Charge Modification at Multiple C-terminal Lysine Residues Regulates p53 Oligomerization and Its Nucleus-Cytoplasm Trafficking

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The tumor suppressor p53 plays critical roles in protecting cells from malignant transformation. Upon its activation in response to a variety of stress stimuli, p53 becomes stabilized and accumulates in the nucleus and induces cell cycle arrest and apoptosis (1). Due to its potent growth inhibition and pro-apoptotic activity, p53 is normally kept latent and at low levels in unstressed cells by its negative regulator MDM2. MDM2 represses p53, in part, by promoting p53 degradation and nuclear export through catalyzing p53 poly- and monoubiquitination on specific lysine residues (2). Although polyubiquitinated p53 is targeted to proteasome for degradation, the exact mechanism by which monoubiquitination promotes p53 nuclear export is not understood.

In contrast to MDM2-mediated ubiquitination, acetylation has been positively linked to both p53 transcriptional activity and p53 stability (reviewed in Refs. 3 and 4). Consistent with this idea, p53 invariably becomes acetylated upon its activation by various p53-activating agents (5). The acetylation of p53, which is catalyzed by the p300/CBP (6) acetyltransferases, occurs on at least six lysine residues clustered at the C terminus (6, 7). It is unclear why so many lysines are modified by acetylation. Nor is it known whether the acetylation of the lysines provides a functional moiety for protein-protein interaction or serves to modify the conformation of p53. One clue as to the function of p53 acetylation lies in the potentially interesting links between acetylation and ubiquitination. Both the acetylation and the ubiquitination machinery modify the amino group of the lysine residue. Furthermore, acetylation and ubiquitination occur on a common set of lysine residues at the C terminus of p53 (7). These observations suggest that MDM2-mediated ubiquitination and p300/CBP-mediated acetylation might functionally antagonize one another to control p53 activity.

Recent studies have highlighted the importance of intracellular trafficking in the regulation of p53 activity (8–10). It is known that p53 actively shuttles between the nucleus and cytoplasm via a mechanism that involves a nuclear export signal (NES) and its receptor Crm-1 (reviewed in Ref. 11). Although its importance is apparent, little is known about how p53 nuclear export is regulated. Recent studies have indicated that the oligomeric status of p53 may control the accessibility of NES to the export machinery (8). Biochemical and structural studies revealed that the C-terminal NES is normally exposed in the inactive monomeric or dimeric forms of p53 that are subject to active nuclear export. Upon its conversion to the active tetrameric form, however, the C-terminal NES becomes buried and inaccessible, resulting in p53 nuclear retention. At present, it is not understood how the oligomerization status of p53 is regulated in response to specific stress signals or modifications, such as ubiquitination.

Here we have presented evidence that lysine acetylation is a novel mechanism that controls p53 oligomerization and subcellular trafficking. We found that p53 hyperacetylation leads to cytoplasmic accumulation of endogenous p53 in response to p53-activating agents. We further showed that overexpression of p300 stimulates cytoplasmic accumulation of p53 in an acetylation-dependent manner. Mechanistically, we have provided evidence suggesting that acetylation promotes the nuclear export of p53 by neutralizing the "charge patch" created by the C-terminal lysine residues. The charge neutralization of the C-terminal lysines prevents p53 oligomerization and therefore exposed the p53 NES, allowing efficient p53 export. The requirement of multiple modified lysines for efficient export suggested a potential threshold mechanism wherein the acetylation level of p53 serves as a signal that promotes p53 export to the cytoplasm.

**MATERIALS AND METHODS**

*Cell Lines and Transfection—*H1299 human cells, p53(−/−/minus)), MDM2(−/−/minus)) mouse embryonic fibroblast (MEF) cells, and A549 cells were maintained in Dulbecco’s modified Eagle’s medium. All cells were grown at 37 °C in the presence of 10% fetal bovine serum and 1% penicillin-streptomycin.
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RESULTS

Acetylation Regulates Subcellular Localization of p53—We have previously shown that p53 becomes acetylated by p300/CBP upon cellular stresses (5). As p53 activation is accompanied by its accumulation in the nucleus, we asked whether p300-mediated acetylation regulates p53 subcellular localization. To test this, we transfected p53-null H1299 cells with expression plasmids for p53 and p300 and then examined p53 subcellular localization. As shown in Fig. 1A, panel a, when expressed alone, p53 resides almost exclusively in the nucleus. To our surprise, upon co-expression with p300, p53 localized to both the nucleus and the cytoplasm in the majority of the cells expressing both transfected p53 and transfected p300 (Fig. 1, A, panel b, and B). Cell counts demonstrated that up to 84% of the cells that expressed both p53 and p300 showed cytoplasmic accumulation of p53 (Fig. 1B). To determine whether the accumulation of p53 in the cytoplasm requires p300 activity, we examined whether an acetyltransferase-deficient p300DY mutant (5) could affect the subcellular localization of p53. As shown in Fig. 1, A, panel c, and B, unlike wild type p300, the acetyltransferase-deficient p300DY mutant did not stimulate p53 accumulation in the cytoplasm. As expected, both p300 and p300DY are localized to the nucleus (Fig. 1A, panels h, i, j, and l), and neither p300 nor the p300DY mutant had an effect on the levels of p53 under the experimental conditions (Fig. 1D) (5). Together, these results indicate that p300-mediated cytoplasmic accumulation of p53 requires p300-acetyltransferase activity.

As p53 subcellular localization is regulated by active nuclear export, we investigated whether acetylation-dependent p53 accumulation in the cytoplasm is dependent on the activation of the nuclear export machinery. As shown in Fig. 1A, panel d, and B, the nuclear export inhibitor leptomycin B (LMB) efficiently blocked the cytoplasmic accumulation of p53 induced by p300. We also evaluated the subcellular localization of a p53 mutant, the C-terminal NES (8) of which is inactivated. As shown in Fig. 1A, panel e, and B, when expressed alone, NES mutant p53 is localized to the nucleus, similar to wild type p53. However, unlike wild type p53, NES mutant did not accumulate in the cytoplasm in response to p300 expression and remained in the nucleus (Fig. 1A, panel f, and B). Importantly, wild type and NES mutant p53 are acetylated to comparable levels. These results demonstrated that p300-mediated cytoplasmic accumulation of p53 requires the C-terminal NES and suggested that acetylation enhances p53 nuclear export.

To further verify the conclusions from the immunolocalization study, we determined the subcellular localization of p53 by biochemical fractionation. As shown in Fig. 1C, p53 is normally a nuclear protein (lanes 1 and 2). However, co-expression of wild type p300, but not the p300DY mutant, induced a marked accumulation (25% of the total p53) of the p53 protein in the cytoplasmic fraction (compare lanes 2, 4, and 6). The p300-dependent cytoplasmic accumulation of p53 could again be largely reversed by LMB treatment (lane 8). These results are in agreement with the data obtained from the immunolocalization, providing further evidence that p300-mediated acetylation affects subcellular localization of p53. The presence of acetylated p53 in the cytoplasmic fraction suggests that p53 can be exported to the cytoplasm after being acetylated (Fig. 1C).

Lastly, we asked whether endogenous p53 accumulates in the cytoplasm during stress stimuli. As shown in Fig. 1E, we observed significant accumulation of endogenous p53 in the cytoplasm in response to UV irradiation or hypoxia when deacetylation of p53 is suppressed (Fig. 1E, compare panels b and d with panels a and c). In agreement with immunolocalization analysis, subcellular fractionation assays also reveal significant accumulation of endogenous p53 in the cytoplasm in response to UV irradiation (Fig. 1F, lane 3). Importantly, cytoplasmic p53 induced by UV irradiation is acetylated, as it can be recognized by an antibody specific for the acetylated p53 (Fig. 1F, lane 3, upper panel). Together, these results support the conclusion that hyperacetylation of p53 leads to its accumulation in the cytoplasm.

p300-mediated Cytoplasmic Accumulation of p53 Is Independent of MDM2—It was reported that MDM2 stimulates p53 nuclear export by promoting p53 ubiquitination (13, 14). As p300 functionally interacts with MDM2 (15), we determined whether p300-induced cytoplasmic accumulation of p53 requires MDM2. To test this idea, we expressed p53 alone or together with p300 in MDM2-deficient MEF cells (16) and assessed p53 subcellular localization. As shown in Fig. 2A, in the absence of MDM2, p300 is still capable of stimulating the cytoplasmic accumulation of p53. In fact, more than 90% of cells overexpressing p300 showed p53 in cytoplasm, which is similar to the effect induced by penicillin/streptomycin in a humidified atmosphere of 5% CO₂. All transfections were performed by the calcium phosphate method as described previously (12).

Plasmids—Wild type human p53 cDNA, wild type human MDM2, wild type human Myc-p300, and human p300DY mutants were described previously (5). p53-5KR and -6KR mutants were generated using site-directed mutagenesis, changing lysines 320, 370, 372, 373, 381, and 382 to arginines. p53-2KA, -3KA, -4KA, and -5KA mutants were also constructed using site-directed mutagenesis to exchange the lysines to alanines. p53-NES(-) mutants were also constructed using site-directed mutagenesis to change the leucines (amino acids 348 and 350) to alanines.

Immunofluorescence—For immunofluorescence staining, cells grown on a glass coverslip were transfected with 0.1 μg of p53 and 1 μg of Myc epitope-tagged p300 expression plasmids. Immunostaining was performed as described (12) using anti-p53 rabbit polyclonal antibody (FL-393, Santa Cruz Biotechnology), anti-HDAC1 monoclonal antibody (H-11, Santa Cruz Biotechnology), and anti-Myc monoclonal antibody 9E10 and anti-MDM2 monoclonal antibody SMP-14 (Santa Cruz Biotechnology). Hoechst 33258 was used to visualize the nucleus.

Fractionation, Immunoprecipitation, and Immunoblotting—The cells were homogenized using a Dounce homogenizer in buffer (25 mM Hepes–HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 5 mM MgCl₂, 50 mM NaF, 1 mM dithiothreitol) supplemented with 5 μM deacetylase inhibitor TSA (Sigma) and protease inhibitors. After centrifugation at 960 × g for 5 min, nuclei pellets were washed with the homogenizer buffer and then lysed in buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) supplemented with 5 μM trichostatin A and protease inhibitors. Lysates were centrifuged at 5000 × g for 5 min to obtain supernatants of the nuclear fraction. Meanwhile, NaCl and Nonidet P-40 were added to cytoplasmic supernatants to bring the concentrations up to 150 mM and 1%, respectively. Immunoprecipitation and immunoblotting were performed as described previously (5). Proteins were detected with one of the following antibodies: anti-human p53 antibody (Ab-6, Calbiochem), anti-human acetylated (Lys-373) p53 antibody and anti-human acetylated (Lys-382) p53 antibody (Calbiochem); anti-α-tubulin antibody (DM1A, Sigma); or anti-green fluorescent protein (GFP) antibody (Roche Applied Science).

Protein Production and Oligomerization Assay—Wild type, 3KA, 4KA, or 4KR p53 C-terminal DNA (amino acid 326–393) including the tetramerization domain was cloned into pGEX-6p-1 vectors. GST fusion constructs were expressed in Escherichia coli and purified with glutathione-Sepharose 4B (Amersham Biosciences) and cleaved with Precision protease (Amersham Biosciences). The oligomerization assay was performed as described previously (8).
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FIGURE 1. Acetylation regulates p53 nuclear export. A, subcellular localization of p53 and p300 following transfection into p53 (-/minus) H1299 cells. Cells were transfected with p53 wild type (wt) alone (panels a, g, and m), p53 wild type and Myc-p300 (panels b, d, h, j, n, and p), p53 wild type and Myc-p300DY (panels c, i, and o), p53-NES (-) mutant alone (panels e, k, and q), or p53-NES (-) mutant and Myc-p300 (panels f, l, and r) as indicated. The localization of p53, p300, and p300DY was determined as described under "Materials and Methods." 10 ng/ml of LMB was added 8 h prior to immunostaining as indicated (panels d, j, and p). Nucleus was visualized by Hoechst 33258 (panels m–r). B, acetylation by p300.
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MDM2 (Fig. 2B). This result indicates that p300-mediated cytoplasmic accumulation of p53 is independent of MDM2.

C-terminal Lysine Residues Are Required for Efficient Acetylation-induced Cytoplasmic Accumulation of p53—The results presented so far have supported the idea that p300 affects subcellular localization of p53 in an acetylation-dependent manner, likely by directly acetylating p53. To further investigate this possibility, we determined whether lysine residues known to be acetylated by p300 are required for acetylation-induced cytoplasmic accumulation of p53. We therefore mutated multiple lysines to arginines (5KR and 6KR, Fig. 3A) that are known targets of acetylation by p300 (7) and evaluated the subcellular distribution of these mutants in response to p300. As shown in Fig. 3B, when expressed alone, the localization of these p53 KR mutants is almost entirely nuclear and indistinguishable from wild type p53. However, in response to ectopically expressed p300, the number of cells that show a cytoplasmic accumulation of the 5KR (51%) and 6KR (30%) p53 mutants is markedly reduced when compared with that of wild type p53 (84%). These data indicated that the lysine residues acetylated by p300 are required for p53 nuclear exit in response to p300.

Neutralization of Positively Charged Lysine Residues in the C Terminus Regulates Subcellular Localization of p53—It was previously shown that acetylation in histone H2A.Z works by neutralizing the charge conferred by multiple lysine residues (17). As acetylation also targets multiple lysine residues in p53, we asked whether acetylation promotes p53 nuclear export by a similar mechanism. To test this, we generated charge-neutralizing mutations by converting lysine residues known to be acetylated and ubiquitinated to alanine, either individually or in combination, and evaluated their subcellular localization. As shown in Fig. 4, the subcellular localization of the 2KA and several 3KA (3KA-1, 3KA-2, and 3KA-3) mutants is similar to that of wild type p53 and is mostly nuclear (Fig. 4, B and C). In contrast, when four lysine residues are mutated in three different combinations (4KA-1, 4KA-2, and 4KA-3), these p53 mutants clearly began to accumulate in the cytoplasm. The conversion of five lysine residues (5KA) led to a further increase in the number of cells with cytoplasmic p53 staining (Fig. 4, B and C). The cytoplasmic localization of the 4KA-1, 4KA-2, 4KA-3, and 5KA p53 mutants was observed in 51, 52, 48, and 64% of transfected cells, respectively, when compared with 19% for wild type p53 (Fig. 4C). Furthermore, the intensity of the wild type p53 protein detected in the cytoplasm was much weaker than that of the 4KA and 5KA mutants (data not shown). Importantly, the percentage of cells with cytoplasmic p53, 100–200 cells from each transfection were scored. Results are an average of three independent experiments. C, cytoplasmic p53 is acetylated. Levels of acetylated p53 in the nuclear (N) and cytoplasmic (C) fractions were determined by immunoprecipitation with anti-p53 polyclonal antibody followed by immunoblotting with anti-acetylated p53 antibody. Levels of total p53, HDAC1, and β-tubulin were assayed by blotting with anti-p53 monoclonal antibody, anti-HDAC1 monoclonal antibody, and anti-tubulin monoclonal antibody. D, p300 does not change the expression level of total p53. GFP was used as internal control and detected by anti-GFP monoclonal antibody. DY, p300DY. E, A549 cells were irradiated to UV(75J/m2)(panels a and b) or treated with deferoxamine (DFX) to induce hypoxia (panels c and d). After UV irradiation or hypoxia treatment, the cells were treated with or without trichostatin A (TSA) and nicotinamide (Nico) for 8 h and then immunostained with anti-p53 antibody. F, cytoplasmic accumulation of endogenous p53 after UV exposure and p300 acetylation were determined as described above for C.
cytoplasmic p53 4KA and 5KA mutants can be effectively eliminated upon treatment with LMB (Fig. 4, B and C), supporting the idea that nuclear export is required for cytoplasmic accumulation of the 4KA and 5KA p53 mutants. Together, these observations indicate that the degree of p53 cytoplasmic accumulation is proportional to the number of lysine residues neutralized and suggest that acetylation modulates p53 subcellular localization by modifying the positive charge of specific lysine residues at the C terminus of p53.

**C-terminal Lysine Charge Determines the Oligomerization Status of p53**—The accessibility of the C-terminal NES to the export machinery has been shown to be regulated by the oligomerization status of p53 (8). We therefore assessed whether modification of the lysine charge activates p53 export by regulating p53 oligomerization status. Based on the observation that the conversion of at least four but not three lysines to alanines promotes p53 cytoplasmic accumulation, recombinant wild type, 3KA, and 4KA (4KA-1, 4KA-2, and 4KA-3, Fig. 4A) mutant polypeptides encompassing the entire p53 tetramerization domain and lysine-rich C terminus (amino acids 326–393) were tested for their ability to oligomerize. A mutant p53 with four lysines converted to arginines (4KR), which would prevent acetylation but preserve the charge of the lysine, was used as an additional control. As shown in Fig. 5, the wild type and 3KA polypeptides dimerized and tetramerized readily (lanes 2 and 4). In contrast, the 4KA-1, 4KA-2, and 4KA-3 polypeptides completely failed to do so (lanes 6, 8, and 10). Importantly, polypeptides from the 4KR mutant, which is a nuclear protein (data not shown), showed a wild type capacity to oligomerize (lane 12). Together, these results demonstrate that the oligomerization status of
p53 can be controlled by the charge conferred by a defined number of lysine residues.

DISCUSSION

In this report, we have provided evidence that acetylation regulates p53 subcellular localization, at least in part, by activating its nuclear export. Our study identified the charge of C-terminal lysine residues targeted by p300 as a regulatory element that controls p53 oligomerization and subcellular localization. Our data suggested that acetylation regulates p53 nucleus-cytoplasm trafficking by neutralizing the lysine charge patch, which in turn controls oligomerization-dependent p53 nuclear export.

The subcellular localization of p53 is believed to be controlled at the levels of its oligomerization status. It was proposed that tetramerization prevents both p53 nuclear import and p53 nuclear export (reviewed in Ref. 18). In the case of nuclear export, as the dominant C-terminal NES is located in the oligomerization domain, it was suggested that the accessibility of the p53 NES is regulated by the oligomerization status (8). This conclusion is supported by the solution and the crystal structure of the oligomerization domain, which demonstrates that the C-terminal NES is exposed in monomeric or dimeric conformations but that it is buried in p53 tetramers (19–21). The interconversion between the tetrameric and monomeric states, which corresponds to p53 transcriptional activity, would therefore determine the availability of the NES and, consequently, the efficiency of p53 export. However, the biochemical basis that controls p53 oligomerization and its regulation is poorly understood. In this report, we have provided experimental evidence that the charge of lysine residues targeted by acetylation and ubiquitination machinery may be a key determinant of p53 oligomerization. This conclusion is supported by the observation that mutations that neutralize four acetylatable lysine residues result in p53 that cannot oligomerize (Fig. 5). Further, these charge-neutralizing mutations also lead to p53 accumulation in the cytoplasm (Fig. 4). Lastly, these cytoplasmic accumulations of p53 can be effectively reversed by the nuclear export inhibitor LMB (Figs. 1 and 4). Our data, however, did not exclude the possibility that the charge neutralization of lysine residues might also affect p53 nuclear import as well. Regardless of which mechanism might play a more dominant role, our results supported the idea that a charge modification of the lysine residues is a critical determinant in regulating p53 subcellular localization.

If C-terminal charge plays a critical role in p53 nuclear export, how is this charge patch regulated? Our study identified acetylation as one potential mechanism that regulates the lysine charge patch. Acetylation occurs at the ε-amino group of a lysine. Thus, acetylation would result in a loss of the positive charge, which in turn would affect p53 oligomerization (Fig. 5) and nucleus-cytoplasm trafficking (Fig. 4). The idea that acetylation regulates p53 export by neutralizing charge is supported by an elegant study on histone H2A.Z in which acetylation was shown to function by modifying a charge patch also made of multiple lysines (17). In this regard, ubiquitin also modifies the ε-amino group of a lysine. However, given that a fusion of monoubiquitin to p53 is sufficient to promote p53 nuclear export (22), acetylation and ubiquitination would likely regulate p53 export through different mechanisms.

Interestingly, although the conversion of different combinations of four lysines known to be targets of acetylation to alanines (4KA mutants) promotes cytoplasmic accumulation of p53, the conversion of only three lysines (3KA) has little effect (Fig. 4). In support of this observation, we show that p53 4KA mutants fail to tetramerize, whereas the 3KA mutants do as efficiently as the wild type (Fig. 5). These results suggest that there might be a threshold for the activation of p53 export, as determined by the overall charge provided by the lysine cluster at the C terminus. Thus, the extent of charge neutralization by acetylation could in theory determine the subcellular distribution of the activated p53. We suspect that, upon stresses, p53 would be stabilized in the nucleus when fewer than three lysine residues are acetylated; however, when more than four lysine residues are acetylated, presumably in response to prolonged or intense insults, p53 becomes hyperacetylated and exported to the cytoplasm.

What is the biological significance of acetylation-mediated p53 nuclear exit? All evidence so far indicates that acetylation positively regulates p53 function (4). Thus, it is logical to speculate that hyperacetylated p53 has a function when it is delivered to the cytoplasmic compartment. Interestingly, recent evidence indicates that p53 can also promote apoptosis by directly interacting with the apoptosis machinery in mitochondria (10). Although a recent study observed very similar profiles in the acetylation level on lysine 382 and 372 of nuclear and mitochondrial p53 and thus concluded that acetylation is not the determining factor for mitochondria targeting (23), it remains possible that the total acetylation of mitochondrial p53 might still be up-regulated via other acetylatable lysine residues. Alternatively, the hyperacetylated cytoplasmic p53 might undergo rapid deacetylation before they reach mitochondria. Although the importance of acetylation-mediated p53 nuclear export in tumor suppression still awaits further investigation, our study identified acetylation as a potential mechanism that controls p53 intracellular trafficking in response to stresses.

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