MDM2 can promote p53 degradation \textit{in trans}

MDM2 Can Promote the Ubiquitination, Nuclear Export, and Degradation of p53 in the Absence of Direct Binding

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Summary

MDM2 can bind the N-terminus of p53 and promote its ubiquitination and export from the nucleus to the cytoplasm, where p53 can then be degraded by cytoplasmic proteasomes. Several studies have reported that an intact MDM2 binding domain is necessary for p53 to be targeted for ubiquitination, nuclear export, and degradation by MDM2. In the current study, we examined whether the MDM2 binding domain of p53 could be provided \textit{in trans} through oligomerization between two p53 molecules. p53 proteins mutated in their MDM2 binding domains were unable to bind MDM2 directly, and were resistant to MDM2-mediated ubiquitination, nuclear export, and degradation when expressed with MDM2 alone. However, these same p53 mutants formed a complex with MDM2 and were efficiently ubiquitinated, exported from the nucleus, and degraded when co-expressed with MDM2 and wild-type p53. Moreover, this effect required MDM2 binding by wild-type p53, as well as oligomerization between wild-type p53 and the MDM2-binding deficient p53 mutants. Taken together, these results support a model whereby MDM2-binding deficient forms of p53 can bind MDM2 indirectly through oligomerization with wild-type p53, and are subsequently targeted for ubiquitination, nuclear export and degradation. These findings may have important implications regarding the DNA damage response of p53.
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Introduction

The tumor suppressor protein p53 is a transcription factor and potent inhibitor of cell growth. Wild-type p53 is expressed at low levels in most cells due to a short protein half-life (1, 2). In contrast, the p53 protein is stabilized and its levels increase in response to various stresses, including DNA damage, hypoxia, and inappropriate oncogene signaling (3). In its active form, p53 is a tetramer that can bind DNA in a sequence-specific manner and activate gene transcription (4). Several genes have been identified which are transcriptionally induced by p53, including p21 and bax (5, 6). Induction of these p53-responsive genes results in either a G1 cell cycle arrest, or activation of an apoptotic cell death program, respectively (4).

p53 levels are regulated in large part by MDM2, the product of a p53-inducible gene. MDM2 can bind the N-terminus of p53 and inhibit the activity of p53 as a transcription factor (7, 8). Importantly, MDM2 binding also promotes the ubiquitination of p53 and its export from the nucleus to the cytoplasm, where p53 is then degraded by cytoplasmic proteasomes (9-11). There is some evidence that MDM2 can function as an E3 ubiquitin ligase which can transfer ubiquitin directly to p53 (12). More recent studies have suggested a link between the ubiquitination of p53 and its export from the nucleus to the cytoplasm. In these studies, mutations that inhibited the ability of MDM2 to ubiquitinate p53 also inhibited p53 nuclear export (13, 14). These findings led to a model in which MDM2-mediated ubiquitination activates a nuclear export signal (NES) in p53, leading to p53 nuclear export.

The stabilization of p53 that occurs in response to oncogene signaling is thought to result from increased levels of p14ARF, a tumor suppressor protein that can form a complex with MDM2 and inhibit the ability of MDM2 to degrade p53 (15-17). In contrast, the mechanisms by which p53 is stabilized in response to different DNA damaging agents have not been fully clarified. Certain DNA damaging agents, including UV radiation, actinomycin D, and the
alkylating agents mitomycin C (MMC) and methylmethane sulfonate (MMS) signal a decrease in MDM2 mRNA and protein levels, and a subsequent decrease in p53 ubiquitination (18, 19). This suggests that the stabilization of p53 in response to these agents results from a specific decrease in MDM2 gene expression. The most widely held view suggests that the DNA damage-induced stabilization of p53 results from phosphorylation of specific N-terminal sites in the p53 protein (3). For example, p53 is phosphorylated at multiple sites within or near the MDM2 binding domain in response to various DNA damaging stresses (3, 20). Phosphorylation at serines -15 and -20 has been reported to inhibit the interaction between p53 and MDM2 (20, 21). These results support a model in which DNA damage-induced phosphorylation at serines -15 and 20 stabilizes p53 by inhibiting p53:MDM2 binding, and thus preventing MDM2 from promoting p53 ubiquitination. The premise of this model is that modifications to p53 that inhibit its direct interaction with MDM2 will stabilize the p53 protein.

The purpose of the current study was to test the notion that direct binding between p53 and MDM2 is required for MDM2 to promote the ubiquitination, nuclear export, and degradation of p53. Towards this end, epitope-tagged p53 mutants were generated which are deficient in their ability to bind MDM2. These mutants were resistant to MDM2-mediated ubiquitination, nuclear export, and degradation when expressed with MDM2 alone. However, these same p53 mutants formed a complex with MDM2 and were efficiently ubiquitinated, exported from the nucleus, and degraded when co-expressed with MDM2 and wild-type p53. Further, this effect required MDM2 binding by the wild-type p53 protein, as well as oligomerization between wild-type p53 and the MDM2-binding deficient p53 mutants. Taken together, these results support a model whereby MDM2-binding deficient forms of p53 can bind MDM2 indirectly through oligomerization with wild-type p53, and are subsequently targeted for ubiquitination, nuclear export and degradation. These findings may have important implications regarding the DNA damage response of p53.
Experimental Procedures

Plasmid DNAs  HA-tagged wild-type p53 expression DNA was obtained from Christine Jost (Dana Farber Cancer Institute, USA). Untagged wild-type p53 and untagged p53 Δ1-42 (22) were from Peter Howley (Harvard Medical School, USA). GFP-tagged wild-type p53 has been described (14) and was obtained from Tyler Jacks (Massachusetts Institute of Technology, USA). Wild-type MDM2 DNA was obtained from Steve Grossmann (Dana Farber Cancer Institute, USA). DNA encoding myc-tagged ubiquitin (23) was obtained from Ron Kopito (Stanford University, USA). HA p53 Δ1-42 was generated by PCR using untagged p53 as a template. The 3’ primer for PCR was the SP6 primer, and the 5’ primer was 5’-GCGAATTCCATGTACCATACGATGTCCAGATTACGCTTTGATGCTGTCCCGGACG-3’, which encodes the HA epitope and p53 sequence initiating at amino acid 43. To construct GFP p53 Δ1-42, the GFP p53 wild-type DNA was used as a PCR template with the following primers; 5’-CCGCTGCAGACCATGTTGATGCTGTCCCGG-3’ as the 5’ primer, and 5’-CGGGGATCCCGGTCTGAGTCAGGCCC-3’ as the 3’ primer. The resulting PCR product was digested with PstI and BamHI, and cloned into the corresponding sites in the pEGFP-N1 vector (CLONTECH laboratories). The HA p53 W23S, untagged p53 Δoligomerization domain (OD) (Δ324-357), and GFP p53 ΔOD DNAs were generated using the Quick Change mutagenesis kit (Stratagene). The following oligonucleotides and their complementary oligonucleotides were used for the mutagenesis. For HA p53 W23S; 5’-CAGGAAGTAGTTTTCGAAGGTTGTCAAAATGTCTTCC-3’. For untagged and GFP p53 ΔOD; 5’-GCCAAAGAAGAAACCACCTCGAGCCAGGGGGAGGAGGG-3’.

Tissue culture and immunofluorescence  Saos-2 or U2OS cells were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin and streptomycin. Transfections were done using the calcium phosphate method in 35 mm dishes when the cells were approximately 80% confluent. Sixteen to 20 hrs. after addition
of the DNA precipitate, cells were washed twice with DMEM minus serum, and refed with DMEM plus 10% FBS. Cell extracts were prepared 8-10 hrs. later. For immunofluorescence staining, cells were plated on glass coverslips, and were transfected, washed and refed as described above. Twenty-four hrs. after transfection, cells were rinsed with phosphate buffered saline (PBS) plus 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$, and fixed with 4% paraformaldehyde for 30 min at 4°C. Paraformaldehyde was then replaced with 50 mM NH$_4$Cl for 5 min, and cells were permeabilized with 0.1% Triton X-100 plus 0.2% bovine serum albumin (BSA). p53 staining was carried out using the anti-HA monoclonal antibody HA.11 (Babco) as the primary antibody, and rhodamine red-conjugated anti-mouse antibody (Jackson Labs) as the secondary antibody. MDM2 staining was carried out using the anti-MDM2 polyclonal antibody N-20 (Santa Cruz) as the primary antibody, and AMCA-conjugated anti-rabbit antibody (Jackson Labs) as the secondary antibody. Specimens were then examined under a fluorescent microscope.

**Immunoblots and immunoprecipitations** Cells were rinsed with PBS and scraped into 500 µl lysis buffer (50 mM Tris [pH 7.5], 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 µg/ml aprotinin, 5 µg/ml leupeptin). The scraped cells were lysed on ice for 30 minutes with occasional light vortexing, followed by 15 minute centrifugation to remove cellular debris. Protein extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a PolyScreen PVDF transfer membrane (NEN Life Science Products). The membrane was probed with either an anti-HA monoclonal antibody (HA.11 from Babco), an anti-MDM2 monoclonal antibody (SMP-14 from Santa Cruz), an anti-p53 monoclonal antibody (PAb1801 [Ab-2] from Oncogene Science), or a monoclonal antibody against GFP (CLONTECH). For immunoprecipitations, 200 µg of transfected cell extract was immunoprecipitated with 0.4 µg of the anti-HA polyclonal antibody Y-11 (from Santa Cruz). The immunoprecipitates were resolved by SDS-PAGE and examined by immunoblot analysis as described above.
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Results

MDM2 can bind p53 and promote its ubiquitination and subsequent degradation by the proteasome (10, 11). The MDM2 binding domain is located in the p53 N-terminus, and mutations within this region can inhibit p53:MDM2 binding and thus inhibit p53 ubiquitination. To disrupt p53:MDM2 binding, we generated p53 mutants in which either the N-terminal 42 amino acids were deleted (p53 Δ1-42), or which contained a single point mutation within the MDM2 binding domain (p53 W23S). Saos-2 cells (p53-null; Figs. 1A and 1C) and U2OS cells (wild-type p53; Figs. 1B and 1D) were transfected with expression DNAs encoding epitope-tagged (HA-tagged) versions of either wild-type p53, p53 W23S or p53 Δ1-42 alone, or co-transfected with an excess of MDM2 DNA. Lysates from the transfected cells were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were examined with an antibody against MDM2. As shown in Figs. 1A and 1B (lower panels), MDM2 was co-immunoprecipitated with HA-tagged wild-type p53, but not with the HA p53 W23S or Δ1-42 mutants. To observe p53 ubiquitination, the transfected cell lysates were then either immunoprecipitated with an anti-HA antibody followed by immunoblot analysis with a second anti-HA antibody (Saos-2 cells), or examined by direct immunoblot analysis with an anti-HA antibody without prior immunoprecipitation (U2OS cells). As shown in Figs. 1A and 1B (upper panels), a high molecular weight ladder of p53 species that are ubiquitin:p53 conjugates was detected in both cell types when HA-tagged wild-type p53 was expressed with MDM2, indicating that MDM2 could promote wild-type p53 ubiquitination. In contrast, neither HA p53 W23S nor HA p53 Δ1-42 were ubiquitinated with MDM2 expression. Further, steady-state levels of HA wild-type p53 were decreased with MDM2 expression, indicative of MDM2-mediated p53 degradation, whereas levels of HA p53 W23S and HA p53 Δ1-42 remained unchanged (Figs. 1C and 1D). These results are consistent with the hypothesis that an intact MDM2 binding domain within the p53 protein is necessary for MDM2 to ubiquitinate and degrade p53.
Because p53 can exist as a homo-oligomer, we wished to determine whether p53 mutants that are deficient for MDM2-binding could bind MDM2 indirectly through oligomerization with wild-type p53. To this end, Saos-2 cells (Figs. 2A and 2C) were transfected with different combinations of HA p53 W23S, MDM2, and untagged p53 that was either wild-type, lacked the C-terminal oligomerization domain (ΔOD), or lacked the N-terminal MDM2 binding domain (Δ1-42). Similarly, U2OS cells (Figs. 2B and 2D) were transfected with various combinations of HA p53 Δ1-42, MDM2, and GFP-tagged p53 that was either wild-type, lacked the oligomerization domain (GFP p53 ΔOD), or lacked the N-terminal MDM2 binding domain (GFP p53 Δ1-42). In these experiments, equal amounts of p53 and MDM2 DNAs were transfected to facilitate detection of p53:MDM2 complexes in spite of p53 degradation. Lysates from the transfected cells were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were examined first with antibodies against either p53 or GFP. As shown in Fig. 2A (lower panel), untagged versions of both wild-type p53 and p53 Δ1-42 were co-immunoprecipitated with HA p53 W23S when these proteins were co-expressed in Saos-2 cells. Likewise, GFP wild-type p53 and GFP p53 Δ1-42 co-immunoprecipitated with HA p53 Δ1-42 when these proteins were co-expressed in U2OS cells (Fig. 2B, lower panel). These results indicate that HA p53 W23S and HA p53 Δ1-42 could oligomerize with wild-type p53 and with forms of p53 which lacked the N-terminal 42 amino acids. In contrast, neither of the HA-tagged p53 mutants formed a complex with the p53 ΔOD mutants, indicating that complex formation required the p53 oligomerization domain (Figs. 2A and 2B, lower panels). The HA immunoprecipitates were next examined with antibodies against MDM2. As shown in Figs. 2A and 2B (upper panels), a large amount of MDM2 co-immunoprecipitated with the HA-tagged p53 mutants only when the p53 mutants were co-expressed with MDM2 and wild-type p53, but not when co-expressed with MDM2 and either p53 Δ1-42 or p53 ΔOD. These results indicate that the MDM2-binding deficient mutants of p53 could bind MDM2 indirectly, and that this occurred through oligomerization with the wild-type p53 protein.
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Given that the HA p53 W23S and Δ1-42 mutants could bind MDM2 indirectly through oligomerization with wild-type p53, we next wished to determine whether these same p53 mutants could be targeted for ubiquitination when co-expressed with MDM2 and wild-type p53. Accordingly, lysates from the transfected cells were examined by immunoblot analysis with an anti-HA antibody. As shown in Fig. 3A, HA p53 W23S was not ubiquitinated when expressed with MDM2 alone in Saos-2 cells, but was efficiently ubiquitinated when expressed with both MDM2 and wild-type p53. Likewise, HA p53 Δ1-42 was not ubiquitinated when expressed with MDM2 alone (see Fig. 1B), but was ubiquitinated when expressed with MDM2 and GFP-tagged wild-type p53 (Fig. 3B). It should be noted that only modest levels of ubiquitinated HA p53 Δ1-42 were observed in these experiments compared to the ubiquitination of HA p53 W23S. We suspect this is due to the relatively low expression level of the HA p53 Δ1-42 mutant, and to the relatively low level of MDM2 binding observed when the HA p53 Δ1-42 mutant was co-expressed with MDM2 and GFP-tagged wild-type p53 (Fig. 2B and data not shown). Nonetheless, ubiquitin conjugates of HA p53 Δ1-42 were clearly evident when this mutant was expressed with GFP-tagged wild-type p53 and MDM2. Importantly, the HA p53 W23S mutant was not ubiquitinated when expressed in Saos-2 cells with either p53 Δ1-42 or p53 ΔOD, and HA p53 Δ1-42 was not ubiquitinated when expressed in U2OS cells with either GFP p53 Δ1-42 or GFP p53 ΔOD (Figs. 3A and 3B). Taken together, these results indicate that p53 mutants that are unable to bind MDM2 directly can be targeted for ubiquitination by MDM2 when co-expressed with both MDM2 and wild-type p53. Further, this ubiquitination requires MDM2 binding by the wild-type p53 protein, and oligomerization between wild-type p53 and the MDM2-binding deficient p53 mutants.

MDM2-mediated ubiquitination is thought to promote the export of p53 from the nucleus to the cytoplasm, where p53 can then be degraded by cytoplasmic proteasomes (13, 14). We next wished to determine whether p53 mutants that are deficient in direct MDM2-binding could also be targeted for nuclear export when expressed with MDM2 and wild-type p53. To monitor
nuclear export, the subcellular localization of HA wild-type p53 and HA p53 Δ1-42 was determined by immunofluorescence staining when expressed alone, or when co-expressed with MDM2. As shown in Figs. 4A and 4B, both HA wild-type p53 and HA p53 Δ1-42 displayed a nuclear localization pattern when expressed alone. In contrast, HA wild-type p53 was relocalized to the cytoplasm in cells in which it was expressed with MDM2, whereas HA p53 Δ1-42 remained nuclear with MDM2 expression. Previous studies have demonstrated that the relocalization of wild-type p53 with MDM2 expression requires a nuclear export signal (NES) in p53, and can be blocked by addition of the nuclear export inhibitor leptomycin B (13, 14 and data not shown). These studies indicate that the cytoplasmic localization displayed when wild-type p53 is expressed with MDM2 results from increased p53 nuclear export. The fact that HA p53 Δ1-42 remains nuclear with MDM2 expression indicates that this mutant is resistant to nuclear export when expressed with MDM2 alone.

To test whether the MDM2 binding domain could be provided through oligomerization for p53 nuclear export, cells were transfected with DNAs encoding HA p53 Δ1-42, MDM2, and GFP-tagged p53s that were either wild-type, lacked the MDM2 binding domain (GFP p53 Δ1-42), or lacked the oligomerization domain (GFP p53 ΔOD). As shown in Figs. 5A and 5B, both HA p53 Δ1-42 and GFP wild-type p53 were nuclear when expressed alone, but were exported from the nucleus and relocalized to the cytoplasm when co-expressed with MDM2. The fact that HA p53 Δ1-42 was exported under these conditions indicates that MDM2 can promote p53 nuclear export in the absence of direct p53:MDM2 binding. In contrast, HA p53 Δ1-42 remained nuclear when expressed with MDM2 and either GFP p53 Δ1-42 or GFP p53 ΔOD, and these GFP-tagged p53 proteins also remained nuclear (Figs. 5A and 5B). These results indicate that the nuclear export of HA p53 Δ1-42 required oligomerization with wild-type p53, and MDM2 binding by the wild-type p53 protein.

Finally, we wished to determine whether a p53 mutant deficient in direct MDM2 binding
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could also be targeted for degradation when expressed with MDM2 and wild-type p53. To this end, HA p53 Δ1-42 was co-expressed with GFP wild-type p53, GFP p53 Δ1-42, or GFP p53 ΔOD either alone, or with MDM2. To facilitate p53 degradation, an expression DNA encoding myc-tagged ubiquitin was also included in these transfections, and the amount of MDM2 DNA transfected was in excess of the p53 DNA. As shown in Fig. 6, steady state levels of both HA p53 Δ1-42 and GFP wild-type p53 were decreased when these two proteins were co-expressed with MDM2, indicating that MDM2 could promote the degradation of both proteins. The fact that HA p53 Δ1-42 was degraded in these experiments indicates that MDM2 can promote p53 degradation in the absence of direct p53:MDM2 binding. Importantly, HA p53 Δ1-42 was not degraded when expressed with MDM2 and either GFP p53 Δ1-42 or GFP p53 ΔOD, and these GFP-tagged p53 proteins were also not degraded. Taken together, these results support a model whereby the HA p53 Δ1-42 mutant of p53 could bind MDM2 indirectly through oligomerization with wild-type p53, and was subsequently targeted for ubiquitination (Fig. 3), nuclear export (Fig. 5) and degradation (Fig. 6).

Discussion

Wild-type p53 functions as a tumor suppressor protein by activating the transcription of genes which mediate either cell cycle arrest or apoptosis (4, 24). Amino acids 1-42 in p53 comprise the transcriptional activation domain, and it is this region that binds MDM2. Residues 102-292 encode the sequence-specific DNA binding domain. p53 binds DNA as a tetramer, and the oligomerization domain is encoded by residues 327-355. In addition to affecting the DNA binding activity of p53, oligomerization has also been reported to affect the susceptibility of p53 to MDM2-mediated ubiquitination. For example, oligomerization-deficient mutants of p53 displayed low binding affinity for MDM2 and were relatively poor substrates for ubiquitination (25, 26). However, efficient ubiquitination was restored when an oligomerization
deficient p53 mutant was fused to the dimerization domain of a heterologous protein (25). These results indicated that oligomerization is required for wild-type p53 to efficiently bind and be targeted for ubiquitination by MDM2.

Several studies have reported that an intact MDM2 binding domain is necessary for p53 to be degraded by MDM2. In the current study, we examined whether the MDM2 binding domain of p53 could be provided in trans through oligomerization between two p53 molecules. p53 proteins mutated in their MDM2 binding domains were unable to bind MDM2 directly, and were resistant to MDM2-mediated ubiquitination, nuclear export, and degradation when expressed with MDM2 alone in either Saos-2 cells (p53-null) or U2OS cells (wild-type p53). However, these same p53 mutants formed a complex with MDM2 and were efficiently ubiquitinated, degraded, and exported from the nucleus when co-expressed with MDM2 and wild-type p53. Moreover, this effect required MDM2 binding by wild-type p53 as well as oligomerization between wild-type p53 and the MDM2-binding deficient p53 mutants. Taken together, these results support a model whereby MDM2-binding deficient forms of p53 can bind MDM2 indirectly through oligomerization with wild-type p53, and are subsequently targeted for ubiquitination, nuclear export and degradation. It should be noted that targeting of the MDM2-binding deficient p53 in U2OS cells required over-expression of the mutant with both wild-type p53 and MDM2, despite the fact that these cells express endogenous, wild-type p53 protein. This could be because of the relatively low concentration of endogenous wild-type p53 compared to the over-expressed mutant, or because the pre-existing p53 in U2OS cells is already oligomerized and unavailable for oligomerization with the newly expressed mutant p53.

The mechanisms which regulate the nuclear export of p53 have not been fully clarified. Original studies suggested that a nuclear export signal (NES) within MDM2 was necessary for MDM2 to promote p53 degradation (27). Based on these findings, MDM2 was proposed to function as a molecular shuttle that would bind p53 and subsequently transport p53 from the
nucleus to the cytoplasm via the MDM2 NES. More recent studies have suggested that the ability of MDM2 to ubiquitinate p53, and not the MDM2 NES, is required for MDM2 to promote the nuclear export of p53. According to this model, the addition of ubiquitin moieties is thought to expose an NES within the p53 C-terminus, leading to the export of p53 from the nucleus to the cytoplasm (13, 14). In the current study, MDM2 could promote the nuclear export of a p53 mutant which lacked the ability to bind MDM2 directly. Moreover, MDM2 remained nuclear under conditions in which either wild-type p53 or an MDM2-binding deficient mutant of p53 was exported to the cytoplasm (Figs. 4 and 5). These results are most consistent with a model in which an activity of nuclear MDM2, and not MDM2 shuttling itself, is necessary to promote p53 nuclear export.

Our results also invite consideration of the mechanisms which regulate p53 in response to DNA damaging stress. Current models contend that the stabilization of p53 that occurs in response to DNA damage results from phosphorylation of p53 at one or more N-terminal sites. These phosphorylations are thought to inhibit p53:MDM2 binding, and therefore stabilize the p53 protein (3, 20, 21, 28). The premise of this model is that modifications to p53 which inhibit its interaction with MDM2 would result in stabilization of the p53 protein. However, p53 can exist as an oligomer of multiple p53 molecules, and it has not been determined how many p53 molecules within a p53 oligomer would need to lose MDM2-binding capacity in order for the whole oligomer to be stabilized. In the current study, p53 mutants deficient in MDM2-binding could oligomerize with wild-type p53 and bind MDM2 indirectly, and both the wild-type and mutant p53 proteins were then susceptible to MDM2-mediated degradation. This scenario may be considered similar to that in which an unmodified p53 molecule that can bind MDM2 oligomerizes with a phosphorylated p53 molecule that has lost MDM2-binding capacity in DNA damaged cells. In light of our results, we predict that the phosphorylated p53 could oligomerize with the unmodified p53 and bind MDM2 indirectly as a result of this oligomerization, and that both the unmodified and phosphorylated p53 molecules would then be susceptible to

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degradation. Accordingly, most if not all of the molecules within a p53 oligomer may have to lose MDM2 binding capacity in order for the entire oligomer to be stabilized. Conversely, dephosphorylation of a single p53 molecule within a stabilized p53 oligomer may allow complex formation between the whole oligomer and MDM2. Based on our results, we predict that all molecules within that p53 oligomer would then be susceptible to MDM2-dependent degradation. This may serve as an effective means to rid the cell of excess p53 during recovery from a DNA damage-induced arrest.
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Figure Legends

**Figure 1**  
**MDM2-binding deficient mutants of p53 are not ubiquitinated and degraded by MDM2.**

Saos-2 (A, C) and U2OS (B, D) cells were transfected with 500 ng of either HA-tagged wild-type p53 (HA p53 wt), HA p53 W23S, or HA p53 Δ1-42 alone, or cotransfected with 3 μg of MDM2. DNA encoding myc-tagged ubiquitin (500 ng) was included in the U2OS cell transfections. In each transfection, the total amount of DNA was adjusted to 4 μg by the addition of salmon sperm DNA. (A, B \textit{Lower panels}) Cell lysates (200 μg) were immunoprecipitated with 0.4 μg of the anti-HA polyclonal antibody Y-11 (Santa Cruz), and the immunoprecipitates were examined by immunoblot analysis with the anti-MDM2 antibody SMP-14 (Santa Cruz). (Upper panels) Ubiquitinated p53 (p53-Ubn) was analyzed by either immunoprecipitation with the anti-HA polyclonal antibody Y-11, followed by immunoblot analysis with the anti-HA monoclonal antibody HA.11 (Babco) (A), or by direct immunoblot analysis with the HA.11 monoclonal antibody (B). (C, D) HA p53 and MDM2 protein levels were determined by immunoblot analysis with the HA.11 monoclonal antibody, and the MDM2 monoclonal antibody SMP-14.

**Figure 2**  
**MDM2-binding deficient mutants of p53 can bind MDM2 indirectly through oligomerization with wild-type p53.**

Saos-2 cells (A, C) were transfected with either HA-tagged wild-type p53 (HA p53 wt) or HA p53 W23S alone, or co-transfected with MDM2 and/or untagged versions of p53 that were either wild-type (p53 wt), lacked the N-terminal 42 amino acids (p53 Δ1-42), or lacked the C-terminal oligomerization domain (p53 ΔOD). U2OS cells (B, D) were co-transfected with HA p53 Δ1-42 and GFP-tagged wild-type p53 (GFP p53 wt), GFP p53 Δ1-42 or GFP p53 ΔOD with/without MDM2. In these transfections, 1.5 μg of each DNA was used,
and the total amount of DNA was adjusted to 4.5 µg by the addition of salmon sperm DNA. (A, B) Cell lysates (200 µg) were immunoprecipitated with 0.4 µg of the anti-HA polyclonal antibody Y-11, and the immunoprecipitates were examined first by immunoblot analysis with an anti-MDM2 antibody SMP-14 (*Upper panel*). The immunoprecipitates were then examined by immunoblot analysis with the anti-p53 monoclonal antibody PAb1801 (A, *Lower panel*), or with an anti-GFP monoclonal antibody (B, *Lower panel*). (C, D) Cell lysates were examined by immunoblot analysis to document expression of the transfected genes with antibodies against p53 (PAb1801), GFP, the HA epitope (HA.11 monoclonal), and MDM2 (SMP-14).

**Figure 3**  
**MDM2-binding deficient mutants of p53 can be ubiquitinated by MDM2 through oligomerization with wild-type p53.**

Lysates from the cells transfected in Fig. 2 were examined for ubiquitination of the MDM2-binding deficient p53 mutants. (A) Ubiquitination of HA p53 W23S in Saos-2 cells was examined by direct immunoblot analysis with the anti-HA monoclonal antibody HA.11. Asterisk (*) indicates a nonspecific background band. (B) Ubiquitination of HA p53 Δ1-42 in U2OS cells was examined by immunoprecipitation from 200 µg of transfected cell lysate using the anti-HA polyclonal antibody Y-11, followed by immunoblot analysis with the anti-HA monoclonal antibody HA.11.

**Figure 4**  
**A MDM2-binding deficient mutant of p53 alone is not exported to the cytoplasm by MDM2.**

U2OS cells were transfected with 1 µg of either HA-tagged wild-type p53 (HA p53 wt) or HA p53 Δ1-42 alone, or with 1 µg of MDM2. The total amount of DNA was adjusted to 3 µg using salmon sperm DNA. p53 immunostaining was carried out using the anti-HA monoclonal antibody HA.11 as the primary antibody, and rhodamine red-conjugated anti-mouse antibody as the secondary antibody. MDM2 staining was carried out using the anti-MDM2 polyclonal antibody N-20 as the primary antibody, and AMCA-conjugated anti-rabbit antibody as the
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secondary antibody, as described in the experimental procedures. (A) Representative samples of p53 and MDM2 colocalization are shown. (B) The staining pattern for p53 was scored for 100 cells in two separate experiments, as described previously (13). Graph shows the percentage of cells with the indicated HA p53 staining patterns. Cells in which MDM2 did not display complete nuclear staining were excluded from the analyses.

**Figure 5**  
A MDM2-binding deficient mutant of p53 can be exported to the cytoplasm by MDM2 when cotransfected with wild-type p53.

U2OS cells were cotransfected with HA-tagged p53 Δ1-42 (HA p53 Δ1-42) and GFP-tagged wild-type p53 (GFP p53 wt), GFP p53 Δ1-42 or GFP p53 Δoligomerization domain (OD), with/without MDM2. In these transfections, 1 µg each of DNAs was used, and the total amount of DNA was adjusted to 3 µg using salmon sperm DNA. (A) Representative colocalization patterns are illustrated. (B) The staining pattern for HA p53 Δ1-42 and the indicated GFP p53 proteins was scored in cells co-expressing both proteins and MDM2 for at least 100 cells in two separate experiments. Graph shows the percentage of cells with the indicated HA p53 and GFP p53 staining patterns. Cells in which MDM2 did not display complete nuclear staining were excluded from the analyses. The results indicate that HA p53 Δ1-42 and GFP wild-type p53 are both exported from the nucleus when co-expressed with MDM2. In contrast, HA p53 Δ1-42 remains nuclear when co-expressed with MDM2 and either GFP p53 Δ1-42 or GFP p53 ΔOD.

**Figure 6**  
A MDM2-binding deficient mutant of p53 is degraded by MDM2 through oligomerization with wild-type p53.

U2OS cells were cotransfected with 125 ng of HA-tagged p53 Δ1-42 (HA p53 Δ1-42) and 375 ng of either GFP-tagged wild-type p53 (GFP p53 wt), GFP p53 Δ1-42 or GFP p53 Δoligomerization domain (OD) with/without 3 µg of MDM2. To facilitate p53 degradation, 500 ng of myc tagged ubiquitin was included in each transfection. The total amount of DNA was...
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adjusted to 4 µg by the addition of salmon sperm DNA. Cell lysates were then examined by immunoblot analysis with an anti-GFP antibody, an anti-HA antibody HA.11, and the MDM2-specific antibody SMP-14.
Fig. 1
Fig. 2
Fig. 3
Fig. 4

A

a. HAp53 wt

b. HAp53 wt + MDM2

c. HAp53 Δ1-42

d. HAp53 Δ1-42 + MDM2

HA

MDM2

B

HA Staining Pattern

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<th>Weak nuclear + strong cytoplasmic</th>
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Fig. 5
**Fig. 6**

**GFP IB**
- GFPp53 wt
- GFPp53 Δ1-42
- GFPp53 ΔOD

**HA IB**
- HAp53 Δ1-42

**MDM2 IB**
- MDM2

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MDM2 can promote the ubiquitination, nuclear export, and degradation of p53 in the absence of direct binding

Tomomi Inoue, Rory K. Geyer, David Howard, Zhong K. Yu and Carl G. Maki

J. Biol. Chem. published online September 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107477200

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