Identification of a Sequence Element from p53 That Signals for Mdm2-Targeted Degradation

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The binding of Mdm2 to p53 is required for targeting p53 for degradation. p73, however, binds to Mdm2 but is refractory to Mdm2-mediated degradation, indicating that binding to Mdm2 is not sufficient for degradation. By utilizing the structural homology between p53 and p73, we generated p53-p73 chimeras to determine the sequence element unique to p53 essential for regulation of its stability. We found that replacing an element consisting of amino acids 92 to 112 of p53 with the corresponding region of p73 results in a protein that is not degradable by Mdm2. Removal of amino acids 92 to 112 of p53 by deletion also results in a non-Mdm2-degradable protein. Significantly, the finding that swapping this fragment converts p73 from refractory to sensitive to Mdm2-mediated degradation supports the conclusion that the amino acids 92 to 112 of p53 function as a degradation signal. We propose that the presence of an additional protein recognizes the degradation signal and coordinates with Mdm2 to target p53 for degradation. Our finding opens the possibility of searching for the additional protein, which most likely plays a critical role in the regulation of p53 stability and therefore function.

Normal mammalian cells respond to DNA damage by undergoing cell cycle arrest, DNA repair, or apoptosis. Failure to respond properly to DNA damage allows the cell to replicate and segregate damaged DNA molecules, which can result in genetic instability and malignant transformation (11, 21, 24, 34). The initiation of DNA damage-induced responses largely depends on the induction of the tumor suppressor p53, which is increased in its stability as well as its specific activity in response to genotoxic stress (19, 27, 31). Despite intensive research, mechanisms that regulate p53 activity are still not completely understood. In an unstressed cell, the tumor suppressor p53 is a short-lived protein due to its high turnover rate and is maintained at a low level. Upon exposure to DNA damage, p53 is activated and accumulated in the absence of apparent changes in mRNA levels (19). It has been shown that an increase in p53 protein levels correlates with a prolonged half-life (25, 27, 33), indicating that control of protein stability is an important mechanism that regulates p53 function, although enhanced translation may also contribute to the rise in p53 protein levels (10, 30).

Mdm2, itself a transcriptional target of p53, plays a critical role in regulation of p53 activity. Knockout of the mdm2 gene is lethal in mice with a functional p53 gene during early embryogenesis (16). Simultaneous deletion of both mdm2 and p53 genes gave rise to mice that developed normally, demonstrating that Mdm2 is essential for negative regulation of the p53 activity during development (16). Mdm2 regulates p53 function by directly binding to the transactivation domain (TAD) of p53 to block the transactivation activity of p53 (2, 4, 5, 6, 29, 32, 36) and by targeting p53 for degradation (12, 13, 22, 28). The p53 protein is degraded by ubiquitin-mediated proteolysis (26). A recent study suggests that Mdm2 is a member of a novel class of E3 ubiquitin ligases and can ubiquitinate p53 in vitro using purified components of the ubiquitin pathway (13). E3 activity by Mdm2 in vivo has not been demonstrated. Given the fact that Mdm2 must binds to p53 in order to target the tumor suppressor for degradation, one way to stabilize p53 in cells is by disrupting the complex between p53 and Mdm2. In line with this notion, p53 is a phosphoprotein containing a number of phosphorylation sites in the vicinity of the N-terminal Mdm2-binding region. Phosphorylation of p53 by protein kinase-dependent DNA in response to DNA damage has been shown to decrease the association of Mdm2 and p53 (35), providing a mechanism for DNA damage-induced accumulation of p53. In more recent studies (1, 3), however, it has been shown that p53 mutants with all potential phosphorylation sites mutated remain responsive to DNA damage-induced activation and to an accumulation of p53, indicating that mechanisms other than phosphorylation can regulate p53 activation and stability in DNA-damaged cells. While Mdm2-mediated degradation represents a key mechanism in regulation of p53 protein levels, stabilization of p53 in response to DNA damage implies that Mdm2-mediated degradation of p53 must be inhibited by a mechanism that is activated by DNA damage. A better understanding of the molecular basis of Mdm2-mediated degradation of p53 will undoubtedly shed light onto the mechanisms responsible for DNA damage-induced activation of p53.

The p53 protein can be divided into several well-characterized domains (24), which include the N-terminal acidic transactivation domain (TAD), which contains the Mdm2-binding motif, a proline-rich domain (PRD) that is important for interaction with SH3-containing proteins, and a central sequence-specific DNA-binding domain (DBD), and the C terminus, which contains the oligomerization domain (OD), nuclear export and localization signals, and a region at the extreme C terminus which is involved in the regulation of the sequence-specific DNA-binding function. Protein degradation is usually determined by the structure of the protein, i.e., the degradation signal and other proteins that are involved in the recognition of the degradation signals. Numerous attempts have been made to investigate the sequence elements involved in the regulation of p53 stability. Using deletion mutants of p53, it has been shown that fusing the first 42 amino acid residues of p53 with Gal4 results in a fusion protein that is necessary and sufficient for the degradation of p53 by the Mdm2-mediated pathway (12). In a similar approach, other
studies have showed that in addition to the N terminus, the OD and the extreme C terminus of p53 also contribute to the regulation of Mdm2-directed degradation of p53 (22, 23). Whereas the results from these studies suggest that the domains of p53 are important for p53 stability, it is still not clear which sequence element of p53 can function as a degradation signal for Mdm2-mediated degradation.

p73, a recently identified member of the p53 family, exhibits high sequence homology to the p53’s TAD, DBD, and OD (18). The structural similarity gives p73 the ability to activate transcription of p53-responsive promoters and induce apoptosis (17, 18). p73, however, is not induced at the protein level in response to DNA damage (18) and is refractory to Mdm2-mediated degradation (9, 38). Thus, we hypothesize that p53 has a unique sequence element that can function as a signal for Mdm2-mediated degradation. This sequence is essential for the control of p53’s stability and function.

MATERIALS AND METHODS
Cell culture and transfections. All cells were maintained in minimal essential medium (GIBCO-BRL) containing 10% fetal bovine serum (Sigma), 100 U of penicillin per mL, and 100 μg of streptomycin per mL. Transfections were performed by the calcium phosphate method (37) for 293T, U2OS, and Saos-2 cells. Luciferase activities were assayed 24 h posttransfection with an enhanced chemiluminescence system (1800K; Analytical Luminescence).

Plasmids. Vectors expressing p73 or p73p were have been reported previously (37). The p53–p73 chimeras were prepared by a two-step PCR using primers carrying a 12-nucleotide tail of the p53 or p73 to be fused. The point mutation mutants of p53 (R273H) and p73 (R293H) were generated by PCR using a 20-nucleotide fragment carrying the mutated nucleotide. The p53 or p73 deletion mutant was prepared by two-step PCR. Restriction enzyme digestion and DNA sequencing confirmed the identity of each construct.

Immunoprecipitation and immunoblot analysis. Immunoprecipitations were performed as described elsewhere (37). Cell lysates were prepared in 0.5% Triton X-100 lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM NaF, 2 mM phenylmethysulfonyl fluoride, 10 μg each of leupeptin and aprotinin per mL) and incubated with anti-Flag agarose beads (M5; Sigma) for 8 to 12 h. Immune complexes and whole lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose filters. The filters were incubated with anti-p53 (Ab-6; Oncogene Science), anti-Flag (M5; Sigma), anti-WAF1 (Ab-3; Oncogene Science), anti-Mdm2 (Ab-1; Oncogene Science), anti-Flag (M5; Sigma) antibodies. Proteins were detected with a normalized protein concentration 24 h posttransfection.

RESULTS
Preparation of p53-p73 chimeras. The fact that despite its high degree of structural homology to p53 (18) and binding to Mdm2, p73 is refractory to Mdm2-mediated degradation (9, 38) implicates the presence of a sequence unique to p53 that is essential for Mdm2-mediated degradation. To identify this p53 sequence, we generated a series of p53-p73 chimeras and tested them for sensitivity to Mdm2-mediated degradation. The high degree of structural homology between p53 and p73 allowed us to exchange each of the p53 domains with the corresponding region of p73 without disturbing the wild-type conformation. The chimeras were prepared (Fig. 1A) by a two-step PCR using primers carrying the 12-nucleotide tail of the molecules to be fused. Restriction enzyme digestion and DNA sequencing confirmed the identity of each chimera (data not shown). To test whether the chimeras retained wild-type function, Flag-pCDNA3 vectors expressing the chimera were prepared and then tested for the ability to induce p21 expression. Each of the vectors was transfected transiently into Saos-2 cells, and the cells were analyzed for induction of p21 by Western blotting 24 h posttransfection. As shown in Fig. 1B, the levels of p21 are induced, though to variable extents, by the expression of the chimeras (Fig. 1B, top panel), indicating their transactivational competence. Immunoblotting with an anti-Flag antibody exhibits comparable levels of expression achieved for the wild-type proteins and chimeras (Fig. 1B, middle panel). Consistent with the results from the Western analysis, transcriptional activity assessed by the luciferase reporter gene with the p21 promoter also demonstrated that the chimeras are transcriptionally active (Fig. 1C).

Role of the OD of p53 in Mdm2-mediated degradation. It has been recently reported (23) that the deletion of p53’s OD resulted in a loss of its sensitivity to degradation, which was suggested to be related to defects in binding to Mdm2. The fact that p73p, which is 42% identical to p53 in the OD, can form an oligomer as well as p53 does (18) but is resistant to degradation by Mdm2 suggests the involvement of some other factor in addition to Mdm2 binding. To assess the role of the OD in Mdm2-mediated p53 degradation, we examined the OD-swapping chimeras (Fig. 2A) for sensitivity to Mdm2-targeted degradation. To determine whether the domain swapping has any impact on oligomerization, we assessed the capability of the chimeras to form oligomers in vivo by examining protein-protein interaction using an immunoprecipitation-Western blotting (IP-Western) analysis. To this end, Flag-tagged p73, p53, or p53-p73 chimeras with switched ODS were coexpressed with
GFP-tagged p53. Lysates were prepared from the transfectants 24 h posttransfection and subjected to immunoprecipitation with an anti-Flag antibody. Anti-GFP immunoblotting analysis of the immunocomplexes demonstrated that GFP-p53 associates with Flag-p53 but not Flag-p73 (Fig. 2B). This observation implies that p53 forms homo-oligomers but not hetero-oligomers in vivo. Interestingly, switching the OD between p53 and p73 results in heterocomplex formation, as evidenced by the finding that p53 was readily detected in the immune complex of p73β and p53 amino acids 319 to 365, a region that contains the OD of p53 [p73β-p53(aa319-364)] (Fig. 2B). This finding indicates that it is the sequence of OD that determines the specificity of oligomerization. Similar results were observed in a parallel experiment where Flag-tagged p73, p53, or the chimeras were coexpressed with GFP-p73. As shown in Fig. 2C, anti-GFP immunoblotting analysis revealed that GFP-p73 associates with Flag-p73 and with the chimera containing the OD of p73 but not with Flag-p53 or the p73 chimera with OD of p53. Taken together, the results indicate that the OD-swapping chimeras are functional in oligomer formation. Furthermore, our results demonstrated that both p53 and p73 can form only homo-oligomers, consistent with what was recently reported (8).

If the OD of p53 is essential for Mdm2-mediated degra-
tion, an alteration of sensitivity to degradation by Mdm2 should result from switching the OD between p53 and p73. To test this, each of the OD-swapping chimeras was transfected into Saos-2 cells with or without Mdm2; protein levels were determined by immunoblotting with an anti-Flag antibody 24 h posttransfection. Wild-type p53 and p73 were included as controls. As expected, p53 but not p73 was degraded by the coexpression of Mdm2 (Fig. 2D, top panel, lanes 1 to 4). Switching the OD between p53 and p73 does not alter their sensitivity to Mdm2-mediated degradation. The p53-p73
\textsuperscript{b}(aa345-390) chimera remained sensitive (Fig. 2D, lanes 5 and 6), and p73
\textsuperscript{b}-p53(aa319-364) was still refractory (Fig. 2D, lanes 7 and 8) to Mdm2-targeted degradation. The result indicates that the OD of p53 does not contain the sequence element essential for Mdm2-mediated degradation.

Role of the N terminus of p53 in Mdm2-mediated degradation. The Mdm2-binding motif of p53 is located at its N terminus and is conserved in p73 (18). The finding that p73 binds to Mdm2 but is refractory to Mdm2-mediated degradation suggests that in addition to binding to Mdm2, another element(s) is required for Mdm2-targeted degradation. It has been reported that a small domain of p53 at the N terminus is sufficient for its degradation by Mdm2 (12). Except for the Mdm2-binding motif, the homology between p53 and p73 at the N terminus is much less pronounced (29% identity), providing a potential structural basis for their distinct response to Mdm2-targeted degradation. To examine this matter, we first assessed the sensitivity of the chimeras p53-p73
\textsuperscript{b}(aa1-131) and p73-p53(aa1-112) (Fig. 3A, top) to Mdm2-mediated degradation. Strikingly, the result demonstrated that switching the N terminus between p53 and p73 is associated with a loss of sensitivity in p53 (Fig. 3A, middle panel, lanes 5 and 6) and gain of sensitivity in p73 (Fig. 3A, middle panel, lanes 7 and 8) to Mdm2-mediated degradation. This finding indicates that the N terminus of p53, consisting of amino acids 1 to 113, is indeed sufficient for Mdm2-targeted degradation. Interestingly, p73-p53(aa1-112) becomes and p53-p73
\textsuperscript{b}(aa1-131) remains capable of ubiquitination, which suggests that both the N and C termini of p53 are involved in the ubiquitination. The finding that p53-p73
\textsuperscript{b}(aa1-131) remains ubiquitinated but is resistant to degradation by Mdm2 suggests that ubiquitination and degradation are separable events.

Amino acids 1 to 112 of p53 can be further divided into the TAD and PRD (24). It was reported (12) that the Gal4-p53(aa1-42) fusion protein, like wild-type p53, was degraded by Mdm2, suggesting this small region of p53 is necessary and
sufficient for Mdm2-mediated reduction in protein levels. If that is the case, one would expect a conversion of p73 from refractory to sensitive to Mdm2-mediated degradation by replacing the TAD of p73 with that of p53. To test this, we assessed the sensitivity of p53-p73\(\text{b}(\text{aa1-54})\) and p73\(\text{b}-\text{p53}(\text{aa1-45})\) (Fig. 3B, top) to Mdm2-targeted degradation. To our surprise, the results demonstrated that the TAD of p53 is dispensable for Mdm2-mediated degradation (Fig. 3B, middle panel), p53-p73\(\text{b}(\text{aa1-54})\) remains sensitive to Mdm2-mediated degradation (Fig. 3B, lanes 5 and 6), and p73\(\text{b}-\text{p53}(\text{aa1-45})\) is still refractory to Mdm2-mediated degradation (Fig. 3B, lanes 7 and 8). However, switching the PRD between p53 and p73, which results in the chimeras p53-p73\(\text{b}(\text{aa55-131})\) and p73\(\text{b}-\text{p53}(\text{aa46-112})\) (Fig. 3B, top), respectively, renders p53 refractory (Fig. 3B, lanes 9 and 10) and p73 sensitive (Fig. 3B, lanes 11 and 12) to Mdm2-mediated degradation. Together, these results indicate that the PRD but not the TAD of p53 is essential for its sensitivity to Mdm2-mediated degradation.

In an effort to map the minimum sequence required for Mdm2-mediated degradation, a more refined swapping at the proline-rich region was carried out to generate the p53-p73 chimeras as shown in Fig. 4A. We then prepared Flag-tagged vectors expressing the chimeras to test their sensitivity to Mdm2-targeted degradation. We first examined whether this refined domain swapping had an effect on transcriptional activity. Each of the vectors was transfected transiently into Saos-2 cells, and the cells were analyzed for induction of p21 by immunoblotting with an anti-p21 antibody 24 h posttransfection. The result demonstrated that chimeras are transcriptionally active, as shown by the induction of p21 levels (Fig. 4B, top panel). Anti-Flag immunoblotting ensured that comparable levels of chimeras proteins were expressed (Fig. 4B, middle panel). To assess their response to Mdm2-mediated degradation, a vector containing the chimera cDNA was transfected into Saos-2 cells with or without coexpression of Mdm2. The result shows that the region from amino acids 92 to 112 of p53 is required for Mdm2-mediated degradation, as demonstrated by the observation that switching amino acids 92 to 112 of p53 with the corresponding region of p73 (amino acids 106 to 131) rendered p73 sensitive (Fig. 4C, lanes 11 and 12) and p53 resistant to Mdm2-mediated degradation (Fig. 4C, lanes 9 and 10). In contrast, substitution of p53’s amino acids 46 to 63 or 64 to 91 with the corresponding p73 amino acids 55 to 75 or 76 to 104, respectively, did not lead to any apparent alteration of their response to Mdm2-mediated degradation (Fig. 4C, lanes 1 to 8). To rule out that the refractory nature of p53-p73\(\text{b}(\text{aa106-131})\) to Mdm2-mediated degradation was due to an impaired binding to Mdm2, interaction of the chimera with Mdm2 was examined by IP-Western analysis. As shown in Fig. 4D, p53-p73\(\text{b}(\text{aa106-131})\), but not Rad52, bound to Mdm2 with an affinity comparable to that of wild-type p53, as did p73\(\text{b}-\text{p53}(\text{aa92-112})\), indicating no apparent effect on the Mdm2 binding from the swapping fusion. To further determine whether the gain of resistance in p53 and loss of resistance in
FIG. 4.

A

B

C

| pCDNA Flag Vector |
|--------------------|
| p53(73aa55-75)     |
| p53(73aa64-63)     |
| p53(73aa76-105)    |
| p53(73aa92-113)    |
| p73β(53aa64-91)    |
| p73β(53aa76-105)   |
| p73β(53aa92-113)   |
| pCMV-Mdm2          |

IB: Anti-p21

IB: Anti-Flag

IB: Anti-GFP

p53(73aa55-75) + +
p73β(53aa46-63) + +
p53(73βaa76-105) + +
p73β(53aa64-91) + +
p53(73βaa106-131) + +
p73β(53aa92-113) + +
pCMV-Mdm2 + + + + + + + +

IB: Anti-Flag

IB: Anti-GFP
FIG. 4. Identification of the region from amino acids 92 to 112 of p53 as an essential element for Mdm2-mediated degradation. (A) The p53-p73 chimeras with a more refined swapping at the PRD were prepared as described in Fig. 1 by switching each segment between p53 and p73 at the indicated position. (B) p21 levels are induced by the expression of the chimeras. A vector (2.5 μg) containing the indicated cDNA was transfected into Saos-2 cells, and the transfectants were analyzed by immunoblotting (IB) with anti-p21 (upper panel) and anti-Flag (lower panel) 24 h posttransfection. (C) The segment from amino acids 92 to 112 of p53 is essential for Mdm2-mediated degradation. The p53-p73 chimeras with more refined swapping at the PRD were tested for sensitivity to Mdm2-mediated degradation as described for Fig. 2. Levels of the chimeras and transfection efficiency were determined by Western analysis with anti-Flag (upper panel) and anti-GFP (lower panel) respectively. (D) The p53-p73 chimeras bind to Mdm2 with an affinity comparable to that of their wild-type counterparts. Flag-tagged vectors expressing wild-type p53, wild-type p73, or the indicated chimeras were coexpressed with pCMV-Mdm2. Cell lysates were prepared 24 h posttransfection and subjected to anti-Flag immunoprecipitation. The immunocomplexes were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and analyzed by immunoblotting with anti-Mdm2 (upper panel) and anti-Flag (lower panel). Flag-tagged Rad52 was included as a control. (E) The p53 mutant lacking amino acids 92 to 112 is no longer sensitive to Mdm2-mediated degradation. The p53(Daa92-112) mutant prepared by a two-step PCR was tested for sensitivity to Mdm2-mediated degradation as described in the legend to Fig. 2. The corresponding deletion mutant of p73 was included as a control. Levels of the proteins and transfection efficiency were determined by Western analysis with anti-Flag (upper panel) and anti-GFP (lower panel), respectively. (F) The p53 or p73 deletion mutants retain the ability to bind to Mdm2. Association of the deletion mutants with Mdm2 was examined as described for panel D.
p73 were due to an inhibitory effect of amino acids 106 to 131 of p73 on degradation, we prepared a p53 deletion mutant lacking amino acids 92 to 112 and a corresponding mutant of p73 lacking amino acids 106 to 131 to test their sensitivity to Mdm2-mediated degradation. The result shows that removal of the amino acids 92 to 112 of p53 is associated with a loss of sensitivity to Mdm2-mediated degradation (Fig. 4E, lanes 5 and 6). Deletion of the corresponding region of p73, however, had no apparent effect on resistance to Mdm2-mediated degradation (Fig. 4E, lanes 7 and 8). IP-Western analysis demonstrated that the deletion mutants remained capable of binding to Mdm2 (Fig. 4F), indicating that loss of sensitivity of the p53 deletion mutant is not due to any defect in Mdm2 binding. Taken together, the results demonstrate that in addition to the N-terminal Mdm2-binding sequence, the 21 amino acid residues 92 to 112 of p53 form the sequence element of p53 that functions as the degradation signal for Mdm2-mediated degradation.

Role of the C terminus and DBD of p53 in Mdm2-mediated degradation. In an unstressed cell, the p53 protein is not only at a very low level but also in an inactive state. The extreme C-terminal region of p53 has been shown to be able to prevent DNA binding through an allosteric mechanism (14, 15). A recent study showed that a small deletion of the C terminus of p53 leads to a decrease of sensitivity to Mdm2-mediated degradation (23), suggesting a contribution of the C terminus of p53 to its stability. Since p53 activity can be allosterically regulated by its C terminus, deletion of this region might result in some degree of alteration in the conformation of p53, which may complicate interpretation of the results. To clarify this issue, we assessed the Mdm2-mediated degradation with the chimeras in which the corresponding region of p73 (Fig. 5A, top panel) had replaced the C terminus of p53. The results show that p53-p73(aa105-131) has a prolonged and p73-p53(aa92-113) has a shortened half-life. Having identified amino acids 92 to 112 of p53 as the degradation signal to Mdm2-mediated degradation, we examined whether the changed sensitivity to Mdm2-mediated degradation corresponded to an altered stability by measuring the half-lives of the proteins. The ability of p53 and p73 to induce growth arrest and apoptosis impedes expression of the wild-type proteins. To overcome this, we generated p53 and p73 mutants by introducing a point mutation into the DNA-binding domain (Arg273-His for p53 or corresponding Arg292-His for p73), which has been shown to result in an abrogation of DNA binding and, therefore, of transcriptional activity (17, 20). When transiently transfected into Saos-2 cells, the mutants failed to induce p21 expression (data not shown). Because cycloheximide inhibits de novo protein synthesis, the half-life of the protein can be determined by Western blot analysis in cells treated with the drug. U2OS cells expressing the indicated vectors were analyzed at 0, 30, 60, 120, 180, and 300 min following addition of cycloheximide. The results demonstrated that replacing amino acids 92 to 112 of p53 with the corresponding region (amino acids 106 to 131) of p73 resulted in a markedly prolonged half-life (Fig. 6A, left middle panel). On the other hand, p73-p53(aa92-112) exhibited a half-life much shorter than that of wild-type p73 (Fig. 6A, right middle panel). A significantly prolonged half-life is also evident in the p53 deletion mutant lacking amino acids 92 to 112 (Fig. 6A, left bottom panel). Deletion of the corresponding region of p73, however, had no apparent effect on its half-life (Fig. 6A, right bottom panel). Densitometric measurement of the bands from Western blots enabled the quantitation of the proteins as a percentage of the total starting levels (Fig. 6B). Together the results demonstrate that the region from amino acids 92 to 112 of p53 is critical for control of p53 stability.

DISCUSSION

Protein degradation is generally determined by the degradation signal derived from the structure of the protein and other proteins that are needed for recognition of the degradation signal. While interaction with Mdm2 is required for targeting p53 for degradation, the observation that p73 binds to Mdm2 but is resistant to degradation by Mdm2 indicates the existence of an additional structural element unique to p53 required for Mdm2-targeted degradation. Using the p53-p73 chimeras generated by switching each of p53’s domains with the corresponding region of p73, we identified amino acids 92 to 112 of p53 as the element that can function as a degradation signal for Mdm2-mediated degradation. Replacement of amino acids 92 to 112 of p53 with the corresponding region of p73 is associated with a loss of its response to Mdm2-mediated degradation even though the chimera retains its capability of binding to Mdm2. In support of this observation, removal of amino acids 92 to 112 of p53 by deletion also results in a loss of response to Mdm2-mediated degradation, indicating that in addition to the Mdm2-binding domain, the region from amino acids 92 to 112 is required for degradation of p53 by Mdm2. The notion that amino acids 92 to 112 of p53 can function as a degradation signal for the Mdm2-mediated pathway is supported by the finding that p73 gains sensitivity to Mdm2-mediated degradation since the sequence spanning amino acids 92 to 112 of p53 is fused to the corresponding region of p73. Interestingly, a BLAST sequence homology search identified no apparent sequence homologue of the degradation signal sequence, indicating its uniqueness to p53. How this sequence element of p53 functions as a degradation signal is not clear. We speculate that an additional protein recognizes the sequence element and coordinates with Mdm2 to target p53 for degradation. Study is under way to search for the potential protein. Inhibition of Mdm2-mediated degradation of p53 has been suggested to be a principal mechanism for stress-induced p53 accumulation (23). Investigation of the response of the degradation signal and its interacting protein to genotoxic stress will likely provide insights into the role for Mdm2-targeted degradation in stress-activated induction of p53.

It has been reported that the OD of p53 participates in regulation of its sensitivity to Mdm2-mediated degradation (23). The results obtained from our study with the chimeras show that switching the OD between p53 and p73 does not have any apparent effect on Mdm2-mediated degradation. This discrepancy could reflect a difference in Mdm2 binding because the OD-swapping chimeras are functional in oligomer formation (Fig. 2) and remain capable of binding to Mdm2 (not shown) but the OD deletion mutant of p53 is impaired in its Mdm2-binding (23). Nevertheless, our result indicates that the OD of p53 does not contain the unique sequence element essential for Mdm2-mediated degradation.

The contribution of the extreme C terminus of p53 to its
FIG. 5. Contribution of the C terminus and DBD of p53 to Mdm2-mediated degradation. (A) The p53-p73 chimeras with their C termini switched (top panel) were tested for sensitivity to Mdm2-mediated degradation as described for Fig. 2. Levels of the chimeras and transfection efficiency were determined by immunoblotting with anti-Flag (middle panel) and anti-GFP (bottom panel), respectively. The p53-p73 chimeras with their DBDs switched (top panel) were tested for sensitivity to Mdm2-mediated degradation as described for panel A.
stability is reflected by a reduced sensitivity of the C-terminal chimera to Mdm2-mediated degradation. There is no significant homology between the C-terminal domains of p53 and p73 (18). Whether the decreased degradation of the C-terminal chimera of p53 by Mdm2 is due to an allosteric regulation remains to be determined.

In summary, we have identified the region from amino acids 92 to 112 of p53 as the element that functions as a degradation signal for Mdm2-mediated degradation. Our finding provides a basis on which to search for some additional protein(s) needed for recognition of the degradation signal. The additional protein(s) should play a critical role in the regulation of p53 stability and will most likely be a potential new therapeutic target for manipulation of p53 activity.

FIG. 6. Correlation of Mdm2-mediated degradation to protein stability. The region from amino acids 92 to 112 of p53 is critical for protein stability. U2OS cells were transfected with 2.5 μg of the indicated vector. The cells were treated with cycloheximide (40 μg/ml) at 24 h posttransfection and then harvested at 0, 30, 60, 120, 180, or 300 min later. Lysates from the cells were analyzed by anti-Flag Western blotting (A). Quantitation of the protein as a percentage of the starting levels was derived from a densitometric measurement of the Western blot signals (B).
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