Dual Role of p300 in the Regulation of p53 Stability*

Received for publication, August 13, 2001, and in revised form, September 20, 2001 Published, JBC Papers in Press, October 8, 2001, DOI 10.1074/jbc.M107770200

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While the function of p300 as a transcriptional co-activator of p53 is well documented, its role in the regulation of p53 stability remains ill-defined since opposite effects of p300 on p53 levels have been reported. We show here that p300 stabilizes both p53 and its negative regulator MDM2, thereby enhancing the p53/MDM2 negative regulatory loop. Binding of p300 is associated with the retention of p53 in the nucleus, which results in the accumulation of p53 in an acetylase-independent manner. Stabilization of MDM2, on the other hand, requires the acetylase activity of p300. Importantly, MDM2, once expressed, is able to reverse the stabilizing effect of p300 on p53. A temperature-permissive p53-expressing cell line enabled us to demonstrate the completely opposite roles of p300 in the regulation of p53 stability, depending on the expression of MDM2. Prior to p53 activation, when MDM2 levels are low, p300 acts as a positive regulator to increase p53 levels. Upon shifting to permissive temperature, however, when MDM2 expression is induced, p300 becomes a negative regulator of p53 by stabilizing MDM2 and thereby augmenting MDM2’s ability to target p53 for degradation.

p300 and CBP1 are transcriptional co-activators originally identified as E1A or CREB-binding proteins, respectively. Both p300 and CBP play an integral role in various cellular events, including cell proliferation, differentiation, and development by interacting with a variety of cellular proteins. In addition, CBP/p300 possess histone acetyltransferase activity that originates either from intrinsic activity and/or from an associated protein, PCAF. Paradoxically, CBP/p300 appear to be capable of contributing to opposite cellular processes since both coactivators participate in various tumor-suppressor pathways as well in the activities of several oncoproteins. Consistent with this notion, CBP and p300 have been shown to promote apoptosis as well as cell proliferation, effects that appear to be highly context-dependent.

The growth suppression functions of CBP/p300 are partially mediated by their interaction with the tumor suppressor p53. Studies have shown that p53 binds to the C-terminal region of CBP/p300 and that this interaction contributes to transcriptional activation of p53-responsive downstream genes including mdm2, p21, and bax (1–3). The finding by Gu and Roeder (2) that p53 could be acetylated by p300 uncovered a previously unrecognized aspect of CBP/p300 function. Acetylation of specific lysine residues in the C terminus of p53 was found to increase DNA binding dramatically, presumably by altering the conformation of an inhibitory regulatory domain. The ability of p53 to be acetylated in vivo was confirmed subsequently by using acetylation-specific antibodies (4, 5). Lambert et al. (6) have recently reported that ionizing irradiation promotes the N-terminal phosphorylation of p53, resulting in an increased affinity for CBP/p300 and an accompanying stimulation of p53 acetylation (6).

CBP/p300 also contributes to the mechanisms that regulate p53 degradation. The p53 protein is stabilized in cells that express adenovirus E1A, which blocks the ability of p53 to induce its target genes, such as MDM2. Because MDM2 is implicated in p53 degradation (7, 8), blocking of MDM2 expression by E1A would be expected to suppress a negative feedback loop (9). Grossman et al. (10) have shown that both p53 and MDM2 bind to the first zinc finger domain of p300. MDM2 mutants incapable of interacting with p300, but still capable of binding to p53, are defective in inducing p53 degradation (10). Similarly, p53 mutants rendered incapable of interacting with p300 escape MDM2-mediated degradation. These findings led to the suggestion that interaction of MDM2 and p53 with p300 is required for p53 turnover. We previously reported that p300 was actually able to stabilize p53 (11). Using cells engineered to express ribosomes directed against either CBP or p300, we showed that p53 induction by DNA damage was compromised in cells deficient in p300. These contrasting findings reveal an unresolved question as to how p300 could exert opposite effects on p53 stability. In the present report, we demonstrate that p300 indeed can differentially control p53 stability, depending on the expression of MDM2.

EXPERIMENTAL PROCEDURES

Cell Culture, Transient Transfection—H1299 cells, 293T cells (American Type Culture Collection) were maintained in Eagle’s minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Sigma). Cells were transfected by a calcium-phosphate method as described (12). Identities of the constructs were verified by restriction digestion and by DNA sequencing (Harvard Cancer Center, Boston, MA).

Preparation of Whole Cell Extracts and Immunoblot Analysis—Cells were transfected in 60-mm plates with 8 μg of DNA and harvested at 48-h post-transfection. Cells were lysed in 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride and protease inhibitors) by incubating on ice for 30 min, and the extracts were centrifuged at 13,000 rpm for 15 min to remove cell debris. Protein concentrations were determined by Bio-Rad protein assay (Hercules, CA). After addition of 5× loading buffer, samples were incubated at 95 °C for 5 min and resolved through SDS-PAGE. Proteins were trans-
ferred to nitrocellulose membranes (Schleicher and Schuell) and probed with anti-FLAG (M5; Sigma), anti-p300 (RW128; Upstate Biotechnology), anti-acetylated p53 (Lys-373 and -382; Upstate Biotechnology), anti-p300 (RW128; Upstate Biotechnology), anti-FLAG (M5; Sigma), anti-p300 (RW128; Upstate Biotechnology), and proteins were identified by Western blot using the indicated antibodies. Anti-β-actin immunoblot was used as a loading control. B, vector-expressing FLAG-p53 was transfected with wild type (lanes 1 and 2) or DY mutant (lanes 3 and 4) of p300 into 293T cells, and proteins were analyzed as described in A. C, plasmid-expressing wild type (lanes 1–4) or 6K/R mutant (lanes 5–8) of p53 was cotransfected into 293T cells with 0, 1, 3, or 5 μg of p300-expressing vector. Proteins were determined as described in A. D, mRNA was isolated from 293T cells and analyzed by RT-PCR using a kit according to the manufacturer’s protocol. E, green fluorescent protein-tagged p53 was cotransfected with a control vector (panel 1) or with a vector expressing wild type (panel 2) or DY mutant (panel 3) of p300 in H1299 cells. Cells were fixed at 36 h post-transfection and green fluorescent protein-positive cells were visualized under a fluorescent microscope. F, green fluorescent protein-positive cells were classified as having p53 distributed exclusively nuclear/strongly nuclear (EN/SN), equally distributed in two compartments/strongly cytoplasmic (ED/SC), or distributed as nuclear aggregates (NA). For each condition, 200 cells from random fields were scored. Values are averages ± the standard error of the mean (S.E.) from two separate experiments.

**Fig. 1.** p300 stabilizes p53 in an acetylase-independent manner. A, plasmid encoding FLAG-p53 was transfected with a HA-empty vector (lanes 1 and 3) or HA-p300 (lanes 2 and 4) into 293T (lanes 1 and 2) or H1299 cells (lanes 3 and 4). The cells were harvested 48 h post-transfection, and proteins were identified by Western blot using the indicated antibodies. Anti-β-actin immunoblot was used as a loading control. B, vector-expressing FLAG-p53 was transfected with wild type (lane 2) or DY mutant (lane 3) of p300 into 293T cells, and proteins were analyzed as described in A. C, plasmid-expressing wild type (lanes 1–4) or 6K/R mutant (lanes 5–8) of p53 was cotransfected into 293T cells with 0, 1, 3, or 5 μg of p300-expressing vector. Proteins were determined as described in A. D, mRNA was isolated from 293T cells and analyzed by RT-PCR using a kit according to the manufacturer’s protocol. E, green fluorescent protein-tagged p53 was cotransfected with a control vector (panel 1) or with a vector expressing wild type (panel 2) or DY mutant (panel 3) of p300 in H1299 cells. Cells were fixed at 36 h post-transfection and green fluorescent protein-positive cells were visualized under a fluorescent microscope. F, green fluorescent protein-positive cells were classified as having p53 distributed exclusively nuclear/strongly nuclear (EN/SN), equally distributed in two compartments/strongly cytoplasmic (ED/SC), or distributed as nuclear aggregates (NA). For each condition, 200 cells from random fields were scored. Values are averages ± the standard error of the mean (S.E.) from two separate experiments.

In **Vitro Acetylation Assay**—Purified p300-HAT domain was incubated with the indicated GST fusion protein in 50 μl of HAT assay buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM TCE, and 1 mM dithiothreitol) in the presence of 0.1 μCi of [3H]acetyl-CoA (PerkinElmer Life Sciences) at 30 °C for 30 min. The reaction products were resolved by SDS-PAGE. After drying, the gel was exposed to film for three days. Subcellular Distribution Assay—Cells were grown on Chamber Slides (Nunc) and transfected with the indicated vector. Cells were washed with cold phosphate-buffered saline 36 h after transfection and fixed with 4% paraformaldehyde (Sigma) for 30 min at 4 °C. Following phosphate-buffered saline washing, the slides were mounted with Fluoromount-G (Southern Biotechnology Associates) containing 2.5 mg/ml n-propyl gallate (Sigma). Specimens were examined under a fluorescent microscope (Zeiss).

**RESULTS**

**P300 Stabilizes p53 in an Acetylase-independent Manner**—Since completely opposite effects of p300 on p53 stability have been reported, we sought to investigate these contrasting findings by asking whether p300 could differentially control p53 stability under different circumstances. In light of our previous observation that p300-deficient MCF-7 cells exhibit an impaired p53 induction in response to DNA damage (11), we first asked whether p300 could stabilize p53 protein when the two proteins are coexpressed. Plasmid expressing FLAG-p53 was cotransfected with HA-p300 into 293T cells, and p53 protein levels were determined by Western analysis 48 h post-transfection. In agreement with our previous observation, p300 appears to contribute to the accumulation of p53 as demonstrated by an increase in p53 protein levels in cells expressing p300 in comparison with the cells expressing vector control (Fig. 1A, lane 1 versus 2). A similar result was obtained in H1299 cells (Fig. 1A, lanes 3 and 4), indicating that this effect of p300 is not cell type-specific.

Consistent with the previously reported finding that p53 is a non-histone substrate of p300, immunoblot analysis with an antibody specific to acetylated p53 demonstrated p300-dependent acetylation of p53 (Fig. 1A, panel 3). We then asked whether acetylation of p53 contributed to increased p53 levels. p300 mutant deficient in acetylase activity (p300DY) was used to test this possibility. In contrast to wild type p300, the p300DY mutant failed to acetylate p53 (Fig. 1B, panel 3, lane 2 versus 3). However, the p53 protein levels were increased to a comparable extend in both wild type and the p300DY mutant-expressing cells (Fig. 1B, panel 2, lane 2 versus 3), revealing an acetylation-independent nature of p53 up-regulation by p300. To further confirm this finding, p300 mutant lacking the acetylation sites (6K/R-p53) was generated. In contrast to the wild type p53 that was acetylated by p300 in a dose-dependent manner (Fig. 1C, panel 3, lanes 1–4), no apparent acetylation was detected in the 6K/R mutant (Fig. 1C, panel 3, lanes 5–8). However, the 6K/R mutant protein levels were induced by p300 as efficiently as those of wild type p53 (Fig. 1C, panel 2). These results lend further support to the notion that p300 increases p53 levels in an acetylase-independent manner.

Up-regulation of p53 protein levels in p300-expressing cells could be attributed to either increased protein synthesis or
reduced degradation. Levels of p53 mRNA were therefore determined using RT-PCR to differentiate between these two possibilities. As shown in Fig. 1D, ectopic expression of p300 did not significantly affect the abundance of p53 mRNA when compared with control vector (Fig. 1D, lane 2 versus 3), indicating that the accumulation of p53 protein is accomplished via post-transcriptional mechanism.

Since interference with p53 nuclear export is associated with the accumulation of p53 protein (13), we asked whether p300 could up-regulate p53 levels by affecting subcellular distribution of p53. Vector-expressing GFP-p53 was transfected with or without HA-p300, and subcellular distribution of GFP-p53 protein was examined. When expressed alone in H1299 cells, a typical distribution pattern of p53 was observed: ~90% of p53 localized to the nucleus, and 10% of the protein was found in the cytoplasm (Fig. 1E, panel 1). Significantly, when p300 was coexpressed, the nuclear population of p53 became remarkably enriched and displayed as bright nuclear aggregates (Fig. 1E, panel 2). To quantify these results, p53 distribution in green fluorescence-positive cells was classified as “exclusive/strong nuclear” (EN/SN), “exclusive/strong cytoplasmic” (EC/SC), or “equally distributed in two compartments” (ED). 200 cells from random fields were scored for each condition. The result demonstrates that although there was no significant difference between the overall nuclear and cytoplasmic fractions of p53, p300 expression was associated with a significant increase in nuclear aggregates (Fig. 1F). A similar result was obtained with the p300DY mutant (Fig. 1E, panel 3).

### p300 Stabilizes MDM2 in an Acetylase-dependent Manner—

Given the fact that p300 and MDM2 physically interact with each other (13), we asked whether complex formation between the two proteins has any impact on the function of MDM2. To this end, MDM2-mediated p53 ubiquitination was analyzed in the presence or absence of p300 expression. In our experimental settings, efficient MDM2-mediated p53 degradation usually requires 2–3 times more MDM2 plasmid DNA than p53. Co-transfection of p53 with less than a 2-fold amount of MDM2 is associated with ubiquitination without significant degradation of p53. We therefore used a relative ratio of 1:1 of MDM2 to p53 to assess the effect of p300 on the ability of MDM2 to ubiquitinate p53. As expected, coexpression of MDM2 resulted in ubiquitination of p53, as demonstrated by the typical ladder of p53 proteins. We therefore used a relative ratio of 1:1 of MDM2 to p53 to assess the effect of p300 on the ability of MDM2 to ubiquitinate p53. As expected, coexpression of MDM2 resulted in ubiquitination of p53, as demonstrated by the typical ladder of p53 proteins. We therefore used a relative ratio of 1:1 of MDM2 to p53 to assess the effect of p300 on the ability of MDM2 to ubiquitinate p53.

Elevated MDM2 E3 ligase activity could be due to an increase in intrinsic enzyme activity or up-regulation of MDM2 protein levels. Anti-DM2 immunoblot analysis revealed significantly elevated MDM2 protein levels in the p300-expressing cells when compared with cells expressing the control vector (Fig. 2A, lane 3 versus 4), suggesting that the induced E3 ligase activity was the result of an up-regulation of MDM2 protein by p300. To confirm this observation, plasmid expressing cDNA of MDM2 was transfected along with either HA-p300 or HA-empty vector. Western analysis showed that p300 indeed caused the induction of MDM2 protein levels (Fig. 2B, lane 1 versus 2). RT-PCR was then performed to determine whether elevated MDM2 levels in p300-expressing cells could be attributed to up-regulated transcription. As shown in Fig. 2C, levels of MDM2 mRNA isolated from either p300-expressing or non-expressing cells are comparable, indicating that the apparent stabilization effect of p300 on MDM2 is a post-transcriptional event.

Since MDM2 is an unstable protein that is degraded by the proteasome-mediated proteolysis, we asked whether p300 could affect the half-life of MDM2. To this end, half-life of MDM2 protein was determined in the presence or absence of coexpression of p300. We utilized cycloheximide to inhibit de novo protein synthesis and monitored the disappearance of MDM2 by Western blot analysis in cycloheximide-treated cells. The 293T cells expressing the indicated vectors were analyzed at 0, 60, 120, and 180 min following the addition of cycloheximide. Coexpression of p300 indeed resulted in a prolonged half-life of MDM2, as demonstrated in Fig. 2D.

Next, to examine whether the stabilization of MDM2 by p300 is acetylase-dependent, MDM2 levels were determined in cells expressing either wild type or DY mutant of p300. Immunoblot analysis with anti-DM2 revealed that the acetylase activity of p300 was required for its stabilization of MDM2, as MDM2 levels were increased in cells expressing wild type, but not mutant p300 (Fig. 3A, lane 2 versus 3). An almost identical result was seen when H1299 cells were used (Fig. 3A, lanes 4–6).

The acetylase-dependent stabilization suggests a possibility that p300 could directly acetylate MDM2. To test this, we arbitrarily divided MDM2 into four fragments to generate GST fusion proteins for testing in an in vitro acetylation assay in which GST-DM2 was incubated with the purified HAT domain of p300 in the presence of 14C-labeled acetyl-CoA. Autoradiography analysis revealed that amino acids 124–246 and 370–491 of MDM2 (Fig. 3B, lanes 14 and 16), but not other regions of the molecule, were acetylated by p300. GST only (Fig. 3B, lane 2 and 10) and p53 C terminus (Fig. 3B, lane 3 and 11) were included as negative and positive controls, respectively.

Expression of MDM2 Converts p300 from a Positive to a Negative Regulator of p53 Stability—p300 stabilizes p53 by retaining it in the nucleus, whereas the up-regulation of MDM2 levels by p300 would result in an increased p53 degradation. To better understand these entirely opposite effects of p300, we...
asked whether MDM2 presence could affect p300's ability to stabilize p53. Vectors expressing p300 and p53 were cotransfected with pCMV-MDM2 or the parental vector, and protein levels were analyzed by Western analysis 48 h post-transfection. Consistent with previous observations, p300 induced p53 accumulation when the two proteins were coexpressed (Fig. 4B, lanes 2 and 3). While at non-permissive temperature the inactive p53 was efficiently stabilized in p300-expressing cells (Fig. 4C, lanes 5 and 6), the ability of the wild type p300, but not the DY mutant, to up-regulate p53 was completely abolished at 32 °C (Fig. 4C, lane 3). Correspondingly, MDM2 was not detectable at 37 °C (Fig. 4C, lanes 4–6) and induced MDM2 levels at 32 °C were further increased in the wild type p300-expressing cells but not the DY mutant-expressing cells (Fig. 4C, lane 2 versus 3).

**DISCUSSION**

We demonstrate here that p300 can stabilize both p53 and its negative regulator MDM2, which explains the completely opposite effects of p300 on p53 stability. MDM2 appears to play a key role in determining whether p300 exerts a positive or negative effect on p53 protein stability.

In unstressed cells, where p53 levels are low and MDM2 is not induced, p300 contributes to the initial activation as well as accumulation of p53. Our previous finding that p53 induction in response to DNA damage is impaired in p300-deficient MCF-7 cells is consistent with this model (11). The p53 nuclear aggregates observed in p300-expressing cells are very similar to those observed in leptomycin B-treated cells and represent a consequence of inhibited p53 nuclear export. Since p300 does not affect mRNA levels of p53 (Fig. 1D), the increased nuclear aggregates in p300-expressing cells likely result from a diminished nuclear export of p53, which leads to increased p53 levels. The exact mechanism of p300-mediated inhibition of p53 nuclear export is presently unknown. A recent study identified a
second p53NES at the N-terminal transactivation domain of p53 (14), which coincides with the p300-binding motif (3). It is therefore possible that binding of p300 to the p53 N terminus results in the concealment of this second p53NES, thereby abrogating its function. Further studies are needed to test this possibility. Overall, it appears when the levels of MDM2 is low, p300 acts as a positive regulator of p53.

However, once MDM2 is induced, p300 is converted into a negative regulator of p53 stability. As we demonstrate in this study, p300 does so by stabilizing MDM2 and thereby augmenting its ability to target p53 for degradation. The finding that p300 promotes p53 degradation in MDM2-expressing cells is consistent with the recent report by Grossman et al. which showed that p300 participates in the MDM2-mediated p53 degradation (10). We, however, extend this observation further by showing that p300 exerts its negative influence on p53 stability by increasing the half-life of MDM2. Experiments conducted in cells expressing temperature-sensitive p53 mutant allowed us to recapitulate precisely the effects of p300 on p53 stability under different conditions, specifically in the presence or absence of induction of endogenous MDM2 expression. These data provided compelling evidence to demonstrate that MDM2 is the key determinant of the p300 effect on p53 stability.

Our finding that MDM2 stabilization by p300 is acetylase-dependent identifies MDM2 as yet another non-histone substrate of p300, an observation consistent with recently reported results (15). MDM2 itself is a very unstable protein that undergoes self-ubiquitination and is degraded by the proteasome-dependent pathway. Whether MDM2 is directly acetylated by p300 in vivo and how p300 acetylase activity contributes to increased MDM2 protein stability is currently under investigation.

Together with findings reported by others, our results suggest a dual function for p300 in the regulation of p53 stability. p300 seems to enhance the opposite processes in the p53/MDM2-negative regulatory feedback loop. In proliferating cells, the p53 protein is maintained at low levels via the action of the p53/MDM2 negative regulatory feedback loop, which is blocked upon exposure to stress signals that activate p53 response. p300 plays a critical role in initial activation and accumulation of p53. After the damaged DNA is repaired or severely compromised cells are removed via apoptosis, it is necessary to down-regulate p53 levels so that cells can resume DNA replication and reenter the cell cycle. p300 ensures the removal of p53 via its stabilizing effects on MDM2. In conclusion, p300 plays an important role in both p53 activation in response to stress, and also contributes to cellular recovery process.

Acknowledgment—We thank Dr. Tso-Pang Yao (Duke University) for acetylase-deficient p300 mutant.

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