reporter assays for the human *hdm2* promoter, the human *p21* promoter, and the murine *cyclin G* promoter.

activity, the acetylation-deficient p53 mutant showed no response (Fig. 6*C*).

Given that PTMA affects p53 acetylation and transcriptional activity, we investigated whether PTMA occupies the promoter of the p53 target gene *p21* using chromatin immunoprecipitation analysis. The *p21* promoter was chosen because mRNA and protein for *p21* are significantly induced in 293 cells in response to overexpressed PTMA (Fig. 5*A* and *B*). Figure 6*D* shows that overexpressed PTMA in 293 cells resulted in increased association of PTMA with the *p21* promoter but did not significantly affect the amount of p53 associated with the *p21* promoter. However, the amount of acetylated p53 at the *p21* promoter increased markedly in the presence of overexpressed PTMA (ac-p53 in Fig. 6*D*). These results indicate that PTMA can occupy a p53-dependent promoter and is likely to affect p53 acetylation at the promoters of p53 target genes.

Discussion

PTMA is a growth-promoting protein, and expression studies of PTMA in human cancers identified PTMA as an oncoprotein that shows higher levels in various cancer tissues compared with adjacent normal tissues (1, 2). In this report, we provide evidence that inappropriate activity of PTMA triggers a cellular response that involves p53 activation. The effect of PTMA on p53 was observed in two *p53*-negative cell lines with exogenous p53 and four cell lines with endogenous wild-type p53 (Figs. 1 to 5). The effect of PTMA on p53 thus seems to be quite general, although it is not universal, based on our negative results for HCT116 cells (see below for further discussion). Our RNA interference experiments for PTMA indicate that not only overexpressed but also endogenous PTMA affects p53 transcriptional activity (Fig. 4). Future studies need to determine whether this result also reflects p53 activation in response to an inappropriate growth stimulus, or whether PTMA has a broader role in regulating p53 activities. Excess PTMA resulted in increased transcript and protein levels for two important endogenous p53 target genes, *hdm2* and *p21* (Fig. 5*A* and *B*). PTMA caused growth suppression of H1299 (transfected with wild-type p53), 293, and U2OS cells (Fig. 5*C* and *D*), suggesting that p53 activation by PTMA, as assessed by us, may also have cellular consequences. Our results for H1299 cells furthermore indicate that PTMA can also have p53-independent effects on cell growth that require further exploration.

What is the underlying mechanism for the PTMA-induced activation of p53? p53 activity is regulated by coactivators and post-translational modifications (20–23). A number of the transcriptional coactivator complexes that bind p53 *in vivo* possess HAT activity, and recent data suggest that p53 is not only modified by these complexes but may also use them to affect chromatin remodeling in a more global sense (39). PTMA has also been shown to change the state of chromatin. It can induce the unfolding of chromatin fibers in a process that involves the interaction with histone H1 (10–12), a known major regulator of chromatin structure (40, 41). Based on these observations, we decided to focus on the role of HATs and p53 acetylation in the p53 response to PTMA, in particular, because both p53 and PTMA are known to interact with the HATs CBP and p300 (14, 15, 20–22, 36).

Our results show that p53 acetylation is an important aspect of p53 activation in response to PTMA. Excess PTMA led to increased p53 acetylation not only by CBP and p300 but also by PCAF, a third HAT that acetylates p53 (Fig. 6*A*). Further supporting a role for PCAF, PTMA-induced activation of p53 was significantly enhanced by coexpression of PCAF (Fig. 6*B*). An acetylation-deficient p53 mutant showed no increase in transcriptional activity in response to excess PTMA (Fig. 6*C*). Considering that p53 is known to directly interact with CBP, p300, and PCAF (20–22), and PTMA directly interacts with CBP and p300 (14, 15), it is very likely that PTMA is in close proximity of p53 when triggering the p53 response. We

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/6/3137/2558822/3137.pdf by guest on 12 April 2024

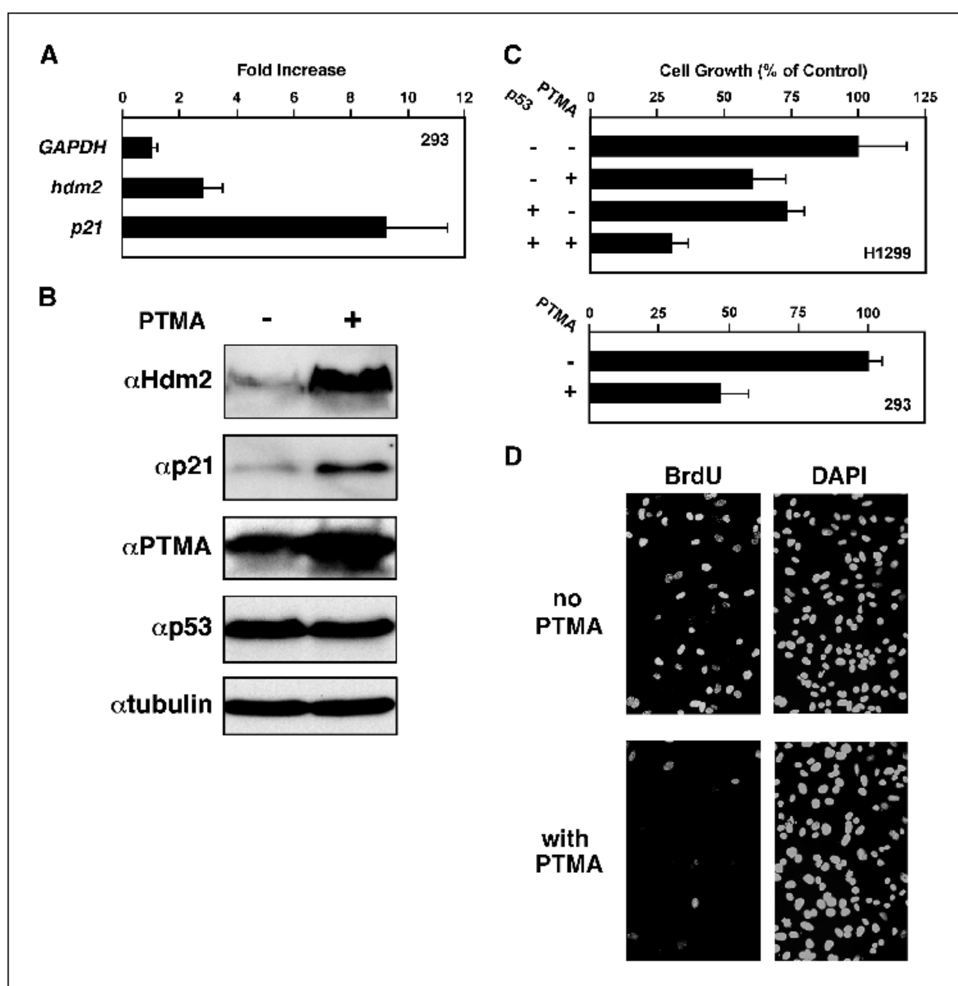

Figure 5. PTMA increases expression of endogenous p53 target genes and causes suppression of cell growth. *A,* PTMA increases endogenous mRNA levels for *hdm2* and *p21*. A quantitative real-time PCR assay determined mRNA levels for *hdm2, p21*, and *GAPDH* in 293 cells. 293 cells were cotransfected with pBpuro and *PTMA* or empty control plasmid. Total RNA for real-time PCR was isolated after puromycin selection. *Bars,* fold induction for each mRNA amount observed with *PTMA* as compared to empty control plasmid. *Columns,* average calculated from three independent experiments, each done in triplicate; *bars,* SD. *B,* PTMA increases endogenous protein levels for Hdm2 and p21. After puromycin selection of transfected 293 cells, immunoblotting was done for Hdm2, p21, PTMA, p53, and control protein α-tubulin in the absence or presence of *PTMA* plasmid. *C,* PTMA suppresses growth in H1299 and 293 cells. H1299 (with or without p53 and/or PTMA), and 293 cells transfected with *PTMA* or empty control plasmid were selected with puromycin, plated in six-well plates at the same densities and cultured in puromycin-containing medium. The cells were collected after 3 days and counted in a hematocytometer. *Columns,* relative numbers of viable cells calculated from four independent experiments; *bars,* SD. *D,* PTMA reduces the fraction of proliferating U2OS cells. After puromycin selection of U2OS cells (endogenous p53) transfected with *PTMA* (*bottom*) or empty control plasmid (*top*), cells were plated in chamber slides at a concentration of 1×10^5 per well and maintained in puromycin containing medium overnight. Proliferating cells were analyzed by BrdUrd (*BrdU*) incorporation (*left*). The nuclei were stained with 4′,6-diamidino-2-phenylindole (*DAPI*; *right*).

have been unable to coimmunoprecipitate p53 and PTMA despite numerous attempts under various conditions.[9] Although not conclusive, it suggests that the effect of PTMA on p53 is indirect and mediated by HATs. However, it is also possible that the general effects of PTMA on chromatin (10–12) are "sensed" by p53 in a more indirect way. Lastly, one has to consider the fact that lysine residues acetylated by CBP, p300, and PCAF can also be modified by other mechanisms (21, 22). It is therefore possible, although much less likely, that PTMA primarily acts by preventing other p53 modifications, thus promoting p53 acetylation indirectly. Future studies need to determine in greater detail how PTMA and the p53 pathway intersect at HATs and their multiprotein complexes. Results for mouse knock-in cells with acetylation-deficient p53 were recently published; this represents a particularly powerful system to stringently determine the contribution of p53 acetylation to p53 activation in response to PTMA (42).

The fact that excess PTMA had the most pronounced effects on p53 in 293 cells also points toward p53 acetylation as an important mechanism of the p53 response to PTMA, although indirectly. 293 cells express the adenoviral protein E1A that directly binds to and inhibits the activity of p53 coactivators CBP, p300, and PCAF (37, 38, 43). Thus, part of the PTMA effect on p53 activity in 293 cells may be release of these crucial p53 coactivators from E1A inhibition. However, there may be additional reasons why PTMA had a stronger effect in 293 cells. For example, PTMA may function by affecting another adenoviral p53-inhibitory protein present in 293 cells, E1B 55K, that has been shown to sequester p53 in the cytoplasm, but to also interact with p53 bound to DNA (44) and to inhibit p53 acetylation by PCAF (45).

Our negative results for HCT116 cells suggest the possibility that HAT activity in HCT116 cells is sufficiently deregulated to prevent a p53 response to overexpressed PTMA. Interestingly, one of the HATs known to acetylate p53, p300, is abnormal in HCT116 cells. HCT116 cells are hemizygous for *p300* and carry a mutation that results in the sole expression of a COOH-terminally truncated protein (1,708 instead of 2,414 amino acids; ref. 46). This truncation does not seem to affect the role of p300 in acetylating and activating p53 after UV-induced DNA damage. In fact, in response to DNA damage, p300, not CBP or PCAF, is the main p53 acetylase in HCT116 cells (47). However, the p300 truncation results in loss of one of two binding regions of p300 for PTMA (15) and thus may affect the response to PTMA.

Our results are consistent with the model that PTMA, as an oncoprotein, results in p53 activation as a cellular response to counteract an inappropriate growth stimulus, similar to p53 responses to overactive Myc, Ras, E2F-1, and β-catenin. Myc, Ras, E2F-1, and β-catenin are known to induce p14ARF that, in turn, inhibits Hdm2, a negative regulator of p53 (48). Recent reports

[9] T. Kobayashi, data not shown.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/6/3137/2558822/3137.pdf by guest on 12 April 2024

indicate that inappropriate proliferative stimuli in early tumorigenesis elicit a DNA damage response, possibly due to "DNA replication stress," that activates p53 and involves p53 phosphorylation (49, 50). Future studies need to address whether the ARF and "DNA replication stress" pathways also contribute to p53 activation in response to excess PTMA or whether PTMA represents a novel pathway that triggers a p53 response.

Many reports clearly show that PTMA induces cell proliferation (1, 2), yet we find that excess PTMA elicits a p53 response and growth suppression. This situation is very reminiscent of results for Myc, Ras, E2F-1, and β-catenin (51) and points toward the particular assay systems as one possible explanation for these apparently different results. The use of cell lines with different genetic alterations are likely to be important, and cell lines with an impaired p53 pathway may predominantly proliferate in response to PTMA. It is furthermore very likely that protein tags significantly alter results for PTMA, such as subcellular localization and biological functions (52, 53). We have tested NH_2-terminally tagged PTMA (FLAG, Myc, and glutathione *S*-transferase tags) in p53 reporter gene assays and found that none were able to stimulate p53 activity, despite expression levels similar to native PTMA.[9] Thus, protein tags may cause significant conformational changes of PTMA that disrupt or alter its normal biological functions.

Our experiments focused mainly on two important p53 target genes, *hdm2*, involved in a negative feedback loop for p53, and *p21*, key mediator of p53-induced cell cycle arrest. We chose these genes because they are the most extensively studied regarding the roles of HATs in p53-mediated transactivation (39). It will be important to extend our studies to additional p53 target genes, in particular those important for p53-mediated apoptosis. Several cofactors of p53 have been shown to differentially affect activation of p53 target genes (54), and the roles of HATs for p53-mediated activation of other target genes have not been defined as well as for *p21* and *hdm2* (39). Thus, it is conceivable that PTMA affects only a subset of p53 target genes.

In the case of apoptosis-related p53 target genes and p53-mediated apoptosis, the interplay between p53 and PTMA is likely to be even more complex. There is significant evidence that p53 not only activates target genes to induce apoptosis but also directly interacts with Bcl-2 family members to ultimately result in cytochrome *c* release from mitochondria (18, 19). Likewise, PTMA does not only function in the nucleus but also has a significant antiapoptotic role in the cytoplasm (4–6) by directly inhibiting the apoptosome, a crucial complex in the execution of apoptosis (5). Thus, p53 and PTMA may functionally intersect at several levels to induce or prevent apoptosis. The p53-mediated induction of p21 may also factor into the cell's decision to undergo or avoid apoptosis in response to inappropriate PTMA activity. Recent studies have pointed out that in addition to being an inhibitor of cell proliferation, p21 acts as an inhibitor of apoptosis in a number of systems, and this may counteract its tumor-suppressive functions as a growth inhibitor (55, 56). Therefore, the induction of p21 may be an important aspect of PTMA's ability to function as an oncoprotein.

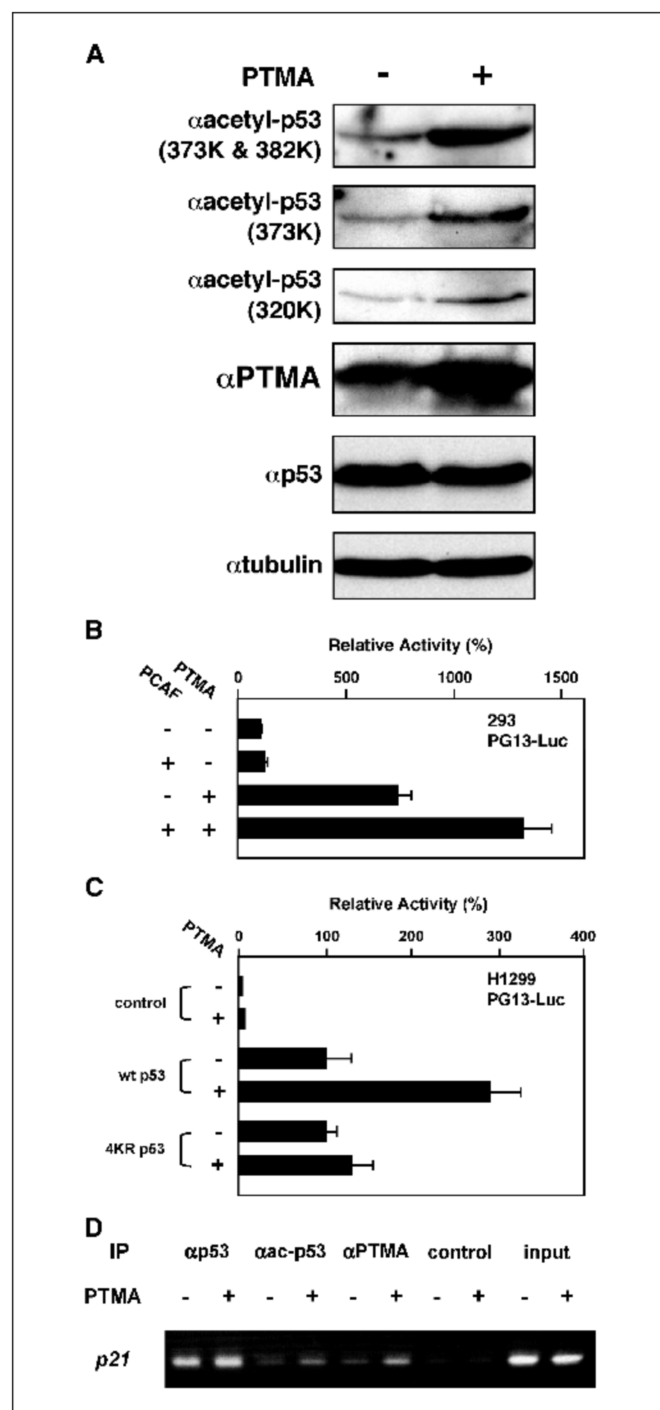

Figure 6. p53 acetylation is an important aspect of p53 activation in response to overexpressed PTMA. *A,* PTMA causes elevated levels of acetylated p53. Immunoblotting with three different antibodies recognizing p53 acetylated at Lys^{373} and Lys^{382}, at Lys^{373} only, or at Lys^{320} only was done to evaluate levels of acetylated p53 in puromycin-selected 293 cells that were transfected with *PTMA* or empty control plasmid. Please note that the Western blots for acetylated p53 were done with the same cell lysates that were used in Fig. 5*B*; they are displayed separately merely for presentation purposes. *B,* the HAT PCAF enhances the PTMA-mediated activation of p53. The HAT PCAF was expressed alone or in combination with PTMA, and transcriptional activity of p53 was assessed with the *luciferase* reporter plasmid PG13-Luc in 293 cells. *C,* an acetylation-deficient p53 mutant shows no increased transcriptional activity in response to PTMA. H1299 cells were transfected with wild-type (*wt*) or mutant (*4KR*) p53 and PTMA, and transcriptional activity of p53 determined using cotransfected PG13-Luc. *D,* chromatin immunoprecipitation analysis shows concomitant association of PTMA and acetylated p53 with the *p21* promoter. Chromatin immunoprecipitation analysis was done in 293 cells with or without overexpressed PTMA with primers specific for the *p21* promoter. Antibodies specific for p53, for p53 acetylated at Lys^{320} (ac-p53), and for PTMA or a control antibody were used for immunoprecipitation (*IP*). The shown experiment is representative of four additional chromatin immunoprecipitation analyses with similar results.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/6/3137/2558822/3137.pdf by guest on 12 April 2024

No studies are currently available that concomitantly assess the PTMA and p53 status of clinical cancer specimen. Such investigations are needed to help elucidate whether tumorigenesis promoted by overexpression of PTMA ultimately requires inactivation of p53 through gene mutations or other mechanisms or whether it only results in a p53-mediated growth arrest that eventually can be overcome through p53-independent mechanisms.

Acknowledgments

Received 6/16/2005; revised 12/6/2005; accepted 1/18/2006.

Grant support: James S. McDonnell Foundation, the Edward Mallinckrodt, Jr. Foundation, and NIH grant CA87468 (R.K. Brachman).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank S. Berger (Gene Expression and Regulation Program, The Wistar Institute, Philadelphia, PA), D. Dean (Department of Ophthalmology & Visual Sciences, University of Louisville, Louisville, KY), H. Land (Department of Biomedical Genetics, University of Rochester, Rochester, NY), Y. Nakatani (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA), J. Nathans (Departments of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD), M. Oren (Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel), and B. Vogelstein (Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD) for generously providing reagents; Y. Tsuda and M. Yamane for research assistance; and M. Yanome for help in preparation of the article.

References

1. Pineiro A, Cordero OJ, Nogueira M. Fifteen years of prothymosin α: contradictory past and new horizons. Peptides 2000;21:1433–46.
2. Segade F, Gomez-Marquez J. Prothymosin α. Int J Biochem Cell Biol 1999;31:1243–8.
3. Manrow RE, Sburlati AR, Hanover JA, Berger SL. Nuclear targeting of prothymosin α. J Biol Chem 1991; 266:3916–24.
4. Lal A, Kawai T, Yang X, Mazan-Mamczarz K, Gorospe M. Antiapoptotic function of RNA-binding protein HuR effected through prothymosin α. EMBO J 2005;24: 1852–62.
5. Jiang X, Kim HE, Shu H, et al. Distinctive roles of PHAP proteins and prothymosin-α in a death regulatory pathway. Science 2003;299:223–6.
6. Evstafieva AG, Belov GA, Rubtsov YP, et al. Apoptosis-related fragmentation, translocation, and properties of human prothymosin α. Exp Cell Res 2003;284:211–23.
7. Martini PG, Delage-Mourroux R, Kraichely DM, Katzenellenbogen BS. Prothymosin α selectively enhances estrogen receptor transcriptional activity by interacting with a repressor of estrogen receptor activity. Mol Cell Biol 2000;20:6224–32.
8. Yang CH, Murti A, Baker SJ, et al. Interferon induces the interaction of prothymosin-α with STAT3 and results in the nuclear translocation of the complex. Exp Cell Res 2004;298:197–206.
9. Karapetian RN, Evstafieva AG, Abaeva IS, et al. Nuclear oncoprotein prothymosin α is a partner of Keap1: implications for expression of oxidative stress-protecting genes. Mol Cell Biol 2005;25:1089–99.
10. Papamarcaki T, Tsolas O. Prothymosin α binds to histone H1 *in vitro*. FEBS Lett 1994;345:71–5.
11. Diaz-Jullien C, Perez-Estevez A, Covelo G, Freire M. Prothymosin α binds histones *in vitro* and shows activity in nucleosome assembly assay. Biochim Biophys Acta 1996;1296:219–27.
12. Karetsou Z, Sandaltzopoulos R, Frangou-Lazaridis M, et al. Prothymosin α modulates the interaction of histone H1 with chromatin. Nucleic Acids Res 1998;26: 3111–8.
13. Sterner DE, Berger SL. Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 2000;64:435–59.
14. Karetsou Z, Kretsovali A, Murphy C, Tsolas O, Papamarcaki T. Prothymosin α interacts with the CREB-binding protein and potentiates transcription. EMBO Rep 2002;3:361–6.
15. Subramanian C, Hasan S, Rowe M, et al. Epstein-Barr virus nuclear antigen 3C and prothymosin α interact with the p300 transcriptional coactivator at the CH1 and CH3/HAT domains and cooperate in regulation of transcription and histone acetylation. J Virol 2002;76: 4699–708.
16. Karetsou Z, Martic G, Tavoulari S, et al. Prothymosin α associates with the oncoprotein SET and is involved in chromatin decondensation. FEBS Lett 2004;577:496–500.
17. Ho J, Benchimol S. Transcriptional repression mediated by the p53 tumour suppressor. Cell Death Differ 2003;10:404–8.
18. Schuler M, Green DR. Transcription, apoptosis and p53: catch-22. Trends Genet 2005;21:182–7.
19. Erster S, Moll UM. Stress-induced p53 runs a direct mitochondrial death program: its role in physiologic and pathophysiologic stress responses *in vivo*. Cell Cycle 2004;3:1492–5.
20. Prives C, Manley JL. Why is p53 acetylated? Cell 2001; 107:815–8.
21. Xu Y. Regulation of p53 responses by post-translational modifications. Cell Death Differ 2003;10:400–3.
22. Appella E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem 2001;268:2764–72.
23. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307–10.
24. Brachmann RK, Yu K, Eby Y, Pavletich NP, Boeke JD. Genetic selection of intragenic suppressor mutations that reverse the effect of common p53 cancer mutations. EMBO J 1998;17:1847–59.
25. Zauberman A, Lupo A, Oren M. Identification of p53 target genes through immune selection of genomic DNA: the cyclin G gene contains two distinct p53 binding sites. Oncogene 1995;10:2361–6.
26. Ogryzko VV, Kotani T, Zhang X, et al. Histone-like TAFs within the PCAF histone acetylase complex. Cell 1998;94:35–44.
27. Morgenstern JP, Land H. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res 1990;18:3587–96.
28. el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993; 75:817–25.
29. Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 1990;249: 912–5.
30. Barlev NA, Liu L, Chehab NH, et al. Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol Cell 2001;8: 1243–54.
31. Karey KP, Sirbasku DA. Glutaraldehyde fixation increases retention of low molecular weight proteins (growth factors) transferred to nylon membranes for Western blot analysis. Anal Biochem 1989;178:255–9.
32. Yokoi H, Saitoh T, Nakazawa Y, Ohno H. An immunoradiometric assay for thymosin α 1. J Immunoassay 1996;17:85–100.
33. Kobayashi T, Wang T, Qian H, Brachmann RK. Genetic strategies in S. cerevisiae to study human tumor suppressor genes. In: El-Deiry WS, editor. Methods in molecular biology. Vol 223. tumor suppressor genes: regulation, function, and medicinal applications. Totowa: Humana Press; 2003. p. 73–86.
34. Wang T, Kobayashi T, Takimoto R, et al. hADA3 is required for p53 activity. EMBO J 2001;20:6404–13.
35. Bunz F, Dutriaux A, Lengauer C, et al. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. Science 1998;282:1497–501.
36. Brooks CL, Gu W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr Opin Cell Biol 2003;15:164–71.
37. Hamamori Y, Sartorelli V, Ogryzko V, et al. Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. Cell 1999;96:405–13.
38. Chakravarti D, Ogryzko V, Kao HY, et al. A viral mechanism for inhibition of p300 and PCAF acetyltransferase activity. Cell 1999;96:393–403.
39. Allison SJ, Milner J. Remodelling chromatin on a global scale: a novel protective function of p53. Carcinogenesis 2004;25:1551–7.
40. Mellor J. The dynamics of chromatin remodeling at promoters. Mol Cell 2005;19:147–57.
41. Bustin M, Catez F, Lim JH. The dynamics of histone H1 function in chromatin. Mol Cell 2005;17:617–20.
42. Feng L, Lin T, Uranishi H, Gu W, Xu Y. Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity. Mol Cell Biol 2005;25:5389–95.
43. Sang N, Avantaggiati ML, Giordano A. Roles of p300, pocket proteins, and hTBP in E1A-mediated transcriptional regulation and inhibition of p53 transactivation activity. J Cell Biochem 1997;66:277–85.
44. Neil JC, Cameron ER, Baxter EW. p53 and tumour viruses: catching the guardian off-guard. Trends Microbiol 1997;5:115–20.
45. Liu Y, Colosimo AL, Yang XJ, Liao D. Adenovirus E1B 55-kilodalton oncoprotein inhibits p53 acetylation by PCAF. Mol Cell Biol 2000;20:5540–53.
46. Gayther SA, Batley SJ, Linger L, et al. Mutations truncating the EP300 acetylase in human cancers. Nat Genet 2000;24:300–3.
47. Iyer NG, Chin SF, Ozdag H, et al. p300 regulates p53-dependent apoptosis after DNA damage in colorectal cancer cells by modulation of PUMA/p21 levels. Proc Natl Acad Sci U S A 2004;101:7386–91.
48. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. Nature 2004;432:307–15.
49. Gorgoulis VG, Vassiliou LV, Karakaidos P, et al. Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature 2005; 434:907–13.
50. Bartkova J, Horejsi Z, Koed K, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 2005;434:864–70.
51. Oren M. Decision making by p53: life, death and cancer. Cell Death Differ 2003;10:431–42.
52. Trumbore MW, Berger SL. Prothymosin α is a nonspecific facilitator of nuclear processes: studies of run-on transcription. Protein Expr Purif 2000;20:414–20.
53. Enkemann SA, Ward RD, Berger SL. Mobility within the nucleus and neighboring cytosol is a key feature of prothymosin-α. J Histochem Cytochem 2000;48:1341–55.
54. Coutts AS, La Thangue NB. The p53 response: emerging levels of co-factor complexity. Biochem Biophys Res Commun 2005;331:778–85.
55. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther 2002; 1:639–49.
56. Roninson IB. Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. Cancer Lett 2002;179:1–14.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/66/6/3137/2558822/3137.pdf by guest on 12 April 2024