p300 Does Not Require Its Acetylase Activity to Stimulate p73 Function*

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We previously reported that p73, like p53, utilizes p300 or cAMP-respons e element-binding protein-binding protein as its coactivator. Here, we extended this work by further examining whether the intrinsic acetylase activity of p300 is necessary for stimulating p73 function. Although p300 acetylated the C-terminal fragment of p73 (amino acids 311–636) in vitro, it was unable to efficiently acetylate the full-length p73. Consistently, p300 did not acetylate p73 in vivo when both the proteins were overexpressed in cells. Also, an acetylase-defective mutant p300 named p300AT2 was able to elevate p73-dependent transcription in cells. p300 associated with p73 when forming DNA-protein complexes and stabilized p73 proteins. These results demonstrate that p300 does not need its acetylase activity to be a coactivator of p73.

p300 and CBP\(^1\) have been shown to mediate transcription by many different transcriptional activators (1), including the tumor suppressor p53 protein (2–4), and to be involved in cell growth control and neoplasia (see Ref. 5 and references therein). p300 and CBP are two individual proteins encoded by two different genes (5–8), but they share a significant homology in their functional domains with similar biochemical functions. However, their roles in development and neoplasia are not redundant (5, 9), indicating that each of them is crucial for cell growth control. Interestingly, both of these coactivators possess intrinsic acetylase activity, which plays a critical role in regulating the functions of some p300/CBP interacting transcriptional activators (10), such as p53 (11, 12). By acetylating p53, p300/CBP stimulates its ability to bind to DNA in a sequence-specific fashion in vitro (11) and enhances its transcription in vivo (2–4), indicating that this group of coactivators participate in the p53 responsive pathway. Indeed, DNA damage signals stimulate p53 acetylation by p300 (12), which can be inhibited by the cellular p53 repressor MDM2 (13). Hence, participating in the p53 pathway represents one role of p300/CBP in preventing neoplasia.

p300 has also been shown to regulate the function of the human p53 homolog p73 (14, 15), which has several alternate splice forms (16). p73 shares the sequence homology and some biochemical activity with p53 (14–17). Although p73 was shown to induce the same subset of genes as p53 does with a minor difference (14, 17, 18), they appear to play different physiological roles (19). p73-deficient mice displayed neurological, pheromonal, and inflammatory defects without spontaneous tumors (19), whereas p53 knockout mice developed normally with an early onset of tumorigenesis (20). However, because of the high mortality of the p73 null mice (19), it is difficult to assess the role of p73 in tumorigenesis. Other studies showed that the oncogenic p53 mutants inhibit p73 function (21, 22), and the alternate splice form of p73 that lacks the N-terminal transactivation domain represses the function of both p53 and p73 (23, 24), suggesting that p73 may also be involved in tumorigenesis. However, this function of p73 may be suppressed by its N-terminal truncated alternates (23, 24), which were found to be expressed at the high level in some primary tumors (25–28).

Distinct roles of p73 and p53 in development and tumorigenesis suggest that these proteins may be differentially regulated through separate mechanisms. Indeed, upon y irradiation, p73 is activated through the c-Abl tyrosine kinase (29–31), whereas p53 is activated through the Ataxia telangiectasia-mutated Chk2 pathway (32–36). Also, MDM2 mediates degradation of p53, but not p73, although it inhibits the functions of both the proteins (14, 37–42). In addition, unlike p53, p73 contains a unique C-terminal SAM (sterile alpha motif)-like domain that may be involved in protein-protein interaction (43, 44). Distinct mechanisms for the regulation of the p53 family members may, at least partially, account for their different physiological roles in development, homeostasis, or tumorigenesis. Thus, unraveling their regulatory mechanisms is crucial for better understanding of their biological roles.

In an attempt to elucidate the mechanisms of p73 regulation, we previously reported that p73, like p53, utilizes p300 or CBP as its coactivator. Here, we extended this work by further examining whether the intrinsic acetylase activity of p300 is necessary for stimulating p73 function. Surprisingly, p300 was unable to acetylate the full-length p73a in vitro. Also, p300 did not acetylate p73a in vivo when both of the proteins were overexpressed in cells. Moreover, an acetylase-defective mutant p300 named p300AT2, like wild type p300, was able to elevate p73a-dependent transcription in cells. These results demonstrate that p300 does not need its acetylase activity to stimulate p73-dependent transcription.

EXPERIMENTAL PROCEDURES

Cell Culture—Human lung small cell carcinoma H1299 cells were cultured as described previously (45).

Antibodies and Reagents—The anti-acetylated lysine antibody and the polyclonal anti-p53 antibody were purchased from Santa Cruz Biotechnology Inc. Monoclonal anti-p53 antibody Pab421 was described previously (14). Polyclonal anti-p73 antibodies were raised specifically
Acetylated proteins (top described (46). This anti-p73 antibody recognizes both p73 and p73B here. Acetylated proteins were detected by autoradiography. Molecular cated in the figure legends, were pre-incubated with antibodies against protein levels.

Inc.). Monoclonal anti-p53 antibody 421, polyclonal anti-p73 antibody, was detected either by autoradiography or Western blot analysis using antibodies specific for acetylated lysines (Upstate Biotechnology, Inc.). The p53 or p73 protein (lower panel) was detected by WB using anti-p53 or anti-p73 antibodies.

against the C terminus (aa 401–636) of p73α and affinity-purified as described (46). This anti-p73 antibody recognizes both p73α and p73β.

Baculovirus harboring human p90Raf mutant AT2 and pBlueScript-p300AT2 were generously provided by W. Lee Kraus (Cornell University, Ithaca, NY) and James T. Kadonaga (University of California, San Diego, CA). The p300AT2 insert with a C-terminal polyhistidine tag was cloned into the HindIII and NotI sites of pcDNA3 vector (Invitrogen).

Purification of Recombinant p300, p300AT2, p53, p73α, and the p73α C Terminus (aa 311–636)—p300AT2 and p300 were purified from baculovirus-infected SF9 insect cells using immunopurification columns as described. His-p53, His-p73α, and the p73α C terminus (aa 311–636) tagged with histidines were purified from bacteria using nickel-nitri-triacetic acid column as described (14, 15).

Acetylation Assay—Acetylation assays were carried out according to the published method (11, 13). 20 μl of reaction mixture contained 50 mm Tris-HCl (pH 8.0), 10% glycerol (v/v), 0.1 mm EDTA, 1 mm dithiothreitol, 10 μm sodium butyrate, [1-14C]Acetyl-CoA, or 500 mm or different concentrations of acetyl-CoA (Sigma), different amounts of p53, p73, the p73 C-terminal fragment (aa 311–636), histones, p300, or p300AT2 (see figure legends for the amounts of the proteins used in each reaction). The mixture was incubated at 30 °C for 60 min and analyzed by SDS polyacrylamide gel electrophoresis. Acetylated p53 was detected either by autoradiography or Western blot analysis using the polyclonal anti-acetylated lysine antibody (Upstate Biotechnology, Inc.). Monoclonal anti-p53 antibody 421, polyclonal anti-p73 antibody, and polyclonal anti-p300 antibody were used to detect corresponding protein levels.

EMSA—This assay was conducted as described. Proteins, as indicated in the figure legends, were pre-incubated with antibodies against p300 or p73 or p63 in the presence or absence of acetyl-CoA, at 30 °C for 30 min, as described above, prior to being mixed with a DNA binding mixture containing 10 μg Hepes buffer (pH 7.5), 4 μm MgCl2, 60 mm NaCl, 0.1 μg poly(dI-dC), 0.1% Nonidet P-40, 0.1 mm EDTA, and 5’32P end-labeled DNA fragments harboring two copies of the p53RE sequence derived from the MDM2 promoter (5,000 cpm; 1.0 ng of DNA per assay). The reaction was incubated at room temperature for 30 min and directly loaded onto a 4% nondenatured gel.

Western Blot Analysis—Transfected cells were harvested for preparation of nuclear extract. Nuclear extracts containing 150 μg of proteins were directly loaded onto an SDS gel, and proteins were detected by ECL reagents (Bio-Rad) after Western blotting using antibodies as indicated in the figure legends.

Transient Transfection and Luciferase Assay—H1299 cells (106 cells/60-mm dish) were transfected with p73α (500 ng) or p53 (500 ng) alone or together wild type (WT) or mutant (AT2) p300 (2.5 μg), as indicated on top. Whole cell extracts (500 μg) of the transfected cells were directly loaded onto an 8% SDS gel. As indicated on left, p73α and p53 were detected by WB using the monoclonal antibodies against p73 and p53 (top panel). The acetylated proteins were detected by WB using polyclonal antibodies against acetylated lysines (bottom panel). Lane 5 denotes the molecular mass markers, and the acetylated bovine serum albumen was detected by the anti-acetylated lysine antibody (bottom panel). B, overexpression of p300 did not influence the mRNA synthesis of either p73α or p53 in cells. Northern blot analysis (NB) of the mRNA levels of p73α and p53 after the same transient transfection as described in panel A is shown. Plasmids used are indicated on top of both panels. 13 μg of total RNAs isolated from the transfected cells were loaded onto a 1% agarose gel. Ethidium bromide staining for total RNA loading was shown on the lower panel. The mRNA for either p73α or p73 was detected by NB using the 32P-labeled p73α or p53 probes (upper panel).
p300 Does Not Acetylate the Full Length of p73a in Vitro—The acetyltransferase activity of p300 has been shown to be important in mediating the activity of the transcriptional activator p53 (11–13). To check whether this is also true for the p53 homolog p73a (16), we conducted in vitro acetylation assays using recombinant p300 purified from baculovirus and histone p73a or his-C terminus of p73a (aa 311–636) purified from bacteria, as well as 14C-acetyl-CoA as substrates. Surprisingly, as shown in Fig. 1A, p300 was unable to acetylate the intact p73a protein (lanes 3 and 4). This was not because of the inactivity of the purified p300 protein, as it was active in acetylating p53 (lane 1), as well as the C-terminal domain of p73a (lanes 5 and 6). As shown in the representative result (Fig. 1B), even though up to 5 μM of acetyl-CoA was used, p300 (200 ng) was still not able to acetylate p73a (lanes 1–7). Also, 1 μg of p300 did not show efficient acetylase activity on p73a (data not shown). In contrast, p300 needed only 100 nM of acetyl-CoA to sufficiently acetylate p53 (lane 11) and ~1 μM acetyl-CoA to reach a plateau level (lanes 8–14). These results indicate that p300 does not utilize the full-length p73a as a substrate in vitro.

p300 Does Not Acetylate p73a in Vivo—Next, we tested whether p300 acetylates p73a in vivo. To this end, human p53 null lung small cell carcinoma H1299 cells were transiently transfected with plasmids encoding wild type, mutant p300 (p300AT2) lacking acetylase activity (48), p53, or p73a, as indicated in Fig. 2A. Acetylation of p53 was detected by Western blot using polyclonal antibodies specifically against acetylated lysines. As expected, p300 but not the p300AT2 mutant acetylated p53 in cells (lanes 6 and 7 of the bottom panel). By striking contrast, p73a acetylation was not detectable in the presence of wild type p300 (lane 2), despite the fact that the p73a protein was well expressed (top panel), and the transfected cells were exposed to UV or γ irradiation (data not shown). This was not because of the inability of the anti-acetylated lysine antibody to recognize the acetylated p73a, as this same antibody was able to immunoreact with the p73 C-terminal region that was acetylated by p300 in vitro (Ref. 13 and data not shown). These results clearly show that p300 does not acetylate p73a in cells.

Consistent with the published studies by others and us (13, 49), the p53 level increased when p300 was cointroduced into the cells (compare lane 7 with lane 8 of the top panel). The acetylase activity is partially responsible for this increase, as the p53 level was elevated to a lesser degree when the p300 mutant, which did not acetylate p53, was cotransfected with p53 (lane 6). This result was reproduced in separate experiments (data not shown). Similarly, the p73a level also in-
increased when cotransfected with p300 (lane 2). However, this was not dependent upon the acetylase activity of p300, as p300 did not acetylate p73α (compare lane 2 with lane 3 of the bottom panel), and overexpression of the acetylase-defect mutant p300AT2 also increased the level of p73 (lane 1). Because the mRNA synthesis of the exogenous p53 or p73α was not affected by the exogenous p300 (Fig. 2B), the increase of either the p53 or p73α protein was not due to transcriptional activation of these exogenous genes by p300. These results, which were reproducible, suggest that although p300 did not acetylate p73α, it may modulate the stability of this transcriptional activator.

p300 Joins and Stabilizes the p73-DNA Complex in Vitro—Because p300 enhances the ability of p53 to bind the specific p53RE motif by acetylating this protein (11, 13), we tested whether p300 affects the sequence-specific DNA binding ability of p73α without acetylating this protein. First, we established a gel mobility shift assay, using the recombinant p73α protein purified from bacteria and the radiolabeled DNA oligomer probes containing the p53RE sequence derived from the p21 promoter (Fig. 3A). The p73α-DNA complex migrated slower than the p53-DNA complex and was specifically inhibited by the nonlabeled p53RE-containing oligomers but not dIdC. Also, this DNA-protein complex was supershifted by antibodies specifically against p73 but not p63 (Fig. 3B).

As expected (11, 13), p300 (100 ng) markedly stimulated p53-DNA complex formation in the presence of acetyl-CoA (lanes 3 and 4 of Fig. 3C). However, this acetylase only caused a marginal increase of the p73α-DNA complex, even when 2-fold more p300 (200 ng) was used (lanes 5–11). This slight effect was not reproduced (data not shown). Rather, p300 participated in the p73-DNA complex, because addition of increasing amounts of p300 supershifted this complex (lanes 6–7), and this complex was further supershifted by the anti-p300 antibody (lanes 9–11). By participating in the p73-DNA complex, p300 may stabilize this complex, because the amount of the complex increased apparently when 300 ng of p300 was added into the reaction (lanes 8 and 11). This increase was also seen in the absence of acetyl-CoA (data not shown). These results suggest that p300 joins and stabilizes the p73-DNA complex through direct protein interaction but not through its acetylase activity.

Stimulation of p73α-dependent Transcription by the Acetylase-defective Mutant p300—to Further Test whether the acetylase-defective p300AT2 mutant is able to influence p73α function in cells, H1299 cells were transfected with the p73α expression plasmid alone or together with plasmids encoding wild type p300 and its two mutants, p300AT2 and the deletion lacking the central portion from aa 242 to aa 1740 (Δ242–1740), in the presence of the p53RE-driven luciferase reporter plasmid. Luciferase activity was measured as an indication of p73α-dependent transcription (Fig. 4C). We previously showed that Δ242–1740 did not bind to p73 and had no effect on its transcription activity (15). Consistently, this mutant did not increase the level of p73α (two upper panels of Fig. 4B), and thus, was used here as a negative control. As expected, this mutant neither acetylated p53 nor increased its protein level (two lower panels of Fig. 4B). In agreement with our previous study (15), p300 markedly stimulated p73α-dependent transcription, whereas the deletion mutant had no effect on this transcription activity (Fig. 4C). The same was true for p53 (data not shown; see Ref. 11). However, the acetylase-defective p300AT2 mutant, like wild type p300, enhanced p73α-mediated transcription markedly. This enhancement would not be due to the general effect on nucleosome modeling or modification, because this mutant was inactive in acetylating histones (Fig. 4A) and did not affect the mRNA level of p73α (Fig. 2B). These results indicate that p300 can stimulate p73α-mediated transcription without involving its intrinsic acetylase activity.

We previously showed that p73 interacts with p300 through the N terminus of p73 and the CH1 domain of p300 (15), and this interaction mediates p73-dependent transcription and apoptosis (15). The study presented here provides several lines of evidence showing that p300 positively affects p73-mediated transcription activation independently of its intrinsic acetylase activity. First of all, p73α was a considerably poor substrate for the acetylase activity of p300 in vitro, in contrast with p53, which is acetylated by p300 in response to DNA damage (12, 13). Consistent with this in vitro result, p300 did not acetylate p73α when both of the proteins were overexpressed in cells. p73β was also not acetylated by p300.2 Moreover, the acetylase-defective mutant p300AT2 was able to stimulate p73-dependent transcription in transient transfected cells. Thus, these results demonstrate that p300 does not need its acetylase activity to activate p73 function. Although our study does not exclude the possibility that p73α may be regulated through acetylation by other acetylases such as CBP or p300/CBP-associated factor (12, 50, 51), this appears less likely, because we were not able to detect acetylated p73 molecules by Western blot using antibodies against the acetylated lysine, even when this protein was overexpressed, or the transfected cells were irradiated with UV or γ ray (Fig. 2 and data not shown).

How does p300 modulate p73 function without acetylating this protein? One possibility would be that p300 may regulate p73 stability and thus enhance its activity. The p73α protein level increased when coexpressed with p300 (Fig. 2 and Fig. 4B). However, the mRNA level of p73α was not affected by p300 (Fig. 2B), suggesting that p73α is regulated at the protein level and probably stabilized by p300. Alternatively, p300 may serve as a bridging protein that links p73 with the RNA polymerase II transcriptional machinery. Supporting this is that p300 interacts with p73 when this transcriptional activator binds to its responsive DNA element sequence (Fig. 3C). Although these two possibilities may coexist, further investigation of these questions is necessary to elucidate the detailed molecular and biochemical mechanism of p73 regulation by p300.

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