Role for p300 in Stabilization of p53 in the Response to DNA Damage*

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The nuclear p300/CBP proteins function as coactivators of gene transcription. Here, using cells deficient in p300 or CBP, we show that p300, and not CBP, is essential for ionizing radiation-induced accumulation of the p53 tumor suppressor and thereby p53-mediated growth arrest. The results demonstrate that deficiency of p300 results in increased degradation of p53. Our findings suggest that p300 contributes to the stabilization and transactivation function of p53 in the cellular response to DNA damage.

In the exposure of cells to ionizing radiation (IR), the formation of DNA double-strand breaks is associated with increases in p53 levels and the transactivation function of p53 (1–3). Activation of p53 in the response to IR induces transcription of the p21 (WAF1, Cip-1) gene (4). Thus, the growth arrest function of p53 is regulated at least in part by p21-mediated inhibition of cyclin-Cdk complexes and the proliferating cell nuclear antigen (PCNA) (5). In addition, p53-dependent induction of the bax gene contributes to the apoptotic response to DNA damage (5). Other genes implicated in p53-induced growth arrest and apoptosis include GADD45 (6), mdm2 (6, 7), cyclin G (8), and IGF-BP3 (9).

Recent work has demonstrated that the DNA-dependent protein kinase (DNA-PK) is necessary but not sufficient for activation of p53 sequence-specific DNA binding (10). Phosphorylation of the p53 N-terminal region by DNA-PK may contribute to the transactivation function and stability of p53 (11, 12). Other studies have shown that the ataxia telangiectasia-mutated (ATM) protein phosphorylates p53 on serine 15 in vitro (13, 14). The findings that the p53 serine 15 site is phosphorylated in IR-treated cells (15, 16) and that this effect is diminished in AT cells (16) have supported a role for ATM in the regulation of p53. The p300/CBP proteins (17–20) have also been implicated as coactivators of the p53 transactivation function (21, 22). The N-terminal domain of p53 interacts with the C-terminal region of p300/CBP. Acetylation of the p53 C-terminal domain by p300/CBP stimulates the DNA binding activity of p53 (23). A dominant negative form of p300/CBP has also been found to inhibit p53-mediated transactivation and the G1 arrest and apoptotic responses (24).

Cells derived from p300-deficient embryos exhibit severe defects in proliferation (25). Consequently, in the present work, we have established cells expressing ribozymes specific for p300 or CBP such that the transfectants are selectively deficient in either protein. Our results demonstrate that p300, and not CBP, is essential for IR-induced increases in both p53 levels and the p53 transactivation function.

MATERIALS AND METHODS

Cell Culture—MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated bovine serum, 2 mM l-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin. The active p300 (p300-R), inactive p300 (p300-RI), active CBP (CBP-R), or inactive CBP (CBP-RI) ribozymes (36) were stably introduced into cells by LipofectAMINE (Life Technologies, Inc.) and selection in G418. Cells were treated with ionizing radiation at room temperature using a Gammacell 1000 (Atomic Energy of Canada) with a 137Cs source emitting at a fixed dose rate of 0.21 Gy/min.

Immunoprecipitation and Immunoblot Analysis—Immunoprecipitations were performed as described (27). Soluble proteins were incubated with anti-p300 (RW128; Upstate Biotechnology Inc.), anti-CBP (06–1110; Upstate Biotechnology Inc.), and anti-PCNA. Proteins were prepared from a cell extract (38) with anti-p21 (Ab-3; Oncogene Science), anti-FLAG (M5; Eastman Kodak Co.) or anti-PCNA (SC-5b; Santa Cruz). Proteins were resolved on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose paper. The filters were incubated with anti-p300, anti-CBP, anti-p300 (Ab-6; Oncogene Science), anti-p21 (Ab-3; Oncogene Science), anti-MDM2, anti-FLAG (M5; Eastman Kodak Co.) or anti-PCNA (SC-5b; Santa Cruz). Proteins were visualized by Enhanced Chemiluminescence (ECL; Amersham Pharmacia Biotech). The filters were exposed to x-ray film for 2–24 h at −80 °C.

Transient Transfections—Cells were cotransfected with: (i) pNF-eB-Luc and pPC-MEKK (Stratagene 219077), (ii) pCRE-Luc and pFC-PKA (Stratagene 219075), (iii) mdm2NA-Luc (28) and HA-p300, or (iv) FLAG-p53 and pFC-MEKK (Stratagene 219077), (v) C-terminal region of p300/CBP. Acetylation of the p53 C-terminus by DNA-PK may contribute to the transactivation function (21, 22). The N-terminal domain of p53 interacts with the p53 transactivation function (23). A dominant negative form of p300/CBP has also been found to inhibit p53-mediated transactivation and the G1 arrest and apoptotic responses (24).

Cells derived from p300-deficient embryos exhibit severe defects in proliferation (25). Consequently, in the present work, we have established cells expressing ribozymes specific for p300 or CBP such that the transfectants are selectively deficient in either protein. Our results demonstrate that p300, and not CBP, is essential for IR-induced increases in both p53 levels and the p53 transactivation function.

RESULTS AND DISCUSSION

Expression of the inactive p300 ribozyme (p300-R) had no detectable effect on p300 levels (Fig. 1A). By contrast, the active p300 ribozyme (p300-R) markedly decreased p300, and not CBP, expression (Fig. 1A). Similar results were obtained in two independently selected cell clones (Fig. 1A). Conversely, expression of the active CBP ribozyme (CBP-R) decreased CBP, but not p300, levels (Fig. 1B). These findings supported specificity of the ribozymes for selective depletion of p300 or CBP.

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† The abbreviations used are: IR, ionizing radiation; PCNA, proliferating cell nuclear antigen; PK, protein kinase; ATM, ataxia telangiectasia-mutated; Gy, gray; BrdUrd, bromodeoxyuridine; GFP, green fluorescence protein; FACS, fluorescence-activated cell sorting.
Also, none of the ribozymes had detectable effects on basal levels of p53 expression (Fig. 1, A and B). The effects of deficiency in p300 or CBP were investigated by assessing activation of the NF-κB and CRE transcription factor pathways. NF-κB activation by MEKK was significantly compromised in the p300-, but not in the CBP-, deficient cells (Fig. 1 C). By contrast, although stimulation of CRE activity by protein kinase A was partially affected by deficiency in p300, CRE activation was markedly attenuated in the CBP-deficient cells (Fig. 1 D). These findings confirm that transcriptional coactivation functions are selectively abrogated in cells deficient in p300 or CBP (18, 29–31).

p53 transcriptional activity was assessed with a reporter construct containing the luciferase gene driven by a p53 enhancer from the MDM2 promoter (28). Despite similar basal levels of p53 in the cell clones, the transactivation function of p53 was markedly decreased in the p300-deficient cells (Fig. 2 A). By contrast, there was no apparent effect of CBP deficiency on p53 transcriptional activity (Fig. 2 A). Similar results were obtained in the two independently selected p300- and CBP-deficient clones (data not shown). To confirm that the deficiency in p300 is responsible for the defect in p53-mediated transactivation, the p300-deficient cells were cotransfected with the luciferase reporter and a vector expressing HA-p300. The results demonstrate that the luciferase activity is restored in a dose-dependent manner by expression of p300 (Fig. 2 B). Cotransfection of p300 with a similar reporter driven by an MDM2 promoter with a mutated p53 binding site was associ-
ated with little if any induction of luciferase activity (data not shown). Whereas IR induces activation of p53, we asked whether p300-mediated p53 transactivation is involved in the responses to IR. The results demonstrate that exposure of p300-RI cells to IR results in activation of p53 transcriptional activity in a time-dependent fashion (Fig. 2C). By contrast, IR-induced p53 transactivation was significantly attenuated in p300-R cells. In concert with the basal transcriptional activity, deficiency in CBP did not appear to have any effect on IR-induced p53 activation. To determine whether p53 associates with the p300/CBP proteins, lysates from wild-type MCF-7 cells were subjected to immunoprecipitation with anti-p300 or anti-CBP, and not CBP, is necessary for the p53-mediated G1 arrest response to IR. A, representative two-dimensional FACS analysis of cells expressing the indicated ribozymes 24 h after exposure to 0- or 5-Gy IR. B, percentage of S phase cells after IR relative to unirradiated cells. Results represent the mean ± S.D. from two experiments each performed in triplicate. C, cells expressing the p300-RI or p300-R ribozymes were transfected with 0.5 μg of pEGFP-Ci (lanes 1 and 2), and cells expressing p300-R were cotransfected with 3 μg of empty vector and 0.5 μg of pEGFP-Ci (lane 3) or HA-p300 and 0.5 μg of pEGFP-Ci (lane 4). After 24 h, the cells were exposed to 0- or 5-Gy irradiation. After an additional 24 h, the cells were harvested, fixed, and the GFP-positive cells were sorted for two-dimensional FACS analysis. Results represent the percentage of S phase cells (mean ± S.D. of two experiments performed in triplicate) after IR relative to unirradiated cells.

**Fig. 3.** p300, and not CBP, is necessary for the p53-mediated G1 arrest response to IR. A, representative two-dimensional FACS analysis of cells expressing the indicated ribozymes 24 h after exposure to 0- or 5-Gy IR. B, percentage of S phase cells after IR relative to unirradiated cells. Results represent the mean ± S.D. from two experiments each performed in triplicate. C, cells expressing the p300-RI or p300-R ribozymes were transfected with 0.5 μg of pEGFP-Ci (lanes 1 and 2), and cells expressing p300-R were cotransfected with 3 μg of empty vector and 0.5 μg of pEGFP-Ci (lane 3) or HA-p300 and 0.5 μg of pEGFP-Ci (lane 4). After 24 h, the cells were exposed to 0- or 5-Gy irradiation. After an additional 24 h, the cells were harvested, fixed, and the GFP-positive cells were sorted for two-dimensional FACS analysis. Results represent the percentage of S phase cells (mean ± S.D. of two experiments performed in triplicate) after IR relative to unirradiated cells.

**Fig. 4.** p300 regulates accumulation of p53. A, cells expressing the indicated ribozymes were left untreated (C) or were exposed to 5-Gy ionizing radiation (IR). Lysates from cells harvested 3 h after IR were subjected to immunoblot analysis with anti-p53, anti-p21, or anti-MDM2. B, p300-deficient cells were cotransfected with 3 μg of control vector or HA-p300. After 24 h, the cells were treated and analyzed as described in A. C, p300-deficient cells were cotransfected with 3 μg of control vector and 1 μg of FLAG-p53 or with 3 μg of HA-p300 and 0.5 μg of FLAG-p53 to achieve comparable FLAG-p53 expression levels. After 24 h, the cells were treated with 10 μg/ml cycloheximide and then harvested at the indicated times. Lysates were subjected to immunoblot analysis with anti-FLAG or anti-PCNA. D, cells expressing the p300-RI or p300-R ribozymes were left untreated (C) or exposed to 5-Gy ionizing radiation (IR) and harvested 1 h later. Lysates were subjected to immunoprecipitation with a limiting amount of anti-MDM2. The immunoprecipitates were analyzed by immunoblotting with anti-p53 and anti-MDM2.
anti-CBP. Analysis of the immunoprecipitates demonstrated binding of p53 to p300 and not CBP (Fig. 2D).

To define the functional consequences of the defect in p53-mediated transactivation in p300-deficient cells, we tested the ability of IR to induce a G1 arrest response. Using BrdUrd labeling and bivariate FACS analysis, we found less irradiated p300-deficient cells arrested in G1 phase compared with similarly treated control cells expressing the inactive ribozyme (Fig. 3A). Over 30% of the p300-deficient cells were in S phase 24 h after receiving 5 Gy IR compared with untreated controls. By contrast, irradiated CBP-deficient cells were less affected, with the S phase population being approximately 10% of untreated samples (Fig. 3, A and B). Similar results were obtained with the independently isolated p300- and CBP-deficient clones (data not shown). To confirm that p300 deficiency is responsible for the defective G1 arrest response, the p300-deficient cells were transfected with the empty vector or HA-p300. The results demonstrate that the response to irradiation was restored by expression of p300 (Fig. 3C). These findings indicate that p300, and not CBP, is necessary for the G1 arrest response to IR.

Whereas IR-induced G1 growth arrest is impaired in p300-deficient cells, we asked whether this defect is due to a decrease in the transactivation function of p53. The results demonstrate that both the accumulation of p53 and the induction of p21 and MDM2 in response to IR are attenuated in the p300-deficient cells, but not in the CBP-deficient or control cells (Fig. 4A). To confirm that the deficiency in p300 is responsible for the impaired p53 response to IR, we transfected the p300-deficient cells with HA-p300. Transfection of HA-p300, but not the empty vector, restored IR-induced accumulation of p53 and the transactivation of p21 and MDM2 (Fig. 4B). These findings suggest that the attenuated accumulation of p53 and the impaired induction of p21 in p300-deficient cells are responsible for the defect in IR-induced G1 arrest. Because DNA damage-induced accumulation of p53 is regulated by a post-translational mechanism (1), the attenuated induction of p53 in irradiated p300-deficient cells prompted studies on p53 stability. FLAG-tagged p53 was cotransfected with a control vector or HA-p300 into p300-deficient cells. Stability of the FLAG-tagged p53 was assessed after the addition of cycloheximide. Comparison of the declines in p53 levels under the different experimental conditions demonstrates that p300 stabilizes the p53 protein (Fig. 4C). MDM2 binds to p53 in the transactivation domain and promotes p53 degradation (32–34). Thus, our findings collectively support a model in which p300 stabilizes p53 and contributes to the transcriptional coactivation of p53 (data not shown). These findings suggest that results obtained in transient overexpression or gain of function studies may confound interpretations of events observed in cells with loss or deficiency of function. In this context, p300-, but not CBP-, deficient cells exhibit defects in induction of p21 and MDM2 in the response to DNA damage. These results are in concert with the observation that p300, and not CBP, associates with p53 in cells. We show that p300, and not CBP, is required for the IR-induced accumulation of p53. The results indicate that p300 functions in the stabilization of p53. Thus, our findings collectively support a model in which p300 stabilizes p53 and contributes to the p53 transactivation function in the growth arrest response to DNA damage.

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