Published OnlineFirst January 19, 2006; DOI: 10.1158/1541-7786.MCR-05-0097

Ubiquitination of p53 at Multiple Sites in the DNA-Binding Domain

Wan Mui Chan, Man Chi Mak, Tsz Kan Fung, Anita Lau, Wai Yi Siu, and Randy Y.C. Poon

Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong

Abstract

The tumor suppressor p53 is negatively regulated by the ubiquitin ligase MDM2. The MDM2 recognition site is at the NH_2-terminal region of p53, but the positions of the actual ubiquitination acceptor sites are less well defined. Lysine residues at the COOH-terminal region of p53 are implicated as sites for ubiquitination and other post-translational modifications. Unexpectedly, we found that substitution of the COOH-terminal lysine residues did not diminish MDM2-mediated ubiquitination. Ubiquitination was not abolished even after the entire COOH-terminal regulatory region was removed. Using a method involving *in vitro* proteolytic cleavage at specific sites after ubiquitination, we found that p53 was ubiquitinated at the NH_2-terminal portion of the protein. The lysine residue within the transactivation domain is probably not essential for ubiquitination, as substitution with an arginine did not affect MDM2 binding or ubiquitination. In contrast, several conserved lysine residues in the DNA-binding domain are critical for p53 ubiquitination. Removal of the DNA-binding domain reduced ubiquitination and increased the stability of p53. These data provide evidence that in addition to the COOH-terminal residues, p53 may also be ubiquitinated at sites in the DNA-binding domain. (Mol Cancer Res 2006;4(1):15–25)

Introduction

Loss of the p53 tumor suppressor function is one of the most common steps in tumorigenesis. Germ-line mutations of *p53* are present in cancer-prone families with Li-Fraumeni syndrome (1), and somatic mutations are found in more than half of all cancer cases (2). In clear demonstration of the role of p53 as a key tumor suppressor, mice nullizygous of *p53* spontaneously develop a broad spectrum of tumors (3).

Received 7/14/05; revised 11/23/05; accepted 12/2/05.
Grant support: Research Grants Council grant HKUST6135/03M and Hong Kong University of Science and Technology grant HIA03/04.SC01 (R.Y.C. Poon).
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Requests for reprints: Randy Y.C. Poon, Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong. Phone: 852-23588703; Fax: 852-23581552. E-mail: bcrandy@ust.hk
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doi:10.1158/1541-7786.MCR-05-0097

The *p53* gene encodes a transcription factor. The majority of the mutations in *p53* are missense point mutations clustered in the DNA-binding domain that disrupt DNA binding (4). The activity of p53 is highly regulated by post-translational mechanisms, including protein-protein interactions, acetylation, neddylation, phosphorylation, sumoylation, and ubiquitination (5). One of the transcriptional targets of p53, MDM2, can regulate both the transcriptional activity and the half-life of p53 in a negative feedback loop (6). MDM2 binds to the NH_2-terminal transactivation domain of p53 and inhibits its transcriptional activity directly. MDM2 also shuttles p53 out of the nucleus by the virtue of the nuclear exporting signal in MDM2. Finally, MDM2 is a ubiquitin ligase that targets p53 for ubiquitin-mediated proteolysis. Ubiquitin-mediated degradation entails linking of ubiquitin by a thioester linkage to a ubiquitin-activating enzyme (E1) before the ubiquitin is transferred to a ubiquitin carrier (E2). E2 acts alone or in conjunction with a ubiquitin ligase (E3) to conjugate ubiquitin to the ε-amino group of lysine residues in substrate proteins to form a glycyl-lysine isopeptide bond (7). Multiple rounds of ubiquitin conjugation form polyubiquitin chains on the substrates, which are then degraded by the 26S proteasome complex.

Ubiquitination seems to serve more complex function than simply targeting p53 to the proteasome. Ubiquitination also contributes to the efficient export of p53 to the cytoplasm (8, 9). Furthermore, whereas export is associated with monoubiquitination and low levels of MDM2, nuclear degradation is associated with polyubiquitination and high levels of MDM2 (10). Another study shows that monoubiquitination is catalyzed by MDM2 alone, whereas polyubiquitination is mediated by MDM2 together with p300 (11). After DNA damage or other stresses, several kinases, including ATM, ATR, CHK1, CHK2, and DNA-PK, phosphorylate the NH_2-terminal region of p53 and disrupt the interaction with MDM2. An extra level of regulation is provided by YY1 (12) and ARF (13), which stimulates and inhibits MDM2, respectively.

MDM2 is not the only protein that can induce ubiquitination of p53. The MDM2-related protein MDMX has been shown to contain ubiquitin ligase activity against p53 (14). Like MDM2, MDMX also contains a RING finger domain found in many ubiquitin ligases. Two other RING finger-containing proteins, COP1 (15) and PIRH2 (16), can also bind and ubiquitinate p53. Finally, human papillomavirus E6 targets p53 for degradation through the recruitment of the cellular ubiquitin ligase E6-AP (17).

Although the function of MDM2 as a ubiquitin ligase for p53 is well established, it is unclear precisely which lysine residues in p53 are the ubiquitin acceptor sites. One prevailing

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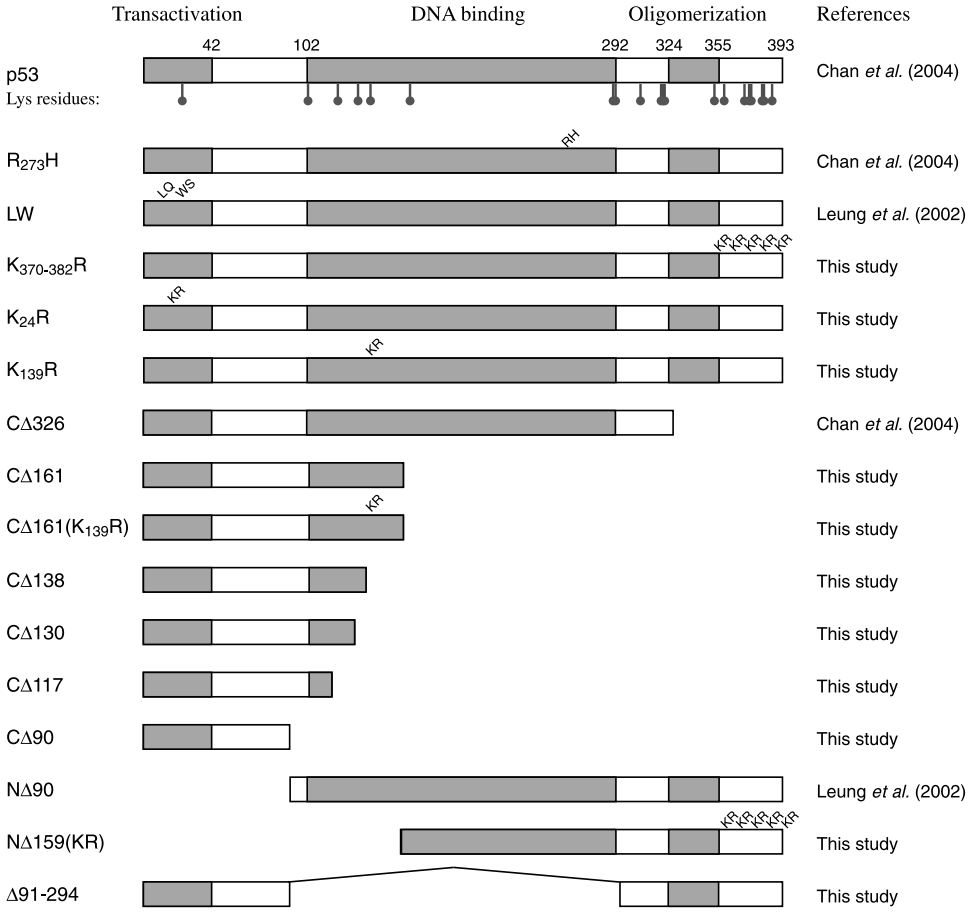

FIGURE 1. Schematic diagram of the p53 constructs used in this study. The positions of the various structural elements are shown to scale. All constructs are FLAG tagged at the NH_2 terminus. The positions of all lysine residues in p53 are indicated.

view is that, in general, multiple lysine residues in proteins can function as redundant ubiquitin acceptor sites as exemplified by cyclin B (18). However, ubiquitination does occur at specific sites in some proteins: for instance, FANCD2 is monoubiquitinated specifically at Lys^{561} (19). Importantly, the precise sites of ubiquitination can be functionally significant. For example, the yeast cyclin-dependent kinase inhibitor Sic1p can be ubiquitinated on multiple lysine residues, but ubiquitin chains attached on different lysines specify degradation by the proteasome at markedly different rates (20). Elucidating the exact sites of ubiquitination is important, as they frequently overlap with other post-translational modifications like sumoylation and acetylation (21-23).

Several studies have investigated the potential ubiquitin acceptor sites in p53. Substitution of the four lysine residues that are involved in acetylation (372, 373, 381, and 382) with alanine reduces ubiquitination of p53 *in vivo* and *in vitro* (24). Another report shows that mutation of the above four lysines plus Lys^{370} reduces the *in vitro* ubiquitination of p53 by ~60% (21). Mutation of the above five lysines plus Lys^{386} significantly reduces (but does not eliminate) the ubiquitination of p53 *in vivo* and *in vitro* (9, 25, 26). Together, the available data suggest that the COOH-terminal lysine residues of p53 are involved in ubiquitination. Notably, these studies also indicate that ubiquitination does not occur exclusively at these sites, as mutants without these residues are still ubiquitinated.

In this study, we found that p53 could be ubiquitinated at the NH_2-terminal portion of the protein. We also found that Lys^{24} in the transactivation domain is not essential for ubiquitination, as substitution with an arginine did not affect MDM2 binding and ubiquitination. In contrast, several conserved lysine residues in the DNA-binding domain are critical for ubiquitination. Thus, we propose that apart from the COOH-terminal region, the DNA-binding domain contains additional ubiquitin acceptor sites.

Results

Ubiquitination of p53 Requires the NH_2-Terminal MDM2-Binding Region but Does Not Require the COOH-Terminal Lysines

Human p53 contains 20 lysine residues. Interestingly, their distribution along the protein is not uniform but is confined to three clusters: a single lysine at the transactivation domain, five at the NH_2-terminal region of the DNA-binding domain, and the rest at the COOH-terminal region of the protein (Fig. 1). To see which of the lysine residue(s) is ubiquitinated, we first set up conditions that recapitulate MDM2-dependent ubiquitination of p53. FLAG epitope–tagged p53 was coexpressed with hemagglutinin (HA)–tagged ubiquitin (HA-ubiquitin) in the p53-null H1299 cells. As expected, coexpression of MDM2 reduced the level of p53 (Fig. 2A).

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To detect p53 ubiquitination, the FLAG immunoprecipitates were subjected to immunoblotting. The proteasome inhibitor LLnL was added to preserve the ubiquitinated forms of p53. Full-length p53 and its monoubiquitinated form could be detected with antibodies against p53 (or FLAG tag). Due to the heterogeneous nature of ubiquitin conjugation, the polyubiquitinated forms of p53 could only be detected with antibodies against the multiple HA epitopes in the ubiquitin chains as large molecular size smear (Fig. 2B). No ubiquitination signal was detected when either HA-ubiquitin or FLAG-p53 was absent (data not shown). Both FLAG-p53 and the associated MDM2 could be detected in the immunoprecipitates with FLAG antiserum but not with control serum (Fig. 2C, *lanes 2* and *5*). Moreover, ubiquitination of p53 was largely dependent on the presence of coexpressed MDM2 (compare *lanes 3* and *6*). On this article, the blots for HA signals in the total lysates were deliberately overexposed to view the p53-specific ubiquitination in the immunoprecipitates. Weaker exposures were also made in all experiments to assess similar expression of total HA-ubiquitin (Fig. 2C; data not shown).

We initially noted that proteasome inhibitor stabilized monoubiquitination of both wild-type p53 and a mutant with five COOH-terminal lysine residues (370, 372, 373, 381, and 382) substituted with arginine ($K_{370-382}R$) (Fig. 3A). This suggested that residues other than the five COOH-terminal ones could contribute to ubiquitination. To examine this hypothesis, we created several p53 mutants in the same backbone and examined their ubiquitination. As expected, disruption of the MDM2-binding site by mutation of Leu^{22} and Tyr^{23} (LW mutant) or deletion of the entire NH_2-terminal region (NΔ90) abolished ubiquitination (Fig. 3B). This confirmed the MDM2 dependence of the ubiquitination. On the other hand, the naturally occurring non-DNA-binding mutant $R_{273}H$ still underwent ubiquitination (Fig. 3C), indicating that DNA binding is not required for ubiquitination. Unexpectedly, the $K_{370-382}R$ mutant was ubiquitinated just as wild-type p53 (Fig. 3B). Not surprisingly, $K_{370-382}R$ lacking the MDM2-binding NH_2-terminal region was not ubiquitinated (Fig. 3C). Remarkably, we found that p53 lacking the entire COOH-terminal region up to residue 326 (CΔ326) or 161 (CΔ161) was still ubiquitinated (Fig. 3B and C). Because CΔ161 does not contain any of the lysine residues in the COOH-terminal cluster (see Fig. 1), these data suggest that p53 can be ubiquitinated at residues in additional to those in the COOH-terminal region.

To be sure that the observed ubiquitination of p53 lacking the COOH-terminal lysine residues was not specific for H1299 cells only, the ubiquitination assays were done in another p53-negative cell line. As expected, p53 was ubiquitinated in Hep3B cells in the presence of MDM2 (Fig. 3D). We found that similar to the results described above both $K_{370-382}R$ and CΔ326 mutants were also readily ubiquitinated.

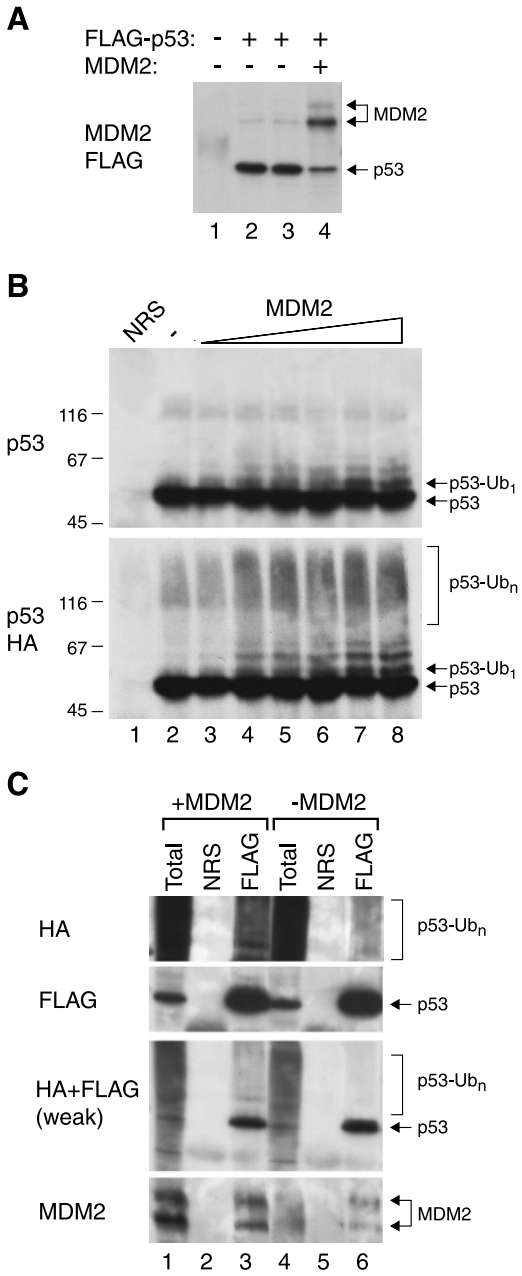

FIGURE 2. MDM2-dependent p53 ubiquitination and degradation assays. **A.** MDM2-dependent degradation of p53. H1299 cells were cotransfected with plasmids expressing FLAG-p53 and MDM2. Cell extracts were prepared at 24 hours after transfection and subjected to immunoblotting for FLAG and MDM2. **B.** Ubiquitination assay of p53. Constant amount of FLAG-p53 and HA-ubiquitin were coexpressed with increasing amounts of MDM2 in H1299 cells and treated with the proteasome inhibitor LLnL for 6 hours before harvest. Cell extracts were prepared and subjected to immunoprecipitation with either control normal rabbit serum (*NRS*) or FLAG antiserum. The immunoprecipitates were immunoblotted with antibodies against p53 (*top*) followed by reprobing for HA (*bottom*). The positions of unmodified, monoubiquitinated, and polyubiquitinated p53 are indicated. Left, positions of molecular size standards (in kDa). **C.** Binding of MDM2 to p53. H1299 cells were cotransfected with FLAG-p53 and HA-ubiquitin in the presence or absence of MDM2. Cells were treated with LLnL for 6 hours to inhibit the proteasome before harvest. Cell extracts were prepared and 100 μg were subjected to immunoprecipitation as in **B**. The immunoprecipitates were then immunoblotted with monoclonal antibodies 12CA5 against HA to detect polyubiquitinated proteins, 2A10 to detect MDM2, and M2 to confirm the expression of FLAG-tagged proteins. Total cell lysates (10 μg) were applied to indicate the inputs. A weaker exposure of a blot for HA and FLAG is shown to provide a better indication of the input levels.

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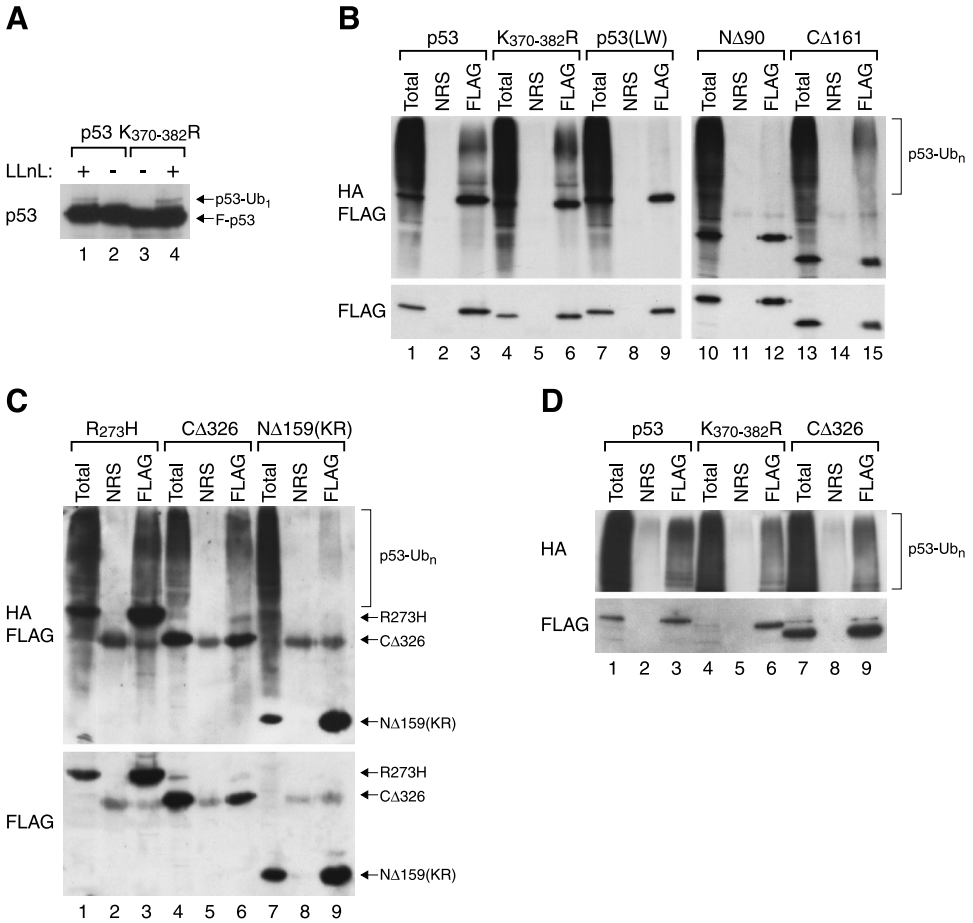

FIGURE 3. Ubiquitination of p53 requires the NH_2-terminal MDM2-binding site but does not require the COOH-terminal lysine residues. **A.** Monoubiquitination of $K_{370\text{-}382}R$ mutant. H1299 cells were cotransfected with plasmids expressing HA-ubiquitin, MDM2, and FLAG-tagged p53 or $K_{370\text{-}382}R$. Cells were left untreated or treated with LLnL for 6 hours before harvest. Cell extracts were prepared and immunoblotted with antibodies against p53. The position of monoubiquitinated p53 is indicated. **B.** Both $K_{370\text{-}382}R$ and CΔ mutants can be ubiquitinated. MDM2 and HA-ubiquitin were coexpressed with FLAG-tagged p53, $K_{370\text{-}382}R$, LW, NΔ90, or CΔ161. Cell extracts were prepared and subjected to immunoprecipitation followed by immunoblotting as in Fig. 2B. The blot was first probed with antibodies against FLAG (*bottom*) before reprobed with antibodies against HA (*top*). **C.** Ubiquitination of p53 does not require active p53. The experiment was done as in **B**, except that FLAG-tagged $R_{273}H$, CΔ326, and NΔ159(KR) were used. **D.** $K_{370\text{-}382}R$ and CΔ326 can be ubiquitinated in Hep3B cells. The experiment was done similar to **B**, except that Hep3B cells were used.

The NH_2-Terminal Portion of p53 Is Ubiquitinated by MDM2

To circumvent the potential problems associated with truncation or substitution of lysines, we used a method that examined ubiquitination in full-length p53. A short 3C-protease cleavage sequence was introduced into various positions of FLAG-p53, and the recombinant protein was subjected to MDM2-induced ubiquitination as before. Following immunoprecipitation with FLAG antiserum, the ubiquitinated p53 was cleaved with 3C-protease. Examination of the presence of ubiquitinated peptide in the pellet or the supernatant fraction (or both) would reveal whether the ubiquitin acceptor site(s) was present at the NH_2- or COOH-terminal side of the 3C-protease site, respectively (see Fig. 4B for summary).

To verify that this method works in principle, a 3C-protease site was first introduced immediately COOH terminal to the FLAG tag and NH_2 terminal to p53 (Fig. 4A). As expected, all the p53-ubiquitin signals were associated with the pellet in the absence of 3C-protease (Fig. 4C, *lanes 4* and *5*). In contrast, p53 (slightly smaller due to the loss of FLAG tag) and p53-ubiquitin conjugates were found exclusively in the supernatant after cleavage with 3C-protease. This experiment also indicated that the FLAG tag itself was not ubiquitinated. All the 3C-protease mutants used in this study retain transcriptional activities and could bind to MDM2 just as wild-type p53 (data not shown).

We next introduced the 3C-protease site NH_2 terminal to the tetramerization domain [FLAG-p53($3C_{303}$)]. Figure 5A shows that the majority of the ubiquitinated peptides were associated with the pellet fraction after cleavage, indicating that ubiquitination occurred NH_2 terminal to the tetramerization domain. Immunoblotting for FLAG epitope indicated that FLAG-p53($3C_{303}$) was efficiently cleaved. Consistent with this, a monoclonal antibody (122) that recognized the COOH-terminal region of p53 (around residue 355) detected p53 in the pellet only in the absence of 3C-protease. A caveat to this method is that although the majority of the small COOH-terminal peptide (NΔ303) was released into the supernatant, a portion remained with the beads.

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Despite this limitation, it can be concluded that the major p53 ubiquitin acceptor site is not COOH terminal to residue 303.

Given that ubiquitination may occur NH_2 terminal to the tetramerization domain, we next introduced the 3C-protease site NH_2 terminal to the DNA-binding domain [FLAG-p53($3C_{90}$)]. In contrast to p53($3C_{303}$), the ubiquitinated peptides of p53($3C_{90}$) were released into the supernatant after cleavage (Fig. 5B), indicating that at least some of the ubiquitination sites were COOH terminal to residue 90. Ubiquitinated peptides were also detected in the pellet fraction, which would suggest multiple ubiquitin acceptor sites on both sides of residue 90. However, we think that this was probably due to some of the cleaved COOH-terminal fragment retaining in the beads [as also in p53($3C_{303}$)] as revealed by immunoblotting with monoclonal 122. To verify this idea, Lys^{24} (which is the only lysine NH_2 terminal to residue 90) was mutated to arginine in p53($3C_{90}$). With this mutant, the ubiquitinated peptides were still detected in the pellet fraction after cleavage (Fig. 5C), suggesting that the apparent ubiquitination of the NH_2-terminal 90 amino acid was probably an artifact due to the stickiness of the COOH-terminal peptide. Collectively, these data indicate that p53 is ubiquitinated at the DNA-binding domain.

Lys^{24} in the Transactivation Domain Is Not Required for MDM2 Binding and Ubiquitination

The above results indicate that Lys^{24} in the transactivation domain may not be involved in ubiquitination. However, Lys^{24} is of potential interest because it resides among residues essential for MDM2 binding (Phe^{19}, Leu^{22}, Tyr^{23}, and Leu^{26}), although Lys^{24} itself has not been implicated. To further examine whether Lys^{24} is involved in ubiquitination, a $K_{24}R$ mutation was introduced into FLAG-p53 before MDM2-dependent ubiquitination was assayed. Figure 5D shows that $K_{24}R$ could still bind MDM2 and be ubiquitinated, indicating that p53 ubiquitination does not require Lys^{24}.

To see whether Lys^{24} is essential for transactivation of target genes, the transcriptional activity of p53 was assayed using a MDM2 promoter. MDM2 promoter could be transactivated by the $K_{24}R$ mutant (Fig. 5E), indicating that Lys^{24} is not required for transactivation. Interestingly, the transcriptional activity of $K_{24}R$ mutant was slightly higher than the wild-type p53, suggesting that the mutation may reduce the basal negative regulation. Taken together, these results indicate that Lys^{24} itself is not a ubiquitin acceptor site and is not necessary for MDM2 binding or ubiquitination of p53.

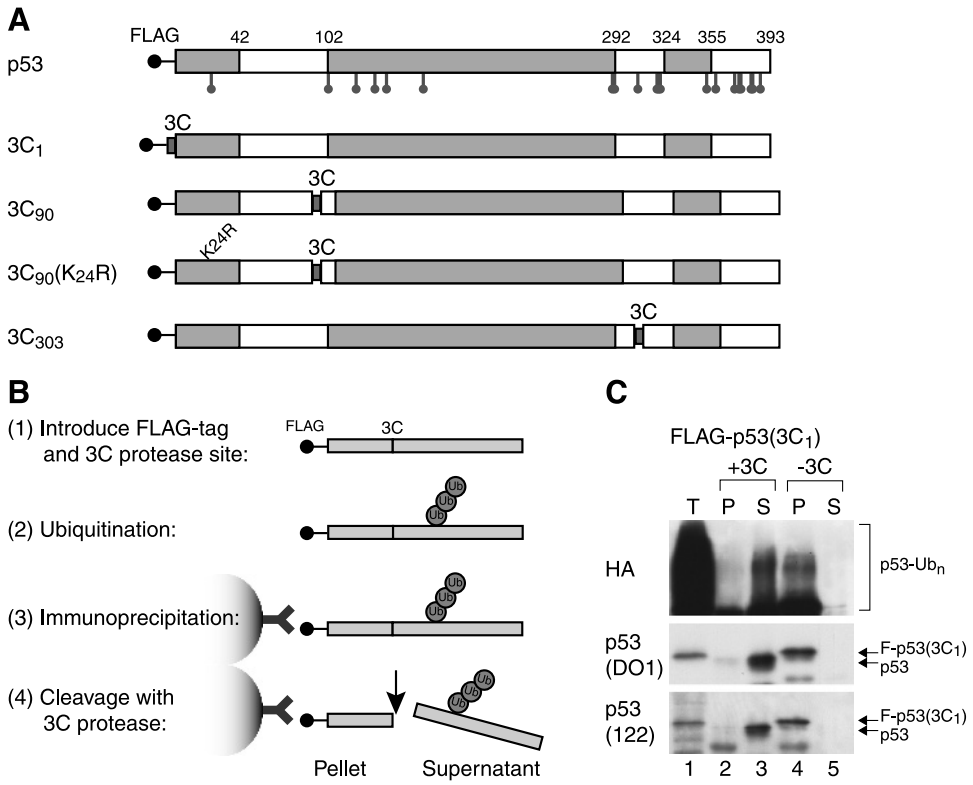

FIGURE 4. Analysis of ubiquitin acceptor sites by introduction of 3C-protease cleavage sequences. **A.** Schematic diagram of 3C-protease constructs used in this study. Top, wild-type p53. The positions of 3C-protease cleavage sites are shown to scale. **B.** Procedures of 3C-protease cleavage assay. See Materials and Methods for details. **C.** Release of ubiquitinated peptides by 3C-protease when the cleavage site is placed immediately COOH terminal to the FLAG tag. FLAG-p53($3C_1$) was coexpressed with HA-ubiquitin and MDM2 in H1299 cells. Cell extracts were prepared and subjected to immunoprecipitation with FLAG antiserum. The immunoprecipitates were either mock-treated or treated with 3C-protease as described in Materials and Methods. The pellet (*P*) and supernatant (*S*) fractions were subjected to immunoblotting for HA and p53 (with both monoclonal antibodies DO1 and 122). Total cell lysates (10 μg) were applied to indicate the input (*T*).

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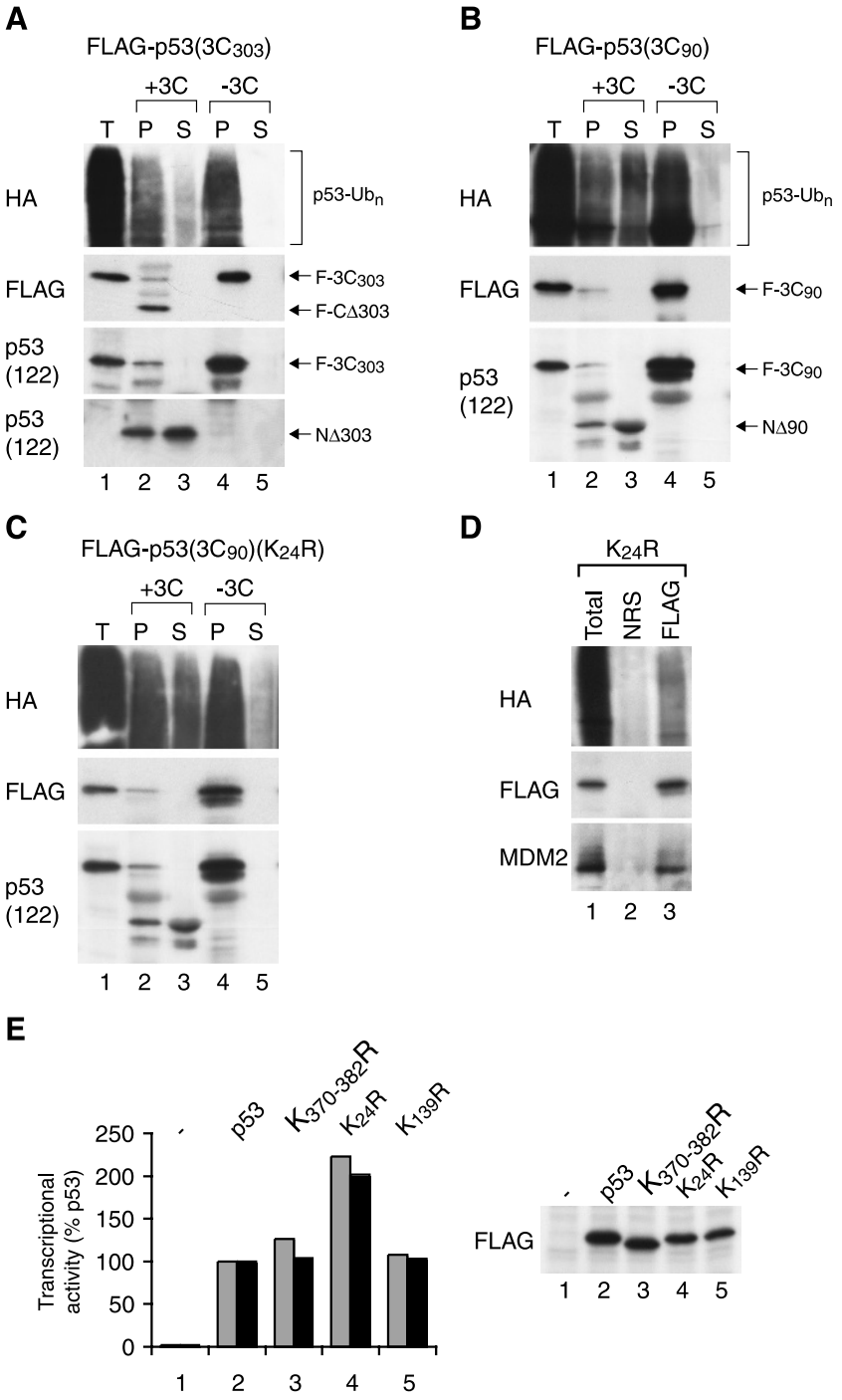

FIGURE 5. p53 is predominantly ubiquitinated at the NH_2-terminal region. **A.** p53 is ubiquitinated NH_2 terminal to residue 303. Experiments were done as in Fig. 4C, except that FLAG-p53($3C_{303}$) was used. The p53 peptides were detected by immunoblotting for FLAG tag and using monoclonal antibody 122. The small NΔ303 fragment was resolved using a separate higher percentage gel. **B.** Protease cleavage of p53($3C_{90}$). Experiments were done as in **A**, except that FLAG-p53($3C_{90}$) was used. **C.** Protease cleavage of p53($3C_{90}$) ($K_{24}R$). Experiments were done as in **A**, except that FLAG-p53($3C_{90}$) containing the $K_{24}R$ point mutation was used. **D.** $K_{24}R$ mutant can bind MDM2 and be ubiquitinated. Experiments were carried out according to the methods described in Fig. 3B, except that FLAG-p53($K_{24}R$) was used. Expression of MDM2 was detected by a monoclonal antibody 2A10. **E.** Transcriptional activities of different p53 KR mutants. H1299 cells were transfected with plasmids expressing a MDM2 promoter luciferase reporter and β-galactosidase. Plasmids expressing wild-type FLAG-tagged p53, $K_{370-382}R$, $K_{24}R$, or $K_{139}R$ were cotransfected. Cell lysates were prepared at 24 hours after transfection. Expression of the proteins was confirmed by immunoblotting with a monoclonal antibody against FLAG epitope (*right*). The luciferase activity was measured, normalized with the β-galactosidase activity to correct for variations in transfection efficiency between samples, and plotted as a percentage of wild-type p53 (*left*). Representative of two independent experiments.

Lysines in the Central DNA-Binding Domain Are Involved in Ubiquitination

The above data suggest that the DNA-binding domain may contain the ubiquitin acceptor sites. Four lysine residues in the DNA-binding domain (Lys^{120}, Lys^{132}, Lys^{139}, and Lys^{164}) are very well conserved from human to *Xenopus* to *Drosophila* (the other, Lys^{101}, is conserved among mammalian p53). As shown above, a protein containing residues 1 to 161 (CΔ161) was still ubiquitinated. We next created a further series of COOH-terminally deleted mutants that aimed to remove one lysine at a time. Figure 6A shows that deletion from the COOH terminus reduced polyubiquitination and monoubiquitination. No ubiquitination was detected after complete removal of all the lysines in the DNA-binding domain (CΔ90) (Fig. 6B).

Although the ubiquitination assay we used was not designed to be quantitative, we noted that there was a relatively large reduction of ubiquitination between CΔ161 and CΔ138

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(Fig. 6A). To further explore this, we next mutated the lysine between residues 138 to 161 (Lys^{139}) to arginine. We found that Lys^{139} was not essential for DNA binding, as $K_{139}R$ substitution did not affect the transcriptional activity of p53 (Fig. 5E). $K_{139}R$ mutation only slightly reduced but not abolished ubiquitination (Fig. 6C). Similarly, mutation of Lys^{139} in the context of CΔ161 also only slightly decreased ubiquitination (Fig. 6D).

We next removed the entire DNA-binding domain, leaving the MDM2-binding domain and the COOH terminus intact (Δ91-294). We found that monoubiquitination of p53 was reduced in Δ91-294 in comparison with p53 (Fig. 7A). Similarly, polyubiquitination of Δ91-294 was significantly subdued in comparison with wild-type p53 (Fig. 7B). Not surprisingly, Δ91-294 was not active as a transcription factor (data not shown). The ineffective ubiquitination of Δ91-294 mutant was not simply due to an inability to bind DNA, as the non-DNA-binding $R_{273}H$ mutant was effectively ubiquitinated (Figs. 3C and 7A). These data further underscore the idea that the lysine residues in the DNA-binding domain are important for p53 ubiquitination.

To see if the defective ubiquitination of Δ91-294 resulted in a stabilization of the protein, we compared the stability of full-length p53 with Δ91-294 in the presence of MDM2. Figure 7C shows that, in contrast to the rapidly degraded p53, Δ91-294 mutant was significantly more stable. Collectively, these data indicate that the DNA-binding domain is required for efficient ubiquitination and destabilization of p53.

Discussion

Ubiquitination of p53 depends on at least two elements in the protein: the sequence recognizes by the ubiquitin ligases (like MDM2) and the sequence that actually receives the ubiquitin conjugation. Conceptually, the two elements can either be at distinct locations or be overlapped, and disruption of either element should abrogate p53 ubiquitination. Both these factors contribute to some difficulties in studying protein ubiquitination in general. It is well established that MDM2 binds the NH_2-terminal region of p53 within the transactivation domain. Indeed, either point mutation or deletion of the MDM2-binding domain abolished ubiquitination (Fig. 3). Both CΔ326 and CΔ161 still displayed ubiquitination (Fig. 3), verifying that the COOH-terminal region did not contain a MDM2-binding site. More importantly, however, these data disagree with the prevailing view that the majority of the ubiquitination occurred at the COOH-terminal lysine residues. Similarly, mutation of five COOH-terminal lysines

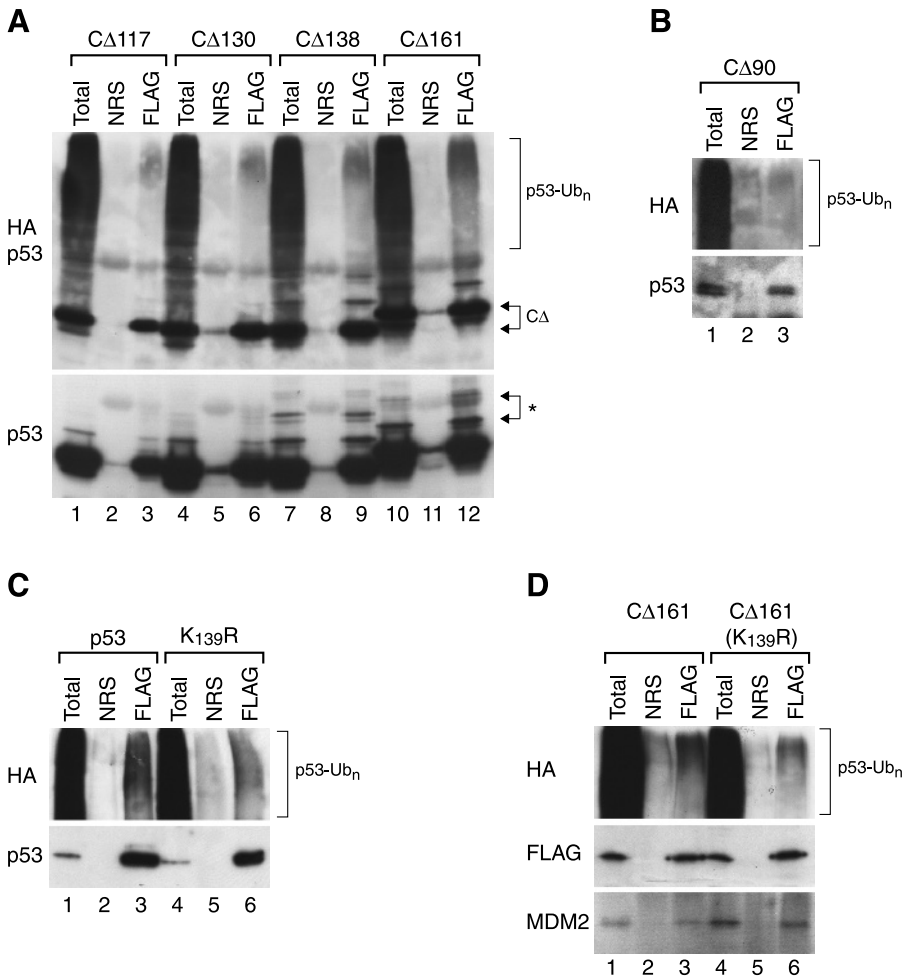

FIGURE 6. The NH_2-terminal region of the DNA-binding domain can be ubiquitinated by MDM2. **A.** *In vivo* ubiquitination assays of FLAG-tagged CΔ117, CΔ130, CΔ138, and CΔ161 mutants were carried out according to Fig. 3B. Bottom, an overexposed blot of p53 indicating the positions of the monoubiquitination and diubiquitination forms (*asterisk*). FLAG-tagged CΔ90 (**B**), p53 and $K_{139}R$ (**C**), and CΔ161($K_{139}R$) and CΔ161 (**D**) were subjected to *in vivo* ubiquitination assays as described in Fig. 3B.

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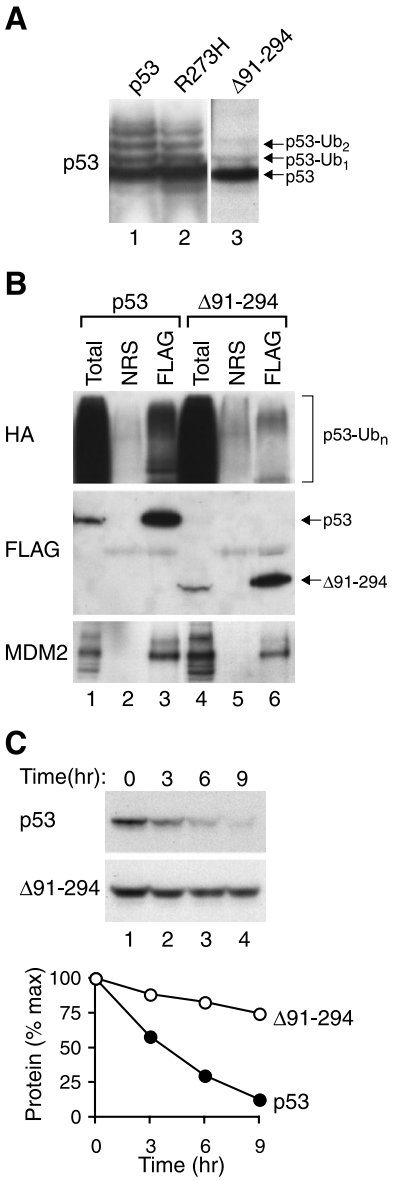

FIGURE 7. The central DNA-binding domain of p53 is required for efficient ubiquitination by MDM2. **A.** FLAG-tagged p53, $R_{273}H$, or Δ91-294 was coexpressed with MDM2 in H1299 cells. Cell extracts were prepared and immunoblotted with antibodies against p53. **B.** *In vivo* ubiquitination assay of FLAG-p53 and FLAG-p53(Δ91-294) was done as described in Fig. 3B. **C.** p53(Δ91-294) is more stable than wild-type p53. FLAG-tagged p53 or Δ91-294 was coexpressed with MDM2 in H1299 cells. At 48 hours after transfection, doxycycline and cycloheximide were added and cell extracts were prepared at the indicated time points. The stability of the FLAG-tagged proteins was examined by immunoblotting. Bottom, intensities of bands quantified using ImageJ software (NIH).

did not affect ubiquitination (Fig. 3B and D). However, our data are not entirely inconsistent with previously published results, because it has not been shown that mutation of the COOH-terminal lysines completely eliminates ubiquitination. It was found recently in a transgenic model that mouse p53 with seven COOH-terminal lysines changed to arginine exhibited a normal half-life (27). This strongly supports our model that the COOH-terminal lysines are not the only residues for ubiquitination.

Several of our experiments suggest that the NH_2-terminal half of p53 contained sites for both MDM2 binding and ubiquitination. In this connection, we could not completely exclude the possibility that p53 is ubiquitinated at the NH_2 terminus. Most of our experiments were done with NH_2-terminally FLAG-tagged p53, and it is possible that untagged p53 may behave differently. The FLAG epitope itself was unlikely to be ubiquitinated, as experiments with FLAG-p53($3C_1$) indicated that ubiquitination did not occur within the FLAG tag (Fig. 4C). Within the NH_2-terminal region of p53, Lys^{24} is a logical candidate for ubiquitination site because of its proximity to the MDM2-binding site. It is noteworthy that Lys^{24} is not well conserved among different species (conserved in most mammalian species but not in bovine, *Xenopus*, or *Drosophila* p53). We found that mutation of Lys^{24} did not have any discernable effect on MDM2 binding and ubiquitination (Fig. 5). Nevertheless, Lys^{24} may be involved in subtle interaction with MDM2 or other transcription machinery because the transcriptional activity of p53($K_{24}R$) was higher than p53 (Fig. 5E). The possible difference in the degree of MDM2 binding is difficult to evaluate, as coexpression of MDM2 reduced the transcriptional activity of both p53 and p53($K_{24}R$) to background level (data not shown). Interestingly, it has been shown that substitution of Lys^{24} with isoleucine reduces the transcriptional activity of p53 (28).

Based on the analysis using truncations and 3C-protease mutants, we hypothesized that residues in the DNA binding are targeted for ubiquitination. In particular, progressive removal of sequences from the COOH terminus eventually eliminated ubiquitination (Fig. 6A and B). Furthermore, deletion of the DNA-binding domain (Fig. 7A and B) disrupted ubiquitination. A caveat is that deletion of the DNA-binding domain did slightly reduce the binding to MDM2 (Fig. 7B). Some ubiquitination was still detected in Δ91-294 possibly due the contribution from the COOH-terminal residues. Furthermore, mutation of several lysine residues in DNA-binding domain to arginine (K101, K120, K132, and K139) did not abolish ubiquitination.[1] We think that although specific residues are normally ubiquitinated (lysines in COOH-terminal region and DNA-binding domain) it is possible that there is a large degree of redundancy in the ubiquitination system. Mutation of normal ubiquitin acceptor sites may allow other sites to be used instead, as in the case for cyclin A (29).

The physiologic importance of ubiquitin acceptor sites in the DNA-binding domain of p53 may be more than for ubiquitin-mediated proteolysis alone. Although the lysine residues in the DNA-binding domain are not mutational hotspots, it is conceivable that monoubiquitination or polyubiquitination of these residues could hinder DNA binding. There may also be potential overlap and competition for conjugation to ubiquitin, SUMO, NEDD8, and acetylation. Several lysines at the COOH-terminal region have been identified as the major sites for these modifications. Lys^{386} is the major sumoylation site (22, 23),

[1] W.M. Chan et al., unpublished data.

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Published OnlineFirst January 19, 2006; DOI: 10.1158/1541-7786.MCR-05-0097

and Lys^{370}, Lys^{372}, and Lys^{373} are the major neddylation sites triggered by MDM2 (30). Acetylation of p53 by p300/PCAF occurred at the COOH-terminal lysines (370, 372, 373, 381, and 382; refs. 31-33). It would be very interesting to evaluate if these post-translational modifications also occur at residues in the DNA-binding domain, as they may alter the DNA-binding affinity of p53. In this connection, it was found recently that the COOH-terminal lysine residues are not essential for p53 regulation but may contribute to a fine-tuning mechanism *in vivo* (27).

An interesting question for future analysis is whether the various ubiquitin ligases for p53 target the same sites for ubiquitination as MDM2. There is yet no report on the ubiquitin acceptor sites of PIRH2 and COP1. Human papillomavirus E6–dependent degradation of p53 does not depend on the COOH-terminal lysines (24, 28) but may involve sites at the NH_2-terminal region (34). Another interesting question is whether the removal of ubiquitin by the p53 deubiquitination enzyme HAUSP (35) acts on all lysines with the same efficacy. It is conceivable that different enzymes regulate p53 differently by targeting different ubiquitin acceptor sites.

Until recently, identification of the precise sites of ubiquitination has not been as prevalent as other post-translation modifications. The question of physiologic relevance as well as the lack of satisfactory methodology could be part of the reasons. One approach is by blunt site-directed mutagenesis of all lysine residues in a protein as in the case for $p21^{CIP1/WAF1}$ (36). A caveat is that ubiquitination may switch to different residues when others are mutated, as we have discovered to be the case for human cyclin A (29). We found that several lysine residues proximal to the D-box (Lys^{37}, Lys^{54}, and Lys^{68}) were ubiquitin acceptor sites in cyclin A. Mutation of these lysines, however, shifted the ubiquitination to other cryptic sites.

The introduction of a 3C-protease cleavage site into p53 provided a simple way to decipher the region of ubiquitination in the absence of other mutation (Fig. 4B). However, there are some shortcomings to consider. This method will not be useful if there are multiple ubiquitin acceptor sites scatter along the whole protein. Depending on the specific location, it is also possible that the 3C-protease recognition sequence could affect the general structure of the protein. Another potential problem is that we found that some cleaved peptides were sticky and retained in the pellet fraction possibly because the peptides were misfolded and precipitated after cleavage. Thus, the results from the supernatant fraction are better indicators than the pellet fraction. We also cannot exclude the possibility that some ubiquitinated proteins were specifically resistant to cleavage. Given these potential limitations, we believe that this approach is still helpful in indicating the positions of the ubiquitin acceptor sites. Similar approach is likely to be more suitable for studying smaller post-translation modifications like acetylation and sumoylation. Finally, another complication is that we cannot exclude the possibility that the high molecular weight ubiquitin smear is coming from a p53-associated protein like MDM2. We think this is unlikely because the monoubiquitinated band and at least a portion of the polyubiquitinated smear were smaller than the size of full-length MDM2.

Materials and Methods

Materials

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

DNA Constructs

Constructs used in previous publications are shown in Fig. 1. MDM2 in pCMV (37), p53 in pRcCMV (37), and HA-ubiquitin in pUHD-P2 (38) were obtained from sources as described previously. Glutathione *S*-transferase 3C-protease in pGEX-KG was a gift from Jane Endicott (University of Oxford, Oxford, United Kingdom). The p53($K_{370-382}R$) construct with substitution of five lysines (370, 372, 373, 381, and 382) to arginine (21) was a gift from Dr. Wei Gu (Columbia University, New York, NY). Based on this clone, FLAG-p53($K_{370-382}R$) in pUHD-P1 was constructed the same way as wild-type FLAG-p53 in pUHD-P1 (39). The *Nco*I fragment of p53 was put into pUHD-P1 to create FLAG-p53(CΔ161) in pUHD-P1. Other CΔ constructs were created from FLAG-p53 in pUHD-P1 by PCR amplification using a forward primer in the vector and a reverse primer (CΔ138: 5′-GGAATTCTCAGGCCAGTTGGCAAA-3′, CΔ130: 5′-AGAATTCTCAGAGGGCAGGGGAGTA-3′, and CΔ117: 5′-AGAATTCTGACCCAGAATGCAAGAA-3′); the PCR products were cut with *Nhe*I and *Eco*RI and ligated into pUHD-P1. A 3C-protease cleavage site (LEVLFQ↑GP) was created COOH terminal to the FLAG tag by ligating the oligonucleotides 5′-CATGCTGGAAGTTCTGTTTCAGGGGCC-3′ and 5′-CATGGGCCCCTGAAACAGAACTTCCAG-3′ into the *Nco*I site of pUHD-P1. The *Nco*I-*Nco*I and *Nco*I-*Eco*RI fragments from FLAG-p53 in pUHD-P1 were ligated into this vector to create FLAG-p53($3C_1$) in pUHD-P1. To introduce a 3C site into position 90, p53 was amplified by PCR using a vector forward primer and 5′-CCGAATTCTGGCCCCTGAAACAGAACTTCCAGGGAGGGGGCTGGTGCAGGGGCCGCCG-3′; the PCR product was cut with *Nco*I-*Eco*RI and ligated into pUHD-P1 to create FLAG-p53(CΔ90); insertion of the *Eco*RI fragment from FLAG-p53(NΔ90) in pUHD-P1 into this construct then created FLAG-p53($3C_{90}$) in pUHD-P1. To introduce 3C site to position 303, p53 was first amplified by two PCR reactions: vector forward primer and 5′-CCCCTGAAACAGAACTTCCAGGCTCCCTGGGGGCAG-3′ and 5′-GAAGTTCTGTTTCAGGGGCCAACTAAGCGAGCACTGCC-3′ and p53 reverse primer (5′-TTTGAATTCTCAGTCTGAGTCAGGCCC-3′); the two PCR products were mixed and another PCR amplification was set using the flanking primers; the product was cut with *Nhe*I-*Eco*RI and ligated into pUHD-P1 to create FLAG-p53($3C_{303}$). Site-directed mutagenesis was carried out with QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) by using the following primers and their antisense: 5′-GACCTATGGAGACTACTTCCTG-3′ ($K_{24}R$) and 5′-TGCCAACTGGCCAGGACCTGCCCT-3′ ($K_{139}R$).

Cell Culture

H1299 (non–small cell lung carcinoma; ref. 40) and Hep3B cells (hepatocellular carcinoma) were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% (v/v) fetal

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bovine serum (Invitrogen, Carlsbad, CA) in a humidified incubator at 37°C in 5% CO_2. H1299 cells were transfected with a calcium phosphate precipitation method (41) and Hep3B cells were transfected with LipofectAMINE (Invitrogen). The amount of total DNA transfected was adjusted to the same level with blank vectors. Cycloheximide (10 μg/mL) and doxycycline (1 μg/mL) were used at the indicated concentrations. Cell-free extracts were prepared as described previously (42). Protein concentration of cell lysates was measured with the bicinchoninic acid protein assay system (Pierce, Rockford, IL).

Transactivation Assays

The transcriptional activity of p53 was assayed using a MDM2 promoter luciferase construct and normalized with the β-galactosidase activity as described previously (37).

In vivo *Ubiquitination Assays*

In vivo ubiquitination assays were done as described previously (38). Briefly, constructs expressing FLAG-tagged proteins were cotransfected with HA-ubiquitin in pUHD-P2. The cells were treated with 50 μmol/L LLnL for 6 hours before they were harvested. Cell extracts prepared from the transfected cells were immunoprecipitated with either normal rabbit serum or FLAG antiserum. The presence of HA-ubiquitin–conjugated proteins in the immunoprecipitates was detected by immunoblotting with the anti-HA monoclonal antibody 12CA5.

Cleavage of Ubiquitinated Proteins with 3C-Protease

Expression of recombinant glutathione *S*-transferase 3C-protease in bacteria and purification with reduced glutathione-agarose chromatography were as described previously (42). FLAG-tagged p53(3C) constructs were coexpressed with HA-ubiquitin in mammalian cells as described above. Cell lysates were prepared and 100 μg were subjected to immunoprecipitation with FLAG antiserum. The immunoprecipitates were washed with buffer A [10 mmol/L HEPES (pH 7.2), 25 mmol/L KCl, 10 mmol/L NaCl, 1.1 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 0.1 mmol/L DTT] and either mock-treated or incubated with 1 μg glutathione *S*-transferase 3C-protease in 20 μL buffer A at 25°C for 1 hour. The supernatant was removed and saved, and the beads were washed twice as in standard immunoprecipitation. Both the supernatant and the bead fractions were mixed with SDS sample buffer and applied onto SDS-PAGE for immunoblotting.

Antibodies and Immunologic Methods

Immunoblotting and immunoprecipitation were done as described previously (42). Rabbit polyclonal antibodies against FLAG tag (43), monoclonal antibody 12CA5 against HA tag (43), M2 against FLAG tag (38), and 2A10 against MDM2 (44) were obtained from sources as described previously. Monoclonal antibody 122 against p53 was a gift from Tony Hunter (The Salk Institute, La Jolla, CA). Monoclonal antibody DO1 against p53 and polyclonal antibodies against p53 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Acknowledgments

We thank Drs. Jane Endicott, Wei Gu, and Tony Hunter for generous gifts of reagents and the members of the Poon Lab for constructive criticism on the article.

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Molecular Cancer Research

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Wan Mui Chan, Man Chi Mak, Tsz Kan Fung, et al.

Mol Cancer Res 2006;4:15-25. Published OnlineFirst January 19, 2006.

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