Acetylation of p53 Inhibits Its Ubiquitination by Mdm2

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Summary

In response to DNA damage, the activity of the p53 tumor suppressor is modulated by protein stabilization and post-translational modifications including acetylation. Interestingly, both acetylation and ubiquitination can modify the same lysine residues at the C-terminus of p53, implicating a role of acetylation in the regulation of p53 stability. However, the direct effect of acetylation on Mdm2-mediated ubiquitination of p53 is still lacking because of technical difficulties. Here, we have developed a method to obtain pure acetylated p53 proteins from cells, and by using an in vitro purified system, we provide the direct evidence that acetylation of the C-terminal domain is sufficient to abrogate its ubiquitination by Mdm2. Importantly, even in the absence of DNA damage, acetylation of the p53 protein is capable to reduce the ubiquitination levels and extend its half-life in vivo. Moreover, we also show that acetylation of p53 can affect its ubiquitination through other mechanisms in addition to the site-competition. This study has significant implications regarding a general mechanism by which protein acetylation modulates ubiquitination-dependent proteasome proteolysis.
Introduction

The p53 tumor suppressor exerts anti-proliferative effects, including growth arrest, apoptosis, and cell senescence, in response to various types of stress (1-4). Inactivation of p53 function appears to be critical to tumorigenesis in all different types of human cancers (5). p53 is a short-lived protein whose activity is maintained at low levels in normal cells (3). Tight regulation of p53 is essential for its effect on tumorigenesis as well as maintaining normal cell growth. The precise mechanism by which p53 is activated by cellular stress is not completely understood; it is generally thought to involve mainly post-translational modifications of p53, including ubiquitination, phosphorylation and acetylation (1-4).

Early studies demonstrated that CBP/p300, a histone acetyl-transferase (HAT), acts as a coactivator of p53 and potentiates its transcriptional activity as well as biological function in vivo; significantly, the observation of functional synergism between p53 and CBP/p300 together with its intrinsic HAT activity led to the discovery of a novel FAT (transcriptional factor acetyl-transferase) activity of CBP/p300 on p53 (5-7). p53 is specifically acetylated at multiple lysine residues (Lys 370, 371, 372, 381, 382) of the C-terminal regulatory domain by CBP/p300. The acetylation of p53 can dramatically stimulate its sequence-specific DNA binding activity, possibly as a result of an acetylation-induced conformational change (6-8). By developing site-specific acetylated p53 antibodies, CBP/p300 mediated acetylation of p53 was further confirmed in vivo by a number of studies (9-11). In addition, p53 can also be acetylated at Lys320 by another HAT cofactor, PCAF, although the in vivo functional consequence needs to be further elucidated (9, 10, 12). Significantly, the steady-state levels of acetylated p53 are stimulated in
response to various types of stress, indicating the important role of p53 acetylation in stress response (11).

By serving as a signal for specific cellular protein degradation, ubiquitination plays a critical role in physiological regulation of many cellular processes (13, 14). The ubiquitination of p53 was first discovered in papilloma virus-infected cells through the functions mediated by the viral E6 protein (15), however, in normal cells, Mdm2 functions as an ubiquitin ligase and plays a major role in p53 ubiquitination and subsequent degradation (16-18). Like in the case of acetylation, the ε-amino group of the substrate lysine residue is also the target for ubiquitination (13-14). Significantly, recent studies have indicated that the lysine residues at the C-terminal domain of p53, five of which are the acetylation sites, play a critical role in Mdm2-mediated ubiquitination and subsequent degradation (19-22). Furthermore, increasing the levels of p53 acetylation with deacetylase inhibitors in the cell also prevents p53 from degradation in vivo (11). Therefore, it is reasonable to speculate that acetylation of p53 may directly regulate its ubiquitination levels in vivo. However, thus far, there is no direct evidence regarding how p53 acetylation affects its ubiquitination, mainly because of the technical difficulties. In this study, we have developed several assays to establish an important mechanism that p53 acetylation is directly involved in the regulation of its ubiquitination and subsequent proteolysis induced by Mdm2.

Experimental Procedures

Plasmid construction To construct the p53, UbcH5c, Mdm2, ubiquitin expression vectors, the DNA sequences corresponding to the full-length proteins, were amplified by PCR from Marathon-Ready Hela cDNA (Clontech) or other templates, and subcloned either into a pGEX (GST) vector, or pET-11(His), or pET-Flag for
expression in bacteria, or a pCIN4 vector for expression in mammalian cells (23-25). Regarding the different mutant constructs, DNA sequences corresponding to different regions were amplified by PCR from above constructs and subcloned into respective expression vectors. Site-directed mutations were generated by using the Gene Edit system (Promega).

**Protein purification of acetylated p53, and other components for in vitro ubiquitination assays.** To prepare the purified components for the in vitro ubiquitination assay, GST-p53, E2 (GST-Ubc5Hc) and E3 (GST-Mdm2) were induced in BL21 cells at room temperature and extracted with buffer BC500 (20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 500 mM KCl, 20% glycerol, 1mM DTT and 0.5 mM PMSF) containing 1% NP-40, and purified on glutathione-Sepharose (Pharmacia). His-Ub was induced in the same manner and purified on the NTA column. The rabbit E1 was purchased as a purified protein from Calbiochem. To improve their activities, each protein was further purified on the Suprose-12 column by the SMART/FPLC system (Amersham/Phamacia). For the purification of acetylated p53 proteins, H1299 cells were co-transfected with Flag-p53 and p300. 20 hrs after the transfection, the cells were treated with 1mM TSA and 5mM nicotinamide for 6 hrs, then lysed in Flag-lysis buffer (50 mM Tris, 137 mM NaCl, 10 mM NaF, 1mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 1 mM DTT, 10% glycerol, pH 7.8 and fresh proteinase inhibitors) with mild sonication, and the cell extracts were immunoprecipitated with Flag monoclonal antibody beads (M2, Sigma). After eluted with the Flag peptide, the total p53 proteins were loaded on the PAb421 antibody column. The unacetylated portion of the p53 protein was depleted by the PAb421 column after the total proteins were passed through the column several times. The unbound proteins were purified acetylated p53 and were further tested for their purity by Western blot with α-p53 (DO-1, PAb421, or Acetylated-p53-specific antibody). To avoid p53 deacetylation by both HDAC1 and
Sir2-mediated deacetylases (23, 24), 1 µM of TSA and 5mM of nicotinamide were added in each step. The labeled p53 substrates were produced in an in vitro transcription/translation system (TNT kit, Promega) with 2 ug of the pCIN4 expression vectors in a standard reaction.

**In vitro ubiquitination assays.** The in vitro ubiquitination assay was performed as previously described (18) with some modifications. For a standard reaction, 10 ng of the bacteria produced GST-p53, or 5 ul of the purified acetylated p53 proteins from H1299 cells, or 5 ul of the labeled substrates from a TNT reaction, were mixed with other purified components including E1 (12 ng), E2 (GST-UbcH5C) (200 ng), E3 (GST-Mdm2) (500 ng), and 2 ug of His-ubiquitin in 20 µl of reaction buffer (40 mM Tris, 5 mM MgCl₂, 2 mM ATP, 2 mM DTT, pH 7.6). The reaction was stopped after 60 min at 37°C by addition of SDS sample buffer, and subsequently resolved by either 6% SDS-PAGE gels or 4 to 12% gradient gels for either western blot analysis with α-p53 (DO-1) or autoradiography.

**Ubiquitination levels of p53 in human cells.** The assay for detecting the ubiquitination levels of endogenous p53 proteins was performed as previously described (25). The human Burkitt lymphoma cells (BL2) were treated with 20µM of MG132 for 4 hr before harvesting, and the cells were lysed in RIPA buffer (1% NP40, 0.1% SDS, Tris-HCl pH 7.8, 150 mM NaCl, 1mM DTT, 0.5 mM EDTA and 1 mM PMSF) with mild sonication. To avoid p53 deacetylation by either HDAC1 or Sir2-mediated deacetylases during the preparation, 1 µM of TSA and 5mM of nicotinamid were added in each step (29,30). For preparing the total p53 protein, the cell extracts were immunoprecipitated with a p53 antibody against the full-length protein (Santa Cruz). For preparing the acetylated p53 protein, the cell extracts were first depleted by the PAb421 antibody and then immunoprecipitated by the acetylated p53-specific antibody (23). The immunoprecipitates were
subsequently resolved on 6% SDS-PAGE gels and analyzed by western blot with α-p53 (DO-1).

**Measuring the half-life of endogenous p53 proteins.** For measuring the half-life of the total p53 protein in unstressed cells, the human Burkitt lymphoma cells (BL2) were maintained in Iscove medium with 10% fetal bovine serum. After pretreated with cyclohexamide (20 uM), BL2 cells were harvested at different time points as indicated, and extracted with the RIPA buffer. For the half-life of the acetylated p53 protein, the cell extracts were immunoprecipitated by the acetylated p53-specific antibody (23). To preserve the acetylation levels of p53 proteins during the treatment with cyclohexamide (20 uM), 1 μM of TSA and 5mM of nicotinamide were also added to prevent the deacetylation by endogenous deacetylases (23, 24). Both crude extracts and immunoprecipitates were subsequently resolved by 6% SDS-PAGE and analyzed by western blot with α-p53 (DO-1) and α-actin.
Results and Discussion

One of the major obstacles in elucidating the precise role of acetylation in the regulation of protein function is obtaining the pure acetylated form. Since the acetylated non-histone proteins are inseparable from the unacetylated forms on the regular SDS-PAGE gel, thus far, most of the functional studies on newly-identified acetylated substrates including p53, are derived from the “acetylated proteins” without any quantitative analysis of their contents (6-12). Therefore, it is very difficult to provide consistent results about the functional differences between the “unacetylated form” and the “acetylated form” of these proteins as the “acetylated form” may be heavily contaminated with the “unacetylated form”. To provide direct evidence that acetylation of p53 can modulate its ubiquitination by Mdm2, we had to obtain the pure form of acetylated p53 from cells. Interestingly, our preliminary data indicated that the acetylated p53 protein reacts poorly to PAb421, one of the p53 antibodies, although it can be recognized perfectly well by several other p53 antibodies including DO-1 (data not shown, also see Fig. 1). Since the PAb421 antibody was raised specifically against the C-terminal domain of p53, overlapping with the sites for acetylation (Fig. 1a), it is very likely that acetylation of the C-terminal domain may prevent p53 from binding to the PAb421 antibody. This piece of information provided an important clue ---that the unacetylated p53 form can be specifically removed from the acetylated p53 fractions through the differential bindings with the PAb421 antibody-- for purification of the acetylated p53 form.

Thus, we applied a two-step strategy to purify the pure form of acetylated p53 from human cells. First, we purified total p53 proteins from cell extracts, and second, we isolated the acetylated p53 from the total p53 proteins by the PAb421 antibody column (Fig. 1b). As indicated in Figure 1c, the acetylation levels of p53
were enhanced significantly when the Flag-tagged p53 protein was co-expressed with p300 in H1299 cells (lane 2 vs. 1), and these transfected cells provided a good resource for the purification of acetylated p53 proteins. By using the M2/Flag column, the total p53 proteins were purified from these cell extracts, but heavily contaminated with unacetylated p53 proteins (data not shown). Then, we loaded them on the PAb421 antibody column to deplete the unacetylated form of p53 (Fig. 1b), and the purified acetylated p53 proteins were finally tested for their purities. As shown in Figure 1d, the purified acetylated p53 was easily detected by the acetylated p53-specific antibody (lane 2 vs. 1, Upper). Both acetylated and unacetylated forms of p53 reacted equally well to the DO-1 antibody (lane 2 vs. 1, middle), which is against the N-terminal domain of p53 (Fig. 1a). Significantly, the purified acetylated p53 protein could not be detected by the PAb421 antibody at all (lane 2 vs. 1, lower). These data indicate that acetylation of the p53 protein by p300 can block the recognition site for the PAb421 antibody. More importantly, we have obtained the pure form of acetylated p53 from cells without obvious contamination from unacetylated p53 proteins.

To provide the direct evidence that acetylation of p53 affects its ubiquitination, we set up a reconstitution system for p53 ubiquitination by Mdm2. As indicated in Figure 2a, the E2 (GST-UbcH5c) and E3 (GST-Mdm2) were expressed in bacteria as GST-fusion proteins and purified to near homogeneity (lanes 5 and 3); ubiquitin was expressed as a His-tagged protein and obtained by a similar manner (lane 7); the E1 was purchased as a purified protein (lane 2). The highly purified in vitro system was used in this assay in order to avoid any possible indirect effect by other factors on p53 ubiquitination. To define the purified system for p53 ubiquitination, we first tested whether all purified components can support Mdm2-dependent ubiquitination of an unmodified form of p53 in vitro. Indeed, as shown in Figure 2b, the bacteria-produced p53 protein was specifically
ubiquitinated only in the presence of Mdm2 (lane 5). Importantly, as shown in Figure 2c, the total p53 protein purified from human cells (Fig. 1), like the bacteria produced p53 protein, was strongly ubiquitinated (lane 2); however, the same amount of the acetylated p53 protein was barely ubiquitinated by Mdm2 under the same conditions (lane 4 vs. 2). These results demonstrate directly that acetylation of p53 strongly inhibits its ubiquitination mediated by Mdm2.

To further confirm the above findings, we searched for an acetylation-mimicking mutant for p53. Interestingly, it was previously reported that the histone H4 protein with the Lys→Gln mutation at the H4 acetylation site functionally behaved as the acetylated form to prevent Sir3p from binding H4 and spreading to form heterochromatin in yeast (26). In fact, a Lys→Gln mutation can mimic the acetylated state of lysine (K) mainly because of the structure similarity between the Gln (Q) residue and the acetylated-lysine residue (Fig. 3a). Thus, a p53(K-Q) mutant, in which all five acetylation sites (K370, K371, K372, K381, K382, see ref. 6) were replaced by the glutamine residues, may mimic the acetylated form of p53. Interestingly, several studies have shown that the Lys→Arg mutation at the C-terminal acetylation sites only partially block Mdm2-mediated ubiquitination of the whole p53 polypeptide (18-19), indicating that there are additional lysine residues that can be ubiquitinated by Mdm2. Thus, the mechanism by which acetylation of p53 inhibits its ubiquitination by Mdm2 may contain two folds. First, acetylation of the p53 C-terminal domain blocks the ε-amino group of these acetylated lysine residues for ubiquitination, and second, acetylation of p53 may also induce protein conformation change to inhibit the ubiquitination of additional lysine residues that are not the acetylation targets. To test the above hypothesis, we examined whether the K→Q mutation can effectively inhibit Mdm2-mediated ubiquitination of the whole p53 protein in vitro. Since the K→R mutation only eliminates the ε-amino group of these acetylation sites for ubiquitination without a neutralization of
positive charges, we included a p53 (K-R) mutant in the same assay as a comparison, in which the five acetylation sites were replaced by arginine residues (Fig. 3b).

For better quantitative analysis of the ubiquitination activities, we used the S<sup>35</sup>-labeled, in vitro translated p53 proteins as substrates for the ubiquitination assay. Thus, similar reactions as described above (Fig. 2b) were set up by incubating labeled p53 substrates, E1, E2 (GST-Ubh5c), E3 (GST-Mdm2) and ubiquitin. As shown in Figure 3c, the wild type p53 protein was strongly ubiquitinated by Mdm2 (lane 2 vs. 1) whereas the p53 (K-R) mutant protein had a significant, but only partial reduction on Mdm2-mediated ubiquitination (~ 60% decrease) (lane 6 vs. 2), consistent with the previously published results (18, 19). Strikingly, however, the KÆQ mutation on the same lysine residues induced a much stronger resistance to Mdm2-mediated ubiquitination, and the ubiquitination levels produced by the p53 (K-Q) mutant protein were almost undetectable under the same conditions (lane 4). These results clearly demonstrate a much more effective inhibitory effect on Mdm2-mediated ubiquitination by the KÆQ mutation than the effect by the KÆR mutation of these acetylation sites of p53. Based on the facts that the KÆQ mutation mimics the acetylated form of the lysine residue whereas the KÆR mutation only eliminates the ε-amino group of the acetylation sites for ubiquitination, our data further suggest that acetylation of p53, in addition to blocking the ubiquitination sites of these acetylated lysine residues, may also inhibit Mdm2-mediated ubiquitination of other lysine residues, possibly through inducing a protein conformational change.

To examine the role of acetylation in the regulation of p53 ubiquitination in vivo, we tested the effect of acetylation on ubiquitination levels of endogenous p53 proteins. The acetylated p53 protein is much more easily obtained from the cells
treated with the DNA damage reagents, however, to avoid other factors (e.g. p53 phosphorylation) that may also be involved in p53 stabilization under the DNA damage conditions (12), we chose to purify the acetylated form of endogenous p53 proteins from unstressed cells. As indicated in Figure 4a, high levels of ubiquitinated p53 proteins were present in the total p53 protein prepared from human Burkitt lymphoma cells (BL2) by immunoprecipitations with a p53 antibody against the full-length protein (lane 2). In contrast, the ubiquitinated p53 was almost undetectable in the acetylated form of the endogenous p53 protein (lane 1, Fig. 4a), which was purified by immunoprecipitations with the anti-acetylated p53-specific antibody from the same cells (see methods). Furthermore, we also examined the effect of acetylation on the half-life of p53 in vivo. As indicated in Figure 4b, the half-life of the total p53 protein was less than 30 min. Strikingly, the acetylated form of p53, which constitutes only a very small portion of the total p53 protein in unstressed cells, appeared very stable; the half-life of the acetylated p53 was more than 2 hr (Fig. 4c). The above results demonstrate that acetylation of p53 can abrogate its ubiquitination and stabilize the p53 protein under physiological conditions.

Thus, with several technical breakthroughs, we have demonstrated for the first time that acetylation of p53 directly inhibits ubiquitination-dependent proteolysis. More importantly, we have also provided further evidence elucidating a novel mechanism for acetylation-induced effects on p53 ubiquitination: acetylation of p53 not only blocks the ε-amino group of the acetylated lysine residues for ubiquitination, but also attenuates Mdm2-mediated ubiquitination of other unacetylated lysine residues, possibly through inducing a protein conformational change. Stabilization of p53 is critical for its effects on cell growth repression and apoptosis (1-5). Numerous studies imply the existence of multiple pathways involved in p53 stabilization in response to DNA damage or other types of stress.
(1-5, 25, 27). It is generally thought that p53 is phosphorylated at multiple sites (mainly Ser 15 or Ser 20), and that these phosphorylation events promote p53 stabilization by preventing the binding with Mdm2 and rendering p53 more resistant to Mdm2-mediated degradation (28, 29). Interestingly, however, several groups recently reported that mutations of these phosphorylation sites on p53 do not significantly inhibit the ability of DNA damage to stabilize p53 (30-33). Furthermore, some genotoxic drugs, such as actinomycin D, can stabilize p53 without provoking either Ser 15 or Ser 20 phosphorylation (33), raising the possibility that p53 can be stabilized without the modification of its N-terminal domain by phosphorylation. Our findings clearly support such notion by providing an alternative mechanism by which p53 can be stabilized through its acetylation.

Acetylation, which modifies the lysine residue of target proteins including histone and non-histone proteins, is now recognized as an important regulatory step in transcriptional regulation (6-8). A large number of the non-histone transcriptional factors have been demonstrated as bona fide substrates for acetyl-transferases, suggesting that acetylation may represent another type of general protein modifications involved in functional regulation of transcriptional factors. Interestingly, both acetylation and ubiquitination modify the ε-amino group of the substrate lysine residues. Moreover, many of these identified acetylated substrates are involved in ubiquitination-dependent proteasome proteolysis and several identified acetylation lysine residues are also potential ubiquitination sites (7-8, 34-35). Thus, this study on p53 acetylation may also establish a general mechanism by which protein acetylation modulates the ubiquitination-dependent protein degradation pathway. It is likely that the cross-talk between the acetylation and ubiquitination pathways cooperatively controls both activities and stabilities of the target proteins.
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References

Figure Legends:

**Figure 1.** Purification of the pure form of acetylated p53 proteins from human cells. (a) Schematic representation of the functional domains, the acetylation sites and the antibody recognition sites. (b) Schematic representation of the purification steps for acetylated p53 proteins from human cells. (c) Enhancement of p53 acetylation levels by p300 in human cells. Western blot analysis of the crude extract from the H1299 cells transfected with Flag-p53 alone (lane 1), or cotransfected with Flag-p53 and p300 (lane 2). (d) Western blot analysis of the purified acetylated p53 (lane 2) and unacetylated p53 (lane 1) proteins with the anti-acetylated p53-specific antibody (upper panel), or the DO-1 antibody (middle panel), or the PAb421 antibody (lower panel).

**Figure 2.** Acetylation of p53 inhibits its ubiquitination by Mdm2 in vitro. (a) Colloidal blue staining of the purified components for in vitro ubiquitination assays. (b) Western blot analysis of the in vitro ubiquitination reactions mediated by Mdm2 with the DO-1 monoclonal antibody. The purified bacteria-produced GST-p53 protein was incubated with different components as indicated. (c) Western blot analysis of the in vitro ubiquitination reactions on the purified acetylated p53 protein (lanes 3, 4) or total p53 protein (lanes 1, 2) with the DO-1 monoclonal antibody. The ubiquitination reactions were performed in the presence of Mdm2 (lanes 2, 4) or in the absence of Mdm2 as controls (lanes 1, 3).
Figure 3. Regulation of Mdm2-mediated ubiquitination of p53 in vitro by the K→Q mutation vs K→R mutation on the acetylation sites. (a) Schematic representation of the similarities between the acetylated-lysine residue and the glutamine residue. (b) Schematic representation of the p53 (K-Q) mutant and p53 (K-R) mutant. (c) In vitro ubiquitination of the wild-type p53 and p53 mutant proteins by Mdm2. The S\textsuperscript{35} -labeled wild-type p53 protein (lanes 1, 2), or p53 (K-Q) mutant protein (lanes 3, 4) or p53 (K-R) mutant protein (lanes 5, 6) were differentially ubiquitinated by Mdm2 in vitro (lanes 2, 4, 6).

Figure 4. The effect of acetylation on ubiquitination and stability of endogenous p53 in human cells (BL2). (a) Western blot analysis of the ubiquitination levels of acetylated p53 and total p53 proteins immunoprecipitated from human BL2 cells. (b) The half-life of the endogenous p53 protein (total). Cell extracts from human BL2 cells harvested at different time points as indicated after pre-treated with cyclohexamide (CHX), were analyzed for p53 protein levels by western blot with anti-p53 (DO-1). (c) The half-life of the acetylated form of the endogenous p53 protein in BL2 cells. The acetylated p53 proteins were immunoprecipitated with the acetylated p53-specific antibody from the BL2 cell extracts harvested at different time points as indicated after pre-treated with cyclohexamide (CHX), and further analyzed for p53 protein levels by western blot with anti-p53 (DO-1).
Cell Extracts (Cotransfected with Flag-p53 and p300)

M2/Flag Column

Purified p53 protein (total)

Anti-p53 Column (PAb421)

Unbound proteins (Acetylated p53)

Bound proteins (Unacetylated p53)

Flag-p53 p300

Acetylated-p53

p53

Unacetylated p53

Acetylated p53

α-Acetylated-p53

α-p53 (DO-1)

α-p53 (PAb421)
a

Lys (K)

\[ \text{H} \text{NH}_3 - \text{(CH}_2\text{)}_4 \text{C} - \text{COO}^- \]

Acetylated Lys

\[ \text{O} \text{C} - \text{NH} - \text{(CH}_2\text{)}_4 \text{C} - \text{COO}^- \]

Gln (Q)

\[ \text{O} \text{C} - \text{CH}_2 - \text{CH}_2 - \text{COO}^- \]

b

Transactivation Domain

DNA Binding Domain

Regulatory Domain

p53 (K-Q)

p53 (K-R)

c

MDM2

- + - + - +

p53

p53-Ub

p53

1 2 3 4 5 6
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